



Research review paper

Microbial production of sialic acid and sialylated human milk oligosaccharides: Advances and perspectives

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ABSTRACT

Sialic acids (SAs) are important functional sugars, and monomers of sialylated human milk oligosaccharides (sialylated HMOs or sialyllactoses), which are crucial for improving infant development and can facilitate infant brain development, maintain brain health, and enhance immunity. The most common form of SA is *N*-acetylneuraminic acid (NeuAc), and the main forms of sialyllactoses are 6'-sialyllactose (6'-SL) and 3'-sialyllactose (3'-SL). As functional food additive, the demand for NeuAc and sialyllactoses will continuously increase due to their wide and important fields of application. However, NeuAc and sialyllactoses produced by traditional extraction methods are inefficient and may cause allergen contamination, and cannot keep up with the rapidly increasing market demand. Therefore, the production of NeuAc and sialyllactoses by sustainable biotechnological methods have attracted increasing attention. In particular, the development of metabolic engineering and synthetic biology techniques and strategies have promoted efficient biosynthesis of NeuAc and sialyllactoses. In this review, we first discussed the application of NeuAc and sialyllactoses. Secondly, metabolic engineering and protein engineering-fueled progress of whole-cell catalysis and *de novo* synthesis of NeuAc and sialyllactoses were systematically summarized and compared. Furthermore, challenges of efficient microbial production of NeuAc and sialyllactoses as well as strategies for overcoming the challenges were discussed, such as clustered regularly interspaced short palindromic repeats interference (CRISPRi)-aided identification of key precursor transport pathways, synergistically debottleneck of kinetic and thermodynamic limits in synthetic pathways, and dynamic regulation of metabolic pathways for balancing cell growth and production. We hope this review can further facilitate the understanding of limiting factors that hampered efficient production of sialic acid and sialyllactoses, as well as contribute to the development of strategies for the construction of efficient production hosts for high-level production of sialic acid and sialyllactose based on synthetic biology tools and strategies.

1. Introduction

Sialic acids (SAs) are a family of nine-carbon amino sugars, with > 40 discovered forms derived from neuraminic acid (Cheng et al., 2017; Schauer, 2000; Zhu et al., 2017a). The combination of various types of groups at the 2, 4, 5, 7, 8, and 9 positions contributes to the diversity of SAs (Angata and Varki, 2002; Kelm and Schauer, 1997; Varki and Varki, 2007). The most common SA molecule is *N*-acetylneuraminic acid (NeuAc), a carboxylated nine-carbon monosaccharide with *N*-acetyl at 5-position (Lundgren and Boddy, 2007). NeuAc is ubiquitous in bacteria and animals, and it is the main form of SA in humans. Furthermore, NeuAc has important roles in regulating biological recognition, cellular immunity, and diseases, and is one of the most important monomers of sialylated human milk

oligosaccharides (sialylated HMOs or sialyllactose) that is crucial for improving infant development (Ten et al., 2014; Chen and Varki, 2010; Drouillard et al., 2010; Hassan et al., 2016; Kang et al., 2012; Maru et al., 2002; Schauer, 2000, 2004; Soundararajan et al., 2009) (Fig. 1a and b). In particular, NeuAc has been certified as a food additive in the European Union in 2017 (Turck et al., 2017). The Food and Drug Administration (FDA) of the United States and China have also approved NeuAc as a new food ingredient (FDA, 2015). NeuAc can be synthesized by *N*-acetylglucosamine 2-epimerase (AGE) and *N*-neuraminic acid aldolase (NanA), or *N*-acetylneuraminic acid synthase (NeuB) from *N*-acetylglucosamine (GlcNAc) and pyruvate (or PEP) as direct precursors, which are derived from the glycolytic pathway with glutamine-fructose-6-phosphate aminotransferase (GlmS) and glucosamine-6-phosphate *N*-acetyltransferase 1 (GNA1) as key pathway enzymes (Berg

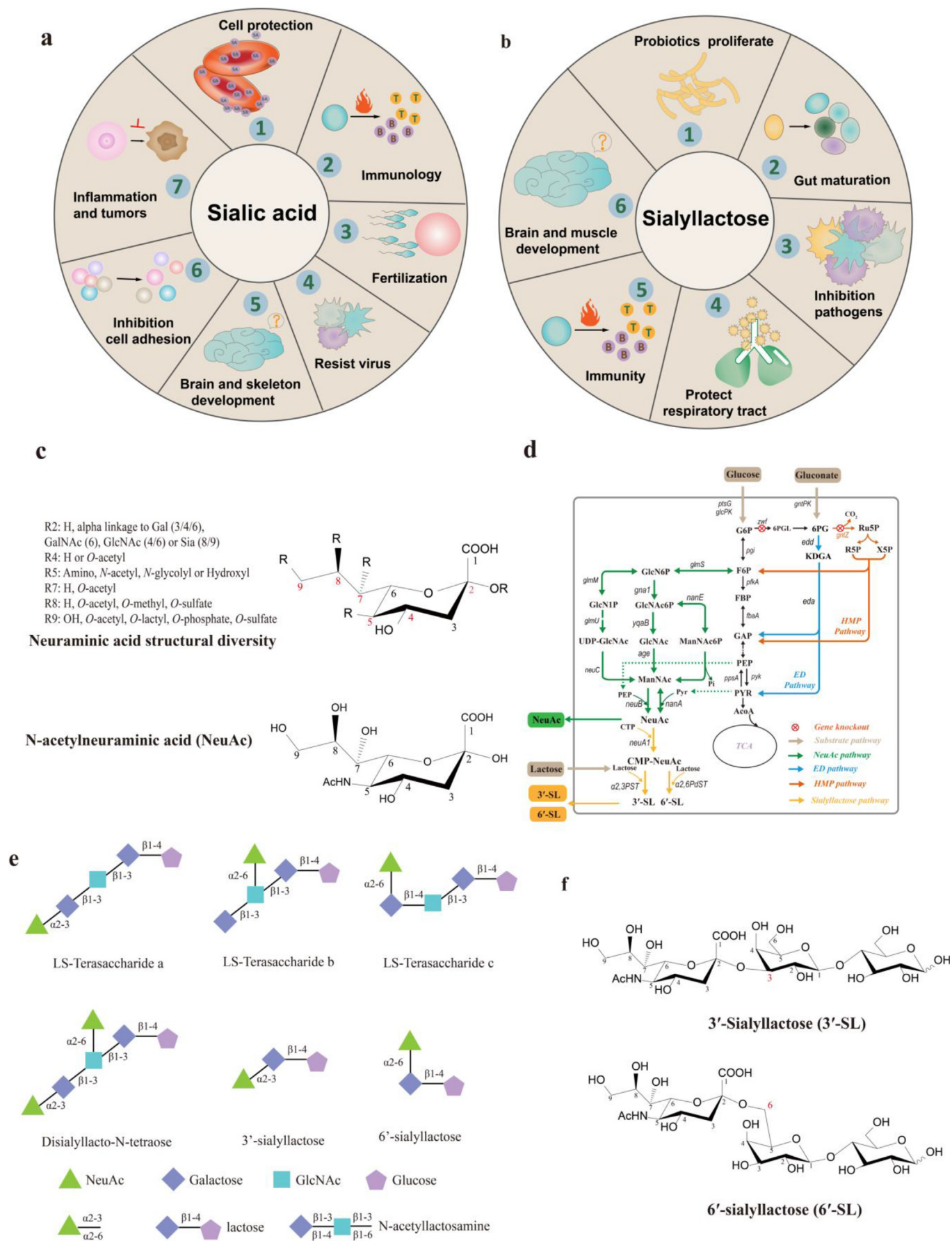
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Fig. 1. a) Physiological function of sialic acid. b) Physiological function of sialyllactose. c) Sialic acid structural diversity of *N*-acetylneuraminic acid (NeuAc). d) Metabolic pathway of NeuAc in *B. subtilis*. Green-colored arrows indicate the construction of the NeuAc biosynthetic pathway, and blue-colored arrows indicate ED pathway. GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvic acid; Pyr, pyruvate; KDPG, 2-dehydro-3-deoxy-6-phosphogluconate; GlcN6P, glucosamine-6P; GlcNAc6P, *N*-acetylglucosamine-6P; ManNAc6P, *N*-acetylmannosamine-6P; GlcN1P, glucosamine-1P; GlcNAc-1P, *N*-acetylglucosamine-1P; UDP-GlcNAc, UDP-*N*-acetylglucosamine; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; NeuAc, *N*-acetylneuraminic acid; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose. *glmS*, encoding glutamine-fructose-6-phosphate aminotransferase; *yqaB*, encoding a phosphatase with high specificity for *N*-acetylglucosamine-6-phosphate; *age*, encoding *N*-acetyl-D-glucosamine 2-epimerase; *neuB*, encoding *N*-acetylneuraminic acid synthase; *edd*, encoding 6-phosphogluconate dehydratase; *eda*, encoding 2-dehydro-3-deoxy-phosphogluconate/4-hydroxy-2-oxoglutarate aldolase; *pyk*, encoding pyruvate kinase; *ppsA*, encoding phosphoenolpyruvate synthetase; *nanE*, encoding *N*-acetylglucosamine-6-phosphate 2-epimerase; *glmM*, encoding phosphogluconate mutase; *glmU*, encoding uridyltransferase/glucosamine-1-phosphate acetyltransferase; *neuC*, UDP-*N*-acetylglucosamine 2-epimerase; *neuA*, encoding CMP-NeuAc synthetase; α -2,3-PST encoding α -2,3-sialyltransferase; α -2,6-PST encoding α -2,6-sialyltransferase. e) The structural of sialylated HMOs. f) The structural of 6'-sialyllactose (6'-SL) and 3'-sialyllactose (3'-SL). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2015; Klein and Ferré-D'Amaré, 2006; Lee et al., 2007a) (Fig. 1 c and 1d). NeuAc is one of the key monomers of sialyllactoses. Among HMOs, approximately 10% to 30% oligosaccharides are sialylated (Günther and Guido, 2008; Kim et al., 2018). Sialylated HMOs mainly include 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL), LS-tetra-saccharide a, LS-tetra-saccharide b, LS-teraccharide c and disialyllacto-N-tetraose (Fig. 1e and f, Bode, 2012). In sialylated HMOs, the content of sialyllactose is the highest, which contains 3'-SL and 6'-SL, reaching 980 nmol/mL following 2'-fucosyllactose (1980 nmol/mL), lacto-N-tetraose (1010 nmol/mL), lacto-N-fucopentaose I (1210 nmol/mL), and lacto-N-fucopentaose II (1180 nmol/mL) (Seppo et al., 2017). The 3'-SL and 6'-SL are oligosaccharides, which are essentially NeuAc bounded to a lactose molecule through α -(2,3)- and α -(2,6)-linkages (Gnoth et al., 2000). The structure of different sialylated HMOs can be identified by nuclear magnetic resonance spectroscopy (NMRS) based on the specific nucleus that can form a nuclear magnetic resonance signal when absorbing the energy provided by a specific frequency radio frequency field and quantified by mass spectrometry (MS) based on molecular weight difference of ionized whole molecule and their fragments (Drouillard et al., 2010; Sakaguchi et al., 2014; Taufik et al., 2013; Wu et al., 2011). NeuAc and lactose are direct precursors of sialyllactose synthesis. First, the cytidine monophosphate-NeuAc (CMP-NeuAc) synthetase catalyzes NeuAc to CMP-NeuAc (Han et al., 2012). Next, the α -2,3-sialyltransferase (α -2,3-PST) and the α -2,6-sialyltransferase (α -2,6-PST) catalyzes CMP-NeuAc and lactose to 3'-sialyllactose and 6'-sialyllactose, respectively (Choi et al., 2014; Kajihara et al., 1996; Sugiarto et al., 2011; Sun et al., 2008; Yu et al., 2005). Therefore, the key to sialyllactose biosynthesis is an efficient supply of NeuAc.

NeuAc has been traditionally prepared from extraction of natural sources and chemical synthesis (Traving and Schauer, 1998; Yang et al., 2017). In cubilose (edible cubilose, a mucilaginous secretion of certain birds), casein, milk, and eggs, the concentration of NeuAc is relatively higher than those in other animal-derived resource (Boddy and Lundgren, 2014; Sun et al., 2013). NeuAc can also be chemically synthesized either with *N*-acetylmannosamine (ManNAc) and α -(bromo-methyl) acrylic acid as substrates under the catalysis of indium in an acidic alcohol solution, or with NeuAc methyl ester as the substrate (Chan and Lee, 1995). However, due to the extremely low content of NeuAc in natural products, the extraction of NeuAc requires acid hydrolysis, neutralization, chromatographic analysis, and concentration, which result in high production costs for NeuAc. The chemical synthesis of NeuAc requires severe reaction conditions and requires expensive and toxic metals as catalysts, which result in high costs of production and environmental pollution. Compared with extraction and chemical synthesis, the biological production of NeuAc, which includes enzyme catalysis, whole-cell catalysis, and *de novo* biosynthesis, offers many advantages, such as mild conditions and low cost (Comb and Roseman, 1960; Gao et al., 2011; Kang et al., 2012). In recent years, applications in metabolic engineering, protein engineering, and synthetic biology techniques have facilitated methods for NeuAc biosynthesis (Chen et al., 2018; Peters et al., 2018; Yang et al., 2017). Although whole-cell catalysis for NeuAc production have been previously summarized, a

systematic discussion on recent advances for the production of NeuAc and sialyllactose fueled by metabolic engineering strategies, enzymatic engineering, and synthetic biology techniques is lacking and therefore relevant (Tao et al., 2010).

To this end, in this review we focus on five years of recent significant progress of biotechnological production approaches for SA and sialylated HMO, especially for NeuAc and sialyllactose, the most important SA and sialylated HMO. Furthermore, we discuss the prospects for the application of NeuAc and sialyllactose. Secondly, metabolic engineering progress of whole-cell catalysis and the *de novo* synthesis of NeuAc and sialyllactose are summarized and compared. Moreover, the strategies for overcoming challenges of microbial *de novo* synthesis of NeuAc and sialyllactose are detailed and analyzed, such as clustered regularly interspaced short palindromic repeats interference (CRISPRi)-aided identification of key precursor transport pathways, analysis the kinetic and thermodynamic limits, and multi-layered dynamic regulation of metabolic pathways for balancing cell growth and production. Finally, we discuss the prospective combination of metabolic engineering and synthetic biology techniques to promote NeuAc production.

2. Applications of SA and sialyllactose

Cubilose is a natural material with the highest content of NeuAc, which contains 7–12% SA and is approximately 50-fold greater than that of egg yolk (0.19%) (Juneja et al., 1991; Yang et al., 2014). Furthermore, cubilose has a long history as a nutraceutical, such as in traditional Chinese medicine. Recently, NeuAc has been certified as a food additive by the European Food Safety Authority, as well as the Food and Drug Administration (FDA) of the United States and China (FDA, 2015; Turck et al., 2017). The SA enters the fetal and infant nervous system and binds to glycosphingolipids to form gangliosides that promote the development of the nervous system (Angata and Fukuda, 2003). However, the SA content in milk is very low (8–25 mg/L), which is much lower than that in human milk (9–155 mg/100 mL) (Carlson, 1985; Claumarchirant et al., 2016; Martin-Sosa et al., 2003; Salcedo et al., 2015; Urashima et al., 2001). The SA content in the brain can be increased by dietary supplementation of SA to improve learning and memory (Wang, 2009; Wang and Brand-Miller, 2003). It has been shown that SA supplementation in the diet has benefits for infant skeletal growth, brain development, and maintenance of elderly brain function (von Itzstein et al., 1993; van Karnebeek et al., 2016; Wang, 2009). SA can also enhance intestinal absorption of minerals and vitamins, and promotes bone development (van Karnebeek et al., 2016).

In addition, SA has shown great value in medical fields in conferring anti-adhesion, anti-virus, and anti-cancer effects. SA peptides prevent intestinal toxins and pathogenic bacteria from binding to intestinal mucosal cells to improve intestinal antibacterial, detoxification, and antiviral ability (Gnoth et al., 2000). SA can adhere to the surface of red blood cells to protect it and help human spermatozoa to better access to the zona pellucida of oocytes (Schauer, 1997). Furthermore, it can be used to treat rheumatoid arthritis because the SA on the cell surface can effectively prevent excessive white blood cell aggregation (Phillips

Table 1
Whole-cell biotransformation for NeuAc and sialyllactose production.

Strains	Characteristics	Substrate concentration	NeuAc/sialyllactose titer	NeuAc yield on GlcNAc/sialyllactose yield on NeuAc (mol/mol)	NeuAc/sialyllactose Productivity	Reference
NeuAc						
<i>E. coli</i> NM522 and <i>Corynebacterium ammoniagenes</i> three-cell coupling catalysis	<i>E. coli</i> NM522 expressing <i>slr1975</i> from <i>Synechocystis</i> sp. PCC6803, and another <i>E. coli</i> NM522 expressing <i>neutB</i> from <i>E. coli</i> K1, coupled with <i>Corynebacterium ammoniagenes</i> to metabolize PEP.	800 mM GlcNAc and 360 mM Glucose	39.7 mM (12.3 g/L)	4.9%	0.56 g/L/h	(Tabata et al., 2002)
<i>E. coli</i> BL21 double cell coupling catalysis	The <i>age</i> gene from <i>Anabaena</i> sp. CH1 and the <i>nanA</i> gene from <i>E. coli</i> . <i>AGE</i> has high activity and requires less ATP to be activated. The cells can be reused for more than eight cycles with a productivity of > 8.0 g/L/h (NeuAc).	1200 mM GlcNAc and 1200 mM pyruvate	395.4 mM (122.3 g/L)	33.3%	10.2 g/L/h	(Lee et al., 2007a)
<i>E. coli</i> BL21 and <i>Pseudomonas stutzeri</i> SDM double cell coupling catalysis	<i>E. coli</i> BL21/pET15b- <i>nanA</i> expressing <i>nanA</i> from <i>E. coli</i> K-12, coupled with <i>Pseudomonas stutzeri</i> SDM to produce LOX for the catalysis of lactate to pyruvate. A chemical method was used to epimerase GlcNAc to ManNAc and lactate instead of using pyruvate as a substrate.	1008 mM GlcNAc and 65.61 g/L lactate	59.2 mM (18.3 g/L)	5.9%	0.92 g/L/h	(Xu et al., 2007)
<i>E. coli</i> DH5α double cell coupling catalysis	The <i>slr1975</i> gene from <i>Synechocystis</i> sp. PCC 6803 and the <i>nanA</i> gene from <i>E. coli</i> . Temperature-inducible promoter replaced the conventional IPTG-inducible promoter.	200 mM GlcNAc and 2200 mM pyruvate	61.9 mM (19.1 g/L)	30.9%	0.55 g/L/h	(Zhang et al., 2010)
<i>E. coli</i> HN0074 single-cell catalyst	The <i>slr1975</i> gene from <i>Synechocystis</i> sp. PCC6803 and the <i>neutB</i> gene from <i>E. coli</i> K1. Glucose was used to supply PEP instead of pyruvate to reduce costs.	540 mM GlcNAc and 277 mM glucose	172 mM (53 g/L)	32.0%	2.4 g/L/h	(Ishikawa and Koizumi, 2010)
<i>E. coli</i> K12 single-cell catalyst	The <i>slr1975</i> gene from <i>Synechocystis</i> sp. PCC6803 and the <i>nanA</i> gene from <i>E. coli</i> K12.	1000 mM GlcNAc and 500 mM pyruvate	191 mM (59.0 g/L)	19.1%	1.64 g/L/h	(Tao et al., 2011)
<i>B. subtilis</i> WB600	The <i>nanA</i> gene from <i>E. coli</i> K-12. Biosynthesis of NeuAc using the alkaline epimerization of GlcNAc to ManNAc and surface-displayed <i>nanA</i> enzyme.	1000 mM GlcNAc and 1000 mM pyruvate	174.3 mM (53.9 g/L)	17.4%	4.9 g/L/h	(Gao et al., 2011)
<i>E. coli</i> K12 single-cell catalyst	The <i>age</i> gene from <i>Anabaena</i> sp. PCC7120 and the <i>nanA</i> gene from <i>E. coli</i> K12. Increased expression of <i>NanA</i> and blocked NeuAc uptake and the competing pathway.	600 M GlcNAc and 800 M pyruvate	240 mM (74.2 g/L)	40%	6.2 g/L/h	(Lin et al., 2013)
<i>E. coli</i> Rosetta (DE3) pLysS single-cell catalyst	The <i>nanA</i> gene from <i>E. coli</i> K12 and <i>age</i> gene from <i>Anabaena</i> sp. CH1. Screening for high-efficiency <i>age</i> enzymes to optimize the expressions of <i>age</i> and <i>nanA</i> to increase NeuAc yield.	800 mM GlcNAc and 1200 mM pyruvate	198 mM (61.3 g/L)	24.8%	1.02 g/L/h	(Sun et al., 2013)
<i>E. coli</i> BL21 double cell coupling catalysis	The <i>nanA</i> gene from <i>E. coli</i> K12, and the <i>slr1975</i> gene from <i>Synechocystis</i> sp. PCC 6803 were used. The <i>slr1975</i> and <i>nanA</i> activity ratio was optimized by varying recombinant amounts of cell biomass. Additionally, 0.4% Triton X-100 was added to increase cell permeability.	600 mM GlcNAc and 1600 mM pyruvate	260 mM (80.4 g/L)	43.3%	1.3 g/L/h	(Zhou et al., 2016)
<i>E. coli</i> Rosetta (DE3) pLysS single-cell catalyst	The <i>nanA</i> gene from <i>C. glutamicum</i> ATCC 13032 and the <i>age</i> gene from <i>Anabaena</i> sp. CH1. The Shine – Dalgarno sequence and aligned spacing distance were optimized to increase NeuAc yield.	800 mM GlcNAc and 1200 mM pyruvate	364.7 mM (112.8 g/L)	45.6%	4.7 g/L/h	(Wang et al., 2017)
<i>E. coli</i> MG1655 single-cell catalyst	The <i>age</i> gene from <i>Anabaena</i> sp. CH1 and the <i>neutB</i> gene from <i>Campylobacter jejuni</i> . Glucose was used to supply PEP instead of pyruvate to reduce costs.	400 mM GlcNAc and 400 mM Glucose	23.3 mM (7.2 g/L)	5.8%	0.099 g/L/h	(Zhu et al., 2017b)
<i>E. coli</i> MG1655 single-cell catalyst	The <i>age</i> gene from <i>Anabaena</i> sp. CH1 and the <i>neutB</i> gene from <i>Campylobacter jejuni</i> . Overexpression of the <i>pck</i> and <i>ppsA</i> genes to increase PEP supply.	400 mM GlcNAc and 400 mM Glucose	27.8 mM (8.6 g/L)	6.9%	0.21 g/L/h	(Zhu et al., 2017a)
<i>Serratia marcescens</i> Db11 single-cell catalyst	The <i>age</i> gene from <i>Anabaena</i> sp. CH1, the <i>slr1975</i> gene from <i>Synechocystis</i> sp. PCC6803, the <i>nanA</i> gene from <i>E. coli</i> MG1655, and the <i>neutB</i> from <i>Campylobacter jejuni</i> NCTC11168. Development of <i>Serratia marcescens</i> Db11 as a	12.5 mM GlcNAc (or 5 g crystal chitin) and 10 mM PEP (or Pyruvate)	1.55 mM (0.48 g/L) for GlcNAc	12.4% for GlcNAc	0.02 g/L/h for GlcNAc	(Yan and Fong, 2018)

(continued on next page)

Table 1 (continued)

Strains	Characteristics	Substrate concentration	NeuAc/sialyl/lactose titer	NeuAc yield on GlcNAc/sialyl/lactose yield on NeuAc (mol/mol)	NeuAc/sialyl/lactose Productivity	Reference
<i>E. coli</i> BL21 single-cell catalyst	host strain and crystal chitin as a cheap substrate. Screens were performed for efficient pathway enzymes, and pathways fluxes were balanced through promoter swapping. The <i>age</i> gene from <i>Anabaena</i> sp. CH1 and the <i>nanaA</i> gene from <i>E. coli</i> . The co-expression system with a single copy of <i>age</i> and two copies of <i>nanaA</i> was established to enhance the <i>nanaA</i> gene expression level.	1200 mM GlcNAc and 1600 mM pyruvate	0.97 mM (0.3 g/L) for crystal chitin 412.6 mM (127.6 g/L)	34.4%	15.9 g/L/h	(Kao et al., 2018)
<i>E. coli</i> BL21 single-cell catalyst	The <i>nanaA</i> gene from <i>E. coli</i> K12, the <i>slr1975</i> gene from <i>Synechocystis</i> sp. PCC 6803 were used. The gene expressions of <i>slr1975</i> and <i>nanaA</i> were balanced using promoter engineering. A structure-guided process was used to screen a more efficient <i>slr1975</i> and <i>nanaA</i> variants by saturating mutations.	600 mM GlcNAc and 800 mM pyruvate	351.8 mM (108.8 g/L)	58.6%	9.07 g/L/h	(Chen et al., 2018)
sialylactos <i>E. coli</i> and <i>C. ammoniagenes</i> four kinds of cell coupling catalysis	CTP was formed from orotic acid by <i>E. coli</i> , CMP-NeuAc was formed from NeuAc by <i>E. coli</i> , 3'-SL was formed from CMP-NeuAc and lactose by <i>E. coli</i> , and UTP was formed from orotic acid by <i>C. ammoniagenes</i> .	129 mM NeuAc 146 mM lactose	(3'-SL) 57 mM (36.0 g/L)	44.2%	3.0 g/L/h	(Endo et al., 2000)
<i>E. coli</i> JM107 single-cell catalyst	The genes for CMP-NeuAc synthase and α -2,3-sialyltransferase from <i>N. meningitidis</i> were used. The <i>lacY</i> and <i>nantT</i> gene expressions were enhanced. Knockouts of the NeuAc degradation <i>nanaA</i> gene and lactose decomposition <i>lacZ</i> gene were performed. The sialylactose titer reached 1.5 g/L in the intracellular fraction, and 1.1 g/L in the extracellular fraction with glycerol and NeuAc as substrate at 22 h.	7.44 mM (2.3 g/L) NeuAc	(3'-SL) 4.1 mM (total) 1.5 g/L (intracellular) and 1.1 g/L (extracellular)	55.1%	0.12 g/L/h	(Priem et al., 2002)

et al., 1990). The uridine and hypoxanthine derivatives of SA can inhibit cancer cell metastasis because it is a strong inhibitor of sialyltransferase (Jefferson et al., 2014; Kijima-Suda et al., 1986; McNicholl and McNicholl, 2001). NeuAc is an intermediate for the antiviral drugs zanamivir and oseltamivir, which are widely used to treat both influenza A and B (von Itzstein et al., 1993; Magano, 2009; Salcedo et al., 2013; Shie et al., 2011). SA and its analogs can also be used to modify drug carriers to better reach their target sites (Bondioli et al., 2011). Therefore, SA has great potential in the field of food additives and medicine (Fig. 1a).

Sialyllactose is a key HMO and consists of a SA bound to lactose at 3 or 6 position, and can promote probiotic proliferate, optimized metabolism in the brain, and facilitate liver and muscle development in infants, and can induce differentiation such as human intestinal epithelial cells (Difilippo et al., 2015; Kuntz et al., 2008; Perdijk et al., 2019). Mouse experiments have shown that sialyllactose can improve the intestinal flora and reduce anxiety (Tarr et al., 2015). Sialyllactose has anti-infective and immuno-modulating effects (Bode, 2012; Hester et al., 2013), and prevents *P. aeruginosa* adhesion to human pneumocytes (Fig. 1b, Marotta et al., 2014). Based on the demonstrated beneficial effects of NeuAc and sialyllactose, biosynthetic methods of NeuAc and sialyllactose were mainly discussed in this review.

3. Biotechnological production of NeuAc and sialyllactose

3.1. Enzymatic production of NeuAc

Enzyme-catalyzed preparation of NeuAc was first used in NeuAc synthesis because the process is environmentally friendly and consists of mild catalytic conditions (Tao et al., 2010). In 1960, the enzymatic catalysis of NeuAc synthesis was first reported (Comb and Roseman, 1960). Subsequently, *N*-acetylglucosamine 2-epimerase (AGE), which catalyzes the reversible reaction of GlcNAc and ManNAc, has been reported (Ghosh and Roseman, 1965). Thus, the expensive precursor material ManNAc was replaced by GlcNAc, which greatly reduces the production cost of NeuAc (Maru et al., 1998). Furthermore, it has become the classical metabolic pathway for the synthesis of NeuAc-AGE and NanA (or NeuB) in a double-enzyme catalytic system (Lee et al., 2004; Maru et al., 2002; Zimmermann et al., 2007). Enzyme immobilization can improve enzyme protein utilization efficiency (Augé et al., 1984; Hu et al., 2010; Wang, 2009), and further analysis of the K_m value, V_{max} value, and the apparent activation energy (E_a) of the immobilization enzyme were used to verify that the key factor of catalytic efficiency was mass-transfer rather than kinetic limit. The maximal titer and conversion yields were 101.45 g/L, respectively, and 82% from 0.4 M GlcNAc and 0.8 M pyruvate. Moreover, the immobilized enzyme still had 75% residual viability after 5 repeated uses (Cheng et al., 2017).

Enzyme catalysis is more suitable for target products with high purity requirements that are difficult to obtain by chemical or biosynthetic methods, and high price (Imanparast et al., 2018). The enzyme catalysis method offers many advantages such as simplicity, mild catalytic conditions, and high conversion rates. However, the purification process of the enzyme and the separation of the product from the substrate are complicated, and the catalytic process requires the addition of the cofactor ATP. Moreover, the substrate GlcNAc and pyruvate are more expensive. Overall, these factors contribute to the high cost of enzyme catalysis and subsequent replacement by whole-cell catalysis technology.

3.2. Whole-cell catalysis for NeuAc production

Whole-cell catalysis is a biosynthesis strategy that efficiently yields the desired product using living cells via the expressions of key enzymes as biocatalyst, which are particularly useful for multi-enzyme reactions that needs expensive cofactors (Both et al., 2016). The whole-cell

catalysis process utilizes a typical AGE and NanA or NeuB double-enzyme catalytic process. Up to now, it can be divided into three parts: 1) multicellular coupling transformation methods, 2) direct precursor single cell transformation method, and 3) indirect precursor single cell transformation method (Table 1). Initially, two strains of *E. coli* expressing the *age* and *neuB* genes were coupled for fermentation with *Corynebacterium ammoniagenes* supplying PEP, and Nymeen S-215 treatment was used to increase cell permeability. The maximal NeuAc titer, GlcNAc conversion yield, and NeuAc productivity were 12.3 g/L, 4.9%, and 0.56 g/L/h, respectively (Tabata et al., 2002). Highly active enzymes are key to whole-cell transformation, and high activity AGE from *Anabaena* sp. CH1 can significantly increase NeuAc production. The maximal NeuAc titer, GlcNAc conversion yield, and NeuAc productivity were 122.3 g/L, 33.3%, and 10.2 g/L/h, respectively (Lee et al., 2007a). Particularly, the NanA enzyme was surface-displayed in *B. subtilis* WB600 to catalyze ManNAc to NeuAc, where ManNAc was prepared through alkaline epimerization. The NeuAc titer, GlcNAc conversion yield, and NeuAc productivity were 53.9 g/L, 17.4%, and 4.9 g/L/h, respectively (Gao et al., 2011). In addition, many optimization strategies have been developed, such as inexpensive lactic acid replacement of pyruvate as a substrate and a temperature-controlled expression system (Xu et al., 2007; Zhang et al., 2010; Zhou et al., 2016).

However, it is limited by multi-cell coupling systems that requires changes in the ratios of multiple strains to achieve the optimal activity ratio of multiple enzyme proteins. Furthermore, the multi-cell system will reduce the substrate transfer rate. In addition, the complex process is not conducive to industrialization, and therefore the single-cell catalytic system has become a trend. Overexpressing *age* and *nanA* genes while blocking the GlcNAc transporter gene *nagE* in the *E. coli* K12 strain results in a NeuAc titer, GlcNAc conversion yield, and NeuAc productivity of 59.0 g/L, 38.2%, and 1.63 g/L/h, respectively (Tao et al., 2011). Subsequently, whole-cell catalysis research have focused on knocking out the *nanTEK* gene of the NeuAc degradation pathway (Lin et al., 2013) Strengthening and balancing the expressions of AGE and NanA results in improvements to NeuAc titer, GlcNAc conversion yield, and NeuAc productivity to 112.8 g/L, 45.6%, and 4.7 g/L/h, respectively (Sun et al., 2013; Wang et al., 2017). In particular, increased expression of NanA by increasing the number of gene copies resulted in an improvement of the NeuAc titer, GlcNAc conversion yield, and NeuAc productivity to 127.6 g/L, 34.4%, and 15.9 g/L/h (Kao et al., 2018). To the best of our knowledge, this has been the highest reported NeuAc productivity and titer (Table 1). Another study analyzed the structure of enzymes encoded by *slr1975* and *nanA*, and screened for high-efficiency enzymes by saturating mutations, which improved the NeuAc titer, GlcNAc conversion yield, and NeuAc productivity to 108.8 g/L, 58.6%, and 9.07 g/L/h. To the best of our knowledge, this has been the highest reported NeuAc productivity (Chen et al., 2018). The synthesis reaction catalyzed by NanA required an excess of pyruvate to increase the NeuAc productivity. However, pyruvate is generally metabolized > 50% by cell metabolic processes in whole-cell catalysis. Since the price of pyruvate is as high as GlcNAc, this can result in a high cost for NeuAc.

Indirect precursor single-cell catalysis based on NeuB catalysis with PEP and ManNAc as substrates may provide a new strategy for whole-cell transformation. Although PEP is more difficult to accumulate than pyruvate, but the K_m for PEP from *E. coli* K12 is just 0.04 m (Vann et al., 1997). Therefore, the accumulation of PEP from glucose can be used to address the high cost due to pyruvate, thereby promoting whole-cell catalysis with the goal of using inexpensive substrates. Overexpression of *age* and *neuB* genes in *E. coli* with GlcNAc and glucose as substrates resulted in an improvement in NeuAc titer, GlcNAc conversion yield, and NeuAc productivity to 53 g/L, 31.8% and 2.41 g/L/h, respectively (Ishikawa and Koizumi, 2010). Metabolic engineering strategies were applied to indirect precursor single-cell catalysis to strengthen the expression of *pck* and *ppsA* genes to enhance PEP supply efficiency (Zhu

et al., 2017b; Zhu et al., 2017a). The chitinolytic bacterium *Serratia marcescens* can metabolize the cheap substrate chitin to replace GlcNAc to synthesize NeuAc (Yan and Fong, 2018).

The whole-cell catalysis is superior to extraction and enzyme catalytic processes because it does not require the addition of expensive cofactors. However, whole-cell catalysis still has many disadvantages in NeuAc biosynthesis. First, the GlcNAc conversion yield is < 60%, which not only increases the cost of GlcNAc recovery but is also detrimental to NeuAc purification. Secondly, whole-cell catalysis requires multiple processes such as cell culture, cell collection, resuspension, and bio-transformation, all of which increases the number of manipulation steps required and costs for production. To solve these problems, metabolic engineering of model microorganisms for *de novo* synthesis of NeuAc by microbial fermentation have been carried out.

3.3. *De novo* NeuAc biosynthesis by metabolic engineering

Escherichia coli K1, *Neisseria meningitidis*, and *Campylobacter jejuni* can naturally produce NeuAc using glucose as a substrate with UDP-GlcNAc as a direct precursor, where UDP-GlcNAc epimerase (NeuC) isomerizes UDP-GlcNAc to ManNAc, and NanA (or NeuB) further catalyzes ManNAc and pyruvate (or PEP) to NeuAc (Peters et al., 2018; Severi et al., 2007). Therefore, the first *de novo* NeuAc biosynthesis was achieved through overexpression of the *neuC* and *neuB* genes from *Neisseria meningitidis*, enhancing *glmS* gene expression, and knocking-out the NeuAc transporter *nanT* gene and reversible pathway *nanA* gene, resulting in a SA titer of 1.70 g/L (Lundgren and Boddy, 2007). Furthermore, rational pathway engineering of *E. coli* NeuAc pathways improved the NeuAc titer to 7.85 g/L by strengthening the NeuAc adolase pathway, blocking the entire NeuAc degradation pathway, and eliminating the production of acetate and lactate byproducts (Kang et al., 2012). In addition, to improve the safety of NeuAc production using a food-grade NeuAc microbial production method, the generally recognized as safe strain *B. subtilis* was modularly engineered for strengthening the NeuAc synthase pathway and balancing the supply of the key precursors GlcNAc and PEP on the basis of previously constructed GlcNAc synthetic pathway, resulting in a production of 2.18 g/L in shake flask fermentation (Liu et al., 2013; Liu et al., 2014a; Liu et al., 2014b; Liu et al., 2017; Zhang et al., 2018).

Although the feasibility of metabolic engineering strategies for improving NeuAc has been demonstrated, the NeuAc titer was still far from being cost-effective for NeuAc production (Table 2). Due to the complexity of the NeuAc synthetic pathway and multi-layers of regulatory mechanisms, rational pathway engineering of the NeuAc pathway may not be sufficient for holistically optimizing NeuAc production. To this end, synthetic biology tools were established and utilized for screening a library of key mutant enzymes and strains for improving NeuAc production.

3.4. Synthetic biology tools to aid improvements of NeuAc production

Metabolite responsive biosensors are important synthetic biology tools, which can be used for efficient laboratory adaptive evolution and high-throughput screening of mutants with improved cellular traits (Eggeling et al., 2015; Johnson et al., 2017; Lin et al., 2017; Xiu et al., 2017; Xu et al., 2014; Xu, 2017). To improve NeuAc production, an *in vivo* NeuAc aptazyme-based biosensor (derived from the *B. subtilis* *glmS* ribozyme) was used, where NeuAc can bind to its ligand, resulting in conformational changes to cause self-cleavage of the ribozyme to reduce the expression of downstream genes. This biosensor has high specificity and affinity ($K_D = 1.35$ nM), which can be applied to NeuAc pathway optimization and key enzyme evolution (Cho et al., 2013). The *tetA* gene causes cells to be more resistant to tetracycline and also more sensitive to NiCl_2 . The NeuAc aptazyme-based biosensor was used to correlate *tetA* gene expression to increase the NeuAc titer of the mutant strain, resulting in a decrease of *tet* gene expression, greater resistant to

NiCl_2 , and improved cell growth. Yang et al. (2017) combined NeuAc aptazyme-based biosensor with a ribosomal binding site (RBS) library for metabolic fine-tuning of *glmS*, *GNA1*, *slr1975*, and *neuB* genes in the NeuAc synthesis pathway. Next, the NeuB mutant library was constructed by error-prone PCR to direct the evolution of NeuB enzyme. Through the above two strategies, the NeuAc titer was increased from 1.58 to 2.61 g/L in the shake flask (Yang et al., 2017).

Besides using ribozymes as metabolite binding and regulatory elements for biosensor construction, a series of naturally available transcription factors were applied to biosensor construction and dynamic regulation (Eggeling et al., 2015; Rogers et al., 2016; Schallmeyer et al., 2014). In *E. coli*, the *nanATEK* operon that catabolizes NeuAc was transcriptionally activated by transcription factor NanR when NeuAc was present, and the binding site sequence was elucidated (Kalivoda et al., 2003). Based on the unraveled regulatory mechanism of NanR, a series of NanR-responsive promoters were used in NeuAc biosensor construction for co-coupling GFP expression levels to NeuAc production in *E. coli* (Peters et al., 2018). Furthermore, a NeuAc biosensor for high-throughput screening of a mutant library of *E. coli* for various NeuAc production levels was performed.

3.5. Bioprocess optimization to improve sialyllactose production

Enzyme catalysis is an important method for producing sialyllactose. Sialidase (Tr6) from *Trypanosoma rangeli* can be efficiently expressed by *Pichia pastoris*, which can be used to catalyze casein glycomacropeptide (cGMP) and lactose to 3'-sialyllactose (Michalak et al., 2014). In order to reduce costs, both cGMP and lactose can be obtained from side streams in the dairy industry (Zeuner et al., 2014). Rational design based on enzyme structure modeling and saturation mutagenesis was carried out for enhancing sialyltransferase activity (Choi et al., 2014). To improve substrate cGMP pretreatment efficiency and reduce sialyllactose production costs, a UF/NF membrane system was constructed (Luo et al., 2014; Nordvang et al., 2014; Zeuner et al., 2014).

To further reduce the production costs of sialyllactose, various microbial fermentation approached for sialyllactose production were developed. First, a sialyllactose fermentation process based on multiple strains was established with *Corynebacterium ammoniagenes* expressing CTP synthase, and two recombinant *E. coli* overexpressed CMP-NeuAc synthetase and sialyltransferase, respectively. Using orotic acid, NeuAc, and lactose as substrates, the sialyllactose titer reached 33 g/L at 11 h (Endo et al., 2000). Next, a single strain for sialyllactose synthesis was developed via overexpression of CMP-NeuAc synthase and α -2,3-sialyltransferase from *N. meningitidis*, strengthening *lacY* and *nanT* expression, and knockout of the NeuAc degradation gene *nanA* and lactose decomposition gene *lacZ*. The sialyllactose titer reached 1.5 g/L intracellularly and 1.1 g/L extracellularly with glycerol and NeuAc as substrates at 22 h (Priem et al., 2002). With glycerol and lactose as substrates, 3'-sialyllactose titer can reach 25 g/L by overexpression of the α -2,3-sialyltransferase, *N*-acetylglucosamine-6-phosphate-epimerase (NeuC), SA synthase (NeuB), and CMP-NeuAc synthetase (NeuA) with knockout of a coding gene of NeuAc aldolase, ManNAc kinase, and β -galactosidase (Fierfort and Samain, 2008). The 6'-sialyllactose titer then reached 30 g/L by a similar strategy as that for 6'-sialyllactose production (Drouillard et al., 2010). Moreover, Guo et al. (2014) found that *Pasteurella multocida* sialyltransferase (PmST) had dual trans-sialidase activities, thereby capable of catalyzing the formation of 3'- and 6'-sialyllactose simultaneously. With glycomacropeptide (cGMP) and lactose as substrates, PmST catalyzes the formation of 3'- and 6'-sialyllactose with different proportions (Guo et al., 2014). In summary, efficient biosynthesis of 3'- and 6'-sialyllactose approaches have been successfully developed with low-cost substrates, such as glycerol and lactose. However, the biosynthesis of NeuAc was not efficient using the low-cost substrate glucose, resulting in a maximal titer of only 8.31 g/L (Yang et al., 2017). Therefore, efforts have to be made for *de novo* NeuAc biosynthesis.

Table 2
De novo biosynthesis of NeuAc and sialyllactoses.

Strains	Characteristics	Key enzymes	NeuAc/sialyllactose titer	ManNAc titer	GlcNAc titer	Reference
NeuAc <i>E. coli</i> K-12	Overexpression of the <i>glms</i> gene, and knockout of the <i>nanT</i> and <i>nanA</i> genes were performed.	<i>neuB</i> and <i>neuC</i> gene from <i>N. meningitidis</i> MCS8 group B	1.7 g/L (shake flask)	N.M	N.M	(Lundgren and Boddy, 2007)
<i>E. coli</i> DH5a	Overexpression of the <i>GnaI</i> and <i>glms</i> [*] genes, and knockout of the <i>nagB</i> , <i>nagA</i> , <i>nanE</i> , <i>nanK</i> , <i>ldhA</i> , <i>poxB</i> , and <i>ackA</i> genes were performed.	<i>slr1975</i> gene from <i>Synechocystis</i> sp. PCC6803, <i>nanA</i> gene from <i>E. coli</i> W3110	1.62 g/L (shake flask) 7.85 g/L (5-L fermentor)	3.49 g/L (5-L fermentor)	15.51 g/L (5-L fermentor)	(Kang et al., 2012)
<i>E. coli</i> DH5a	Overexpression of the <i>GnaI</i> and <i>glms</i> [*] genes. Knockout of the <i>nagB</i> , <i>nagA</i> , <i>nanE</i> , <i>nanK</i> , <i>ldhA</i> , <i>poxB</i> , <i>ackA</i> , and <i>nanA</i> genes. An aptazyme-based biosensor was used to optimize the synthetic pathway to balance and evolve the NeuB enzyme.	<i>slr1975</i> gene from <i>Synechocystis</i> sp. PCC6803, <i>neuB</i> gene from <i>E. coli</i> K1	2.61 g/L (24-well microplates) 8.31 g/L (two-stage fermentation*)	about 2.5 g/L (two-stage fermentation*)	about 11 g/L (two-stage fermentation*)	(Yang et al., 2017)
<i>B. subtilis</i> 168	Modular pathway engineering of the GlcNAc and PEP pathways and balancing was performed. NeuAc biosynthesis and cell growth was assessed.	<i>age</i> from <i>Anabaena</i> sp. CH1, <i>neuB</i> from <i>E. coli</i> K12	2.18 g/L (shake flask)	0 g/L	0 g/L	(Zhang et al., 2018)
sialyllactoses <i>E. coli</i>	The expensive substrate NeuAc was synthesized from glycerol, and knockouts of NeuAc aldolase, ManNAc kinase, and β -galactosidase were performed.	the α -2,3-sialyltransferase gene from <i>Neisseria gonorrhoeae</i> , the α -2,6-sialyltransferase from <i>Photobacterium</i> sp. JT-ISH-224, <i>neuC</i> from <i>Campylobacter jejuni</i> , <i>neuB</i> and <i>neuA</i> from <i>C. jejuni</i> ATCC 43438.	25 g/L (3'-SL) 30 g/L (6'-SL)	/	/	(Drouillard et al., 2010; Fierfort and Samain, 2008)
<i>E. coli</i>	<i>Pasteurella multocida</i> sialyltransferase (PmST) had dual transsialidase activities and was capable of catalyzing the formations of 3'- and 6'-sialyllactose.	the sialyltransferase from <i>Pasteurella multocida</i>	3'-SL 1.74 g/L (2.75 mM) 6'-SL 2.11 g/L (3.33 mM)	/	/	(Guo et al., 2014)

N.M:Not mentioned.

glms^{*}: Multi-site mutagenesis of *glms* (mutant sites: E14K, D386V, S449P and E524G) to remove RNA ribozyme regulation (Heckman and Pease, 2007).
Two-step fermentation*: After initial growth stage, cells were transferred to production medium (Yang et al., 2017).

Table 3NeuAc and sialyllactose production costs (calculated in 30 m³ fermenter with 18 m³ fermentation broth).

Production and price	<i>De novo</i> synthesis of NeuAc	Whole-cell catalysis of NeuAc	<i>De novo</i> synthesis of 6'-sialyllactose
Current titer of NeuAc and sialyllactose (g/L)	8.3	127.6	30.0
Production of fermentation (kg)	132.9	204.1	540
Production after isolation and purification (kg)	61.0	89.0	235.4
Yield of isolation and purification	45.8%	43.6%	43.5%
Production costs (USD/kg)	121.9	140.0	46.9
Market price (USD/Kg)	774.2	774.2	Not available

3.6. Economic analysis of microbial SA and sialyllactose production

We use a 30 m³ fermenter as the calculation model, and the data source is the three articles with the highest NeuAc and sialyllactose titer of *de novo* synthesis and whole cell catalysis (Drouillard et al., 2010; Kao et al., 2018; Yang et al., 2017). Firstly, we assume that the volume of the fermentation is lossless during the treatment, at the same time, we introduce the product loss value to calibrate the total titer of the isolation and purification process. All raw material prices are from Alibaba (<http://www.1688.com/>, an international website for purchasing industrial raw materials), and electricity and labor costs are based on local averages. Other process price and loss values refer to the average of similar products in the local fermentation factory. Secondly, we divide the cost of entire industrial fermentation into medium, feeding solutions, PBS buffer, product isolation and purification, and others (Table 3, Supplementary table 1). Finally, whole cell transformation requires cell collection (10% w/v), therefore the cell volume is concentrated 10 folds.

The cost of *de novo* synthesis and whole cell catalysis of NeuAc are 121.9 and 140.0 USD/kg, respectively, and the current market price is about 774.2 USD/kg (Table 3). Based on the calculation the current market price, 8.3 g/L production for *de novo* synthesis and 127.6 g/L production for whole cell catalysis should be feasible to commercially produce SA. For *de novo* synthesis of NeuAc, lower yields result in higher costs, such as fermentation broth concentration, storage, and labor costs. For whole-cell catalysis, lower conversion rates, high substrate costs, and removal of residual GlcNAc are the main reasons for its high cost. The cost of *de novo* synthesis of sialyllactose is 46.9 USD/kg, but we did not find the market price of sialyllactose, which is probably not commercially available currently.

4. Challenges and perspectives of NeuAc microbial production

4.1. Identifying exporters of GlcNAc, ManNAc, and NeuAc

GlcNAc and ManNAc are intermediates in the synthesis of NeuAc, but the export mechanisms of GlcNAc and ManNAc have not yet been revealed. However, a large amount of intracellular GlcNAc and

ManNAc can be exported, which resulted in decreased a NeuAc titer and yield (Table 2). Although overexpression of the GlcNAc-specific phosphotransferase gene (*nagP*) can result in the import of extracellular GlcNAc and ManNAc into the cell to reduce purification costs, the importation requires energy consumption reactions (Table 2). In addition, inefficient NeuAc exportation may cause intracellular NeuAc accumulation; however, specific exporters of NeuAc have not been identified yet. Therefore, the export of NeuAc needs to be studied and regulated to fine-tune NeuAc biosynthesis and NeuAc exportation.

CRISPRi can be used for gene expression regulation based on a RNA-guided DNA recognition system and a catalytically dead Cas9 lacking endonuclease activity (dCas9). Furthermore, CRISPRi has been used to systematically repress the expressions of *B. subtilis* essential genes for unraveling interactions of essential gene regulations (Qi et al., 2013). Genes encoding exporters of GlcNAc, ManNAc, and NeuAc can be potentially identified based on systematically investigations of transporter functions via CRISPRi. For example, the construction of a sgRNA library that inhibits the expression of multiple genes can be used to screen all potential genes encoding transport systems and unknown functions from the *B. subtilis*. Cells containing the sgRNA library and NeuAc biosensor coupled with GFP were sorted according to the intracellular concentration of NeuAc by flow cytometry. Then the sgRNA library of the sorted cells that contained high concentrations of intracellular NeuAc was sequenced to analyze the NeuAc transport pathway.

4.2. Overcoming kinetic and thermodynamic bottlenecks in NeuAc biosynthetic pathway

AGE and NeuB are key enzymes in the *de novo* synthesis pathway of NeuAc (Tables 4 and 5). However, AGE catalyzes reversible reactions between GlcNAc and ManNAc, which has a higher affinity to ManNAc than GlcNAc. However, only the reaction towards the formation of ManNAc using GlcNAc as a substrate is desirable for NeuAc production. Therefore, insufficient substrate affinity and catalytic activity of AGE constrains NeuAc production, which led to a low conversion rate of NeuAc from GlcNAc. Although efforts have been made to optimize the kinetic properties of AGE, the ManNAc conversion rate using GlcNAc as a substrate is still lower than 60% (Chen et al., 2018). Therefore, the

Table 4Comparison of *N*-acetylglucosamine 2-epimerase (AGE) kinetics originating from different organisms.

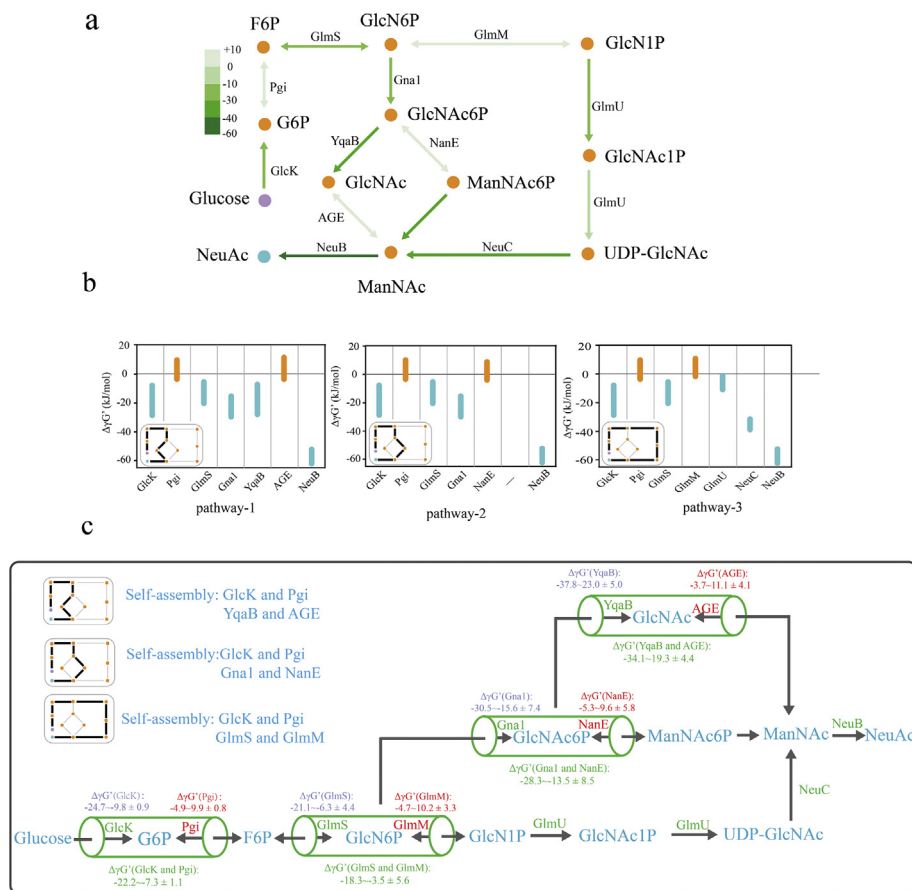
Source	GlcNAc			ManNAc			Reference
	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (M ⁻¹ s ⁻¹)	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (M ⁻¹ s ⁻¹)	
<i>Anabaena</i> sp. CH1	25.4	119	4×10^3	15.6	367	2×10^4	(Lee et al., 2007b)
<i>Bacteroides ovatus</i> ATCC 8483	31.9	128	4×10^3	75	117	2×10^3	(Sola-Carvajal et al., 2012)
<i>Synechocystis</i> sp. PCC6803	6.94	N.M	N.M	4.76	N.M	N.M	(Maru et al., 1996)
Porcine	7.4	N.M	N.M	6.3/13.7	19.5	1.4×10^3	(Maru et al., 1996; Takahashi et al., 2006)
Human	21.3	N.M	N.M	12.8/13.6	19.7	1.5×10^3	(Takahashi et al., 1983; Takahashi et al., 2006)
Rat	N.M	N.M	N.M	7.6	10.6	1.4×10^3	(Takahashi et al., 2006)
<i>Anabaena variabilis</i> ATCC 29413	16.3 ± 2.2	N.M	N.M	16.7 ± 2.2	N.M	N.M	(Klermund et al., 2013)
<i>Nostoc</i> sp. PCC 7120	24.6 ± 2.5	N.M	N.M	19.7 ± 2.7	N.M	N.M	(Klermund et al., 2013)
<i>Nostoc punctiforme</i> PCC 73102	16.6 ± 2.8	N.M	N.M	16.6 ± 1.8	N.M	N.M	(Klermund et al., 2013)

N.M:Not mentioned.

Table 5Comparison of *N*-acetylneuraminic acid synthase (NeuB) kinetics originating from different organisms.

Source	ManNAc			PEP			Reference
	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (M ⁻¹ s ⁻¹)	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (M ⁻¹ s ⁻¹)	
<i>E. coli</i> K1	5.6	N.M	N.M	4.0×10^{-2}	N.M	N.M	(Vann et al., 1997)
<i>Neisseria meningitidis</i> serogroup B	11.6/9.4	49.8	71.55	N.M	NM/0.25	N.M	(Gunawan et al., 2005; Hao et al., 2005)
<i>Neisseria meningitidis</i> Grop C	6.25	N.M	N.M	0.042	N.M	N.M	(Blacklow and Warren, 1962)
<i>Campylobacter jejuni</i> AJ000855	17.6	19.9	1.08	7.3	19	2.6	(Sundaram et al., 2004)
<i>Moritella viscosa</i>	18 ± 5	223	N.M	0.8 ± 0.2	225	N.M	(Berg et al., 2015)
<i>Aliivibrio salmonicida</i>	333	22.46	N.M	0.059	19.9	N.M	(Gurung et al., 2013)

N.M:Not mentioned.

**Fig. 2.** The Gibbs free energy ($\Delta G'$) of various NeuAc synthesis pathways. a) Heat-map representation of.

$\Delta G'$ in the three NeuAc synthesis pathways. b) The thermodynamic bottleneck ($\Delta G' > 0$) in three NeuAc synthesis pathways. c) Pathway enzyme self-assembly to construct the substrate channel. Self-assembly of GlcK and Pgi, YqaB and AGE, and GnaI and NanE. The $\Delta G'$ was calculated at pH 7.0 and ion strength of 0.1 M. Hypothetical concentrations: G6P, 8 mM; F6P, 10 mM; GlcN6P, 15 mM; GlcNAc6P, 20 mM; ManNAc6P, 20 mM; GlcNAc, 23 mM; ManNAc, 11 mM; PEP, 0.2 mM; NeuAc, 10 mM; GlcN1P, 15 mM; GlcNAc1P, 10 mM; and UDP-GlcNAc, 10 mM.

kinetic properties of AGE need to be engineered for strengthening the reaction efficiency towards ManNAc formation and weakening the reaction towards GlcNAc formation. In addition, based on thermodynamic calculations, the Gibbs free energy ($\Delta G'$) of the reaction for ManNAc formation with GlcNAc as the substrate was 1.9 ± 0.2 kJ/mol (calculated at pH 7.0 and an ion strength of 0.1 M; intracellular GlcNAc and ManNAc concentrations were 23 and 11 mM, respectively; <http://equilibrator.weizmann.ac.il/>), which created a thermodynamic bottleneck that adversely affected the forward reaction during NeuAc synthesis (Zhang et al., 2018). However, the investigation on debottlenecking thermodynamic constraints in the NeuAc synthesis pathway has not been carried out. Therefore, the kinetic and thermodynamic bottlenecks for AGE should be relieved by more efficient methods and strategies.

Phage-assisted continuous evolution (PACE) is an efficient method for high-throughput screens of mutant enzymes with desire properties

by coupling the phage growth rate to enzyme properties (Esvelt et al., 2011). This was performed with three helper plasmids, including the selection phage plasmid (SP) containing the target gene, the accessory plasmid (AP) containing the gene III (phage replication essential gene), and the mutagenesis plasmid (MP) containing DNAQ926 and the error-prone repair DNA polymerase pol V genes (DNA replication and error-prone systems) (Esvelt et al., 2011). The throughput of PACE reaches 2.5×10^9 mutants/round selection, which covers all the 9×10^6 theoretical possible double mutations of AGE in a 100 mL lagoon, providing an efficient approach for solving the AGE kinetics bottleneck. In addition, to diagnose and identify other bottlenecks in the synthetic pathway, kinetic modeling in combination with dynamic metabolomics can be used (Liu et al., 2016). At present, the kinetic model of the *B. subtilis* glycolytic pathway and GlcNAc synthetic pathway have been established, which provide an important basis for further construction of a NeuAc kinetic model (Buffing et al., 2018). Comparing the dynamic

metabolomics data with kinetic simulation with different kinetic bottlenecks may help to identify potential rate-limiting steps in the NeuAc synthetic pathway.

In general, AGE is a commonly used enzyme and pathway for the *de novo* synthesis of NeuAc (Yang et al., 2017; Zhang et al., 2018). To estimate the change Gibbs free energy ($\Delta G'$ value) in various condition with different concentrations of intracellular GlcNAc and ManNAc, the possible physiologically-relevant metabolite concentration range for both GlcNAc and ManNAc was set to be from 1 mM to 20 mM based GlcNAc concentration in GlcNAc-producing *B. subtilis* (Liu et al., 2016). However, $\Delta G'$ for the conversion of GlcNAc to ManNAc are ranging from 3.7 kJ/mol to 11.1 $\pm$ 4.1 kJ/mol by AGE (calculated at pH 7.0 and an ion strength of 0.1 M; intracellular GlcNAc and ManNAc concentrations were from 1 mM to 20 mM, respectively, <http://equilibrator.weizmann.ac.il/>), which adversely affected the forward reaction during NeuAc synthesis at the thermodynamic level. Furthermore, two strategies have been developed to address the thermodynamic constraints in metabolic pathways. The first strategy replaced the thermodynamically favorable metabolic pathway (Du et al., 2018; Nagai et al., 2018). Three pathways can be used to synthesize NeuAc from glucosamine-6P, through the key enzymes YqaB, NanE, and GlmM (Fig. 1d and 2a). However, there are thermodynamic bottlenecks in all of the alternative pathways, where the $\Delta G'$ of AGE, NanE, and GlmM were 3.7 kJ/mol to 11.1 $\pm$ 4.1 kJ/mol, -5.3 kJ/mol to 9.6 ± 5.8 kJ/mol, and -4.7 kJ/mol to 10.2 ± 3.3 kJ/mol, respectively. Therefore, the thermodynamic bottlenecks in the NeuAc synthesis pathway cannot be solved by changing the different synthetic pathway (Fig. 2b). In addition, another strategy is to construct a substrate channel by self-assembly of enzymes, where the self-assembly of multiple enzymes were constructed with protein–protein interaction (domain and ligand, such as GBD, SH3, and PDZ) (Dueber et al., 2009; Price et al., 2016). In the NeuAc synthesis pathway, the $\Delta G'$ for the conversion of GlcNAc-6P to GlcNAc was -37.8 kJ/mol to -23.0 ± 5.0 kJ/mol by YqaB enzyme (calculated at pH 7.0 and an ion strength of 0.1 M; intracellular GlcNAc-6P and GlcNAc concentrations were 1 mM to 20 mM, respectively). Under the same conditions, the $\Delta G'$ value that combines the two catalytic processes of YqaB and AGE was -34.1 kJ/mol to 19.3 ± 4.4 kJ/mol, which indicated a highly favorable reaction for the synthesis of NeuAc. Similarly, the $\Delta G'$ value that combined the two catalytic processes of GlcK and Pgi, GnaI and NanE, GlmS and GlmM were -22.2 kJ/mol to -7.3 ± 1.1 kJ/mol, -28.3 kJ/mol to -13.5 ± 8.5 kJ/mol, and -18.3 kJ/mol to -3.5 ± 5.6 kJ/mol, indicating that these enzymes can be used to solve the thermodynamic bottlenecks in the NeuAc pathway (Fig. 2c).

4.3. Fine-tuning NeuAc synthesis and cell growth

NeuAc synthesis needs several important shared precursors from central carbon and nitrogen metabolic pathways, including fructose-6-phosphate, acetyl-coenzyme A, glutamine, and PEP. The supply of multiple precursors and balance between NeuAc production and cell growth needs to be fine-tuned. Modular pathway engineering of the direct precursor GlcNAc and PEP supply, and optimization of pathway enzyme expression levels were implemented for static regulation of NeuAc synthesis (Kang et al., 2012; Zhang et al., 2018). However, accumulation of intermediates and reduced cell growth during NeuAc were observed, which suggested an imbalance of precursor supply as well as an imbalance between NeuAc synthesis and cell growth. Dynamic regulation of key gene expression via a synthetic metabolite-responsive biosensor has become an effective method to balance the metabolic pathway, which can potentially be used for NeuAc production (Green et al., 2017).

Dynamic regulation was developed as a strategy for real-time control of key gene expression levels or cellular functions responding to changes in intracellular or extracellular signal molecules, and is based

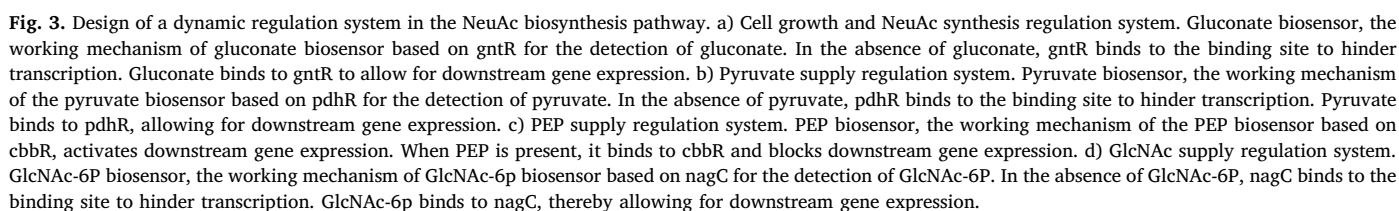
on the various signal molecule-responsive biosensors that respond to changes in host or environmental conditions (Farmer and Liao, 2000; Gupta et al., 2017; Xu et al., 2012; Xu et al., 2013; Zhang et al., 2012). The dynamic balance between precursor supply, and the balance between cell growth and NeuAc synthesis are key to improving the efficiency of NeuAc synthesis. Therefore, we propose the following strategies for systematic dynamic regulation. Dynamic control of the supply of multiple precursors requires multiple metabolite-responsive biosensors. Fortunately, transcriptional factors and corresponding binding site sequences of key precursor for GlcNAc-6P, PEP, and pyruvate have been identified, which can be used for GlcNAc-6P, PEP, and pyruvate biosensor construction (Le Bihan et al., 2017; Plumbridge, 2001; Plumbridge and Kolb, 1991). Moreover, the already developed NeuAc biosensor and gluconate biosensor can be used for dynamic control of the NeuAc pathway (Kalivoda et al., 2003; Peters et al., 2018). Cell growth and NeuAc biosynthesis can be decoupled using a gluconate biosensor, and when the gluconate was added exogenously during the logarithmic growth phase, the circuit was triggered, which led to downregulated gene expressions of *pyk* and *pfkA* through CRISPRi. The expression of *age* and *neuB* genes were activated for NeuAc biosynthesis at the same time. Therefore, the carbon flux of Embden-Meyerhof-Parnas (EMP) pathway and tricarboxylic acid cycle (TCA) were limited, and siphoned glucose and gluconate into the NeuAc pathway (Fig. 3a). In the pyruvate supply regulation system, the pyruvate biosensor was triggered by high concentrations of pyruvate, and led to the expression activation of phosphoenolpyruvate synthetase (*ppsA* gene) from *E. coli*, which can be used to catalyze pyruvate to PEP. Furthermore, the higher concentration of pyruvate could further activate sgRNA expression to downregulate *ptsG* gene expression through CRISPRi (Fig. 3b). In the PEP supply regulation system, the PEP biosensor was triggered by a high concentration of PEP, and led to downregulated gene expressions of *glcP* (a sugar transporter) and *glcK* (glucokinase). This could increase the efficiency of PTS system in the consumption of PEP, and the higher concentration of PEP could further contribute to the downregulation of *edd* and *eda* to limit PEP supply through the Entner–Doudoroff pathway (Fig. 3c). In the GlcNAc supply regulation system, the GlcNAc-6P biosensor was triggered by a high concentration of GlcNAc-6P, and led to activated expressions of *edd* and *eda* genes (two copies in the genome) to increase the supply of PEP, and the higher concentration of GlcNAc-6P could downregulate the gene expression of *glmS* to limit the supply of GlcNAc-6P (Fig. 3d). Overall, a synthetic pathway interaction response mechanism was constructed to achieve intelligent and dynamic regulation of NeuAc synthesis through a dynamic regulation system.

5. Concluding remarks

Significant advances in the biological production of SA and sialyllactate has been made and driven by metabolic engineering, protein engineering, and synthetic biology tools and strategies. Approaches for the efficient biosynthesis of sialyllactoses have been successfully developed with low-cost substrates; however, the biosynthesis NeuAc remains inefficient, and needs to be further investigated. The identification of key NeuAc precursor transport pathways, synergistically debottleneck of kinetic and thermodynamic limits in synthetic pathways, and dynamic regulation of metabolic pathways for balancing cell growth and production are promising methods for developing efficient NeuAc production processes.

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immobilization of AGE and NAL enzymes onto functional amino resin as recyclable and high-performance biocatalyst. *Bioprocess Biosyst. Eng.* 40, 331–340.

Cho, S., Lee, B.-R., Cho, B.-K., Kim, J.-H., Kim, B.-G., 2013. In vitro selection of sialic acid specific RNA aptamer and its application to the rapid sensing of sialic acid modified sugars. *Biotechnol. Bioeng.* 110, 905–913.

Choi, Y.H., Kim, J.H., Park, J.H., Lee, N., Kim, D.-H., Jang, K.-S., et al., 2014. Protein engineering of alpha 2,3/2,6-sialyltransferase to improve the yield and productivity of in vitro sialyllactose synthesis. *Glycobiology*. 24, 159–169.

Claumarchirant, L., Sanchez-Siles, L.M., Matencio, E., Alegría, A., Lagarda, M.J., 2016. Evaluation of sialic acid in infant feeding, contents and bioavailability. *J. Agric. Food Chem.* 64, 8333–8342.

Comb, D.G., Roseman, S., 1960. The sialic acids I. the structure and enzymatic synthesis of *N*-acetylneuraminic acid. *J. Biol. Chem.* 235, 2529–2537.

Difilippo, E., Willems, H.A.M., Vendrig, J.C., Fink-Gremmels, J., Gruppen, H., Schols, H.A., 2015. Comparison of milk oligosaccharides pattern in colostrum of different horse breeds. *J. Agric. Food Chem.* 63, 4805–4814.

Drouillard, S., Mine, T., Kajiwara, H., Yamamoto, T., Samain, E., 2010. Efficient synthesis of 6'-sialyllactose, 6,6'-disialyllactose, and 6'-KDO-lactose by metabolically engineered *E. coli* expressing a multifunctional sialyltransferase from the *Photobacterium* sp JT-ISH-224. *Carbohydr. Res.* 345, 1394–1399.

Du, B., Zielinski, D.C., Monk, J.M., Palsson, B.O., 2018. Thermodynamic favorability and pathway yield change as evolutionary tradeoffs in biosynthetic pathway choice. *Proc. Natl. Acad. Sci.* 115, 11339–11344.

Dueber, J.E., Wu, G.C., Malmirchegini, G.R., Moon, T.S., Petzold, C.J., Ullal, A.V., et al., 2009. Synthetic protein scaffolds provide modular control over metabolic flux. *Nat. Biotechnol.* 27, 753.

Egging, L., Bott, M., Marienhagen, J., 2015. Novel screening methods—biosensors. *Curr. Opin. Biotechnol.* 35, 30–36.

Endo, T., Koizumi, S., Tabata, K., Ozaki, A., 2000. Large-scale production of CMP-NeuAc and sialylated oligosaccharides through bacterial coupling. *Appl. Microbiol. Biotechnol.* 53, 257–261.

Esvelt, K.M., Carlson, J.C., Liu, D.R., 2011. A system for the continuous directed evolution of biomolecules. *Nature*. 472, 499.

Farmer, W.R., Liao, J.C., 2000. Improving lycopene production in *Escherichia coli* by engineering metabolic control. *Nat. Biotechnol.* 18, 533.

FDA, 2015. GRAS Exemption Claim for *N*-Acetyl-D-Neuraminic Acid (NANA).

Fierfort, N., Samain, E., 2008. Genetic engineering of *Escherichia coli* for the economical production of sialylated oligosaccharides. *J. Biotechnol.* 134, 261–265.

Gao, C., Xu, X., Zhang, X., Che, B., Ma, C., Qiu, J., et al., 2011. Chemoenzymatic synthesis of *N*-acetyl-D-neuraminic acid from *N*-acetyl-D-glucosamine by using the spore surface-displayed *N*-acetyl-D-neuraminic acid aldolase. *Appl. Environ. Microbiol.* 77, 7080–7083.

Ghosh, S., Roseman, S., 1965. The sialic acids V. *N*-acetyl-D-glucosamine 2-epimerase. *J. Biol. Chem.* 240, 1531–1536.

Gnoth, M.J., Kunz, C., Kinne-Saffran, E., Rudloff, S., 2000. Human milk oligosaccharides are minimally digested in vitro. *J. Nutr.* 130, 3014–3020.

Green, A.A., Kim, J., Ma, D., Silver, P.A., Collins, J.J., Yin, P., 2017. Complex cellular logic computation using ribocomputing devices. *Nature*. 548, 117.

Gunawan, J., Simard, D., Gilbert, M., Lovering, A.L., Wakarchuk, W.W., Tanner, M.E.,

- et al., 2005. Structural and mechanistic analysis of sialic acid synthase NeuB from *Neisseria meningitidis* in complex with Mn^{2+} , phosphoenolpyruvate, and N-acetylmannosaminol. J. Biol. Chem. 280, 3555–3563.
- Günther, B., Guido, M., 2008. Structural and functional aspects of prebiotics used in infant nutrition. J. Nutr. 138, 1818S.
- Guo, Y., Jers, C., Meyer, A.S., Arnous, A., Li, H., Kirpekar, F., et al., 2014. A Pasteurella multocida sialyltransferase displaying dual trans-sialidase activities for production of 3'-sialyl and 6'-sialyl glycans. J. Biotechnol. 170, 60–67.
- Gupta, A., Reizman, I.M.B., Reisch, C.R., Prather, K.L.J., 2017. Dynamic regulation of metabolic flux in engineered bacteria using a pathway-independent quorum-sensing circuit. Nat. Biotechnol. 35, 273.
- Gurung, M.K., Ræder, I.L.U., Altermark, B., Smalås, A.O., 2013. Characterization of the sialic acid synthase from *Aliivibrio salmonicida* suggests a novel pathway for bacterial synthesis of 7-O-acetylated sialic acids. Glycobiology. 23, 806–819.
- Han, N.S., Kim, T.-J., Park, Y.-C., Kim, J., Seo, J.-H., 2012. Biotechnological production of human milk oligosaccharides. Biotechnol. Adv. 30, 1268–1278.
- Hao, J., Balagurusamy, P., Sarilla, S., Sundaramoorthy, M., 2005. Cloning, expression, and characterization of sialic acid synthases. Biochem. Biophys. Res. Commun. 338, 1507–1514.
- Hassan, M.I., Lundgren, B.R., Chaumun, M., Whitfield, D.M., Clark, B., Schoenhofen, I.C., et al., 2016. Total biosynthesis of legionaminic acid, a bacterial sialic acid analogue. Angew. Chem. Int. Ed. 55, 12018–12021.
- Heckman, K.L., Pease, L.R., 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. Nat. Protoc. 2, 924.
- Hester, S.N., Chen, X., Li, M., Monaco, M.H., Comstock, S.S., Kuhlenschmidt, T.B., et al., 2013. Human milk oligosaccharides inhibit rotavirus infectivity in vitro and in acutely infected piglets. Br. J. Nutr. 110, 1233–1242.
- Hu, S., Chen, J., Yang, Z., Shao, L., Bai, H., Luo, J., et al., 2010. Coupled bioconversion for preparation of N-acetyl-D-neuraminic acid using immobilized N-acetyl-D-glucosamine-2-epimerase and N-acetyl-D-neuraminic acid lyase. Appl. Microbiol. Biotechnol. 85, 1383–1391.
- Imanparast, S., Hamed, J., Faramarzi, M.A., 2018. Enzymatic esterification of acylglycerols rich in omega-3 from flaxseed oil by an immobilized solvent-tolerant lipase from *Actinomyces sediminis* UTM 2870 isolated from oil-contaminated soil. Food Chem. 245, 934–942.
- Ishikawa, M., Koizumi, S., 2010. Microbial production of N-acetylneuraminic acid by genetically engineered *Escherichia coli*. Carbohydr. Res. 345, 2605–2609.
- Jefferson, T., Jones, M.A., Doshi, P., Del Mar, C.B., Hama, R., Thompson, M., et al., 2014. Neuraminidase inhibitors for preventing and treating influenza in healthy adults and children. Sao Paulo Med. J. 132, 256–257.
- Johnson, A.O., Gonzalez-Villanueva, M., Wong, L., Steinbuechel, A., Tee, K.L., Xu, P., et al., 2017. Design and application of genetically-encoded malonyl-CoA biosensors for metabolic engineering of microbial cell factories. Metab. Eng. 44, 253–264.
- Juneja, L.R., Koketsu, M., Nishimoto, K., Kim, M., Yamamoto, T., Itoh, T., 1991. Large-scale preparation of sialic acid from chalaza and egg-yolk membrane. Carbohydr. Res. 214, 179–186.
- Kajihara, Y., Yamamoto, T., Nagae, H., Nakashizuka, M., Sakakibara, T., Terada, I., 1996. A novel α -2, 6-sialyltransferase, transfer of sialic acid to fucosyl and sialyl trisaccharides. J. Organomet. Chem. 61, 8632–8635.
- Kalivoda, K.A., Steenbergen, S.M., Vimr, E.R., Plumbbridge, J., 2003. Regulation of sialic acid catabolism by the DNA binding protein NanR in *Escherichia coli*. J. Bacteriol. 185, 4806–4815.
- Kang, J., Gu, P., Wang, Y., Li, Y., Yang, F., Wang, Q., et al., 2012. Engineering of an N-acetylneuraminic acid synthetic pathway in *Escherichia coli*. Metab. Eng. 14, 623–629.
- Kao, C.-H., Chen, Y.-Y., Wang, L.-R., Lee, Y.-C., 2018. Production of N-acetyl-D-neuraminic acid by recombinant single whole cells co-expressing N-acetyl-D-glucosamine-2-epimerase and N-acetyl-D-neuraminic acid aldolase. Mol. Biotechnol. 60, 427–434.
- Kelm, S., Schauer, R., 1997. Sialic acids in molecular and cellular interactions. Int. Rev. Cytology-a Survey Cell Biol. 175, 137–240.
- Kijima-Suda, I., Miyamoto, Y., Toyoshima, S., Itoh, M., Osawa, T., 1986. Inhibition of experimental pulmonary metastasis of mouse colon adenocarcinoma 26 sublines by a sialic acid, nucleoside conjugate having sialyltransferase inhibiting activity. Cancer Res. 46, 858–862.
- Kim, D., Gurung, R.B., Seo, W., Lee, A.W., Woo, J., 2018. Toxicological evaluation of 3'-sialyllactose sodium salt. Regulat. Toxicol. Pharmacol. RTP 94, 83–90.
- Klein, D.J., Ferré-D'Amaré, A.R., 2006. Structural basis of glmS ribozyme activation by glucosamine-6-phosphate. Science. 313, 1752–1756.
- Klermund, L., Groher, A., Castiglione, K., 2013. New N-acyl-D-glucosamine 2-epimerases from cyanobacteria with high activity in the absence of ATP and low inhibition by pyruvate. J. Biotechnol. 168, 256–263.
- Kuntz, S., Rudloff, S., Kunz, C., 2008. Oligosaccharides from human milk influence growth-related characteristics of intestinally transformed and non-transformed intestinal cells. Br. J. Nutr. 99, 462–471.
- Le Bihan, G., Sicard, J.-F., Garneau, P., Bernalier-Donadille, A., Gobert, A.P., Garrivier, A., et al., 2017. The NAG sensor NagC regulates LEE gene expression and contributes to gut colonization by *Escherichia coli* O157: H7. Front. Cell. Infect. Microbiol. 7, 134.
- Lee, J.-O., Yi, J.-K., Lee, S.-G., Takahashi, S., Kim, B.-G., 2004. Production of N-acetylneuraminic acid from N-acetylglucosamine and pyruvate using recombinant human renin binding protein and sialic acid aldolase in one pot. Enzym. Microb. Technol. 35, 121–125.
- Lee, Y.-C., Chien, H.-C.R., Hsu, W.-H., 2007a. Production of N-acetyl-D-neuraminic acid by recombinant whole cells expressing *Anabaena* sp. CH1 N-acetyl-D-glucosamine 2-epimerase and *Escherichia coli* N-acetyl-D-neuraminic acid lyase. J. Biotechnol. 129, 453–460.
- Lee, Y.-C., Wu, H.-M., Chang, Y.-N., Wang, W.-C., Hsu, W.-H., 2007b. The central cavity from the (alpha/alpha) 6 barrel structure of *Anabaena* sp. CH1 N-acetyl-D-glucosamine 2-epimerase contains two key histidine residues for reversible conversion. J. Mol. Biol. 367, 895–908.
- Lin, B.-X., Zhang, Z.-J., Liu, W.-F., Dong, Z.-Y., Tao, Y., 2013. Enhanced production of N-acetyl-D-neuraminic acid by multi-approach whole-cell biocatalyst. Appl. Microbiol. Biotechnol. 97, 4775–4784.
- Lin, J.-L., Wagner, J.M., Alper, H.S., 2017. Enabling tools for high-throughput detection of metabolites, metabolic engineering and directed evolution applications. Biotechnol. Adv. 35, 950–970.
- Liu, Y., Liu, L., Shin, H.-d., Chen, R.R., Li, J., Du, G., et al., 2013. Pathway engineering of *Bacillus subtilis* for microbial production of N-acetylglucosamine. Metab. Eng. 19, 107–115.
- Liu, Y., Zhu, Y., Li, J., Shin, H.-d., Chen, R.R., Du, G., et al., 2014a. Modular pathway engineering of *Bacillus subtilis* for improved N-acetylglucosamine production. Metab. Eng. 23, 42–52.
- Liu, Y., Zhu, Y., Ma, W., Shin, H.-d., Li, J., Liu, L., et al., 2014b. Spatial modulation of key pathway enzymes by DNA-guided scaffold system and respiration chain engineering for improved N-acetylglucosamine production by *Bacillus subtilis*. Metab. Eng. 24, 61–69.
- Liu, Y., Link, H., Liu, L., Du, G., Chen, J., Sauer, U., 2016. A dynamic pathway analysis approach reveals a limiting futile cycle in N-acetylglucosamine overproducing *Bacillus subtilis*. Nat. Commun. 7, 11933.
- Liu, Y., Li, J., Du, G., Chen, J., Liu, L., 2017. Metabolic engineering of *Bacillus subtilis* fueled by systems biology, recent advances and future directions. Biotechnol. Adv. 35, 20–30.
- Lundgren, B.R., Boddy, C.N., 2007. Sialic acid and N-acyl sialic acid analog production by fermentation of metabolically and genetically engineered *Escherichia coli*. Org. Biomol. Chem. 5, 1903–1909.
- Luo, J., Nordvang, R.T., Morthensen, S.T., Zeuner, B., Meyer, A.S., Mikkelsen, J.D., et al., 2014. An integrated membrane system for the biocatalytic production of 3'-sialyllactose from dairy by-products. Bioresour. Technol. 166, 9–16.
- Magano, J., 2009. Synthetic approaches to the neuraminidase inhibitors zanamivir (Relenza) and oseltamivir phosphate (Tamiflu) for the treatment of influenza. Chem. Rev. 109, 4398–4438.
- Marotta, M., Ryan, J.T., Hickey, R.M., 2014. The predominant milk oligosaccharide 6'-sialyllactose reduces the internalisation of *Pseudomonas aeruginosa* in human pneumocytes. J. Funct. Foods 6, 367–373.
- Martin-Sosa, S., Martin, M.-J., García-Pardo, L.-A., Hueso, P., 2003. Sialyloligosaccharides in human and bovine milk and in infant formulas, variations with the progression of lactation. J. Dairy Sci. 86, 52–59.
- Maru, I., Ohta, Y., Murata, K., Tsukada, Y., 1996. Molecular cloning and identification of N-acyl-D-glucosamine 2-epimerase from porcine kidney as a renin-binding protein. J. Biol. Chem. 271, 16294–16299.
- Maru, I., Ohnishi, J., Ohta, Y., Tsukada, Y., 1998. Simple and large-scale production of N-acetylneuraminic acid from N-acetyl-D-glucosamine and pyruvate using N-acyl-D-glucosamine 2-epimerase and N-acetylneuraminic acid lyase. Carbohydr. Res. 306, 575–578.
- Maru, I., Ohnishi, J., Ohta, Y., Tsukada, Y., 2002. Why is sialic acid attracting interest now? Complete enzymatic synthesis of sialic acid with N-acylglucosamine 2-epimerase. J. Biosci. Bioeng. 93, 258–265.
- McNicholl, I.R., McNicholl, J.J., 2001. Neuraminidase inhibitors, zanamivir and oseltamivir. Ann. Pharmacother. 35, 57–70.
- Michalak, M., Larsen, D.M., Jers, C., Almeida, J.R.M., Willer, M., Li, H., et al., 2014. Biocatalytic production of 3'-sialyllactose by use of a modified sialidase with superior trans-sialidase activity. Process Biochem. 49, 265–270.
- Nagai, H., Masuda, A., Toya, Y., Matsuda, F., Shimizu, H., 2018. Metabolic engineering of mevalonate-producing *Escherichia coli* strains based on thermodynamic analysis. Metab. Eng. 47, 1–9.
- Nordvang, R.T., Luo, J., Zeuner, B., Prior, R., Andersen, M.F., Mikkelsen, J.D., et al., 2014. Separation of 3'-sialyllactose and lactose by nanofiltration, a trade-off between charge repulsion and pore swelling induced by high pH. Sep. Purif. Technol. 138, 77–83.
- Perdijk, O., van Baarlen, P., Fernandez-Gutierrez, M.M., van den Brink, E., Schuren, F.H.J., Brugman, S., et al., 2019. Sialyllactose and Galactooligosaccharides promote epithelial barrier functioning and distinctly modulate microbiota composition and short chain fatty acid production in vitro. Front. Immunol. 10.
- Peters, G., de Paepe, B., de Wannemaeker, L., Duchi, D., Maertens, J., Lammertyn, J., et al., 2018. Development of N-acetylneuraminic acid responsive biosensors based on the transcriptional regulator NanR. Biotechnol. Bioeng. 115, 1855–1865.
- Phillips, M.L., Nudelman, E., Gaeta, F.C., Perez, M., Singhal, A.K., Hakomori, S.-i., et al., 1990. ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Lex. Science. 250, 1130–1132.
- Plumbbridge, J., 2001. DNA binding sites for the Mlc and NagC proteins, regulation of nagE, encoding the N-acetylglucosamine-specific transporter in *Escherichia coli*. Nucleic Acids Res. 29, 506–514.
- Plumbbridge, J., Kolb, A., 1991. CAP and nag repressor binding to the regulatory regions of the nagE and manX genes of *Escherichia coli*. J. Mol. Biol. 217, 661–679.
- Price, J.V., Chen, L., Whitaker, W.B., Papoutsakis, E., Chen, W., 2016. Scaffoldless engineered enzyme assembly for enhanced methanol utilization. Proc. Natl. Acad. Sci. 113, 12691–12696.
- Priem, B., Gilbert, M., Wakarchuk, W.W., Heyraud, A., Samain, E., 2002. A new fermentation process allows large-scale production of human milk oligosaccharides by metabolically engineered bacteria. Glycobiology. 12, 235–240.
- Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., et al., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell. 152, 1173–1183.

- Rogers, J.K., Taylor, N.D., Church, G.M., 2016. Biosensor-based engineering of biosynthetic pathways. *Curr. Opin. Biotechnol.* 42, 84–91.
- Sakaguchi, Y., Hayama, T., Yoshida, H., Itoyama, M., Todoroki, K., Yamaguchi, M., et al., 2014. Liquid chromatography/tandem mass spectrometry with fluoros derivatization method for selective analysis of sialyl oligosaccharides. *Rapid Commun. Mass Spectrom.* 28, 2481–2489.
- Salcedo, J., Barbera, R., Matencio, E., Alegría, A., Lagarda, M.J., 2013. Gangliosides and sialic acid effects upon newborn pathogenic bacteria adhesion, an *in vitro* study. *Food Chem.* 136, 726–734.
- Salcedo, J., Gormaz, M., López-Mendoza, M.C., Nogarroto, E., Silvestre, D., 2015. Human milk bactericidal properties, effect of lyophilization and relation to maternal factors and milk components. *J. Pediatr. Gastroenterol. Nutr.* 60, 527–532.
- Schallmeyer, M., Frunzke, J., Eggeling, L., Marienhagen, J., 2014. Looking for the pick of the bunch, high-throughput screening of producing microorganisms with biosensors. *Curr. Opin. Biotechnol.* 26, 148–154.
- Schauer, R., 1997. Chemistry, biochemistry and biology of sialic acids. *Glycoproteins II* 29, 243–402.
- Schauer, R., 2000. Achievements and challenges of sialic acid research. *Glycoconj. J.* 17, 485–499.
- Schauer, R., 2004. Sialic acids, fascinating sugars in higher animals and man. *Zoology.* 107, 49–64.
- Seppo, A.E., Autran, C.A., Bode, L., Järvinen, K.M., 2017. Human milk oligosaccharides and development of cow's milk allergy in infants. *J. Allergy Clin. Immunol.* 139, 708–711 (e5).
- Severi, E., Hood, D.W., Thomas, G.H., 2007. Sialic acid utilization by bacterial pathogens. *Microbiology.* 153, 2817–2822.
- Shie, J.-J., Fang, J.-M., Lai, P.-T., Wen, W.-H., Wang, S.-Y., Cheng, Y.-S.E., et al., 2011. A practical synthesis of zanamivir phosphonate congeners with potent anti-influenza activity. *J. Am. Chem. Soc.* 133, 17959–17965.
- Sola-Carvajal, A., Sánchez-Carrón, G., García-García, M.I., García-Carmona, F., Sánchez-Ferrer, A., 2012. Properties of BoAGE2, a second N-acetyl-D-glucosamine 2-epimerase from *Bacteroides ovatus* ATCC 8483. *Biochimie.* 94, 222–230.
- Soundararajan, V., Tharakaraman, K., Raman, R., Raguram, S., Shriver, Z., Sasisekharan, V., et al., 2009. Extrapolating from sequence—the 2009 H1N1 swine influenza virus. *Nat. Biotechnol.* 27, 510.
- Sugiarto, G., Lau, K., Li, Y., Khedri, Z., Yu, H., Le, D.-T., et al., 2011. Decreasing the sialidase activity of multifunctional *Pasteurella multocida* α 2–3-sialyltransferase 1 (PmST1) by site-directed mutagenesis. *Mol. Biosyst.* 7, 3021–3027.
- Sun, M., Li, Y., Chokhawala, H.A., Henning, R., Chen, X., 2008. N-terminal 112 amino acid residues are not required for the sialyltransferase activity of *Photobacterium damsela* α 2, 6-sialyltransferase. *Biotechnol. Lett.* 30, 671–676.
- Sun, W., Ji, W., Li, N., Tong, P., Cheng, J., He, Y., et al., 2013. Construction and expression of a polycistronic plasmid encoding N-acetylglucosamine 2-epimerase and N-acetylneuraminic acid lyase simultaneously for production of N-acetylneuraminic acid. *Bioresour. Technol.* 130, 23–29.
- Sundaram, A.K., Pitts, L., Muhammad, K., Jing, W.U., Betenbaugh, M., Woodard, R.W., 2004. Characterization of N-acetylneuraminic acid synthase isoenzyme 1 from *Campylobacter jejuni*. *Biochem. J.* 383, 83–89.
- Tabata, K., Koizumi, S., Endo, T., Ozaki, A., 2002. Production of N-acetyl-D-neuraminic acid by coupling bacteria expressing N-acetyl-D-glucosamine 2-epimerase and N-acetyl-D-neuraminic acid synthetase. *Enzym. Microb. Technol.* 30, 327–333.
- Takahashi, S., OHSAWA, T., MIURA, R., MIYAKE, Y., 1983. Purification of high molecular weight (11MW) renin from porcine kidney and direct evidence that the HMW renin is a complex of renin with renin binding protein (RnBP). *J. Biochem.* 93, 265–274.
- Takahashi, S., Hori, K., Ogasawara, H., Hiwatashi, K., Sugiyama, T., 2006. Effects of nucleotides on the interaction of renin with GlcNAc 2-epimerase (renin binding protein, RnBP). *J. Biochem.* 140, 725–730.
- Tao, F., Zhang, Y., Ma, C., Xu, P., 2010. Biotechnological production and applications of N-acetyl-D-neuraminic acid, current state and perspectives. *Appl. Microbiol. Biotechnol.* 87, 1281–1289.
- Tao, F., Zhang, Y., Ma, C., Xu, P., 2011. One-pot bio-synthesis, N-acetyl-D-neuraminic acid production by a powerful engineered whole-cell catalyst. *Sci. Rep.* 1, 142.
- Tarr, A.J., Galley, J.D., Fisher, S.E., Chichlowski, M., Berg, B.M., Bailey, M.T., 2015. The prebiotics 3'Sialyllactose and 6'Sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations, evidence for effects on the gut-brain axis. *Brain Behav. Immun.* 50, 166–177.
- Taufik, E., Sekii, N., Senda, A., Fukuda, K., Saito, T., Eisert, R., et al., 2013. Neutral and acidic milk oligosaccharides of the striped skunk (Mephitidae, Mephitis mephitis). *Anim. Sci. J.* 84, 569–578.
- Ten, Bruggencate S.J.M., Bovee-Oudenhoven, I.M.J., Feitsma, A.L., van Hoffen, E., Schoterman, M.H.C., 2014. Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. *Nutr. Rev.* 72, 377–389.
- Traving, C., Schauer, R., 1998. Structure, function and metabolism of sialic acids. *Cell. Mol. Life Sci.* 54, 1330–1349.
- Turck, D., Bresson, J.-L., Burlingame, B., Dean, T., Fairweather-Tait, S., Heinonen, M., et al., 2017. Safety of Synthetic N-Acetyl-D-Neuraminic Acid as a Novel Food Pursuant to Regulation (EC) No 258/97.
- Urashima, T., Saito, T., Nakamura, T., Messer, M., 2001. Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconj. J.* 18, 357–371.
- van Karnebeek, C.D.M., Bonafé, L., Wen, X.-Y., Tarailo-Graovac, M., Balzano, S., Royer-Bertrand, B., et al., 2016. NANS-mediated synthesis of sialic acid is required for brain and skeletal development. *Nat. Genet.* 48, 777.
- Vann, W.F., Tavarez, J.J., Crowley, J., Vimr, E., Silver, R.P., 1997. Purification and characterization of the *Escherichia coli* k12 neu B gene product N-acetylneuraminic acid synthetase. *Glycobiology.* 7, 697–701.
- Varki, N.M., Varki, A., 2007. Diversity in cell surface sialic acid presentations, implications for biology and disease. *Lab. Invest.* 87, 851.
- von Itzstein, M., Wu, W.-Y., Kok, G.B., Pegg, M.S., Dyason, J.C., Jin, B., et al., 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature.* 363, 418.
- Wang, B., 2009. Sialic acid is an essential nutrient for brain development and cognition. *Annu. Rev. Nutr.* 29, 177–222.
- Wang, B., Brand-Miller, J., 2003. The role and potential of sialic acid in human nutrition. *Eur. J. Clin. Nutr.* 57, 1351.
- Wang, Z., Zhuang, W., Cheng, J., Sun, W., Wu, J., Chen, Y., et al., 2017. In vivo multi-enzyme complex coconstruction of N-acetylneuraminic acid lyase and N-acetylglucosamine-2-epimerase for biosynthesis of N-acetylneuraminic acid. *J. Agric. Food Chem.* 65, 7467–7475.
- Wu, S., Grimm, R., German, J.B., Lebrilla, C.B., 2011. Annotation and structural analysis of sialylated human milk oligosaccharides. *J. Proteome Res.* 10, 856–868.
- Xiu, Y., Jang, S., Jones, J.A., Zill, N.A., Linhardt, R.J., Yuan, Q., et al., 2017. Naringenin-responsive riboswitch-based fluorescent biosensor module for *Escherichia coli* co-cultures. *Biotechnol. Bioeng.* 114, 2235–2244.
- Xu, P., 2017. Production of chemicals using dynamic control of metabolic fluxes. *Curr. Opin. Biotechnol.* 53, 12–19.
- Xu, P., Qiu, J.H., Zhang, Y.N., Chen, J., Wang, P.G., Yan, B., et al., 2007. Efficient whole-cell biocatalytic synthesis of n-acetyl-d-neuraminic acid. *Adv. Synth. Catal.* 349, 1614–1618.
- Xu, P., Vansiri, A., Bhan, N., Koffas, M.A., 2012. ePathBrick, a synthetic biology platform for engineering metabolic pathways in *E. coli*. *ACS Synth. Biol.* 1, 256.
- Xu, P., Gu, Q., Wang, W., Wong, L., Bower, A.G.W., Collins, C.H., et al., 2013. Modular optimization of multi-gene pathways for fatty acids production in *E. coli*. *Nat. Commun.* 4, 1409.
- Xu, P., Li, L., Zhang, F., Stephanopoulos, G., Koffas, M., 2014. Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. *Proc. Natl. Acad. Sci. U. S. A.* 111, 11299.
- Yan, Q., Fong, S.S., 2018. Design and modularized optimization of one-step production of N-acetylneuraminic acid from chitin in *Serratia marcescens*. *Biotechnol. Bioeng.* 115, 2255–2267.
- Yang, M., Cheung, S.-H., Li, S.C., Cheung, H.-Y., 2014. Establishment of a holistic and scientific protocol for the authentication and quality assurance of edible bird's nest. *Food Chem.* 151, 271–278.
- Yang, P., Wang, J., Pang, Q., Zhang, F., Wang, J., Wang, Q., et al., 2017. Pathway optimization and key enzyme evolution of N-acetylneuraminic acid biosynthesis using an *in vivo* aptazyme-based biosensor. *Metab. Eng.* 43, 21–28.
- Yu, H., Chokhawala, H., Karpel, R., Yu, H., Wu, B., Zhang, J., et al., 2005. A multi-functional *Pasteurella multocida* sialyltransferase, a powerful tool for the synthesis of sialoside libraries. *J. Am. Chem. Soc.* 127, 17618–17619.
- Zeuner, B., Luo, J., Nyffenegger, C., Aumala, V., Mikkelsen, J.D., Meyer, A.S., 2014. Optimizing the biocatalytic productivity of an engineered sialidase from *Trypanosoma rangeli* for 3'-sialyllactose production. *Enzym. Microb. Technol.* 55, 85–93.
- Zhang, Y., Tao, F., Du, M., Ma, C., Qiu, J., Gu, L., et al., 2010. An efficient method for N-acetyl-D-neuraminic acid production using coupled bacterial cells with a safe temperature-induced system. *Appl. Microbiol. Biotechnol.* 86, 481–489.
- Zhang, F., Carothers, J.M., Keasling, J.D., 2012. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat. Biotechnol.* 30, 354.
- Zhang, X., Liu, Y., Liu, L., Wang, M., Li, J., Du, G., et al., 2018. Modular pathway engineering of key carbon-precursor supply-pathways for improved N-acetylneuraminic acid production in *Bacillus subtilis*. *Biotechnol. Bioeng.* 115, 2217–2231.
- Zhou, J., Chen, X., Lu, L., Govender, A., Yang, H., Shen, W., 2016. Enhanced production of N-acetyl-D-neuraminic acid by whole-cell bio-catalysis of *Escherichia coli*. *J. Mol. Catal. B Enzym.* 125, 42–48.
- Zhu, D., Wu, J., Zhan, X., Zhu, L., Zheng, Z., Gao, M., 2017a. Phosphoenolpyruvate-supply module in *Escherichia coli* improves N-acetyl-D-neuraminic acid biocatalysis. *Biotechnol. Lett.* 39, 227–234.
- Zhu, D., Zhan, X., Wu, J., Gao, M., Zhao, Z., 2017b. Efficient whole-cell biocatalyst for Neu5Ac production by manipulating synthetic, degradation and transmembrane pathways. *Biotechnol. Lett.* 39, 55–63.
- Zimmermann, V., Hennemann, H.-G., Daußmann, T., Kragl, U., 2007. Modelling the reaction course of N-acetylneuraminic acid synthesis from N-acetyl-D-glucosamine—new strategies for the optimisation of neuraminic acid synthesis. *Appl. Microbiol. Biotechnol.* 76, 597–605.

Supplementary Files

Microbial production of sialic acid and sialylated human milk oligosaccharides: advances and perspectives

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Supplementary table 1 NeuAc and sialyllactose production costs (calculated in 30 m³ fermenter with 18 m³ fermentation broth)^a

Medium	Price ^b (USD)	<i>De novo</i> synthesis of NeuAc		Whole-cell catalysis of NeuAc		<i>De novo</i> synthesis of 6'-sialyllactose	
		Dosage (kg)	Cost (USD)	Dosage (kg)	Cost (USD)	Dosage (kg)	Cost (USD)
Glucose	595.4	1080	643.0	1080	643.0	0	0
Soybean dregs	669.8	900	602.8	900	602.8	900	602.8
Corn syrup	446.5	216	96.5	216	96.5	216	96.5
CaCl ₂	297.7	16.2	4.8	16.2	4.8	16.2	4.8
MgSO ₄ ·7H ₂ O	223.3	27	6.1	27	6.1	27	6.1
(NH ₄) ₂ SO ₄	297.7	108	32.2	108	32.2	108	32.2
Defoamer	8186.7	9	74.4	9	74.4	9	74.4
K ₂ HPO ₄	1190.8	216	257.2	216	257.2	216	257.2
KH ₂ PO ₄	1190.8	45	53.6	45	53.6	45	53.6
Glycerol	1190.8	0	0	0	0	315	375.1
Lactose	1339.6	0	0	0	0	90	120.6
Total medium costs (USD)		1770.6		1770.6		1623.2	
Feeding solutions	Price (USD)	<i>De novo</i> synthesis of NeuAc		Whole-cell catalysis of NeuAc		<i>De novo</i> synthesis of 6'-sialyllactose	
		Dosage (kg)	Cost (USD)	Dosage (kg)	Cost (USD)	Dosage (kg)	Cost (USD)
Glucose	595.4	2160	1286.1	0	0	0	0
Pyruvate	11908.0	0	0	253.6	3020.0	0	0
GlcNAc	7442.5	0	0	477.8	3556.0	0	0
Glycerol	1190.8	0	0	0	0	1620	1929.1
Lactose	1339.6	0	0	0	0	270	361.7
Total feeding solution costs (USD)		1286.1		6576.0		2290.8	
PBS buffer (0.01M, pH=7.4)	Price (USD)	<i>De novo</i> synthesis of NeuAc		Whole-cell catalysis of NeuAc		<i>De novo</i> synthesis of 6'-sialyllactose	
		Dosage (kg)	Cost (USD)	Dosage (kg)	cost (USD)	Dosage (kg)	Cost (USD)
NaCl	342.4	0	0	14.4	4.9	0	0
KCl	1488.5	0	0	0.36	0.5	0	0
KH ₂ PO ₄	774.0	0	0	0.36	0.3	0	0

Na ₂ HPO ₄	1161.0	0		0		5.22		6.1		0		0	
Total PBS buffer costs (USD)		0				11.8				0			
Product isolation and purification	Price (USD)	De novo synthesis of NeuAc				Whole-cell catalysis of NeuAc				De novo synthesis of 6'-sialyllactose			
		Dosage	Cost (USD)	Volume (t)	Yield (kg)	Dosage	Cost (USD)	Volume (t)	Yield (kg)	Dosage	Cost (USD)	Volume (t)	Yield (kg)
Remove bacteria (10000 rpm)	0.9 /h	8	7.2	16	132.9	1	0.9	1.6	204.1	8	7.2	17	510
Remove bacteria by ceramic membrane 50 nm (5% loss)	4.5 /t	16	72.0	16	126.2	1.6	7.2	1.6	193.8	17	76.5	17	484.5
Remove Protein by membrane 3000 Da (5% loss)	6.0 /t	16	96.0	16	119.8	1.6	9.6	1.6	184.1	17	102.0	17	460.2
Desalting by reverse osmosis membrane (5% loss)	4.5 /t	16	72.0	16	113.8	1.6	7.2	1.6	174.9	17	76.5	17	437.1
Desalting by cation exchange resin D001 (10% loss)	3.0 /t	16	48.0	16	102.4	1.6	4.8	1.6	157.4	17	51.0	17	393.3
Concentrate to 100 g/L by nanofiltration membrane (5% loss)	6.0 /t	15.1	90.6	0.97	97.2	0	0.0	1.6	157.4	13.2	79.2	3.8	373.6
Concentrate to 200 g/L by multi-effect evaporator (5% loss)	11.9 /t	0.48	5.7	0.48	92.3	0.58	6.9	1.02	149.5	1.9	22.6	1.9	354.9
Remove GlcNAc and ManNAc by anionic resin (10% loss)	3.0 /t	0	0.0	0.48	92.3	1.02	3.1	1.02	134.5	0	0.0	0	354.9
Decolorization by 5% activated carbon (5% loss)	1190.8 /t	0.024 t	28.6	0.48	87.6	0.051 t	60.7	1.02	127.7	0.095 t	113.1	1.9	337.2
Purification by 0.1 volume of ethanol	1041.9 /t	0.048 t	50.0	0.52	87.6	0.1 t	104.2	1.12	127.7	0.19 t	198.0	2.1	337.2
Crystallization by 3 volumes of acetic acid (recycle 1 time)	1041.9 /t	1.56 t	812.7	2.08	65.7	3.36 t	1750.4	4.48	95.7	6.3 t	3282.0	8.4	252.9
Centrifugation (10000 rpm) (2% loss)	0.9 /h	1	0.9	2.08	64.3	2.2	2.0	4.48	93.7	4	3.6	8.4	247.8
Constant temperature drying (5% loss)	0.7 /h	8	5.6	—	61.0	17	11.9	—	89.0	32	22.4	—	235.4
Total product isolation and purification costs (USD)		1289.3				1968.9				4034.1			

Other	Price (USD)	De novo synthesis of NeuAc		Whole-cell catalysis of NeuAc		De novo synthesis of 6'-sialyllactose	
		Dosage	Cost (USD)	Dosage	Cost (USD)	Dosage	Cost (USD)
Wage	4.5 /h	120	535.9	60	267.9	120	535.9
Machine depreciation and repair	4.5 /h	85	379.6	48	214.3	85	379.6
Electricity	0.1 /kw·h	2000	297.7	1000	148.8	2000	297.7
Compressed air	0.1 /kw·h	6000	893.1	2000	297.7	6000	893.1
Sewage treatment	44.7 /t	20	893.1	25	1116.4	20	893.1
Steam	29.8 /m ³	3	89.3	3	89.3	3	89.3
Total other costs (USD)		3088.6		2134.5		3088.6	
Production costs (USD/kg)		121.9		140.0		46.9	

^a The production data is from the three articles with the highest NeuAc and sialyllactose titer of *de novo* synthesis and whole cell catalysis (Drouillard et al. 2010; Kao et al. 2018; Yang et al. 2017).

^b The raw material prices are from Alibaba (<https://www.1688.com/>, an international website for purchasing industrial raw materials), and electricity and labor costs are based on local averages. Other process price and loss values refer to the average of similar products in the local fermentation factory.

References

Drouillard S., Mine T., Kajiwarra H., Yamamoto T., Samain E., 2010. Efficient synthesis of 6'-sialyllactose, 6,6'-disialyllactose, and 6'-KDO-lactose by metabolically engineered *E. coli* expressing a multifunctional sialyltransferase from the *Photobacterium* sp JT-ISH-224. Carbohydr. Res. 345, 1394–1399.

Kao C.-H., Chen Y.-Y., Wang L.-R., Lee Y.-C., 2018. Production of *N*-acetyl-D-neuraminic acid by recombinant single whole cells co-expressing *N*-acetyl-D-glucosamine-2-epimerase and *N*-acetyl-D-neuraminic acid aldolase. Mol. Biotechnol., 60, 427–434.

Yang P., Wang J., Pang Q., Zhang F., Wang J., Wang Q. et al., 2017. Pathway optimization and key enzyme evolution of *N*-acetylneuraminate biosynthesis using an *in vivo* aptazyme-based biosensor. Metab. Eng. 43, 21–28.