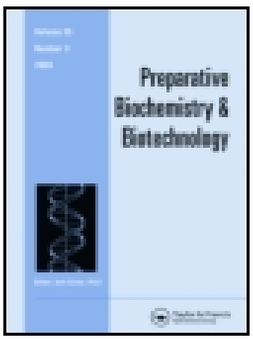

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# Efficient chemoenzymatic synthesis of uridine 5'-diphosphate *N*-acetylglucosamine and uridine 5'-diphosphate *N*-trifluoacetyl glucosamine with three recombinant enzymes

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## Abstract

Uridine 5'-diphosphate *N*-acetylglucosamine (UDP-GlcNAc) is a natural UDP-monosaccharide donor for bacterial glycosyltransferases, while Uridine 5'-diphosphate *N*-trifluoacetyl glucosamine (UDP-GlcNTFA) is its synthetic mimic. The chemoenzymatic synthesis of UDP-GlcNAc and UDP-GlcNTFA was attempted by three recombinant enzymes. Recombinant *N*-acetylhexosamine 1-kinase (NahK) was used to produce GlcNAc/GlcNTFA-1-phosphate from GlcNAc/GlcNTFA. *N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU) from *E.coli* K12 MG1655 was used to produce UDP-GlcNAc/GlcNTFA from GlcNAc/GlcNTFA-1-phosphate. Inorganic pyrophosphatase (PPase) from *E.coli* K12 MG1655 was used to hydrolyze pyrophosphate to accelerate the reaction. The above enzymes were expressed in *E.coli*

BL21 (DE3) and purified respectively, and finally mixed in one-pot bioreactor. The effects of reaction conditions on the production of UDP-GlcNAc and UDP-GlcNTFA were characterized. To avoid the substrate inhibition effect on the production of UDP-GlcNAc and UDP-GlcNTFA, the reaction was carried out with fed batch of substrate. Under the optimized conditions, high production of UDP-GlcNAc (59.51 g/L) and UDP-GlcNTFA (46.54 g/L) were achieved in this three-enzyme one-pot system. The present work is promising to develop an efficient scalable process for the supply of UDP-monosaccharide donors for oligosaccharide synthesis.

**KEYWORDS:** Biosynthesis; *N*-acetylhexosamine 1-kinase; *N*-acetylglucosamine-1-phosphate uridyltransferase; Uridine 5'-diphosphate *N*-acetylglucosamine; Uridine 5'-diphosphate *N*-trifluoacetyl glucosamine; one-pot system





## INTRODUCTION

Heparan sulfate (HS) is a unique class of macromolecules mostly presented on mammalian cell surface and in the extracellular matrix. It consists of a repeating disaccharide unit containing glucuronic acid (GlcUA) or iduronic acid (IdoUA) and glucosamine, both capable of carrying sulfo groups [1]. To investigate the important roles of heparin/HS in protein-binding interactions, structurally defined heparin/HS oligosaccharides such as

antithrombin-binding pentasaccharide has been obtained via chemical total synthesis. But the synthesis of oligosaccharides larger than octasaccharide is extremely difficult, especially in case of biological evaluation which required multiple target structures [2]. Alternatively, the enzymatic biosynthesis has been applied to obtain heparin and heparin oligosaccharides, through which the biologically active HS oligosaccharides have been achieved from bacteria capsular polysaccharides [3–5]. However, the mixture of polysaccharides with various sizes and sulfation patterns could sometimes interfere the structure and activity relationship studies.

Recently, the biosynthesis of oligosaccharides with different sulfation patterns and sizes from a disaccharide building block using glycosyltransferases, HS C5-epimerase, and sulfotransferases has been reported [6]. This method offers a generic approach to prepare HS oligosaccharides possessing predictable structures. The critical step in such method is to utilize bacterial glycosyltransferases and specialized starting molecules (Uridine 5'-diphosphate *N*-acetylglucosamine (UDP-GlcNAc) and Uridine 5'-diphosphate *N*-trifluoacetyl glucosamine (UDP-GlcNTFA)) to build an oligosaccharide backbone from a disaccharide. Furthermore, 10- and 12-step chemoenzymatic syntheses of two structurally homogeneous and biologically active ULMW heparins (MW = 1778.5 and 1816.5) based on this method have been reported with 45% and 37% overall yield respectively [7]. However, the difficulty to obtain these expected starting materials has

made the scalable synthesis of these structurally defined HS oligosaccharides very impractical.

Comparing to the only 15% overall yield of five-step chemical synthesis [8], various enzymatic syntheses afforded superior capability to efficiently produce UDP-GlcNAc under certain conditions. For example, a yeast-based method using 5'-UMP and *N*-acetylhexosamine (GlcNAc) [9], or enzyme co-immobilized agarose beads [10], or reconstituted biosynthetic pathway in a cell-free system [11], were applied to synthesis UDP-GlcNAc. In addition, UDP-GlcNAc has been produced via microbial fermentation of combined recombinant *Escherichia coli* and *Corynebacterium ammoniagenes* [12], *Saccharomyces cerevisiae* [13], and the probiotic bacterium *Lactobacillus casei* strain BL23 [14]. However, the production efficiency of these methods is not high enough. Similarly, the chemical synthetic yield of UDP-GlcNTFA is also very low [15]. The enzymatic synthesis of UDP-GlcNTFA from GlcNTFA-1-phosphate was catalyzed by *N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU), in which GlcNTFA-1-phosphate was prepared from costly substrate $GlcNH_2$ 1-phosphate [6]. To avoid the limited origin of $GlcNH_2$ 1-phosphate, *N*-acetylhexosamine 1-kinase (EC 2.7.1.162, NahK) cloned from *Bifidobacterium longum* JCM1217 [16] was applied to catalyze the direct addition of ATP-derived phosphate to the anomeric position of GlcNAc/GlcNTFA, forming an important phosphate intermediate. Substrate specificity study showed that NahK could tolerate diverse modifications at C2 of GlcNAc [17–19]. On

the other hand, the N-terminal domain of GlmU (amino acids from M1 to R299) from *Escherichia coli* K12 could also catalyze the formation of UDP-GlcNAc from GlcNAc-1-P and UTP. Subsequent substrate evaluation suggested that only analogs with an amide linkage at the C2 nitrogen could be recognized by GlmU [20]. Based on these work, an elaborate route with two-step enzymatic reactions has been designed to accomplish the production of UDP-GlcNAc using common substrates (GlcNAc, ATP and UTP) [21]. For enzymatic production processes it is of interest to use high initial substrate concentrations to obtain higher yields. However, a high concentration of initial substrates (GlcNAc, ATP and UTP) [22] or the pyrophosphate by-product [23] formed in the enzymatic reactions had a large negative effect on the enzyme activity and affect the yield of the desired product.

In this work, recombinant NahK GlmU and PPase are used in one-pot to synthesize UDP–GlcNAc/GlcNTFA (Fig. 1). PPase is added for hydrolyzing the by-product pyrophosphate (PPi) to drive the reaction forward. The effects of temperature, $Mg^{2+}$ concentration, pH and substrate concentration on the productivity of UDP–GlcNAc/GlcNTFA have been investigated respectively. Finally, a fed-batch approach is used for controlling concentrations of substrates which affect the yield of UDP–GlcNAc/GlcNTFA.

## EXPERIMENTAL

### *Strains, Plasmids, And Materials*

*Escherichia coli* K12 substr. MG1655 (ATCC-700926) was used as the original strain to amplify *PPase* and *GlmU* genes; Live Combined *Bifidobacterium*, *Lactobacillus* and *Enterococcus* oral powder (commercial brand PeiFeiKang) was purchased from Sinepharm (Shanghai, China) as the origin to amplify *NahK* gene. *E. coli* DH5α and *E. coli* BL21 (DE3) were purchased from Invitrogen (Carlsbad, CA, USA). The restriction enzymes *Nco* I and *Xho* I, as well as LA Taq DNA polymerase and T4 DNA ligase, were purchased from Takara (Dalian, China). GlcNAc was purchased from Aladdin (Shanghai, China). Ethyl trifluoroacetate (ETFA) and the standard GlcNTFA were purchased from J&K Scientific Ltd. (Beijing, China).

### *Plasmids Construction*

The target genes of NahK, GlmU and PPase were amplified from corresponding microbial genomic DNA, respectively. The primers were shown in Table 1. The introduced restriction sites *Nco* I and *Xho* I were underlined. Each target gene was cloned into pET28a(+) vector via *Nco* I/ *Xho* I and transformed into *E. coli* DH5α competent cells. The resulting expression plasmids were characterized by restriction mapping and DNA sequencing (Sangon, Shanghai, China).

### *Enzyme Expression And Purification*

Validated expression plasmid was transformed into *E. coli* BL21 (DE3). A fresh clone of recombinant *E. coli* strain was grown in 5 mL LB medium containing 30 mg/L kanamycin at 37$^{o}$C and 200 rpm for 12 h, then inoculated into 50 mL fresh LB medium containing 30 mg/L kanamycin and continued culturing at the same conditions. When the $OD_{600}$ of culture was reached 1.0, 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) was added to induce the expression for additional 12 h at 25$^{o}$C. After harvested by centrifugation at 12,000 × g for 10 min, cells were resuspended in 20 mM sodium phosphate (pH 7.4) and sonicated on ice. The resulting cell lysate was centrifuged at 12,000 ×g and 4$^{o}$C for 10 min, and the supernatant was applied into a nickel affinity chromatography column packed with 10 mL Ni-NTA resin (Qiagen, Hilden, Germany). After washing column with washing buffer (20 mM sodium phosphate, pH 7.4, 100 mM NaCl, 10 mM imidazole), NahK was eluted with elution buffer I (20 mM sodium phosphate, pH 7.4, 100 mM NaCl, 500 mM imidazole), and then desalted by HiTrapTM Desalting HP column with 50 mM Tris-HCl buffer, pH 7.5. The purified fractions were concentrated with Amicon Ultra and stored in 20% glycerol (50 mM Tris-HCl, pH 7.5) at -20$^{o}$C. For purification of PPase and GlmU, cells were resuspended in buffer (20 mM Tris-HCl, pH8.0, 500 mM NaCl, 20 mM imidazole) and sonicated on ice. Similarly, target enzyme was eluted with elution buffer II (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 250 mM imidazole). The fractions containing PPase were pooled and desalted by HiTrapTM Desalting HP column with 20 mM Tris-HCl (pH 8.0) and 100 mM KCl, and stored in storage buffer I (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, and 50% (v/v) glycerol) at -20$^{o}$C. The fractions

containing GlmU were pooled and desalted by HiTrapTM Desalting HP column with 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl, and stored in storage buffer II (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2 mM DTT, 150 mM NaCl, and 20% (v/v) glycerol) at -20$^{o}$C.

### *Quantification Of Purified Protein*

The concentration of target enzyme was determined in a 96-well plate using bicinchoninic acid (BCA) Protein Assay Kit (Sangon, Shanghai, China), with bovine serum albumin as the standard. The absorbance of each sample was measured at 562 nm by a spectrophotometer (Varioskan Flash, Thermo Scientific (Waltham, MA, USA)). For PPase, 1 U is the amount of enzyme that will generate 1 μmol of phosphate per minute from PPi under standard reaction conditions (a 10 min reaction at 25 $^{o}$C in 100 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$ and 2 mM PPi in a reaction volume of 0.5 mL). The phosphate concentration in the mixture was measured as described by Heinonen [24].

### *Chemical Synthesis Of Glcntfa*

The chemical synthesis of GlcNTFA is shown as in Fig. 1A. 1.02 g Glucosamine HCl (4.73 mmol) was dissolved in 10 mL MeOH, then 1.00 g $Na_2CO_3$ (9.46 mmol) and 1.34 mL ETFA (9.46 mmol) were sequentially added into the solution. The reaction mixture was stirred at 45$^{o}$C overnight and the GlcNTFA was purified by silica gel column

chromatography (volume ratio, EtOAc: MeOH=100:1). GlcNTFA samples were analyzed by HPLC.

***Synthesis Of Udp-Glcnac And Udp-Glcntfa***

The reaction mixture for synthesis of UDP-GlcNAc/UDP-GlcNTFA contained 1 mM GlcNAc/GlcNTFA, 1 mM UTP, 1 mM ATP, 10 mM $MgCl_2$, 0.2 mg NahK, 0.2 mg GlmU, 5 U PPase , and 50 mM Tris-HCl (pH 8.0). 1 ml mixture was incubated at 37$^{o}$C for 30 min, then boiled for 5 min and centrifuged to remove residues. To optimize the reaction condition, single factor analysis was executed to investigate the effects of temperature, $Mg^{2+}$ concentration, pH on the biosynthesis of UDP-GlcNAc and UDP-GlcNTFA.

Preparative-scale synthesis of UDP-GlcNAc and UDP-GlcNTFA were respectively performed in 10 mL 200 mM Tris-HCl (pH 8.5) containing different equal dose (20 mM, 30 mM, 40 mM, and 50 mM) of GlcNAc/GlcNTFA, UTP and ATP; 5 mM $MgCl_2$, 2 mg NahK, 2 mg GlmU and 50 U PPase. The reaction mixture was incubated at 37$^{o}$C and monitored by HPLC analysis every two hours.

To avoid possible substrate inhibition effect, a substrate feeding strategy was applied on the production of UDP-GlcNAc and UDP-GlcNTFA. The reaction was performed in 100 mL 200 mM Tris-HCl solution containing 2 mmol GlcNAc or 2 mmol GlcNTFA, 2 mmol ATP,

2 mmol UTP, 5mM $MgCl_2$, 20 mg NahK, 20 mg GlmU, and 500 U PPase, and the pH was adjusted to 8.5 using 12M HCl**.** The reaction was monitored by HPLC every half hour. Another dose of substrates (1 mmol GlcNAc or 1 mmol GlcNTFA, 1 mmol ATP, and 1 mmol UTP) were added when reaction was nearly completed, and the pH was adjusted to 8.5 using 2M NaOH. The reaction volume increased by 2 mL each time as a result of the addition of substrates and the pH adjustment. The reaction was finally stopped when no UDP-GlcNAc or UDP-GlcNTFA was produced.

***Products Analysis***

Samples were detected by the Agilent 1100 Series HPLC equipped with a TC-C18 column (4.6×250 mm, 5 μm) and a UV detector. GlcNTFA was detected at 217 nm with isocratic mobile phase (MeOH: $H_2O$=60:40) and 0.5 mL/min flow rate; UDP-GlcNAc was analyzed at 254 nm using 1 mL/min isocratic flow of 50 mM ammonium formate (pH 4.5) containing 5% menthol; UDP-GlcNTFA was also analyzed at 254 nm using 1 mL/min isocratic flow of 50 mM ammonium formate (pH 3.5) containing 5% menthol. The program was performed at 30 °C for 15 min, and the expected nucleotide sugars UTP and ADP/ATP were detected at 254 nm. ESI-MS analysis was carried out using a Bruker LC/MSD Trap mass spectrometer (Bruker, Massachusetts, USA), the collected data was processed by HP Chemstation. ESI-MS analysis of UDP-GlcNAc was carried out in the

positive ion detection mode and ESI-MS analysis of UDP-GlcNTFA was carried out in the negative ion detection mode.

### *Separation Of Udp-Glcnac And Udp-Glcntfa Using A Preparative Hplc*

The separation was performed by an Agilent 1200 Series HPLC equipped with an Agilent Eclipse XDB C18 (9.4×250mm, 5 μm) and a UV detector. For UDP-GlcNAc, 2 mL/min isocratic flow of 20 mM ammonium acetate (pH 4.5) containing 5% menthol was used. For UDP-GlcNTFA, 2 mL/min isocratic flow of 0.1% formic acid containing 5% menthol was used. The resultant products were lyophilized and the purity was validated by HPLC analysis.

## RESULTS AND DISCUSSION

### *Cloning, Expression, And Purification Of Enzymes*

In order to reconstruct the enzymatic synthesis pathway of UDP-GlcNAc/GlcNTFA in vitro, three plasmids pET28a-*NahK*, pET28a-*GlmU* and pET28a-*PPase* were constructed. The subsequent DNA sequencing has validated the correctness of *GlmU* and *PPase* genes. The nucleotide BLAST result suggested that the *NahK* amplified from Peifeikang was identical to *NahK* from *Bifidobacterium longum* subsp. JDM301 (GenBank no. CP002010.1). After enzyme expression and purification, up to 185 mg, 400 mg and 205

mg of NahK, GlmU and PPase could be obtained from one liter of *E. coli* culture, respectively. The SDS-PAGE analysis showed purified proteins around 41, 50 and 20 kDa (Fig. 2), which were well matched with the calculated molecular weight of His6-tagged fusion proteins of NahK, GlmU and PPase (41.1, 50.5 and 19.7 kDa, respectively).

***Enzymatic Synthesis Of Udp-Glcnac And Udp-Glcntfa***

The synthesis of UDP-GlcNAc and UDP-GlcNTFA mainly involve two steps: the formation of GlcNAc-1-P or GlcNTFA-1-P catalyzed by NahK, and the transfer of UDP catalyzed by GlmU. UDP-GlcNAc was detected at the retention time of 3.261 min (Fig. 3A), and subsequent LC-MS analysis gave the signal of UDP-GlcNAc at m/z 607.9 (m/z calculated for $C_{17}H_{27}N_3O_{17}P_2$ (M+H) is 608.1) (Fig. 4A). UDP-GlcNTFA was detected at the retention time of 4.64 min (Fig. 3B), and the subsequent LC-MS analysis gave the signal of UDP-GlcNTFA at m/z 660.0 (m/z calculated for $C_{17}H_{24}F_3N_3O_{17}P_2$ (M-H) is 660.1) (Fig. 4B).

After the purification using a preparative HPLC, the HPLC analysis of final UDP-GlcNAc showed 98% purity based on the HPLC peak area; while the HPLC analysis of final UDP-GlcNTFA showed only 85.34% purity based on the HPLC peak area, with ADP as the major impurity (data not shown). ADP is not easy to remove efficiently by only using preparative liquid chromatograph. In previous reports, calf intestinal alkaline phosphatase

(CIAP) was used to hydrolyze nucleotides for UDP-GlcNAc production [25] and ATP regeneration system [26–28] also could be introduced to avoid the accumulation of ADP.

### *The Effects Of Different Reaction Conditions On The Synthesis Of Udp-Glcnac And Udp-Glcntfa*

#### *The Effect Of Temperature*

The temperature not only affects the enzyme activity, but also affects the stability of the enzyme. The effect of temperature on the synthesis of UDP-GlcNAc/GlcNTFA was shown in Fig. 5. The fractional conversion increased with the increase of temperature under 37$^{o}$C and dramatic decrease was observed when the temperature increased from 40 to 50$^{o}$C. Although NahK remains half of its activity during incubation for 30 min at 50°C [16], the GlmU is extremely unstable [29] so that no UDP-GlcNAc/GlcNTFA is generated at 50$^{o}$C. The final determined optimal temperature was 37$^{o}$C. Overall, the fractional conversion of UDP-GlcNAc is higher than that of UDP-GlcNTFA at the temperature range of 20-50$^{o}$C.

#### *The Effect Of $Mg^{2+}$ Concentration*

Both NahK and GlmU required magnesium ions for activity. As shown in Fig. 6, the optimum $Mg^{2+}$ concentration for the synthesis of UDP-GlcNAc/GlcNTFA was 5 mM. The fractional conversion in the presence of 5 mM $Mg^{2+}$ was about 4~5 times of those in the

presence of 0.5 mM $Mg^{2+}$ and 1mM $Mg^{2+}$. Increasing the concentration of $Mg^{2+}$ from 5 mM to 20 mM caused a slight decrease of fractional conversion.

### *The Effects Of Ph And Buffer System*

The effects of pH and buffer system on the synthesis of UDP-GlcNAc/GlcNTFA were shown in Fig. 7A and Fig. 7B. Both conversion ratios of UDP-GlcNAc and UDP-GlcNTFA were highest after 5 min incubation at pH 8.5 in Tris-HCl buffer. The synthesis of UDP-GlcNAc had a broad pH adaptability while the synthesis of UDP-GlcNTFA had a narrow one. The conversion ratio dropped quickly with either decrease of the pH to below 7.5 or an increase of the pH to above 9.5. About 50% of the optimal activity was observed at pH 6.0 in $NaH_2PO_4/Na_2HPO_4$ buffer and at pH 10.5 in Glycine-NaOH buffer for the synthesis of UDP-GlcNAc. In comparison, 66.3% of the optimal activity was observed at pH 7.5 in Tris-HCl buffer and 60.4% of the optimal activity was observed at pH 9.0 in Glycine-NaOH buffer for the synthesis of UDP-GlcNTFA.

### *The Effect Of Substrate Concentration*

The time-course of UDP-GlcNAc (Fig. 8A) production at different initial substrate concentrations showed that complete conversion of substrate to UDP-GlcNAc took 1h, 3 h, 8h and 18 h, respectively at 20 mM, 30 mM, 40 mM and 50 mM substrate concentration. The accumulation of UDP-GlcNAc in 0.5 h and 1 h increased with the initial substrate

concentrations from 20 mM to 50 mM. At 30 mM substrate concentration, the reaction rate increased over time, which was also observed at 40 mM and 50 mM substrate concentration. This results suggested that substrate inhibit the enzyme activity at high concentration

The time-course of UDP-GlcNTFA (Fig. 8B) production at different initial substrate concentrations showed that complete conversion of substrate to UDP-GlcNAc took 1 h, 4 h and 13h, respectively at 20 mM, 30 mM and 40 mM substrate concentration. Only 24.5% of substrate was converted to UDP-GlcNTFA in 16 h when the initial substrate concentration was 50 mM. The accumulation of UDP GlcNTFA in 0.5 h and 1 h also increased with the initial substrate concentrations from 20 mM to 50 mM. The reaction rate increased over time at 20mM, 30mM and 40mM substrate concentration. All the evidences suggested substrate inhibition effect.

***Scaled-Up Production Of Udp-Glcnac And Udp-Glcntfa With Fed Batch Of Substrate***

To remove the substrate inhibition effect on the production of UDP-GlcNAc and UDP-GlcNTFA , the scaled-up production with fed batch of substrate was executed (Fig. 9A, B). The conversion of initial GlcNAc, ATP and UTP (20 mM) to UDP-GlcNAc took 1 h and the earlier dose of GlcNAc, ATP and UTP was converted to UDP-GlcNAc quickly. The UDP-GlcNAc production slowed down with $Mg^{2+}$ consumption after 4.25 h reaction.

After 47 mg $MgCl_2$ was added at 5.25 h, the synthesis of UDP-GlcNAc accelerated. The last dose reaction lasted for approximately 3 h, which might be due to the product inhibition effect and/or the loss of enzyme activity. Eventually, the final concentration of produced UDP-GlcNAc reached 98.04 mM (59.51 g/L) after 14.75 h reaction. The productivity of UDP-GlcNAc is 4.03 $g \cdot L^{-1} \cdot h^{-1}$ and the conversion ratio was 92.0%.

Similarly, the conversion of initial GlcNTFA, ATP and UTP (20 mM) to UDP-GlcNTFA also took 1 h and the earlier dose of GlcNTFA, ATP and UTP was converted to UDP-GlcNTFA quickly in 1 h. After 47 mg $MgCl_2$ was added at 5.0 h, the synthesis of UDP-GlcNTFA accelerated. The last dose of GlcNTFA, ATP and UTP took 3.5 h to convert to UDP-GlcNTFA. The final concentration of produced UDP-GlcNTFA reached 70.41 mM (46.54 g/L) after 14 h reaction. The productivity of UDP-GlcNTFA is 3.32 $g \cdot L^{-1} \cdot h^{-1}$ and the conversion ratio was 75.5%. The conversion ratio could not reach as high as that of UDP-GlcNAc because some UDP-GlcNTFA was degraded.

Fed-batch of substrate greatly improves the productivity of UDP-GlcNAc and UDP-GlcNTFA. The accumulation of UDP-GlcNAc and UDP-GlcNTFA slowed down as time went by, which might be caused by the product inhibition effect and the loss of enzyme activity. In the future, immobilization of these enzymes together, such as on the surface of $Ni^{2+}$-functionalized beads, can be utilized to further improve the efficiency of enzyme catalysis and reduce the total cost for large-scale production.

## CONCLUSIONS

The aim of the present study was to achieve high production of UDP-GlcNAc and UDP-GlcNTFA. GlcNTFA was chemically synthesized from GlcNAc and ethyl ETFA. The key enzymes essential for the efficient production of UDP-GlcNAc and UDP-GlcNTFA were expressed and purified, and finally mixed in one-pot bioreactor. The effects of reaction conditions on the productivity of UDP-GlcNAc and UDP-GlcNTFA were characterized. To avoid the substrate inhibition effect on the production of UDP-GlcNAc and UDP-GlcNTFA, the reaction was carried out with fed batch of substrate. Under the optimized conditions, high production of UDP-GlcNAc (59.51 g/L) and UDP-GlcNTFA (46.54 g/L) were achieved in this three-enzyme one-pot system. In this study, large-scale production of UDP-GlcNAc/GlcNTFA at low cost can be achieved, which provides the basis for the industrialization of biosynthesis of ultralow molecular weight heparin.



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Table 1. Oligonucleotides used in this study. The restriction sites *Nco* I and *Xho* I are underlined.

| Primer | Sequence（5'to3') |
|---|---|
| NahK F | CGCCCATGGGTATGACTGAAAGCAATGAAGTTTTATTCGG |
| NahK R | CGCCTCGAGCCTGGCAGCCTCCATGATGTCGG |
| GlmU F | CATGCCATGGAAATGTTGAATAATGCTAT |
| GlmU R | CCGCTCGAGCTTTTTCTTTACCGGACG |
| PPase F | GCGCCATGGGAATGAGCTTACTCAACGTCCC |
| PPase R | CGCCTCGAGTTTATTCTTTGCGCGC |

Figure 1. Chemoenzymic synthesis schedule of UDP-GlcNAc and UDP-GlcNTFA. (**A**) Chemical synthesis of GlcNTFA. (**B**) Enzymatic synthesis of UDP-GlcNAc and UDP-GlcNTFA. NahK, *N*-acetylhexosamine 1-kinase cloned from Combined *Bifidobacterium*、*Lactobacillus* and *Enterococcus* Powder; GlmU, *N*-acetylglucosamine-1-phosphate uridylyltransferase cloned from *E.coli* K12 MG1655; PPase, *E.coli* K12 MG1655 inorganic pyrophosphatase.

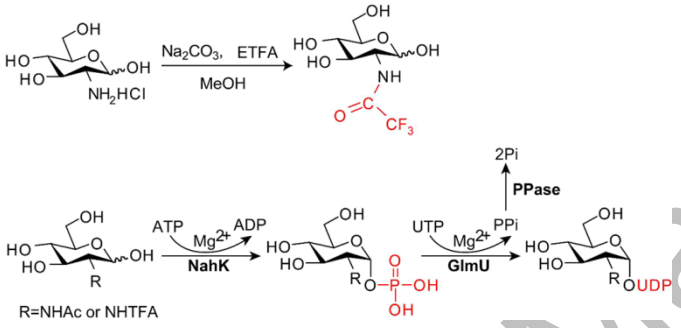


Figure 2. SDS-PAGE analysis of NahK, GlmU and PPase. Lane 1, NahK lysate after induction; Lane 2, purified NahK; Lane 3, GlmU lysate after induction; Lane 4, purified GlmU; Lane 5, PPase lysate after induction; Lane 6, purified PPase.

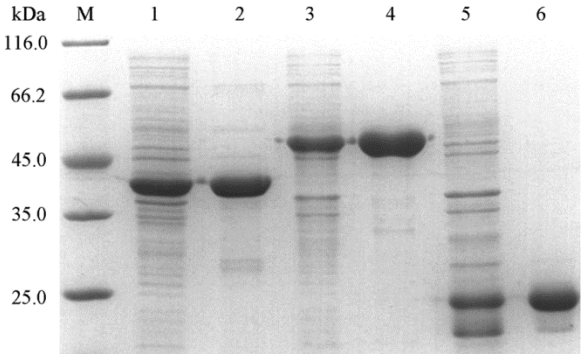


Figure 3. HPLC analysis of UDP-GlcNAc and UDP-GlcNTFA from GlcNAc and UDP-GlcNTFA. **(A)** Enzymatic synthesis of UDP-GlcNAc from GlcNAc. **(B)** Enzymatic synthesis of UDP-GlcNTFA from GlcNTFA.

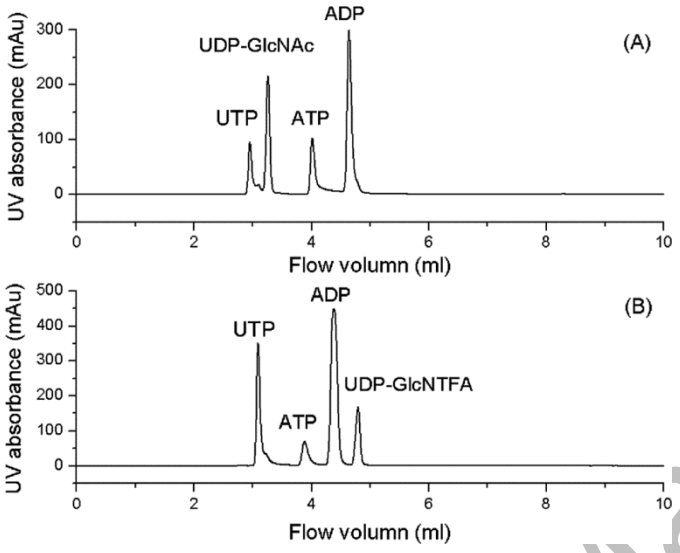


Figure 4. ESI-MS analysis of the product. **(A)** The spectrum of UDP-GlcNAc (*m/z* 607.9).

**(B)** The spectrum of UDP-GlcNTFA (*m/z* 660.0).

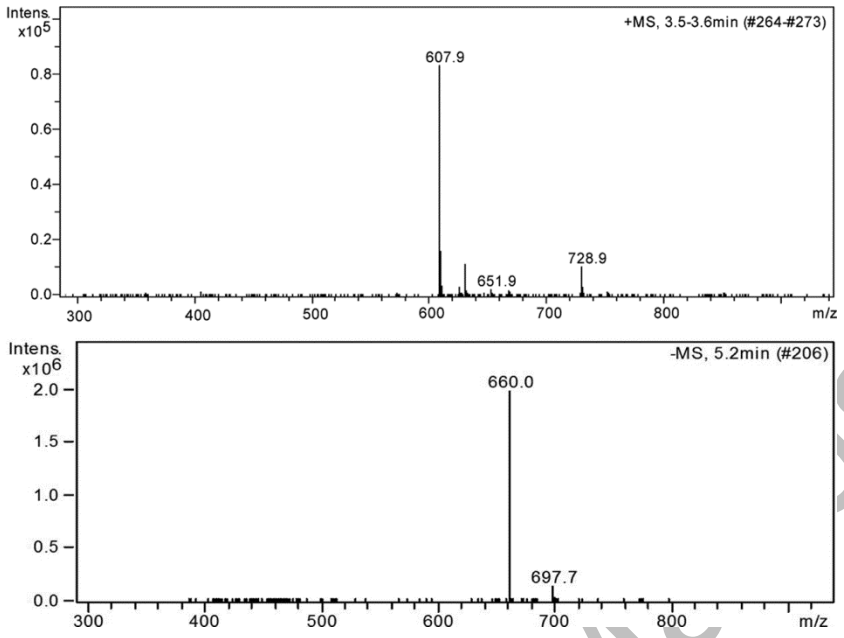


Figure 5. Effect of temperature on the synthesis of UDP-GlcNAc/GlcNTFA. The concentration of ATP, UTP, GlcNAc/GlcNTFA and $Mg^{2+}$ is 5 mM, the reaction lasts 15 min in 50 mM Tris-HCl buffer (pH 8.0). All experiments were performed in triplicate.

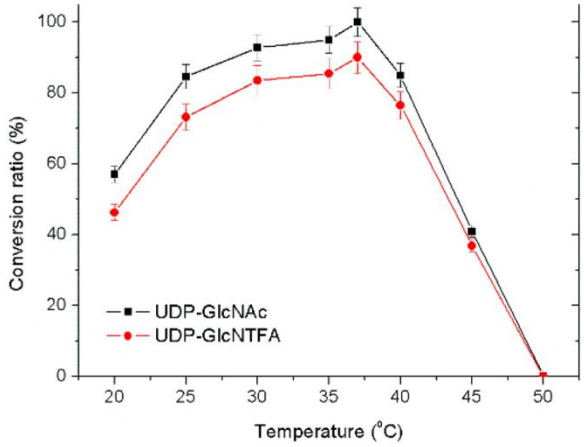


Figure 6. The effect of $Mg^{2+}$ concentration on the synthesis of UDP-GlcNAc/GlcNTFA. The concentration of ATP, UTP, GlcNAc/GlcNTFA is 5mM, the reaction lasts 15min at 37$^{o}$C water bath in 50 mM Tris-HCl buffer (pH8.0). All experiments were performed in triplicate.

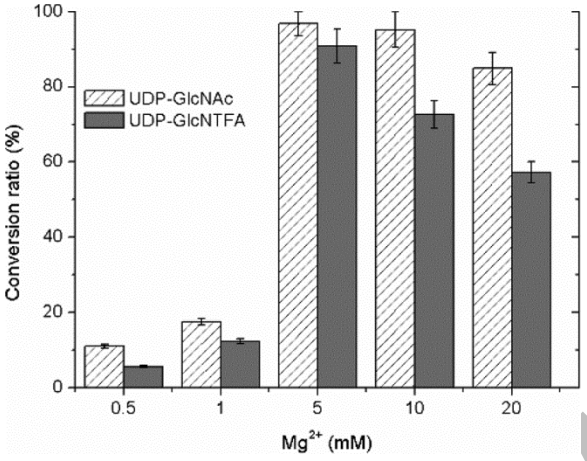


Figure 7. Effect of pH and buffer systems on the synthesis of UDP-GlcNAc (A) and UDP-GlcNTFA **(B)**. —■—, $NaH_2PO_4/Na_2HPO_4$ buffer; —●—, Tris-HCl buffer; —▲—, Glycine/NaOH buffer. The concentration of ATP, UTP, GlcNAc/GlcNTFA and $Mg^{2+}$ is 5 mM, the reaction lasts 5 min at 37°C water bath. All experiments were performed in triplicate.

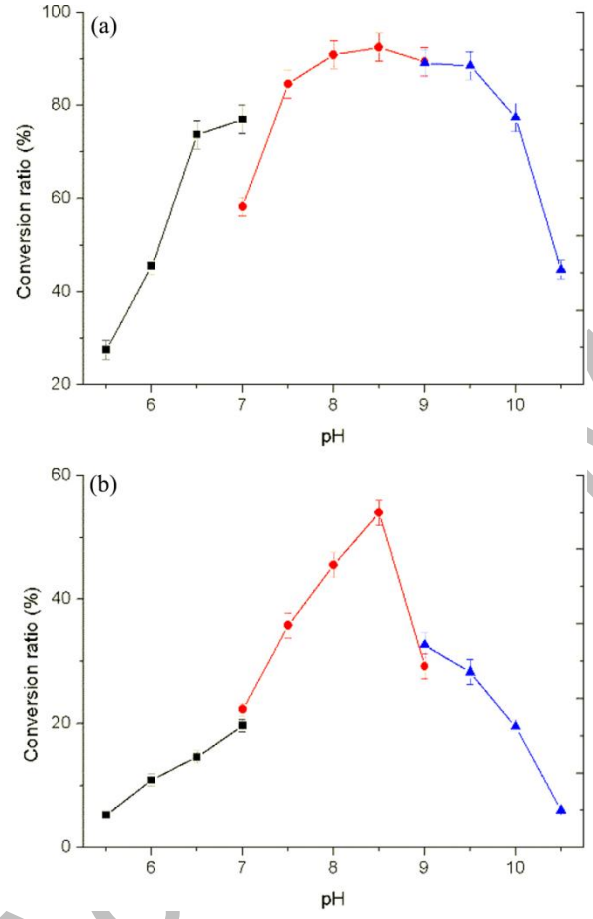
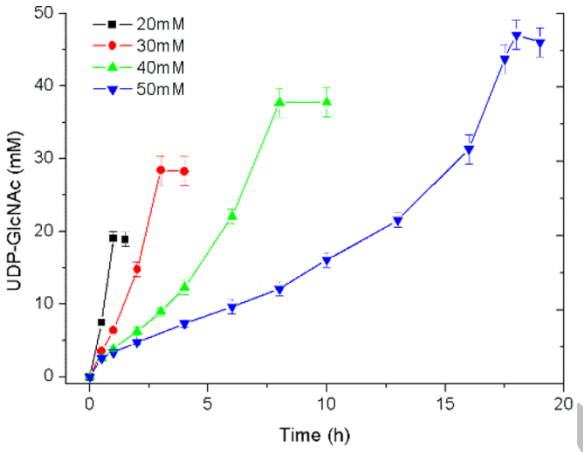
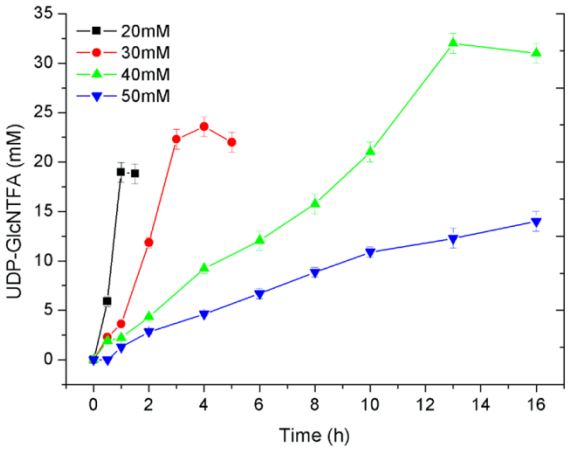


Figure 8. Time-course production of UDP-GlcNAc **(A)** and UDP-GlcNTFA **(B)** at different substrate concentration: 20 mM (■), 30 mM (●), 40 mM (▲) and 50 mM (▼). The concentration of $Mg^{2+}$ is 5 mM, the reaction is performed at 37°C water bath in 200 mM Tris-HCl buffer (pH 8.5). All experiments were performed in triplicate.

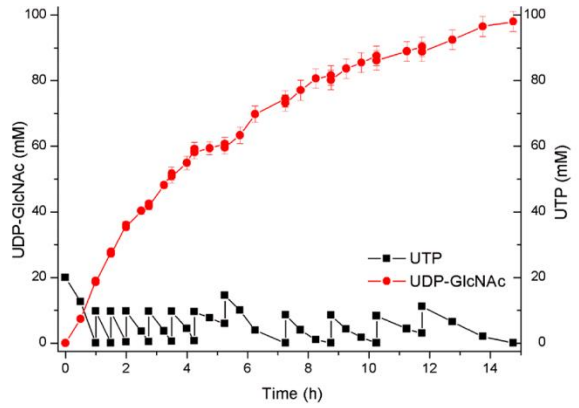



(a)

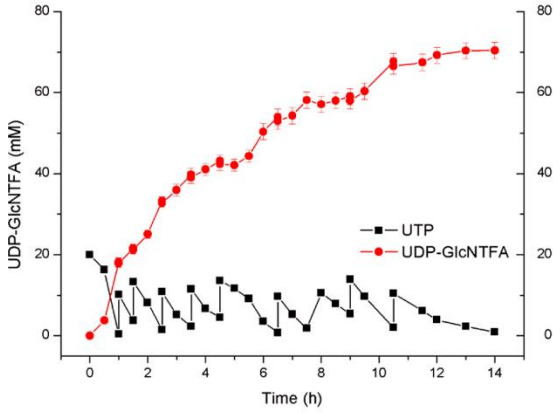



(b)

Figure 9. Time-course production of UDP-GlcNAc **(A)** and UDP-GlcNTFA **(B)** under the optimized conditions with fed batch of substrate. **(A)** UTP (■), UDP-GlcNAc (●). **(B)** UTP (■), UDP-GlcNTFA (●). All experiments were performed in triplicate.




(a)




(b)