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Biocatalytic Production of Glucosamine from *N*-Acetylglucosamine by Diacetylchitobiose Deacetylase

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Glucosamine (GlcN) is widely used in the nutraceutical and pharmaceutical industries. Currently, GlcN is mainly produced by traditional multistep chemical synthesis and acid hydrolysis, which can cause severe environmental pollution, require a long prodution period but a lower yield. The aim of this work was to develop a whole-cell biocatalytic process for the environment-friendly synthesis of glucosamine (GlcN) from *N*-acetylglucosamine (GlcNAc). We constructed a recombinant *Escherichia coli* and *Bacillus subtilis* strains as efficient whole-cell biocatalysts via expression of diacetylchitobiose deacetylase (Dac_{ph}) from *Pyrococcus furiosus*. Although both strains were biocatalytically active, the performance of *B. subtilis* was better. To enhance GlcN production, optimal reaction conditions were found: *B. subtilis* whole-cell biocatalyst 18.6 g/l, temperature 40°C, pH 7.5, GlcNAc concentration 50 g/l and reaction time 3 h. Under the above conditions, the maximal titer of GlcN was 35.3 g/l, the molar conversion ratio was 86.8% in 3-L bioreactor. This paper shows an efficient biotransformation process for the biotechnological production of GlcN in *B. subtilis* that is more environmentally friendly than the traditional multistep chemical synthesis approach. The biocatalytic process described here has the advantage of less environmental pollution and thus has great potential for large-scale production of GlcN in an environment-friendly manner.

Keywords: Whole-cellbiocatalyst, glucosamine, diacetylchitobiose, deacetylase, *Bacillus subtilis*

Introduction

Glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) is an amino sugar obtained by substitution of a hydroxyl group in glucose with an amino group. GlcN has been widely used in food, cosmetics, and pharmaceutical industries, especially in osteoarthritis treatment [1, 2]. In humans, GlcN is a precursor of the disaccharide units of glycosaminoglycans (such as hyaluronic acid, chondroitin sulfate, and keratan sulfate), which are necessary to repair and maintain healthy cartilage and joint function [3, 4]. Currently, GlcN is mainly produced by acid hydrolysis of chitin and chitosan extracted from crab and shrimp shells. On the other hand, this extraction method poses some problems such as severe environmental pollution and potential allergic reactions in consumers. Recently, a new microbial fermentation procedure for the production of GlcN and GlcNAc was developed. The strains used for GlcN and GlcNAc production include fungi, viz., *Aspergillus* sp. BCRC 31742 [5, 6], genetically modified *Escherichia coli* [7, 8], and genetically modified *Bacillus subtilis* [9]. For fermentation by means of filamentous fungi, the GlcN production from the fungal cell wall requires acid hydrolysis and a long culture period. Regarding the engineered *E. coli* and *B. subtilis*, these methods have a high yield of GlcNAc but a lower yield of GlcN. As reported [7, 8], the production of GlcN requires acid hydrolysis; therefore, it also entails serious pollution. The low productivity and drawbacks of these approaches weaken their economic competitiveness. Besides, chitin could be hydrolyzed by chitinase to monomeric GlcNAc. As a result, some productive methods have been proposed for obtaining monomeric GlcNAc, but

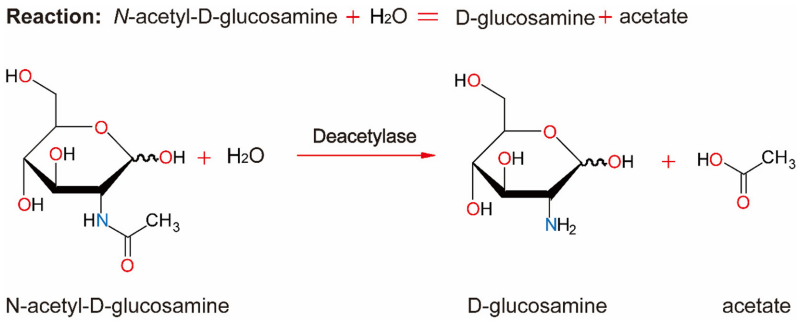

Fig. 1. The deacetylation reaction of diacetylchitobiose deacetylase (Dac_{ph}) from *P. horikoshii*.

there isno high-efficiency method for the production of GlcN. Therefore, to solve the above problems, much attention has been given to manufacturing GlcN by an environmentally friendly method and to the development of a biocatalytic process for the environment-friendly synthesis of GlcN from GlcNAc.

Deacetylase has been mentioned in the literature in this regard, but has some disadvantages, such as poor stability, low tolerance of its substrate, low activity, and a low yield. A novel pathway of chitin degradation in *Pyrococcus furiosus* has been reported [10, 11]. In Archaea, the diacetylchitobiose deacetylase plays an important role in the unique chitin degradation pathway. The enzyme in question, diacetylchitobiose deacetylase, can remove the *N*-acetyl group of the GlcNAc, yielding GlcN. It can also work in combination with glucosaminidase to hydrolyze diacetylchitobiose to GlcN [12]. For this reason, it is called diacetylchitobiose deacetylase (Dac_{ph}). It can hydrolyze monomeric *N*-acetylglucosamine efficiently. The deacetylase reaction (Fig. 1) showed the elements in the catalyst process, as well as the changes of chemical structures between GlcNAc and GlcN. The *Dac*$_{ph}$ gene is 819 bp long and encodes a 273–amino acid protein, and theoretical molecular mass of $Dac_{ph}$is estimated to be 31.6 kDa on the web site http://web.expasy.org/compute pi/. The recombinant Dac_{ph} is tolerant to heat treatment at 85°C for 30 min. This phenomenon is consistent with previous reports; it is derived from hyperthermophilic archaea, and may also be useful for stable biotransformation [13].

In the present study, we expressed diacetylchitobiose deacetylase from *P. horikoshii* (Dac_{ph}) in *E. coli* BL21(DE3) and *B. subtilis* WB600 to produce GlcN in one step from GlcNAc using the *E. coli* and *B. subtilis* strains engineered for whole-cell biocatalysis. The engineered strains were tested as whole-cell catalysts for deacetylation of GlcNAc to GlcN. As compared with *E. coli*, the expression of Dac_{ph} and the whole-cell biocatalytic activity of the *B. subtilis* were higher. In addition, *B. subtilis* is the best-characterized gram-positive bacterium and has unique advantages as a host for large-scale industrial production of enzymes [11, 14, 15], such as bacteriophage resistance factors and has the generally recognized as safe (GRAS) status granted by the United States Food and Drug Administration [16–19]. Therefore, we selected *B. subtilis* for further experiments. Finally, the reaction conditions of the proposed bioconversion were optimized, leading to enhanced production of GlcN from GlcNAc.

Materials and Methods

Materials, Plasmids, and Strains

Bacterial strains, plasmids, and primers used in this study are listed in Table 1. *E. coli* JM109 was employed for cloning and construction of genes and plasmids. *E. coli* BL21(DE3) and *B. subtilis* WB600 served as the biocatalytic hosts. Plasmid pP43NMK was a generous gift from Dr. Zhang (Department of Biological Systems Engineering, Virginia Tech, USA) [20]. The SanPrep Column Plasmid Mini-Preps Kit and ClonExpress MultiS One Step Cloning Kit were purchased from Sangon (China), whereas the DNA purification kit and restriction enzymes from Takara (China). The standard sample of GlcN was acquired from Sigma-Aldrich (USA), whereas ampicillin came from Amresco (USA) and isopropyl-β-D-1-thiogalactopyranoside (IPTG) came from Merck (Germany). All other chemicals were purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (China). Primer synthesis and DNA sequencing were performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. *E. coli* and *B. subtilis* seed cultures were initiated in the Luria–Bertani (LB) medium (LB: 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl), and the growth of whole-cell biocatalysts were incubated in Terrific Broth (TB) (TB: 12 g/l tryptone, 24 g/l yeast extract, 4 ml/l glycerol, 2.31 g/l KH_2PO_4, and 12.54 g/l K_2HPO_4) with the same antibiotic.

Recombinant-Plasmid Construction and Bacterial Transformation

The genes encoding Dac_{ph} (PH0499) were synthesized by Sangon Biotech Co., Ltd. (China) and were optimized for

Table 1. Strains, plasmids, and primers used in this study.

Strain, plasmid or primer	Description or sequence (5′-3′)	Source
Strains		
E. coli JM109	Cloning strain	Takara, Ostu, Japan
E. coli BL21(DE3)	Expression strain	Invitrogen,Carlsbad, CA
B. subtilis WB600	Expression strain	Lab stock
E. coli-Dac	*E. coli* BL21(DE3) containing a pET-28a-Dac	This work
B. subtilis-Dac	*B. subtilis* WB600 containing a pP43NMK-Dac	This work
Plasmids		
pET-28a(+)	Kan^r	Invitrogen, Carlsbad, CA
pP43NMK	Kan^r ; Amp^r	Virginia Tech, USA
pET-28a-Dac	pET-28a(+) containing *Dac*	This work
pP43NMK-Dac	pP43NMK containing *Dac*	This work
Primers		
Dac_{ph}-F1	GTGGACAGCAAATGGGTCGC*GGATCC*ATGGTAGTGAACATGTTCGAAG	
Dac_{ph}-R1	CAGTGGTGGTGGTGGTGGTG*CTCGAG*GATAAGGTCAGTAAACGGTGT	
Dac_{ph}-F2	GTAAAATATAAAGTGATAGC*GGTACC*ATTATAGGTAAGAGAGGAAT	
Dac_{ph}-R2	ACCATGATTACGCCAAGCTT*CTGCAG*TCAGATCAGGTCCGTAAACG	

Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.
Italic letters represent the restriction enzyme sites, and underlined letters represent homologous sequences for cloning.

expression in *E. coli* and *B. subtilis*, respectively (Fig. S1). For expression in *E. coli*, plasmid pET-28a(+) was digested with BamHI and XhoI and then subjected to column purification. The *Dac_{ph}-E* gene was amplified by PCR with gene-specific primers: Dac_{ph}-F1 and Dac_{ph}-R1. The PCR product was processed by gel purification and ligated into the pET-28a(+) vector with the clon Express One Step Cloning Kit (Vazyme Biotech Co., Ltd., China), yielding plasmid pET-28a-Dac. The recombinant plasmid was transformed into the host strain *E. coli* BL21(DE3) to obtain the engineered strain *E. coli*–Dac. Plasmid pET-28a(+) without the insert was transformed into *E. coli* BL21(DE3) cells as a control.

For expression in *B. subtilis*, plasmid pP43NMK was digested with KpnI and PstI and subjected to column purification. The *Dac_{ph}–B* gene was amplified by PCR with gene-specific primers: Dac_{ph}-F2 and Dac_{ph}-R2. The amplicon was processed by gel purification and ligated into the pP43NMK vector by the same recombination method as described above, thereby yielding recombinant plasmid pP43NMK-Dac. The recombinant plasmid was transformed into host strain *B. subtilis* WB600 to obtain engineered strain *B. subtilis*–Dac. Plasmid pP43NMK without the insert was transformed into *B. subtilis* WB600 as the control. All the constructs were confirmed by restriction analysis and DNA sequencing. *E. coli* transformation was performed as described by Sambrook and Russell (2001), and *B. subtilis* transformation was carried out according to the manufacturer's protocol (MoBiTec).

Biocatalyst Preparation

The engineered *E. coli* and *B. subtilis* strains were inoculated into the LB medium containing kanamycin at a final concentration of 50 and 25 mg/l, respectively. The bacteria were cultivated at 37°C overnight on a rotary shaker (220 rpm) to prepare the seed cultures. For preparation of a biocatalyst from *E. coli*, 2% of the seed culture was then inoculated into the fermentation medium(TB) containing 50 mg/l kanamycin and was cultivated at 37°C with shaking at 220 rpm. When optical density at 600 nm (OD_{600}) reached 0.5, which was found to be the optimal time point for Dac_{ph} induction in pilot experiments, IPTG was immediately added to the broth to a final concentration of 0.05 mM [21]. Next, the bacteria were cultivated at 37°C with shaking at 220 rpm for 5 h. For preparation of a biocatalyst from *B. subtilis*, 2% of the seed culture was inoculated into the fermentation medium(TB) supplemented with 25 mg/l kanamycin and was cultivated at 37°C and 220 rpm for 18 h without any inducer. After that, the cells were harvested by centrifugation at 8,000 ×*g* for 10 min at 4°C and washed twice with sterilized water. After that, the cell pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.5) and kept at 4°C for the subsequent biotransformation analysis and further experiments.

Cell Density and Biocatalytic Assays

OD_{600} was measured every 2 h in the growth culture until the stationary phase to construct the growth curve. OD_{600} was determined on a UVmini-1240 spectrophotometer (Shimadzu, Japan) and was converted to dry cell weight (DCW) according to the following equation: DCW (g/l) = (0.4442 × OD_{600}) − 0.021.

Next, the reaction rate was determined in a whole-cell

biocatalytic reaction. Namely, 50 g/l GlcNAc and 18.6 g/l whole-cell biocatalyst were mixed in 50 mM sodium phosphate buffer (pH 7.5). The reaction proceeded in a 250-ml shaking flask and incubated on a rotary shaker at 30°C for 0.5 h. To determine the conversion ratio of GlcNActo GlcN, different concentrations of GlcNAc were added into the sodium phosphate buffer containing 18.6 g/l cells. The reaction was carried out in 250-ml shaking flasks and incubated on a rotary shaker at 30–90°C and pH 5.0–9.0 for 0.5–10 h. The reaction was stopped by adding HCl to the system to a final concentration of 0.1 M, then centrifuged at 8,000 ×*g* for 10 min. The supernatant was recovered for the quantification of GlcNAc and GlcN by HPLC, as described below.

Optimization of pH, Cell Density, Substrate Concentration, and Temperature

For optimization of all the variables, the reaction was conducted in 250-ml shaking flasks containing 20 ml of the reaction mixture and incubated on a rotary shaker for 0.5 h. For pH optimization, the reaction was conducted at 30°C in sodium phosphate buffer (pH 5.0–9.0) containing 50 g/l GlcNAc. For DCW optimization, the conditions were 50 g/l GlcNAc, 30°C, and pH 7.5. To optimize the substrate concentration, the reaction was conducted at pH 7.5, 30°C, with the GlcNAc concentration ranging from 10 to 80 g/l. For temperature optimization, the reaction was carried out at pH 7.5, with temperatures varying between 30°C and 90°C. To optimize the catalytic time, the reaction was carried out at different temperatures (30−90°C) and pH 7.5 for 0.5−5 h. The resulting samples were centrifuged, and the supernatant was tested by HPLC.

Production of GlcN in 3-L Bioreactor

The production of GlcN was performed in 3-L bioreactor (BioFlo 115, New Brunswick Scientific Co., USA) using a 1.4-L reaction mixture. Reactions were performed using the standard whole-cell biocatalytic and the conditions based on the optimal conditions in shaking flask, as described above. Agitation was provided by 2 6-bladed disk turbines. The pH was automatically kept at 7.5 via the addition of 5 M NaOH, and the temperature was maintained at 40°C. The aeration rate and agitation speed were 1.5 vvm and 300 rpm, respectively.

The Stability of GlcN and GlcNAc under the Conversion Conditions

The determination of GlcN and GlcNAc stability is carried out under the optimal conversion conditions. The conditions were 40°C, pH 7.5 (50 mM sodium phosphate buffer) and 50 g/l GLcNAc for incubating 0−5 h without cells. As for the stability of GlcN, the experiments were performed at 40°C, pH 7.5 (50 mM sodium phosphate buffer) and 50 g/l GLcN for 0−5 h without cells. The concentration of GlcN and GlcNAc were measured every 1 h to construct the stability curve were tested by HPLC.

Analytical Methods

GlcN were quantified by HPLC (Agilent1260 series, USA) on a Thermo ODS-2 HYPERSIL C_{18} column (250 mm × 4.0 mm, USA) by the *ortho*-phthalaldehyde (OPA) precolumnde rivatization method [22, 23]. The supernatant of each reaction system was passed through a membrane filter with 0.22-μm pore size, and 10 μl of the filtrate was injected into the HPLC column. The concentrations of GlcNAc and acetate in the supernatant were measured by HPLC on an instrument equipped with an HPX-87H column (Bio-Rad, USA) and a refractive index detector. HPLC was carried out with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min and 40°C. Pure substances served as standards.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% running gel (Bio-Rad Laboratories, USA). The target proteins were released from the cells by sonication, then, samples were incubated in denaturing buffer at 100 40°C for 10 min. The prepared samples and marker were loaded into wells and separated by electrophoresis in MES SDS running buffer, and resolved proteins were visualized by staining with Coomassie Brilliant Blue G250.

Protein concentration was measured by Bradford method (Bradford 1976) using bovine serum albumin as the standard.

The biocatalyst activity was calculated according to the following equation:

$$\text{Activity} = C_{(GlcN)}/(DCW \times T)$$

where $C_{(GlcN)}$ is the concentration (g/l) of GlcN that was generated by the reaction, DCW is expressed in g/l, and T denotes reaction time (min).

The reaction rate is expressed as

$$\text{Reaction rate} = C_{(GlcN)}/T$$

where $C_{(GlcN)}$ is the concentration (g/l) of GlcN that was generated by the reaction, and T represents reaction time (h).

The conversion yield is given by

$$\text{Conversion ratio (\%)} = (M_2/M_1) \times 100$$

where M_1 is the amount (mmol) of GlcNAc before transformation, M_2 is the amount of GlcN that was generated by the reaction.

Statistical Analysis

All the experiments were conducted at least three times, and the results were expressed as mean ± standard deviation (n = 3).

Results and Discussion

Expression of Diacetylchitobiose Deacetylase in *B. subtilis* and *E. coli*

The Dac_{ph} gene (from *P. horikoshii)* after codon optimization was cloned into expression vectors pET-28a (+) and pP43NMK and sequenced, respectively (Fig. S1). The recombinant

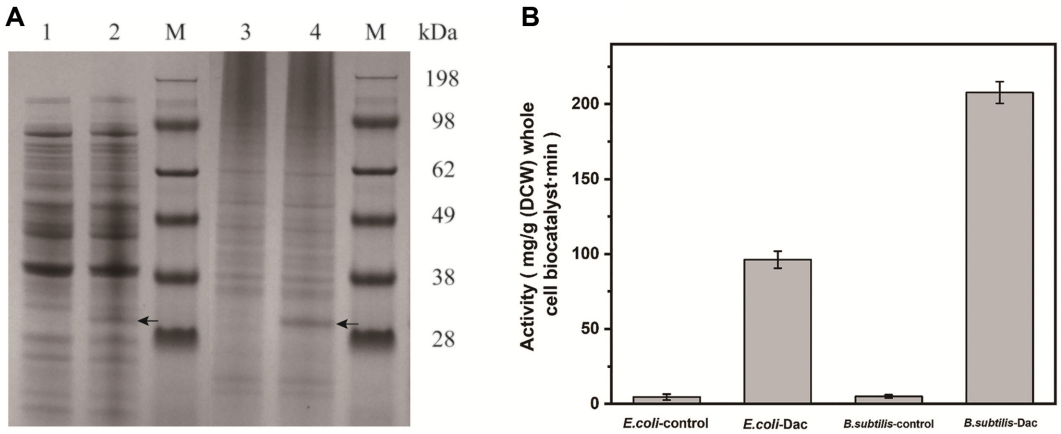

Fig. 2. SDS-PAGE and deacetylase activity analysis of recombinant strains.
(**A**) SDS-PAGE analysis of Dac_{ph} expression in the recombinant strains. M: protein marker, 1: *E. coli*-Control (harboring blank pET-28a (+) plasmid); 2: *E. coli*-Dac (expressing Dac_{ph} enzyme in *E. coli* BL21(DE3)); 3: *B. subtilis*-control (harboring blank pP43NMK plasmid); 4: *B. subtilis*-Dac (expressing Dac_{ph} enzyme in *B. subtilis* WB600). (**B**) Comparison of the whole-cell biocatalytic deacetylase activity of recombinant *E. coli* BL21(DE3) and *B. subtilis* WB600 expressing Dac_{ph} enzyme. *E. coli*-Control, harboring blank pET-28a(+) plasmid; *B. subtilis*-control, harboring blank pP43NMK plasmid; *E. coli*-Dac, expressing Dac_{ph} enzyme in *E. coli* BL21(DE3); *B. subtilis*-Dac, expressing Dac_{ph} enzyme in *B. subtilis* WB600; The data shown are mean values from triplicates with error bars indicating the standard deviation.

plasmids were next transformed into *E. coli* BL21(DE3) and *B. subtilis* WB600, respectively. The expression systems were under the control of the T7 promoter and p43 promoter, respectively. Dac_{ph} was expressed successfully in both host strains (data not shown). The cells were harvested on stationary phase (as shown in Fig. S2). SDS-PAGE analysis showed that both recombinant strains expressed a 32-kDa protein successfully. Obviously, *B. subtilis*-Dac_{ph} had a thicker 32-kDa Dac_{ph} band in the whole-cell fraction (Fig. 2A). Dac_{ph} activity was measured in the whole-cell biocatalysts and in the cytosolic fraction. The recombinant Dac_{ph} activity was 2.2-fold higher in the whole-cell preparation of *B. subtilis* in comparison with *E. coli* (Fig. 2B). On the basis of these results, we proceeded to the biochemical analysis of the whole-cell biocatalytic activity of recombinant-Dac_{ph}-expressing *B. subtilis*.

The Influence of Dac_{ph} Overexpression on Cell Growth

Next, we determined whether the expression of Dac_{ph} affects cell growth of *E. coli* and *B. subtilis*. The cell growth curves of *E. coli* and *B. subtilis* are shown in Fig. S2. Readers can see that the engineered Dac_{ph}-expressing strains grew faster than the control strains (not expressing Dac_{ph}), and the biomass values of the engineered strains were ~2.1-fold greater than those of the control. These data indicated that overexpressed Dac_{ph} from *P. horikoshii* had positive effects on the strains under study in terms of cell growth. The reason for this phenomenon is not clear. Further studies are needed to explain this phenomenon.

Effects of Key Variables on GlcN Production by the Whole-Cell Biocatalysts

The enzymatic properties were evaluated to optimize the key variables of GlcN production by each whole-cell biocatalyst. The effects of pH on GlcN production were evaluated by analyzing the whole-cell biocatalyst reaction rate in Na_2HPO_4–NaH_2PO_4 buffers at pH levels ranging from 5.0 to 9.0 (Fig. 3A). The highest biocatalyst reaction rate (17.9 $g \cdot l^{-1} \cdot h^{-1}$) was obtained when bioconversion was performed at pH 7.5, generally in agreement with one report showing that the optimal pH for GlcNAc catalysis is 8.0 [13]. The effect of the biocatalyst concentration on biocatalyst reaction rate was evaluated next. The highest reaction rate was 17.8 $g \cdot l^{-1} \cdot h^{-1}$ at 18.6 g/l cells Meaning needs verification (Fig. 3B). To determine the optimal substrate concentration for bioconversion, the reactions were run at GlcNAc concentrations ranging from 10 to 80 g/l. The highest reaction rate was 18.9 $g \cdot l^{-1} \cdot h^{-1}$ at 50 g/l substrate (Fig. 3C). Fig. 3D shows the influence of reaction temperatures ranging from 30°C to 90°C. The reaction rate increased with the increase of temperature. The highest reaction rate was 49.6 $g \cdot l^{-1} \cdot h^{-1}$ at 80°C. This finding is consistent with results of a report showing that Dac_{ph} has excellent thermal stability, retaining activity at 85°C for

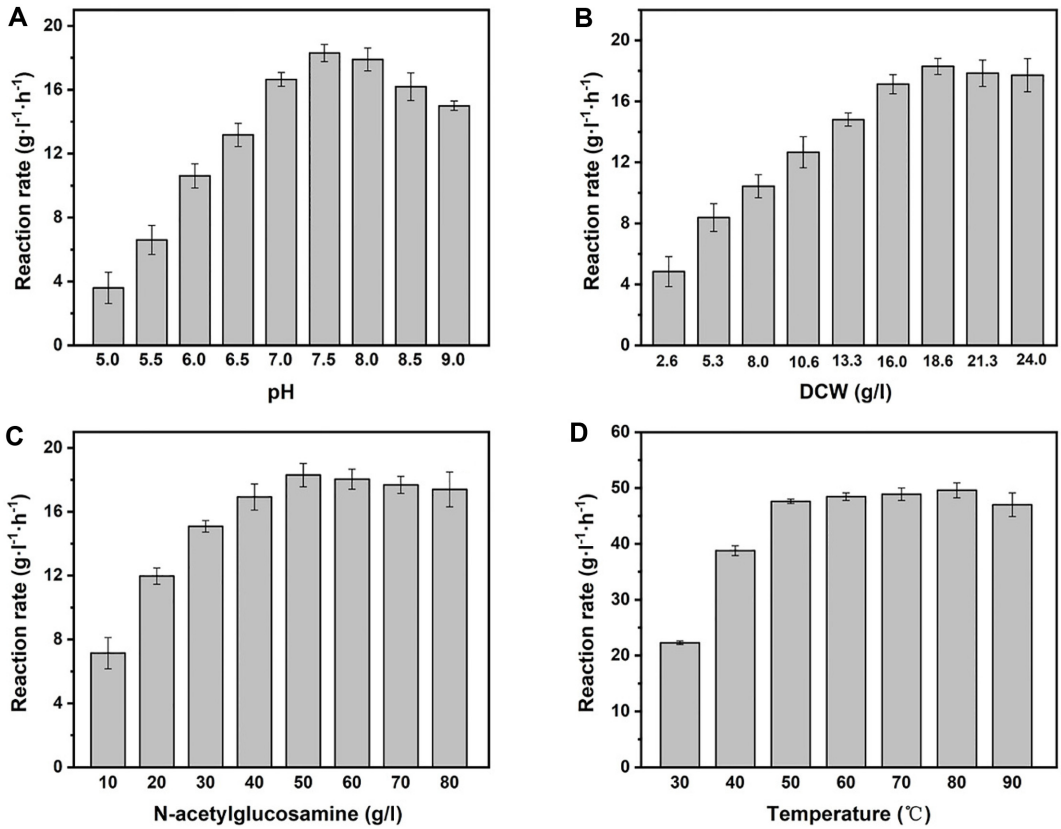

Fig. 3. The effect of pH, DCW, substrate and temperature on whole-cell biotransformation.
(**A**) The reaction was conducted at 30°C in sodium phosphate buffer (pH 5.0–9.0) containing 50 g/l GlcNAc. (**B**) The reaction was performed at the condition of 50 g/l GlcNAc, 30°C, and pH 7.5. (**C**) The reaction was conducted at pH 7.5, 30°C, with the GlcNAc concentrations ranging from 10 to 80 g/l. (**D**) The reaction was carried out at pH 7.5, with temperatures varying between 30°C and 90°C. Results were obtained with recombinant *B. subtilis* whole-cell biocatalyst. The data shown are mean values from triplicates with error bars indicating the standard deviation.

more than 30 min [21]. Product inhibition of Dac_{ph} was assessed by measuring GlcN production. The biocatalyst reaction rate decreased sharply as GlcN concentration increased (Fig. S3).

The stability of GlcN was influenced by the temperature and pH. According to one study, the degradation of GlcN increases with the increasing temperature and pH [8]. As shown in Fig. 3, we measured the GlcN concentration in the reaction system to construct the curve of yield of GlcN over time at different conversion temperatures (ranging from 30°C to 90°C).

According to the Fig. 3D, the highest reaction rate was 49.6 $g \cdot l^{-1} \cdot h^{-1}$ when the temperature was higher than 50°C. But as shown in Fig. 4, the yield of GlcN decreased sharply at higher temperatures. Therefore, this bioconversion wasted a lot of GlcN when performed at a higher temperature. This phenomenon is reasonable. One possible explanation for the phenomenon is that the stability of GlcN was obviously influenced by the temperature at pH 7.5; the degradation of GlcN increases with the increasing temperature. When the reaction was performed at a higher temperature, the production of GlcN occurs at a higher reaction rate. However, the GlcN, deacetyled from GlcNAc, was unstable in the current high temperature environment, and can be converted into other derivatives. As shown in Fig. 4, the yield of GlcN steadily increased when the reaction temperature was lower than 40°C.

In addition, we measured the stability of substrate (GlcNAc) and product (GlcN) under the optimal bioconversion conditions, the experiments were carried out with standard substrate and product in the reaction buffer solution (50 mM sodium phosphate buffer, pH 7.5) without cells at 40°C. According to the Fig. S4, the substrate (GlcNAc) was stable at the optimal bioconversion conditions. When the incubation time was 3 h, the end of biocatalytic production of GlcN, the concentration of GlcNAc decreased from 50 to

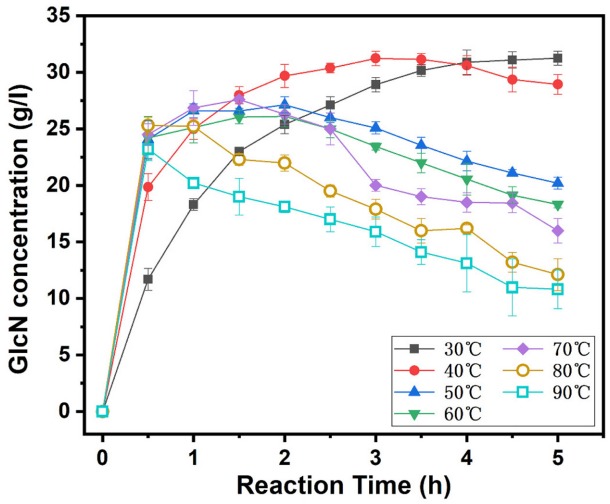

Fig. 4. Time profile of the GlcN production (g/l) under different reaction temperatures (30–90°C).

Symbol denotes: ■, 30°C; ●, 40°C; ▲, 50°C; ▼, 60°C; ◆, 70°C; ○, 80°C; □, 90°C. Results were obtained with recombinant *B. subtilis* whole-cell biocatalyst. The reactions were conducted at pH 7.5 in sodium phosphate buffer containing 50 g/l GlcNAc and 18.6 g/l cells. The data shown are mean values from triplicates with error bars indicating the standard deviation.

49.3 g/l and only 1.4% reduction. When the incubation time was extended to 5 h, the concentration of GlcNAc was 48.6 g/l and decreased by 2.8%. As shown in Fig. S4, under the optimal reaction conditions, the GlcN was relatively stable in the first 3 h. The concentration of GlcN decreased from 50 to 48.1 g/l and only 3.8% reduction. When the incubation time was extended, the concentration of GlcN decreased rapidly, the concentration of GlcN was 40.75 g/l at 5 h and decreased by 18.9%.

Production of GlcN by the Whole-Cell Biocatalysts

To use the whole-cell biocatalyst for GlcN production, the conversion ratio and the yield of GlcN were evaluated under different conditions. According to the above data, we obtained 30.2 g/l GlcN when the reaction was performed at 40°C, pH 7.5, 18.6 g/l cells, and 50 g/l GlcNAc in 250-ml shaking flask. In order to optimize the reaction time and determine the trend of pH in the catalytic system, bioconversion was evaluated in a shaking flask containing 18.6 g/l cells and 50 g/l GlcNAc at 40°C and pH 7.5. The highest yield of GlcN was obtained at 3 h and then decreased. The pH value in the catalytic system was gradually decreased from 7.5 to 6.3. For the conversion efficiency and economical industrial application, 3 h seems to be the ideal operation duration. Overall, the optimal conditions were 18.6 g/l cells at 40°C, pH 7.5 for 3 h (Fig. 5A).

To attain a higher titer of GlcN, amplification experiments of GlcN production were carried out in 3-L bioreactor

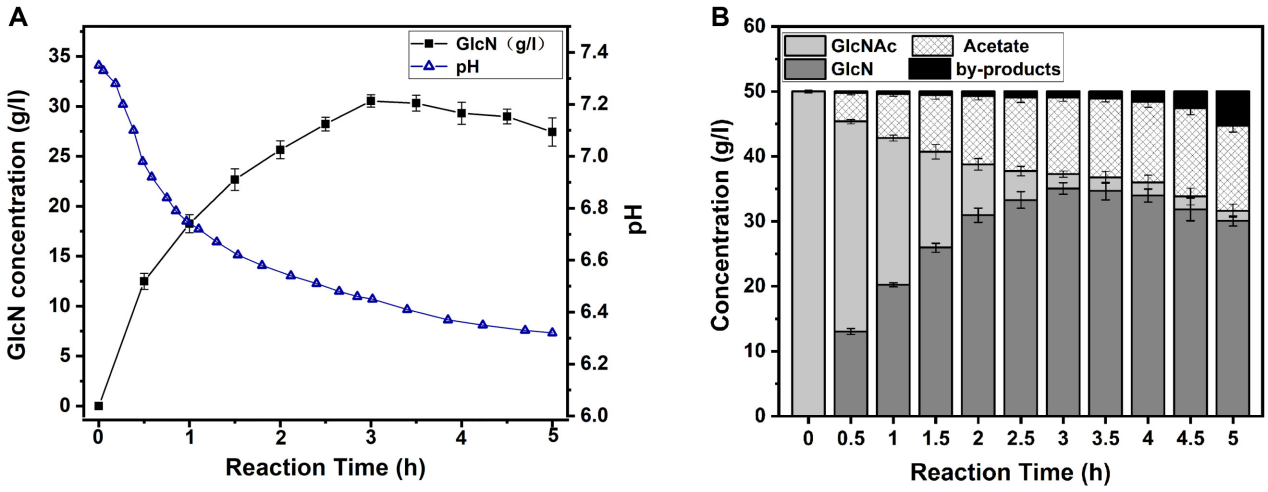

Fig. 5. Time profile of various parameters in different catalytic systems.

(**A**) Time course of GlcNproduction (■) and pH value (△) catalyzation in a 250-ml shaking flask. Results were obtained with recombinant *B. subtilis* whole-cell biocatalyst. The reaction was performed at 40°C in sodium phosphate buffer (pH 7.5) containing 50 g/l GlcNAc and 18.6 g/l cells. The data shown are mean values from triplicates with error bars indicating the standard deviation. (**B**) Time profile of various substances concentrations in the 3-L bioreactor catalytic system. The reaction was performed in 3-L bioreactor under the optimal conditions. The pH was automatically kept at 7.5 via the addition of 5 M NaOH, and the temperature was maintained at 40°C. Results were obtained with recombinant *B. subtilis* whole-cell biocatalyst. The data shown are mean values from triplicates with error bars indicating the standard deviation.

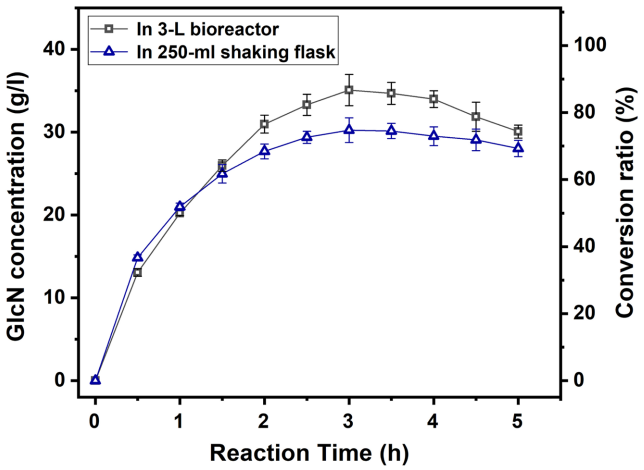

Fig. 6. Comparison of the GlcNproduction during the conversion between catalyzed in 3-L bioreactor (□) and 250-ml shaking flask (△).

Both reactions were performed under the optimal conditions, 40°C, pH 7.5, 18.6 g/l cells and 50 g/l GlcNAc. The data shown are mean values from triplicates with error bars indicating the standard deviation.

based on the optimal conditions in shaking flask. The concentration of various substances in the catalytic system varies with time were determined. The trend was generally consistent with shaking flask. The highest yield of GlcN (35.3 g/l) was obtained at 3 h and then decreased (Fig. 5B). The yield of GlcN and conversion ratio in 3-L bioreactor were improved, where environmental conditions are well-controlled. The maximal titer of GlcN increased from 30.2 to 35.3 g/l, and the molar conversion ratio of GlcNAc to GlcN increased from 74.7% to 86.8% (Fig. 6).

In summary, we successfully devised a whole-cell biotransformation method for GlcN production by expressing diacetylchitobiose deacetylase from *P. horikoshii* in *E. coli* and *B. subtilis*. By means of the latter engineered strain as a biocatalyst, the maximal titer of GlcN was 35.3 g/l after incubation of the engineered *B. subtilis* (18.6 g/l) with 50 g/l GlcNAc for 3 h at 40°C and pH 7.5. The conversion ratio of GlcNAc was 86.8% under these conditions. Further improvement of the biocatalyst activity is necessary to obtain a higher titer of GlcN for industrial production of GlcN. The performance of the biocatalyst may be improved in the future by modifying the catalytic domain of Dac_{ph}. As for substrate binding and product inhibition, it is possible to create highly active and product-noninhibited enzyme mutants by genetic and protein engineering. Moreover, bio-based production provides attractive alternatives to overcome drawbacks in the process of chemical synthesis, like unstable intermediates, multistep reactions, and complex process control, to name a few, it is worthwhile to develop such an environmentally friendly bio-based approach for industrial production of GlcN.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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