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Nitric oxide mediates positive regulation of *Nostoc flagelliforme* polysaccharide yield via potential S-nitrosylation of G6PDH and UGDH

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Abstract

Based on our previous findings that salicylic acid and jasmonic acid increased *Nostoc flagelliforme* polysaccharide yield by regulating intracellular nitric oxide (NO) levels, the mechanism through which NO affects polysaccharide biosynthesis in *Nostoc flagelliforme* was explored from the perspective of S-nitrosylation (SNO). The addition of NO donor and scavenger showed that intracellular NO had a significant positive effect on the polysaccharide yield of *N. flagelliforme*. To explore the mechanism, we investigated the relationship between NO levels and the activity of several key enzymes involved in polysaccharide biosynthesis, including fructose 1,6-bisphosphate aldolase (FBA), glucokinase (GK), glucose 6-phosphate dehydrogenase (G6PDH), mitochondrial isocitrate dehydrogenase (ICDH), and UDP-glucose dehydrogenase (UGDH). The enzymatic activities of G6PDH, ICDH, and UGDH were shown to be significantly correlated with the shifts in intracellular NO levels. For further validation, G6PDH, ICDH, and UGDH were heterologously expressed in *Escherichia coli* and purified via Ni^{+}-NAT affinity chromatography, and subjected to a biotin switch assay and western blot analysis, which revealed that UGDH and G6PDH were susceptible to SNO. Furthermore, mass spectrometry analysis of proteins treated with S-nitrosoglutathione (GSNO) identified the SNO modification sites for UGDH and G6PDH as cysteine 423 and cysteine 249, respectively. These findings suggest that NO modulates polysaccharide biosynthesis in *N. flagelliforme* through SNO of UGDH and G6PDH. This reveals a potential mechanism through which NO promotes polysaccharide synthesis in *N. flagelliforme*, while also providing a new strategy for improving the industrial production of polysaccharides.

Keywords Nitric oxide, *Nostoc flagelliforme*, Polysaccharides, S-nitrosylation

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Introduction

Polysaccharides are secondary metabolites that play a crucial role in the survival of microorganisms [1]. *Nostoc flagelliforme* is a terrestrial cyanobacterium that secretes abundant extracellular polysaccharides to protect itself from external stresses [2, 3]. In addition, studies have shown that *N. flagelliforme* polysaccharides exhibit a multitude of physiological effects, mainly including immunomodulation, anti-aging, blood pressure reduction, lipid level modulation, antitumor, antiviral, radioprotective, antioxidant, and anti-inflammatory activities [4]. Nevertheless, the limited yield of polysaccharides remains a significant challenge, hindering the wider application of these compounds in the field of biomedicine [5, 6]. Many studies aiming to improve the yield of polysaccharides in *N. flagelliforme* investigated the effect of various stress conditions [2, 7, 8]. A previous study found that salicylic acid (SA) and jasmonic acid (JA) significantly increased the accumulation of polysaccharides in *N. flagelliforme* by increasing the intracellular nitric oxide (NO) levels, but the mechanism of action was not clear [9].

NO is known to mediate a variety of physiological and pathological processes, including cell proliferation, differentiation, and inflammation. Moreover, studies have shown that NO can directly promote the synthesis of polysaccharides [9–11]. Liu et al. found that the addition of exogenous NO donors significantly increased the production of extracellular and intracellular polysaccharides during the deep fermentation process of *Ganoderma lucidum* [12]. However, there is still no comprehensive analysis of glycolytic intermediates and the potential link between NO signaling and polysaccharide biosynthesis.

Protein S-nitrosylation (SNO) is a reversible post-translational modification that has emerged as an important mechanism capable of finely regulating protein activity and functionality [13]. SNO proceeds via the addition of a nitroso group to the reactive sulfhydryl group of cysteine to form S-nitrosothiols for NO-mediated signaling. Extensive studies have also shown that SNO is capable of storing, transporting, and releasing NO in organisms, which plays a crucial role in the physiological and metabolic regulation of cells [14–17]. This modification allows cells to protect themselves against oxidative stress, while also mediating vascular tone regulation, immune regulation and anti-inflammatory effects through various signaling pathways [13, 18]. Here, we explored the mechanism through which intracellular NO affects polysaccharide biosynthesis in *N. flagelliforme* from the perspective of SNO.

A comparison of the effects of the respective addition of NO donors and scavengers on intracellular NO content and polysaccharide yield showed that intracellular NO levels had significant positive effects on the polysaccharide yield of *N. flagelliforme*. To explore the underlying mechanism, enzymes related to the polysaccharide synthesis pathway of *N. flagelliforme* were analyzed. Previous studies on the effects of culture conditions on polysaccharide yield and polysaccharide synthesis activity in *N. flagelliforme* showed that UDP-glucose dehydrogenase (UGDH) is the key enzyme regulating the polysaccharide yield [2]. Furthermore, it was found that SA and JA had a significant effect on the enzymatic activities of fructose 1,6-bisphosphate aldolase (FBA), glucokinase (GK), glucose 6-phosphate dehydrogenase (G6PDH), and mitochondrial isocitrate dehydrogenase (ICDH). Crucially, it was also shown that SA and JA could also increase the polysaccharide yield by regulating NO levels [9]. Therefore, FBA, GK, G6PDH, ICDH and UGDH were selected for SNO analysis, the latter three of which were screened out based on the relationship between their enzymatic activities and intracellular NO levels. Subsequently, G6PDH, ICDH and UGDH were heterologously expressed and purified, after which the SNO levels of the three enzymes were determined using a biotin switch assay and western blot analysis. In addition, the modification sites of S-nitrosoglutathione (GSNO) modified proteins were further determined by mass spectrometry. These findings reveal the mechanism through which NO regulates polysaccharide biosynthesis in *N. flagelliforme*. This study therefore provides new perspectives and research directions for further exploring the mechanism of polysaccharide biosynthesis.

Materials and methods

Materials and reagents

The *N. flagelliforme* TCCC11757 used in this study was provided by the Tianjin Key Laboratory of Industrial Microbiology (Tianjin, China).

Carboxy-PTIO (C-PTIO), sodium nitroprusside (SNP), isopropyl β-D-thiogalactoside (IPTG), proteinase inhibitor (PMSF), BCA protein assay kit, and GSNO were purchased from Solarbio (Beijing, China). The fluorescent probe DAF-FM DA NO was purchased as part of a kit from Beyotime (Shanghai, China). Kaumas brilliant blue, bromophenol blue, sodium ascorbate, and glutathione were purchased from Maclean (Shanghai, China). Biotin-HPDP was purchased from Thermo Fisher (Massachusetts, USA). Anti-biotin rabbit IgG was purchased from Cell Signaling Technology (Boston, USA). Goat anti-rabbit IgG was purchased from Anjoron Bio Co (Beijing, China). Unless specified otherwise, all chemicals were of analytical grade.

Cell culture and determination of intracellular NO content, polysaccharide content, and enzyme activity

N. flagelliforme was cultured in BG-11 medium ($BG11_0$ with 1.5 g/L $NaNO_3$ as the nitrogen source) [19]. The cells were cultured in 500 mL shake flasks containing 200

mL of BG-11 medium under continuous illumination of 1000 lx at 25℃ for 16 days. The initial cell suspension in the flask was adjusted to an optical density at 750 nm of approximately 1.0 as a seed suspension [20]. The effects of different sources of NO on the enzyme activity were screened out according to a previously published method [9]. On the sixth day of cultivation, C-PTIO (80 μmol/L) or SNP (125 μmol/L) was added, while no inducer was added in the control group.

The intracellular NO content was determined using the fluorescent probe DAF-FM DA NO assay kit. The cells from 1 mL of culture broth were collected by centrifugation at 4000 rpm and 4 °C for 10 min. Then, 200 μL of 5 μM DAF-FMDA was added and incubated at 37 °C for 40 min in the dark. Subsequently, the DAF-FMDA that did not enter the cells was removed by washing three times with PBS. Then, the cells were resuspended in 200 μL of PBS and the fluorescence intensity was measured (excitation wavelength 495 nm, emission wavelength 515 nm). Polysaccharide content was determined using the phenol sulfate method [21]. The polysaccharide sample, distilled water, 6% phenol and concentrated sulfuric acid were shaken in a vortex mixer at a volume ratio of 1:1:1:5. After standing for 30 min, the absorbance was measured at 490 nm. The values were compared to a standard curve to calculate the polysaccharide concentration.

To determine the activity of enzymes related to polysaccharide biosynthesis in *N. flagelliforme*, a crude enzyme solution was obtained according to a previously described method [22]. To obtain the crude enzyme solution, on the day of the end of the *N. flagelliforme* cell culture cycle, the cyanobacteria were collected, disrupted, and centrifuged to collect the supernatant. The protein concentration was determined using the BCA protein kit. Then, the enzyme activity was measured using a UV spectrophotometer as described before [23]. One unit of enzyme activity was defined as the amount of protein that generates 1 μmol of NADH per minute. The enzyme activity was calculated using the formula:

$$1\mathrm{U}\left(\frac{\mu\,\mathrm{mol}}{\mathrm{min}}\right)=\frac{\Delta A}{\Delta t}\times\frac{V_1\times\ \text{dilution factor}}{V_2\times\ \epsilon\ \times\ d\times\ Cpr}$$

where V_1 is the total volume of the reaction system (0.25 mL), V_2 is the volume of the enzyme solution added (0.03 mL), ε is the molar extinction coefficient (6.22×10^6 mL/mol/cm for NADH), d is the light path (0.5 cm), Δt is the reaction time, and Cpr is the protein concentration (mg/mL).

Construction of recombinant plasmids, protein expression and purification

The coding sequences of the *icdh*, *g6pdh*, and *ugdh* genes were amplified from the genome of *N. flagelliforme* CCNUN1 using specific primers (Supplemental Table 1), after which the purified PCR fragment was digested cloned into pET-28a (+) [24]. Recombinant plasmids were transferred into competent cells of *E. coli* BL21 (DE3). Protein expression was induced by adding IPTG [25]. The cells were collected by centrifugation at 8000 rpm and 4 °C for 15 min, suspended in disruption buffer supplemented with lysozyme and the protein inhibitor PMSF, and disrupted by sonication. The supernatant containing crude protein was collected. The crude protein was loaded onto a Ni^+-NAT agarose column (Qiagen, Shanghai, China) and eluted with different concentrations of imidazole. Aliquots of protein fractions were analyzed by SDS-PAGE, and selected fractions were pooled and concentrated using Amicon ultra centrifugal filter devices (Millipore, Massachusetts, USA.) [26]. Purified protein was stored at -20℃ after lyophilization. The protein concentration was determined using a BCA protein assay kit.

SNO detection of ICHD, G6PDH and UGDH

SNO was detected using a previously published in vitro biotin switch assay [27, 28]. GSH and GSNO were respectively added into the protein sample in the dark to a final concentration of 200 μmol/L (GSH treatment was used as the negative control, and two replicates were prepared for GSNO treatment). The above samples were put into a thermoblock and reacted at 23℃ and 800 rpm for 30 min. Then, protein samples were resuspended in 200 μL blocking buffer, placed in a thermoblock, and reacted at 50℃ and 1200 rpm for 40 min. Proteins were collected by adding 10 μL (500 mmol/L) sodium ascorbate and 10 μL (4 mmol/L) biotin HPDP (including one sample without sodium ascorbate but with 10 μL HENS buffer as another negative control for GSNO treated samples) and incubated for another 1 h at 23 °C and 800 rpm. Biotin-HPDP was removed by the acetone precipitation method after biotinylation. The protein was re-suspended in the HENS buffer, and an SDS loading buffer without a reducing agent was added. An aliquot of the samples was subjected to western blot analysis [29]. The SDS-PAGE protein bands were electroblotted to a nitrocellulose membrane at 220 V and 65 mA for 2 h. The membrane was then incubated with a rabbit anti-biotin primary antibody (1/1000) overnight at 4℃, followed by incubation with the secondary antibody (1/5000) for 1 h [30]. The specific protein bands were visualized using an Odyssey Infrared Imaging System (Gene Company Limited).

Detection of SNO modification sites in UGDH and G6PDH

The appropriate protein band was hydrolyzed with chymotrypsin and trypsin, and the peptide fragments were desalted using a self-filling desalination column. Then, the solvent was volatilized in a vacuum centrifuge at

45℃. Subsequently, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was used to detect the SNO sites of the proteins. The LC-MS/MS conditions are detailed in the supplementary materials. Lastly, bionic was used to analyze and retrieve information from the original target protein database. Parameters were set as follows: the protein modifications were set to aminomethylation (C) (variable), acetyl (protein N-term), oxidation (M) (variable), and SNO (C) (variable). The enzyme specificities were set to chymotrypsin, trypsin, and Asp-N with a maximum leak rate of 3. The mass tolerance of the precursor ions was set at 20 ppm and the MS/MS tolerance was set to 0.02 Da. Only peptides with high-confidence identification were selected for downstream protein identification analysis.

Statistical analysis

Each experiment was repeated three times to ensure the reproducibility of the results. The significance of differences was assessed using a paired samples t-test in SPSS 20.0 [31], with $p<0.01$ (**) considered a highly significant difference, $p<0.05$ (*)a significant difference, and $p>0.05$ as a non-significant difference.

Results

Effect of intracellular NO on polysaccharide biosynthesis in *N. flagelliforme*

In experimental studies, SNP is commonly used as an NO donor and C-PTIO as a scavenger [32, 33]. To verify the effect of intracellular NO content on polysaccharide biosynthesis in *N. flagelliforme*, SNP and C-PTIO were added exogenously, after which the NO content and polysaccharide yield were measured. Figure 1A and

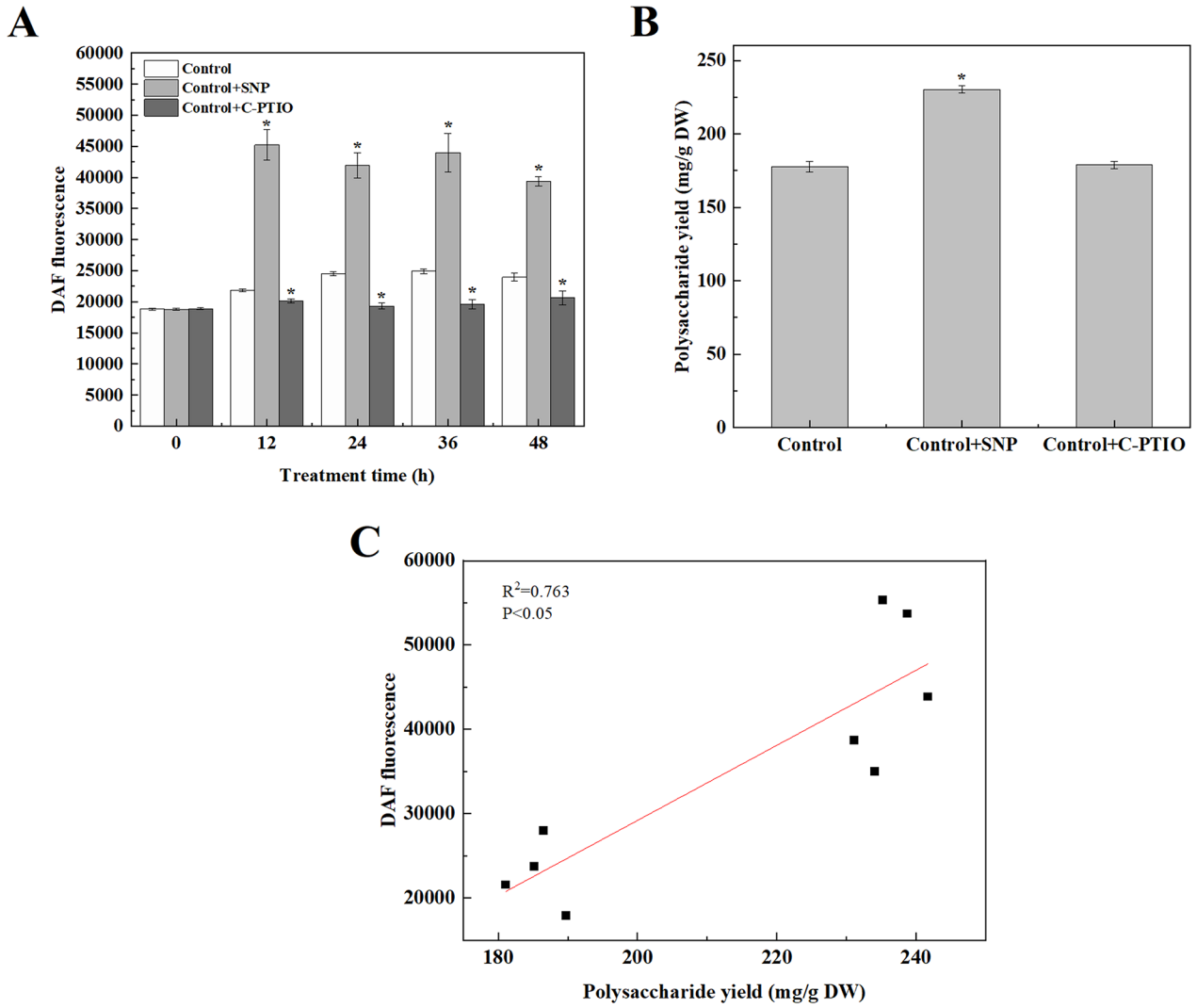

Fig. 1 Effect of SNP and C-PTIO on the NO content (**A**) and polysaccharide yield (**B**) of *N. flagelliforme*, as well as their correlation (**C**). * $p<0.05$, compared with the control group

B show the effects of SNP and C-PTIO on NO content and polysaccharide yield in *N. flagelliforme*, respectively. Compared with the control group, the NO content and polysaccharide yield of *N. flagelliforme* increased with the addition of SNP. However, the NO content and polysaccharide yield of *N. flagelliforme* did not change significantly with the addition of C-PTIO. Further statistical analysis showed a positive correlation between intracellular NO content and polysaccharide yield in *N. flagelliforme* (Fig. 1C), which was consistent with previous finding [9].

Effects of NO on the enzymatic activities of polysaccharide biosynthesis in *N. flagelliforme*

The correlation between five key enzymes related to polysaccharide synthesis (FBA, GK, G6PDH, ICDH, and UGDH) and the intracellular NO content was evaluated, and three enzymes (G6PDH, ICDH, and UGDH) were singled out for in-depth investigation. Figure 2 shows the correlation between the enzymatic activity of enzymes involved in polysaccharide biosynthesis and alterations of intracellular NO levels influenced by different inducers and scavengers, including JA, SA, sodium nitroprusside (SNP), as well as combined salicylic acid and C-PTIO (SA-C), or combined jasmonic acid and C-PTIO (JA-C). Previous research demonstrated that intracellular NO can be modulated by SA, JA, SNP, and C-PTIO [9]. Moreover, SNP has gained significant recognition as an exogenous NO donor, while the NO scavenger C-PTIO is widely utilized to investigate the role of NO in plant stress tolerance by effectively reducing NO concentrations in living tissues [34]. As shown in Fig. 2A and B, the enzyme activities of FBA and GK were not significantly correlated with intracellular NO levels. However, the enzymatic activity of G6PDH respectively increased by 33%, 36%, and 40% under the influence of SA, JA, and SNP (Fig. 2C). Similarly, the enzymatic activity of ICDH was enhanced by 90%, 200%, and 160%, respectively (Fig. 2D). Furthermore, the enzyme activities of G6PDH and ICDH were inhibited by SA-C and JA-C (Fig. 2C and D). At the same time, the activity of UGDH decreased by 18%, 41%, and 17% (Fig. 2E), which was significantly different from that of the control. In summary, changes of intracellular NO content influenced the enzyme activity levels of G6PDH, ICDH, and UGDH.

Construction of recombinant plasmids and protein expression

To investigate the SNO levels of the aforementioned three enzymes, the proteins were heterologously expressed in in *E. coli* and purified. Figure S1 illustrates the gel electrophorograms of the corresponding recombinant plasmids (ICDH, UGDH, and G6PDH), depicting their construction and protein expression profiles. The coding sequences of *icdh*, *ugdh*, and *g6pdh* were obtained by PCR, and the amplicons had the expected sizes (Figures S1A, B, and C). The purified PCR fragments were cloned into pET-28a, after which the constructs were confirmed by Sanger sequencing and comparison with the corresponding sequences in the NCBI database.

For efficient protein expression, the recombinant plasmids were introduced into *E. coli* BL21 (DE3). Single colonies were picked and verified by colony PCR. As illustrated in Figures S1E, F, and G, gel electrophoresis revealed sharp and well-defined bands, confirming the successful retention of the heterologous sequences in the BL21 (DE3) host.

The expression and purification of ICDH, UGDH, and G6PDH was performed according to the method described in 2.3. The soluble proteins were separated by SDS-PAGE electrophoresis, as depicted in Figures S1H, I, and J. The molecular weights of the purified proteins consistently matched their anticipated sizes, thus validating the successful purification of ICDH, UGDH, and G6PDH.

SNO modifications in recombinant ICDH, UGDH, and G6PDH

To better understand the mechanism through which NO regulates enzyme activity, the SNO levels of three enzymes were studied using a biotin switch assay and western blot analysis. The biotin switch assay has been widely employed in previous studies for the assessment of SNO levels [35]. GSNO is a bioactive NO donor used to induce SNO in recombinant proteins [36]. In this assay, sodium ascorbate is employed as a reducing agent to convert S-nitrosated modifications into free sulfhydryl groups, which are subsequently labeled with biotin [28]. In the control group, glutathione (GSH) treatment of GSNO did not influence the SNO levels of the enzymes, while a reduced sample without ascorbate served as the negative control. Each purified protein contained a hexahistidine tag, allowing the quantification of spiked protein levels through the use of antibodies tailored to bind specifically to the tag. As shown in Figure 3, UGDH and G6PDH exhibited varying degrees of SNO, while ICDH did not undergo SNO modification.

Determination of the modified sites in SNO-UGDH and SNO-G6PDH

UGDH and G6PDH proteins were treated with GSNO for MALDI-MS analysis, which identified the sites of S-nitrosylation. In the case of the GSNO-treated recombinant UGDH protein, Cys423 was identified as the SNO site within the trypsin fragment (Fig. 4A). Similarly, for the GSNO-treated recombinant G6PDH protein, Cys249 was pinpointed as the SNO residue in the trypsin fragment (Fig. 4B). These results indicate that the Cys423 residue of UGDH and the Cys249 residue of G6PDH are

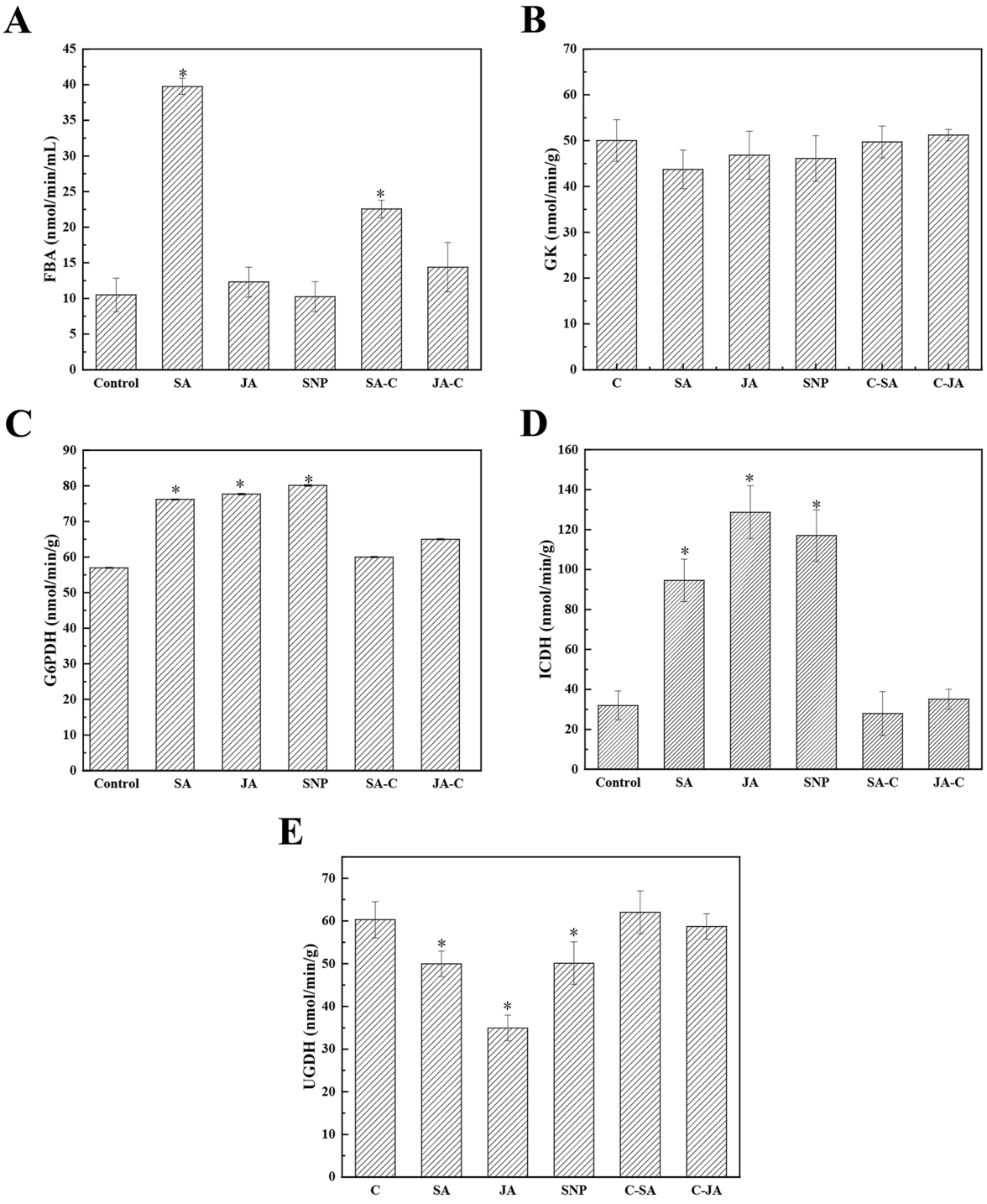

Fig. 2 Effect of NO on the enzymatic activities of enzymes related to polysaccharide biosynthesis in *N. flagelliforme*. (**A**) FBA enzyme activity, (**B**) GK enzyme activity, (**C**) G6PDH enzyme activity, (**D**) ICDH enzyme activity, and (**E**) UGDH enzyme activity. * $p < 0.05$, compared with the control group

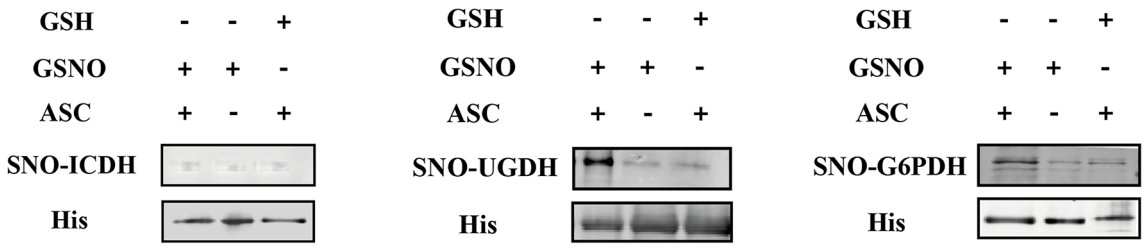

Fig. 3 The SNO modification levels of ICDH, UGDH, and G6PDH were assessed by western blot analysis. Recombinant proteins were supplemented with GSH or GSNO and harvested by adding 500 mM sodium ascorbate and 4 mM biotin-HPDP (one replicate without sodium ascorbate was included as a negative control) and incubated for a further 1 h at 23 °C and 800 rpm. Proteins were resolved by non-reducing SDS-PAGE and transferred to nitrocellulose membranes for western blotting with anti-biotin antibodies. The blot has been cropped for presentation purposes, the full uncropped blots images are included in the Supplementary materials

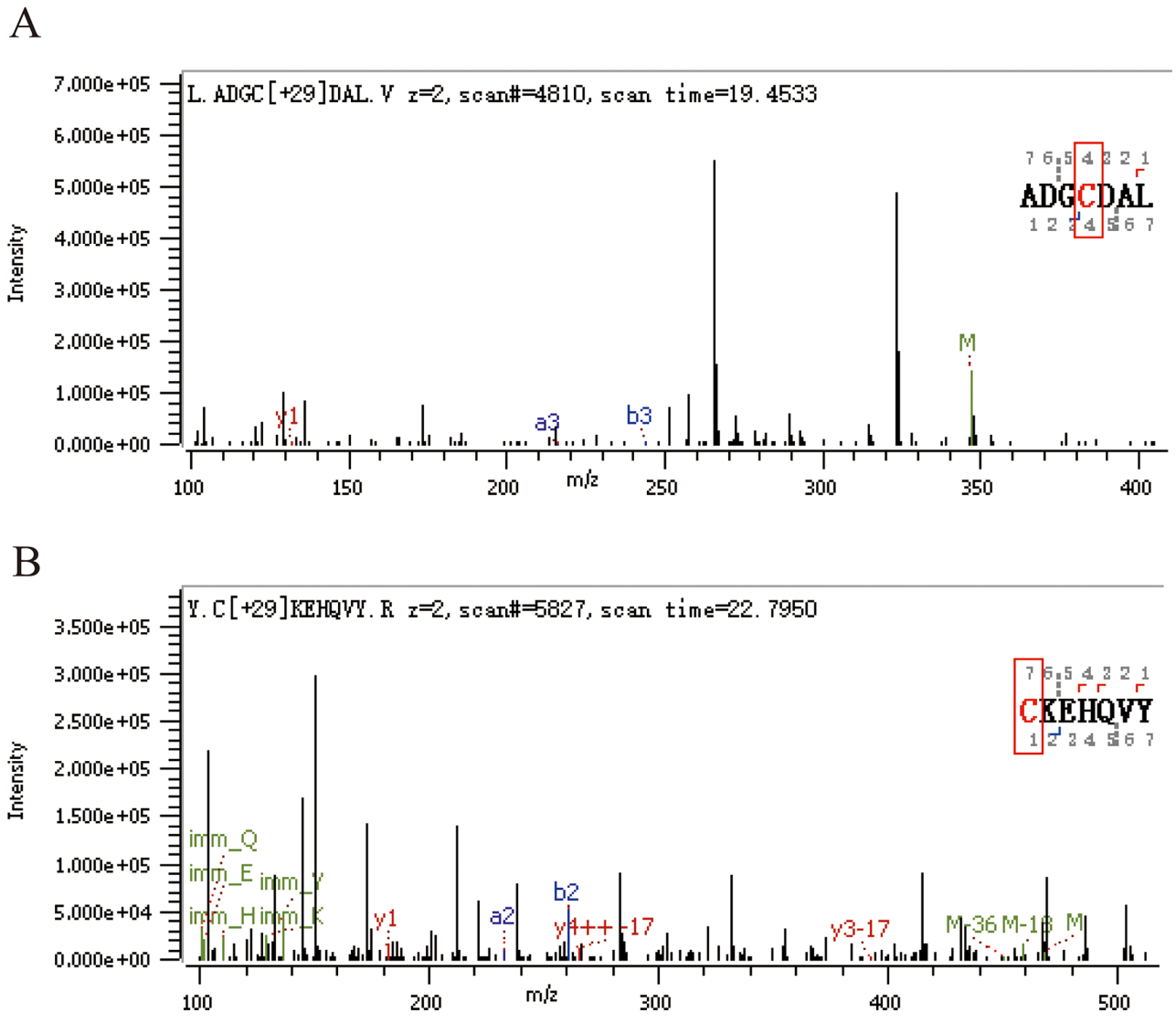

Fig. 4 MS identification of SNO sites of UGDH (**A**) and G6PDH (**B**)

modification sites for SNO, and the molecular weight of both proteins increased by 29 Da after S-nitrosylation, consistent with the expected value.

Discussion

It was shown that the intracellular NO content of *N. flagelliforme* was positively correlated with the polysaccharide yield. However, the underlying mechanism was poorly understood. In recent years, more attention has been given to S-nitrosylation, a redox-based posttranslational protein modification based on covalently linking an NO group to the reactive thiol of a cysteine residue. Lin et al. revealed the molecular mechanisms by which NO affects plant responses to salt stress, whereby NO regulates vesicular transport and ion homeostasis through S-nitrosylation of RAB7, thereby influencing its interaction with PI4P [37]. Here, the mechanism through which NO affects polysaccharide biosynthesis in *N. flagelliforme* was explored from the perspective of S-nitrosylation.

Three enzymes (G6PDH, ICDH and UGDH) were screened out as having a significant correlation with changes of intracellular NO levels by determining the relationship between the activities of enzymes related to polysaccharide biosynthesis in *N. flagelliforme* and intracellular NO levels. It was previously shown that NO increased G6PDH activity and expression in soybean roots under drought stress [38]. In addition, NO can stimulate the pentose phosphate pathway, and under its influence, the enzymatic activity of G6PDH was found to be enhanced, leading to a redistribution of carbon fluxes in the central metabolism of *N. flagelliforme* [39]. This shift in turn increased the conversion of carbon into polysaccharides, which accumulate intracellularly. These polysaccharides may serve diverse cellular functions, such as maintaining energy homeostasis, resisting environmental stresses, and storing energy. Studies likewise suggested a correlation between ICDH enzyme activity and intracellular NO levels [40, 41]. However, the reduction of UGDH activity due to intracellular NO may result from structural alterations in UGDH or changes in its catalytic mechanism. Recently, the oxidative stress agent methyl viologen was shown to enhance the activities of enzymes related to polysaccharide biosynthesis and significantly increase polysaccharide accumulation in cyanobacteria [42]. This is similar to the phenomenon observed in this study, in which the intracellular NO content affected enzyme activity and thus increased the polysaccharide yield of *N. flagelliforme.* Therefore, the subsequent experiments were primarily centered on these three enzymes.

The G6PDH, ICDH, and UGDH proteins were heterologously expressed in *E. coli* and purified. Subsequently, the SNO modification levels of the three proteins were detected using a biotin switch assay and western blot analysis, which showed that UGDH and G6PDH could undergo SNO. In 2021, Francisco et al. also showed that G6PDH can be modified by SNO when NO regulates the NADPH-generating enzyme system in higher plants [43]. Similarly, Smolinski et al. proposed that G6PDH can undergo some degree of SNO. In the goldenrod gall fly *Eurosta solidaginis*, this enzyme promotes cryoprotectant synthesis by undergoing nitrosylation to modify the structure and function of the enzyme to adapt to the increased demand for NADPH at low temperatures. [44]. In addition, it was found that the protein function of G6PDH is regulated through SNO modification [44, 45]. Our results are consistent with these previous findings. Accordingly, we propose for the first time that UGDH protein of *N. flagelliforme* can undergo SNO modification. These results indicate that NO may react with specific sulfhydryl residues in UGDH and G6PDH to form SNO products, thereby altering their activity and function, potentially affecting key metabolic pathways such as glycogen synthesis and glucose metabolism. Interestingly, this investigation revealed that ICDH does not undergo SNO modifications, suggesting that it may possess distinct structural or mechanistic features compared to UGDH and G6PDH. This implies that ICDH likely employs a unique cellular mechanism to maintain the stability and functionality of the citric acid cycle. These results clarified that SNO could further affect cellular metabolism by regulating enzyme activity. SNO modification may constitute a part of the intricate regulatory mechanism of cells, which is associated with cellular adaptation and responses to various physiological and pathological conditions. To better understand the potential role of SNO in the function of UGDH and G6PDH, we also aimed to identify the specific modification sites through mass spectrometry analysis. Notably, this study discerned a notable increase of 29 Da in the molecular weight of specific fragments of both proteins following the SNO modification process. It is well known that the introduction of chemical groups can have a significant impact on protein structure and function [46]. The changes in the molecular weight of UGDH and G6PDH proteins can likewise be explained by the introduction of chemical groups due to SNO.

Based on these findings, it can be inferred that SNO potentially exerts significant effects on both protein structure and function, thus regulating key metabolic pathways. This study revealed that the mechanism by which NO influences the biosynthesis of polysaccharides in *N. flagelliforme* involves the SNO modification of G6PDH and UGDH. Nonetheless, it is important to acknowledge that this study is not without its limitations. Further investigations are essential to gain a more comprehensive understanding of how this modification impacts the functionality of UGDH and G6PDH, as well

as its implications for cellular metabolic processes. This discovery opens up a novel perspective that advances our comprehension of the regulation of polysaccharide biosynthesis. Subsequent research should focus on exploring its molecular mechanism, which can lay the foundation for the development of innovative drugs and functional foods.

Conclusion

In this study, intracellular NO levels were found to have a significant positive effect on the polysaccharide yield, as illustrated by comparing the effects of respective addition of NO donors and scavengers to *N. flagelliforme* cultures. The three enzymes G6PDH, ICDH, and UGDH were screened to be significantly correlated with intracellular NO levels by correlation analysis. These enzymes were then heterologously expressed in *E. coli* and purified. Subsequently, the SNO modification levels of the three proteins were detected using a biotin switch assay and western blot analysis, which showed that UGDH and G6PDH could undergo SNO. Moreover, the SNO modification sites of UGDH and G6PDH were determined to be Cys423 and Cys249 by mass spectrometry analysis of GSNO-treated proteins, respectively. These results indicate that intracellular NO affects polysaccharide biosynthesis in *N. flagelliforme* through SNO modification of UGDH and G6PDH. This provides important clues for further understanding the mechanism of NO action in cellular regulation and polysaccharide biosynthesis, as well as a new strategy for improving polysaccharide yields for industrial production.

Supplementary Information

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Supplementary Material 1

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Author contributions
M.Y.-L. provided concept, methodology, situational analysis and interviews, writing - original manuscript preparation and writing - review and editing. Y.R.-L. Conceptualisation, writing - original manuscript preparation and writing - review and editing. C.F.-H. Conducting conceptual, methodological and situational analyses and surveys. J.Z. provides the method. R.Y.-Z. provided writing-reviewing and editing. Y.Z. and J.L. Conducted situation analyses and surveys. S.R.-J. was supervised. P.P.-H. conducted access to funds and resources. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
This manuscript has been read and approved by all the authors, and they have approved to submit and publish it in this journal.

Competing interests
The authors declare no competing interests.

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