Impact of overexpressing NADH kinase on glucoamylase production in Aspergillus niger

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Abstract: Glucoamylase has a wide range of applications in the production of glucose, antibiotics, amino acids, and other fermentation industries. Fungal glucoamylase, in particular, has attracted much attention because of its wide application in different industries, among which Aspergillus niger is the most popular strain producing glucoamylase. The low availability of NADPH was found to be one of the limiting factors for the overproduction of glucoamylase. In this study, 3 NADH kinases (AN03, AN14, and AN17) and malic enzyme (maeA) were overexpressed in acomial A. niger by CRISPR/Cas9 technology, significantly increasing the size of the NADPH pool, resulting in the activity of glucoamylase was improved by about 70%, 50%, 90%, and 70%, respectively; the total secreted protein was increased by about 25%, 22%, 52%, and 26%, respectively. Furthermore, the combination of the mitochondrial NADH kinase (AN17) and the malic enzyme (maeA) increased glucoamylase activity by a further 19%. This study provided an effective strategy for enhancing glucoamylase production of A. niger.

Keywords: Glucoamylase, NADH kinase, NADPH, Malic enzyme

Introduction

Aspergillus niger is an important industrial microorganism with extreme environmental tolerance, high production economy, strong protein secretion capacity, and high food safety (GRAS designation by FDA of the US) (Dong et al., 2020; Li et al., 2020; Nourouzian et al., 2006; van Dijk et al., 2003). It has a wide range of applications in the fields of enzyme preparation, heterologous protein expression, organic acids, etc., and is one of the main cell factories for homologous or heterologous protein production in today’s industry (Cairns et al., 2018). To effectively enhance the protein production of A. niger, cellular resources, such as amino acids and cofactors accumulated during primary metabolism, require to be allocated coordinately to cell growth and product biosynthesis (Cao et al., 2020).

Many biosynthetic reactions and biotransformations are limited by the low availability of NADPH, which plays a large role in many anabolic processes, such as amino acid, lipid, and nucleotide biosynthesis (Hou et al., 2009). Cytoplasmic NADPH supply, which is essential for maintaining intracellular redox balance, is the driving force for efficient amino acid biosynthesis (Sui et al., 2020). NADPH also provides major anabolic reducing power for biomass growth and natural product biosynthesis (Nielsen, 2019). Efficient control and coordination of redox states are critical in multiple metabolic processes within the cell (Turner et al., 2005). Using ¹³C metabolic flux analysis, Driouch et al. found that after the expression of fructofuranosidase, the metabolic flux of the recombinant strain was redistributed, and the pentose phosphate (PP) pathway and malic enzyme were activated (Driouch et al., 2012). For glucoamylase production by A. niger, Lu et al. reported that carbon flux to the (PP) pathway was increased in a high glucoamylase-producing strain (Lu et al., 2015). In addition, NADPH regeneration and consumption were imbalanced in wild-type strains. This indicated that the supply of NADPH is necessary for efficient protein production in A. niger. Many previous studies have attempted to increase NADPH levels in Escherichia coli (Canonaco et al., 2001; Lim et al., 2002), Yarrowia lipolytica (Wasylenko et al., 2015), and A. niger (Poulsen et al., 2005; Sui et al., 2020) through metabolic engineering of the PP pathway. Poulsen et al. overexpressed the PP pathway gene gcdA (6-phosphoglucuronate dehydrogenase), resulting in a nine-fold increase in intracellular NADPH concentration (Poulsen et al., 2005). However, any correlation between NADPH supply and enzyme overproduction was not further investigated. After that, Sui et al. predicted and overexpressed NADPH-producing enzymes using a genome-scale metabolic network model and found that overexpression of 6-phosphoglucuronate dehydrogenase (gcdA) or malic enzyme (maeA) increased the intracellular NADPH pool by 45% and 66%, and the production of GluA by 65% and 30%, respectively (Sui et al., 2020). Notably, the introduction of exogenous cytosolic NADP+-dependent malic enzyme in Y. lipolytica effectively increased NADPH pool size and lipid production (Qiao et al., 2017).

However, a feature of these metabolic engineering approaches is to target central metabolic pathways for redox rebalancing, which may lead to large changes in local metabolic fluxes (Hou et al., 2009). In addition, the enhancement of PPP flux may lead to excessive consumption of 1C (CO₂) due to the coupling of PPP and CO₂ production. Only when the carbon flux enters the Embden–Meyerhof–Parnas (EMP) pathway can the carbon economy be effectively guaranteed (Lee et al., 2010; Sui et al., 2020). A more efficient approach to achieving cofactor balance is not directly affect any metabolic pathways but to introduce a natural response to cofactors, where changes in metabolic flux are the result of changes in cofactor concentration (Hou et al., 2009). In cells, NADPH is produced by the reduction of NADP+, and NADP+ is

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mainly produced by phosphorylation of NAD+ under the catalysis of NAD+ kinase (Lee et al., 2010). This enzyme is considered to be the only enzyme that produces NADP and is a key enzyme in regulating NADP levels and NADP-dependent biosynthetic pathways in cells (Kawai et al., 2001). NADH kinase can catalyze the direct conversion of NAD+/NADH to NADP+/NADPH without directly affecting the metabolic pathway, the direct conversion of NAD+/NADH to NADP+/NADPH may be beneficial for efficient engineering due to the lack of CO2 overconsumption (Hou et al., 2009; Lee et al., 2010). NADH kinase has been extensively studied in E. coli (Kawai et al., 2001; Lee et al., 2009, 2010), yeasts (Kawai et al., 2001; Qiao et al., 2017), and A. nidulans (Panagiotou et al., 2009), but to our knowledge, no one has studied the effect of NADH kinase on protein production in A. niger. There are three NADH kinases in A. niger and the corresponding accession numbers in the Aspergillus Genome Database (AspGD) are An03g05090, An14g06430, and An17g02020 (Fig. 1) (all uncharacterized, hereinafter referred to as AN03, AN14, and AN17, respectively), which exhibit kinase activity to NAD+ or NADH. Two NADH kinases (AN03 and AN14) are localized in the cytoplasm (cytoplasmic NADH kinase, NADH kinase.c), and one NADH kinase (AN17) is localized in the mitochondria (mitochondrial NADH kinase, NADH kinase.m). We speculate that overexpression of these three NADH kinases is beneficial for efficient protein production from A. niger.

The aims of this study are as follows: (1) using the aconidial A. niger SH-2 as the host, to study the effects of the overexpression of three NADH kinases (AN03, AN14, and AN17) and malic enzyme (maeA) on NADPH supply and glucoamylase production in A. niger; (2) study the effects of malic enzyme and NADH kinase combination on glucoamylase production. Here, our study offers the possibility to improve protein production in A. niger by increasing the availability of limited intracellular NADPH.

**Materials and Methods**

**Strains and Culture Conditions**

The laboratory-deposited aconidial A. niger SH-2 (Δku, ΔpyrG) was used as the host for gene expression. Cultured in Czapek-Dox medium (CD) (2% glucose, 0.3% NaNO3, 0.1% KH2PO4, 0.05% MgSO4·7H2O, 0.05% CaCl2·2H2O, 0.001% FeCl3·6H2O, 0.001% ZnSO4·7H2O, 0.001% MnCl2·4H2O, 0.001% CuSO4·5H2O, and 0.001% CoCl2·6H2O) supplemented with 2% sucrose, 0.5% peptone, and 0.5% yeast extract. The cultures were maintained at 30°C for 48 h in a moist chamber of 95% humidity under a 12-h light/dark cycle (2000 lux) on an orbital shaker (Environa MS50, Germany) at 220 rpm.
Table 1. Aspergillus Niger Strains and Plasmids Used in This Study

| Name       | Genotype                          | Reference               |
|------------|-----------------------------------|-------------------------|
| WT         | SH-2 pyrG—Δku                     | Laboratory preservation |
| K1         | SH-2 pyrG—Δku/AfpymG              | This study              |
| OLEAN03    | SH-2 pyrG—Δku/AN03 AfpymG         | This study              |
| OLEAN14    | SH-2 pyrG—Δku/AN14 AfpymG         | This study              |
| OLEAN17    | SH-2 pyrG—Δku/AN17 AfpymG         | This study              |
| OEmmaA     | SH-2 pyrG—Δku/maeaA AfpymG        | This study              |
| OEmm       | SH-2 pyrG—Δku/maeA ANpyrG         | This study              |
| OEm03      | SH-2 pyrG—Δku/AN03 ANpyrG         | This study              |
| OEm17      | SH-2 pyrG—Δku/AN17 ANpyrG         | This study              |
| OEMP       | SH-2 pyrG—Δku/maeaA ANpyrG        | This study              |

Plasmids

| Name       | Genotype                          | Reference               |
|------------|-----------------------------------|-------------------------|
| pFC330     | Cas9-AMA1-AfpymG/ampR             | Laboratory preservation |
| pFC330 sgRNA | Cas9-sgRNA-AMA1-AfpymG/ampR      | This study              |
| pFC332     | Cas9-AMA1-hygB/ampR               | Laboratory preservation |
| pFC332 sgRNA | Cas9-sgRNA-AMA1-hygB/ampR       | This study              |
| PgpdA-UEV  | PMD18T-PgpdA-Tef                  | This study              |
| PgpdA-AN03 | PMD18T-PgpdA-AN03-Tef             | This study              |
| PgpdA-AN14 | PMD18T-PgpdA-AN14-Tef             | This study              |
| PgpdA-AN17 | PMD18T-PgpdA-AN17-Tef             | This study              |
| PgpdA-maeA | PMD18T-PgpdA-maeA-Tef             | This study              |
| Ptef-UEV   | PMD18T-Ptef-Tef-ANpyrG            | This study              |
| Ptef-AN03  | PMD18T-Ptef-AN03-Tef-ANpyrG       | This study              |
| Ptef-AN17  | PMD18T-Ptef-AN17-Tef-ANpyrG       | This study              |
| Ptef-maeA  | PMD18T-Ptef-maeA-Tef-ANpyrG       | This study              |
| Ptef-ANpyrG| PMD18T-ANpyrG                     | This study              |

Construction of Recombinant Plasmids

A schematic diagram of the construction of cas9 plasmids pFC330 sgRNA and pFC332 sgRNA targeting the integration site is shown in Supplementary file 1 (Supplementary data, Fig. S1). Plasmids pFC330 (marked by AfpymG) and pFC332 (marked by hygB) were digested with restriction enzyme PacI, and sgRNAs were assembled into pFC330 or pFC332 linear fragments by NEBuilder HiFi DNA Assembly Master Mix (NEB). A universal expression vector (PgbaA-UEV) was constructed by seamless connection with NEB together with PgpdA, Tef, and PMD18T (TaKaRa), and another universal expression vector (Ptef-UEV) was constructed by seamless connection with Ptef, Tef, ANpyrG, and PMD18T (TaKaRa) (Supplementary data, Fig. S2). The universal expression vectors PgbaA-UEV and Ptef-UEV were linearized by the restriction enzymes EcoRV and PacI, respectively. Genes for three NADH kinases (AN03, AN14, and AN17) and malic enzyme (maeA) were amplified by PCR using A. niger SH-2 genomic DNA as a template. These four genes were cloned into linearized PgbaA-UEV (Supplementary data, Fig. S2), resulting in PgbaA-AN03, PgbaA-AN14, PgbaA-AN17, and PgbaA-maeA plasmids, respectively (Table 1). Afterward, AN03, AN17, and maeA were cloned into linearized Ptef-UEV (Supplementary data, Fig. S2) to obtain Ptef-AN03, Ptef-AN17, and Ptef-maeA plasmids, respectively (Table 1), and the complementary ANpyrG plasmid Ptef-ANpyrG was constructed (as shown in Supplementary data, Fig. S2). All restriction enzymes used in this study were purchased from TaKaRa (Japan). Supplementary data, Table S1 lists all primers used in this study.

Construction of Recombinant Strains

A total of 2–3 ml of A. niger SH-2 seed liquid in CD medium was inoculated into DPY medium, and cultured for 3 days at 30°C, 200 rpm to prepare protoplasts. PEG-mediated protoplast transformation was described by Huang et al. (2021), the ApaI-digested linear expression cassettes (except PgbaA-AN17) were co-transformed with pFC330 sgRNA, respectively, and the expression cassettes were integrated into the genome of A. niger by homologous recombination (Supplementary data, Fig. S3). PgbaA-AN17 was digested into a linear expression cassette using EcoRI and Ssal. The strain obtained by a single transformation of the pFC330 sgRNA plasmid was the control strain K1 (Table 1). Using AfpymG in the pFC330 sgRNA episomal plasmid as a selectable marker, positive transformants were selected on CD solid medium without uridine. The overexpression mutants with homologous
integration were subjected to upstream and downstream localization analysis by PCR amplification (Supplementary data, Fig. S3), recombinant strains OEN03, OEAN14, OEAN17, OEmaeA, and control strain K1 were obtained (Table 1). Then, the linear expression cassettes digested with Pst-AN03, Pst-AN17, Pst-maeA, and Pst-ANpyrG plasmids were co-transformed with pFC332 sgRNA into OEmaeA strain (Supplementary data, Fig. S3). The positive transformants were screened and identified on CD plates supplemented with 0.1 mg/mL hygB to obtain recombinant strains OEm03, OEm17, OEmmA, and control strain OEmp (Table 1).

Recycle the Episomal Cas9 Plasmid
When recycling the episomal pFC330 sgRNA plasmid in the overexpression strain, it is necessary to replace the carbon source glucose in the CD medium with N-Acetyl-D-glucosamine to prepare the N-CD medium. Inoculate 4 mL of seed solution in N-CD culture at 30°C, 200 rpm for 3–4 days, filter with four layers of Miracloth (EMD Millipore, USA), wash twice with sterile water, and use a microscope for microscopic examination. Dilute the spore-like propagules by appropriate times, cultured, and identified positive strains on screening plates supplemented with 1 mg/mL 5-fluoroorotic acid (5-FOA) and 10 μM uridine.

Determination of Glucoamylase Activity and Total Protein Level
The positive transformants identified were fermented in the medium of starch corn syrup at 30°C and 200 rpm by shaking flask. At the specified time point, a 1 mL sample was extracted from the shaking flask, centrifuged at 12,000 rpm for 5 min at room temperature, and the supernatant was taken for testing. The glucoamylase activity detection method uses 4-nitrophenyl-α-D-glucopyranoside (pNPG) as the substrate, and the glucoamylase activity in the culture medium is determined by spectrophotometry (Pedersen et al., 2000). Briefly, pH 4.6, 0.1 M sodium acetate buffer was used as the solvent for sample dilution and substrate dissolution, and the concentration of p-nitrophenol (λ = 405 nm) was used as a measure of glucoamylase activity in the sample. Set up one control per sample. A total of 20 μL of diluted sample or standard was prewarmed at 37°C for 20 min, then incubated with 230 μL of pNPG (2 g/L) for 20 min at 37°C, and 100 μL of 0.3 M NaCO₃ was added immediately to stop the reaction, and the absorbance was measured at OD₄₅₀. Total extracellular proteins in culture supernatants were determined using the BCA Protein Assay Kit (GBCBio, Guangzhou, China) according to the manufacturer's manual. Enzyme activity assays were performed in triplicate.

Analysis of NADH Kinase Activity in Cytoplasm
Inoculate 4 mL of NADH kinase-positive transformant seed solution into 100 mL of DYP medium and culture at 30°C and 200 rpm. The cells cultured to the late stage of exponential growth were collected by a vacuum filter pump, washed twice with sterile water and 0.8 M NaCl, respectively. The washed cells were stored at −80°C until further analysis. Grind the mycelia into powder in liquid nitrogen and take 0.1 g to 1 mL of the extract. Afterward, the NAD Kinase (NADK) Activity Assay Kit (Boxbio Science, Beijing, China) was used to analyze the NADK activity in the cytoplasm of recombinant strains according to the manufacturer’s manual. Unit definition: One unit of enzyme activity is defined as the production of 1 nmol NADP per minute per g tissue in the reaction system. All assays were performed in duplicate.

Detection of NADP+ and NADPH Content in Cytoplasm
The mycelium stored at −80°C was ground into powder in liquid nitrogen, then NADP+ and NADPH in the samples were extracted with acidic and alkaline extraction solutions, respectively. After that, according to the manufacturer’s manual, the Coenzyme II NADP (H) Content Assay Kit (Boxbio Science, Beijing, China) was used to detect the content of NADP+ and NADPH in the cytoplasm. When directly determining the total content of NADP+ and NADPH, we used the NADP+/NADPH Assays Kits (Beyotime, Beijing, China), according to the manufacturer’s manual. All assays were performed in duplicate.

RNA Extraction and qRT-PCR
As previously described (Huang et al., 2021), the collected exponentially late bacteria were thoroughly ground, and then total RNA was extracted using the HiPure Fungal RNA Kit (Magen, China). Reverse transcription was performed using the PrimeScript RT-PCR Kit with gDNA Eraser (TaKaRa, Japan), and approximately 1 μg of total RNA was used for cDNA synthesis. Quantitative real-time PCR (qRT-PCR) was performed using Applied Biosystems™ QuantiStudio™ 1; gpdA and Actin (actA) were used as reference, and each reaction was performed in triplicate.

Statistical Analysis
Data were expressed as mean value ± standard deviation. The one-way ANOVA was used to compare groups, and p < 0.05 was considered statistically significant.

Results
Characterization of Recombinant Strains
To achieve overexpression of NADH kinases (AN03, AN14, and AN17) and malic enzyme (maeA) in the aconidial host A. niger SH-2 (Aku and ΔpyrG), we used CRISPR/Cas9 gene-editing technology. As described in the method, the linear expression cassettes were co-transformed with the pFC330 sgRNA plasmid, respectively, and recombinant strains overexpressing NADH kinase and malic enzyme were obtained: OEN03, OEAN14, OEAN17, and OEmaeA. The strain obtained by single transfection of the pFC330 sgRNA plasmid was used as the control strain K1. As shown in Fig. 2a, there was no significant difference in the growth phenotype between the recombinant strain and the control strain on solid medium. The analysis of qRT-PCR showed that the transcription levels of the corresponding genes in the recombinant strain were significantly higher than those in the control strain K1 (Fig. 2). Compared with the gpdA reference, there was no significant difference using actA as a reference. The transcription level of AN03, AN14, and maeA increased by about 15-fold, 8-fold, 23-fold, and 16-fold, respectively, compared with the control strain K1 (Fig. 2). Collectively, these results indicated that under the control of the strong constitutive promoter FpdA, NADH kinases and malic enzyme were normally overexpressed in A. niger SH-2.

Effects of Overexpression of NADH Kinases and Malic Enzyme on Glucoamylase Production
The glucoamylase activity of the recombinant strains and the control strain constructed in this study was determined by shaking flask fermentation in a starch corn syrup medium, and the glucoamylase activity of each strain reached the maximum value for comparison. Compared with the control strain K1, the
overexpression of NADH kinases in both cytoplasm and mitochondria significantly increased the production of glucoamylase (Fig. 3a), especially the overexpression of AN17, which had the most significant increase in the activity of glucoamylase. Overexpression of cytoplasmic NADH kinase (NADH kinase c) (AN03, AN14) and mitochondrial NADH kinase (NADH kinase mn) (AN17) recombinant strains OEAN03, OEAN14, and OEAN17 increased the total protein secretion by approximately 25%, 22%, and 52%, respectively (Fig. 3b). The glucoamylase activities were increased by about 70%, 50%, and 90%, respectively (Fig. 3a). Similar to previous studies (Sui et al., 2020), strains overexpressing malic enzyme (maeA) significantly increased the activity of glucoamylase. The total protein secretion and glucoamylase activity of the OEmaeA strain increased by about 26% and 70%, respectively (Fig. 3a and b).

Functional overexpression of NADH kinases was further confirmed by in vitro enzyme activity assays (Fig. 3f). Compared with the control strain K1, the NADH kinase activity was increased by about 80% in the OEAN03 and OEAN14 strains, but there was little change in the NADH kinase activity in the strain OEAN17, which overexpressed NADH kinase mn (Fig. 3f). The results indicated that the overexpression of mitochondrial NADH kinase had no effect on the activity of NADH kinase in the cytoplasm. In addition, we speculated that the liquid nitrogen grinding method used in the experiment may not support our detection of substances in A. niger mitochondria. Similarly, as shown in Fig. 3c and d, when the contents of NADP+ and NADPH in the cytoplasm were detected in vitro, although the OEAN17 strain had no effect on the NADH kinase activity in the cytoplasm, compared with the control strain K1, the contents of NADP+ and NADPH in the cytoplasm were significantly decreased by the OEAN17 strain. The decrease of NADP+ and NADPH in the cytoplasm caused by the overexpression of mitochondrial NADH kinase (AN17) may be attributed to the consumption of cytoplasmic NADP+ and NADPH by OEAN17 strain during the excessive synthesis of glucoamylase and total protein (Fig. 3a and b). This demonstrated that NADPH availability was the limiting factor for protein overproduction in A. niger. After overexpression of NADH kinase c, OEAN03 and OEAN14 strains significantly increased the cytoplasmic NADP+ and NADPH contents (Fig. 3c–e). NADP+ contents increased by 40% and 68%, and NADPH contents increased by 60% and 40%, respectively. Although NADP+, the precursor of NADPH, increased more in OEAN14 than in OEAN13 (Fig. 3c), the activity of glucoamylase decreased by about 15% in OEAN14 compared with the OEAN03 strain. We speculate that the overexpression of AN14 in the OEAN14 strain may lead to an overload of NADP+ levels in the cytoplasm, making the redox environment unfavorable for the accumulation of glucoamylase. To confirm this, we analyzed the transcription levels of another strain, OEAN14-30, which overexpressed AN14 but had decreased glucoamylase (Supplementary data, Fig. S4). Compared with the OEAN14 strain, the glucoamylase level of OEAN14-30 was reduced by 19%, but the transcription level increased by 70% (Supplementary data, Fig. S4), and the total content of NADP+ and NADPH was slightly increased. The OEmaeA strain appears to improve the availability of NADPH through the conversion of NADP+ while improving the redox balance of NADPH and NADP+ (Fig. 3c and d, Table 2), resulting in a significant increase in glucoamylase production. These results suggest that, although important for glucoamylase production by increasing NADPH availability, the balance between NADPH and NADP+ appears to be more important. Compared with the control strain K1, the transcription level of glaA in the OEAN17 strain was increased by about two-fold (Fig. 3g), which may be partly responsible for the most significant increase of glucoamylase in strain OEAN17, but there was no significant change in the transcription level of glaA in strain OEAN03.
Fig. 3. Comparison of glucoamylase activity, total protein level, NADP+ and NADPH content between recombinant strains and control strains. (a) Relative levels of glucoamylase activity, (b) relative levels of total secreted proteins, (c) NADP+ content, (d) NADPH content, (e) total NADPH and NADP+ content, (f) NADH kinase activity, and (g) relative transcription levels of glaA. *Indicates a significant difference compared with the control strain K1 (p < .05).

N-Acetyl-D-Glucosamine Induced OEmaeA Strain as Secondary Transformation Host

Single-gene overexpression experiments increased the NADP+, NADPH, or NADPH/NADP+ ratio to varying degrees, as well as the production of glucoamylase and the level of total protein (Fig. 3). Considering the excellent performance of malic enzyme overexpressed strain OEmaeA in glucoamylase production and NADPH/NADP+ redox rebalancing, we use OEmaeA as the host for the secondary transformation. Based on the above results, the AN03, AN17, and maeA genes were selected as candidate genes to study the cumulative effect of multiple genetic manipulations on these characteristics by combining the overexpression
of multiple genes to promote the further accumulation of glucoamylase.

To facilitate subsequent genetic manipulations, we need to recycle the episomal pFC330 sgRNA plasmid in OEmaeA strain. In a previous study in our laboratory, it was found that the aconidial A. niger SH-2 can produce spore-like propagules under the induction of N-acetyl-D-glucosamine (not yet published). After 3 days of induction in N-CD, the culture medium of the OEmaeA strain turned from colorless and transparent to a brownish yellow (Fig. 4a), and the mycelia were induced to produce spore-like propagules (Fig. 4b). After purification and dilution, the spore-like propagules were cultured on screening plates supplemented with 1 mg/mL 5-FOA and 10 μM uridine, and positive strains were identified (Fig. 4c and d). It was also verified that the OEmaeA strain still had maeA overexpression after recycling pFC330 sgRNA (Fig. 4e), from which we obtained a host for secondary transformation. Then, as described in the method, the linear expression cassettes digested with PlasF AN03, PlasF AN17, PlasC maeA, and PlasF AN pyrG plasmids were co-transformed with pFC332 sgRNA into OEmaeA strain to obtain the secondary recombinant strains OEm03, OEm17, O Emm, and control strain OEmp (Table 1).

### Table 2. The Ratio of NADPH and NADP+ in the Recombinant Strain and the Control Strain

| Strains   | NADPH/NADP+ a |
|-----------|---------------|
| K1        | 0.2078        |
| OEm03     | 0.2361        |
| OEm14     | 0.1744        |
| OEm17     | 0.2199        |
| OEmp      | 0.4409        |

*Values are the mean from two independent cultures.

### Effects of Double Mutant Strains on Glucoamylase Production

Our study shows that it was important to improve the supply of cofactor NADPH during glucoamylase production; likewise, improving the balance between NADP+ and NADPH was critical. However, contrary to expectations, the strain OEm03 overexpressing AN03 in OEmaeA not only did not further increase the production of glucoamylase, but reduced the activity of glucoamylase by about 12% compared with the control strain OEmp, and the total protein level also decreased by around 10% (Fig. 5a and b). It may be the combination of maeA and AN03 that causes more complex changes in intracellular metabolism, creating an environment that is not conducive to protein accumulation. Although the transcription level of maeA was further increased in the O Emm strain (Fig. 2, Supplementary data, Fig. S5), there were no significant changes in glucoamylase activity and total protein level compared with the control strain OEmp (Fig. 5a and b). Surprisingly, although the extracellular total protein level in the OEm17 strain was almost unchanged compared with OEmp, the glucoamylase activity was increased by about 20% (Fig. 5a and b), indicating that the combination of maeA and AN17 is an effective strategy for further accumulation of glucoamylase. Consistent with the trend of overexpression of NADH kinases alone in A. niger SH-2, overexpression of cytoplasmic NADH kinase (NADH kinase c) (AN03) in OEmaeA significantly increased cytoplasmic NADP+ and NADPH content, while overexpression of mitochondrial NADH kinase (NADH kinase m) (AN17), the contents of NADP+ and NADPH in

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**Fig. 4.** Process of recycle of episomal pFC330 sgRNA plasmid in OEmaeA strain. (a) Phenotype of OEmaeA strain in shake flasks after 3 days of N-acetylglucosamine induction. (b) microscopic results of spore-like propagules, (c) positive strains grown on screening plates containing 1 mg/mL 5-FOA and 10 μM uridine, (d) PCR amplification of the ApyrG fragment from the episomal pFC330 sgRNA plasmid to verify that the episomal pFC330 sgRNA plasmid has been recycled, 1 means after recycling, 2 means water, 3 means before recycling, and (e) analysis of upstream and downstream localization of overexpressed maeA. ul means the upstream localization, dl means the downstream localization.
the cytoplasm decreased (Figs. 3, 5c and d). However, part of the reason for the increased glucoamylase activity after overexpression of AN17 may be the result of the increased relative transcription level of glaA (Fig. 3g).

**Discussion**

Glucoamylase is a major enzyme that hydrolyzes starch to produce glucose. It is widely used in the fermentation industries of antibiotics, amino acids, glucose, and organic acids (Kumar & Satyanarayana, 2009), and is one of the important enzymes in industrial production. It has been extensively studied over the past few decades. The glucoamylase secreted by *A. niger* has high thermal stability and heat resistance and is the main strain for the industrial production of glucoamylase (Krijgsheld et al., 2013). Introduction of additional copies of the glucoamylase-encoding gene glaA is a commonly used genetic engineering strategy to increase the yield of *A. niger* glucoamylase (An et al., 2019; Verdoes et al., 1993). However, the introduction of excess glaA copies may overload the host cell and fail to increase glucoamylase production (Cao et al., 2020). The processes of folding, post-translational modification, and secretion of complex proteins require many resources, especially NADPH (Delic et al., 2012; Tomas-Gamisans et al., 2020). Previously, by metabolomic analysis, Lu et al. found the imbalance in NADH regeneration and consumption and the low availability of NADPH may be a bottleneck for the overproduction of glucoamylase (Cao et al., 2020; Sui et al., 2020). NADH kinase can directly convert NAD+/H to NADP+/H without directly affecting any metabolic pathways (Hou et al., 2009), which may be beneficial for efficient engineering.

In this study, we investigated the effects of NADH kinases and malic enzyme overexpression on glucoamylase production, and as expected, overexpression of all three NADH kinases had a positive effect on glucoamylase production. Overexpression of the cytoplasmic NADH kinase-encoding genes AN03 and AN14 both increased glucoamylase production by significantly increasing the content of precursor NADP+ (Fig. 3 and 5). They showed little activity in NADH, significantly increased NADP+ also increased

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**Fig. 5.** Analysis of glucoamylase activity, total protein level, NADP+ and NADPH content in OEmp, OEm03, and OEm17 strains. (a) Comparison of glucoamylase activity, (b) analysis of total protein level, (c) detection of intracellular NADP+ content, and (d) detection of intracellular NADPH content. OEmp, OEm03, OEm17, and OEmm, respectively represent the supplementation of ANpyrG, the overexpression of the NADH kinase c AN03, the overexpression of the NADH kinase m AN17, and the introduction of an additional copy of maEA in the OEmaEA host strain. The significance is indicated by * (p < 0.05).
NADPH content. The enzyme that has a specific effect on NAD+ is called NAD+ kinase, and the enzyme that phosphorylates both NAD+ and NADH is called NADH kinase (Shi et al., 2005). Therefore, we speculated that AN03 and AN14 are NADH kinases. Although cytoplasmic NADH kinase (NADH kinase c) (AN14) has the most significant increase in precursor NADP+, it may be due to overproduction that disrupts the redox equilibrium environment required for glucoamylase production, resulting in a decrease in glucoamylase production compared to NADH kinase c AN03. Perhaps, overexpression of AN14 will have unexpected benefits for the production of heterologous proteins that require NADP+ or NADPH. In E. coli (Lee et al., 2010; Sauer et al., 2004), membrane-bound transhydrogenase (pmtAB) can convert NAD to NADP when intracellular NADP levels are low, while soluble transhydrogenase (udhA) catalyzes NADP to NAD when NADP levels are high. Thus, the role of udhA is critical for growth under metabolic conditions that lead to excessive NADP formation. Introducing it into A. niger in combination with NADH kinase c would have important implications for NADPH-requiring processes and excessive NADPH cycling processes. However, the introduction of a membrane-bound transhydrogenase (pmtAB) from E. coli into Saccharomyces cerevisiae catalyzes the conversion of NADPH to NADH (Anderlund et al., 1999; Hou et al., 2009). Overexpression of NADH kinase m AN17 had the most significant increase in glucoamylase activity and total protein level (Fig. 3), but unlike NADH kinase c, the mechanism by which overexpression of AN17 increased glucoamylase was unclear. The OEAN17 strain reduced the levels of NADP+ and NADPH in the cytoplasm, and a two-fold increase in the transcription level of glaA may be partly responsible for the most significant increase of glucoamylase in OEAN17 strain. In addition, overexpression of mitochondrial NADH kinase (NADH kinase m) in A. niger resulted in a significant increase in biomass yield on glucose and the maximum specific growth rate (Panagiotou et al., 2009). This may be another explanation for the overexpression of NADH kinase m AN17 to increase glucoamylase production. The production of glucoamylase in the OEAN03 strain increased significantly, but the transcription level of glaA did not change significantly, which may be due to the increased availability of NADPH to meet the production of glucoamylase in the process of glucoamylase production. Model strains overexpressing NADH kinase c AN03 may also be suitable for heterologous protein production. Similar to the results of previous studies (Sui et al., 2020), overexpression of malic enzyme maeA can significantly increase the activity of glucoamylase, which may have a significant effect on satisfying NADPH supply and increasing NADPH/NADP+.

We also investigated the combined effect of the malic enzyme and NADH kinase. It was found that the combination of AN03 and maeA not only did not increase the accumulation of glucoamylase and total protein but decreased their levels. Compared with the control strain OEm, the OEm03 strain showed a 12% decrease in glucoamylase and a 10% decrease in total protein levels. Only the combination of maeA and AN17 increased the activity of glucoamylase by about 19%, but the level of total protein was almost unchanged. Similar results were obtained in Y. lipolytica by Qiao et al. (2017), where three NAD+ kinases combined with exogenous NADP+-dependent maeA in Y. lipolytica, but only one NAD+ kinase interacting with maeA further enhanced lipid production content. Although the combination of maeA and AN03 is undesirable for glucoamylase production, the combination of NADH kinase c AN03 and NADH kinase m AN17 may be an interesting phenomenon.

Expect overexpression of NADH kinase or malic enzyme, hyperbranched mycelium tip (Fiedler et al., 2018), and increased supply of amino acid precursors (Cao et al., 2020) are also effective genetic engineering approaches to improve the production of glucoamylase in A. niger. It is worth mentioning that NADPH availability is also a bottleneck in amino acid synthesis (Sui et al., 2020). A combination of NADH kinase and aspartate aminotransferase may be more promising. Of course, the overexpression of these functional genes needs to find a suitable site, that is, an integration site that has little effect on the metabolism of A. niger after the site is knocked out, such as neutral sites (Cai et al., 2021). To quickly realize the multiple genetic manipulations of related genes in aconidial A. niger, a method is also mentioned in this paper, using AfpyrG-tagged cas9 plasmid for homologous recombination (Fig. 3) and N-acetylglucosamine-induced recycle of the episomal cas9 plasmid strategy (Leynaud-Kieffer et al., 2019) (data not shown), these properties provide a convenient means for multiple genetic manipulations of aconidial A. niger.

Conclusion

In this study, we significantly increased the availability of NADPH by overexpressing three NADH kinases and malic enzymes in A. niger, promoting the accumulation of glucoamylase and total protein. In addition, the combination of the malic enzyme (maeA) and the mitochondrial NADH kinase (NADH kinase m) (AN17) could further accumulate glucoamylase. Here, we also provided a rapid marker-free integration method in aconidial A. niger to facilitate multiple genetic manipulations. Future work will focus on investigating the specific mechanism by which mitochondrial NADH kinase (AN17) increases glucoamylase production and further genetic improvements, such as through aspartate aminotransferase (An16g03570) gene expression (Cao et al., 2020) or Rho GTPase RacA knockout (Fiedler et al., 2018), to further enhance the protein secretion capacity of A. niger and achieve high titer, yield, and productivity.

Supplementary Material

Supplementary material is available online at JMB (www.academic.oup.com/jmb).

Author Contributions

L.L. was responsible for conception and design, performed the experiments, acquisition of the data, analysis, and interpretation of the data, and drafting of the article. L.Y. provided a new approach and was responsible for data analysis and interpretation. B.W. and L.P. designed the study, analyzed the data, and drafting of the article. All authors read and approved the final manuscript.

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Ethical Approval
This paper does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest
The authors declare that no competing interests exist.

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