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 aconidial *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; Norouzian et al., 2006; van Dijck 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 ^{13}C 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, NADH 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 *gndA* (6-phosphogluconate 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-phosphogluconate dehydrogenase (*gndA*) or malic enzyme(*maeA*)increased the intracellular NADPH pool by 45% and 66%, and the production of GlaA 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 (CO_2) due to the coupling of PPP and CO_2 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 to 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
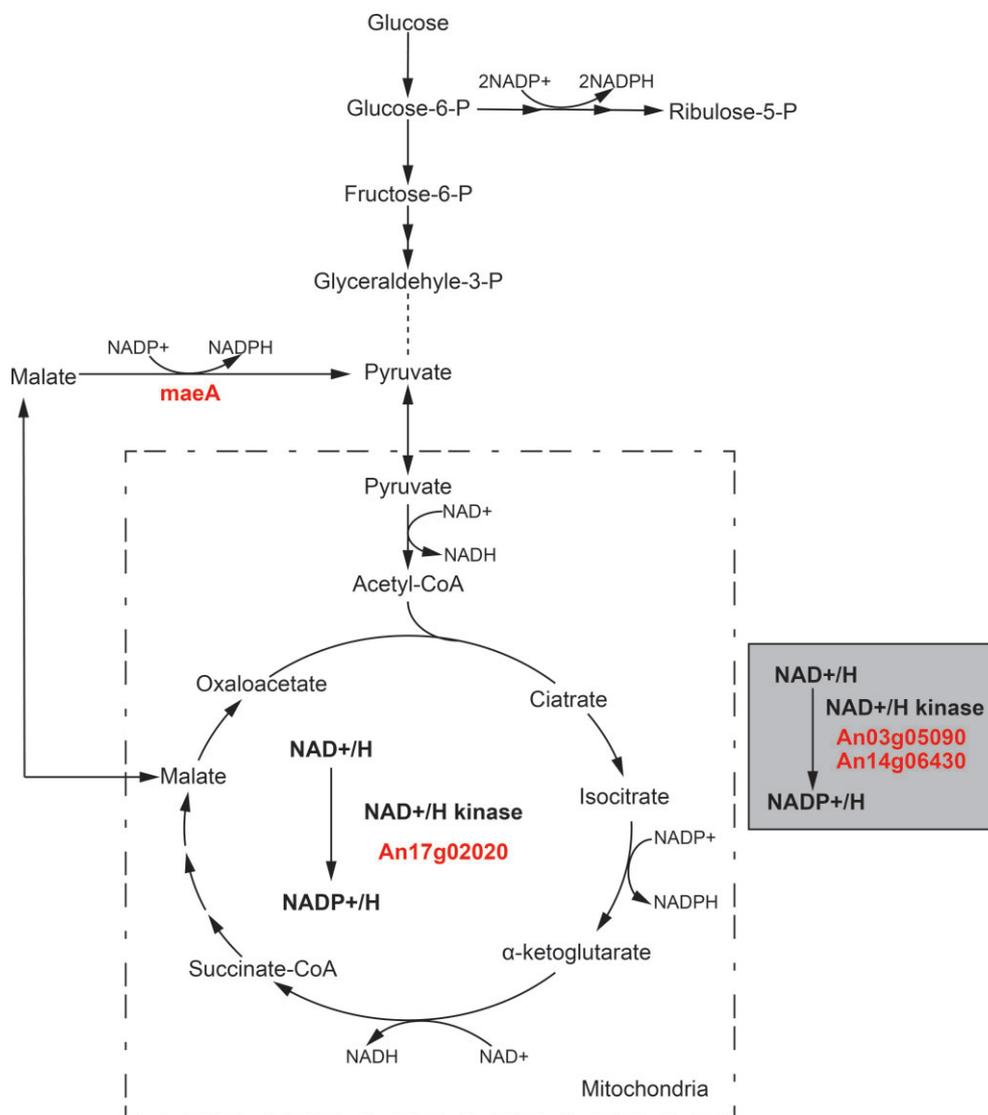
Fig. 1. Cofactor metabolism of NAD+/H kinase (*AN03*, *AN14*, and *AN17*) and malic enzyme (*maeA*) in *A. niger*. The four genes modified in this study are highlighted in red. The dotted box represented the internal metabolism of mitochondria, and the shaded area represented the catalytic mechanism of NADH kinase in the cytoplasm.

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 CO_2 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 (Δ*ku*, Δ*pyrG*) was used as the host for gene expression. Cultured in Czapek-Dox medium (CD) (2% glucose, 0.3% $NaNO_3$, 0.1% KH_2PO_4,

Table 1. *Aspergillus Niger* Strains and Plamisds Used in This Study

Strains		
Name	**Genotype**	**Reference**
WT	SH-2 pyrG—△ku	Laboratory preservation
K1	SH-2 pyrG—△ku/AfpyrG	This study
OEAN03	SH-2 pyrG—△ku/AN03 AfpyrG	This study
OEAN14	SH-2 pyrG—△ku/AN14 AfpyrG	This study
OEAN17	SH-2 pyrG—△ku/AN17 *AfpyrG*	This study
OEmaeA	SH-2 pyrG—△ku/maeA *AfpyrG*	This study
OEmm	SH-2 pyrG—△ku/mmaeA ANpyrG	This study
OEm03	SH-2 pyrG—△ku/maeA AN03 ANpyrG	This study
OEm17	SH-2 pyrG—△ku/maeA AN17 ANpyrG	This study
OEmp	SH-2 pyrG—△ku/maeA ANpyrG	This study
Plasmids		
pFC330	Cas9-AMA1-AfpyrG/ampR	Laboratory preservation
pFC330 sgRNA	Cas9-sgRNA-AMA1-AfpyrG/ampR	This study
pFC332	Cas9-AMA1-hygB/ampR	Laboratory preservation
pFC332 sgRNA	Cas9-sgRNA-AMA1-hygB/ampR	This study
PgpdA-UEV	PMD18T-PgpdA-Ttef	This study
PgpdA-AN03	PMD18T-PgpdA-AN03-Ttef	This study
PgpdA-AN14	PMD18T-PgpdA-AN14-Ttef	This study
PgpdA-AN17	PMD18T-PgpdA-AN17-Ttef	This study
PgpdA-maeA	PMD18T-PgpdA-maeA-Ttef	This study
Ptef-UEV	PMD18T-Ptef-Ttef-ANpyrG	This study
Ptef-AN03	PMD18T-Ptef-AN03-Ttef-ANpyrG	This study
Ptef-AN17	PMD18T-Ptef-AN17-Ttef-ANpyrG	This study
Ptef-maeA	PMD18T-Ptef-maeA-Ttef-ANpyrG	This study
Ptef-ANpyrG	PMD18T-ANpyrG	This study

0.05% $MgSO_4·7H_2O$, 0.2% KCl, and 0.01% $FeSO_4·7H2O$) to support mycelial growth (Huang et al., 2021), adding 10 μM uracil when necessary. The solid CD was used for positive transformant growth, and 0.1 mg/mL hygromycin B (*hygB*) or 10 μM uracil was added if necessary. In DPY medium (2% glucose, 1% peptone, 0.5% yeast extract, 0.5% KH_2PO_4, and 0.05% $MgSO_4·7H_2O$) at 30°C and 200 rpm for protoplast preparation, RNA extraction, NADPH content detection, and NADK activity analysis. All mutants were obtained by protoplast-mediated transformation of *A. niger* SH-2 (Dong et al., 2020). Table 1 lists the recombinant strains and plasmids used in this study. Fermentation of positive transformants was performed in a starch corn syrup medium at 30°C and 200 rpm as previously described (Dong et al., 2020). Using conical flasks with breathable sealing film (Huaxin, Guangdong, China) to maintain aeration. *Escherichia coli* Match1 T1 was used for plasmid cloning in Luria–Bertani (LB) medium (1% NaCl, 1% peptone, and 0.5% yeast extract) supplemented with 100 μg/mL ampicillin at 37°C, cultivated at 220 rpm.

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 *AfpyrG*) 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 (P_{gpdA}-UEV) was constructed by seamless connection with NEB together with PgpdA, Ttef, and pMD18T (TaKaRa), and another universal expression vector (P_{tef}-UEV) was constructed by seamless connection with Ptef, Ttef, AN-

pyrG, and pMD18T (TaKaRa) (Supplementary data, Fig. S2). The universal expression vectors P_{gpdA}-UEV and P_{tef}-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 P_{gpd}A-UEV (Supplementary data, Fig. S2), resulting in P_{gpdA}-AN03, P_{gpdA}-AN14, P_{gpdA}-AN17, and P_{gpdA}-maeA plasmids, respectively (Table 1). Afterward, *AN03*, *AN17*, and *maeA* were cloned into linearized P_{tef}-UEV (Supplementary data, Fig. S2) to obtain P_{tef}-AN03, P_{tef}-AN17, and P_{tef}-maeA plasmids, respectively (Table 1), and the complementary ANpyrG plasmid P_{tef}-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 P_{gpdA}-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). P_{gpdA}-AN17 was digested into a linear expression cassette using *EcoRI* and *SdaI*. The strain obtained by a single transformation of the pFC330 sgRNA plasmid was the control strain K1 (Table 1). Using *AfpyrG* 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 OEAN03, OEAN14, OEAN17, OEmaeA, and control strain K1 were obtained (Table 1). Then, the linear expression cassettes digested with P_{tef}-AN03, P_{tef}-AN17, P_{tef}-maeA, and P_{tef}-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, OEmm, 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, cultured 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-fuoroorotic 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 ($\lambda = 405$ 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 pretreated at 37°C for 10 min, then incubated with 230 μL pNPG (2 g/L) for 20 min at 37°C, and 100 μL of 0.3 M NaCO$_3$ was added immediately to stop the reaction, and the absorbance was measured at OD$_{405}$. 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 DPY 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 Kit (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™ QuantStudio™ 1; *gpd*A and Actin (*act*A) 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 $p < .05$ 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 (Δku and $\Delta pyrG$), 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: OEAN03, 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 *gpd*A reference, there was no significant difference using *act*A as a reference. The transcription level of *AN03*, *AN14*, *AN17*, 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 PgpdA, 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
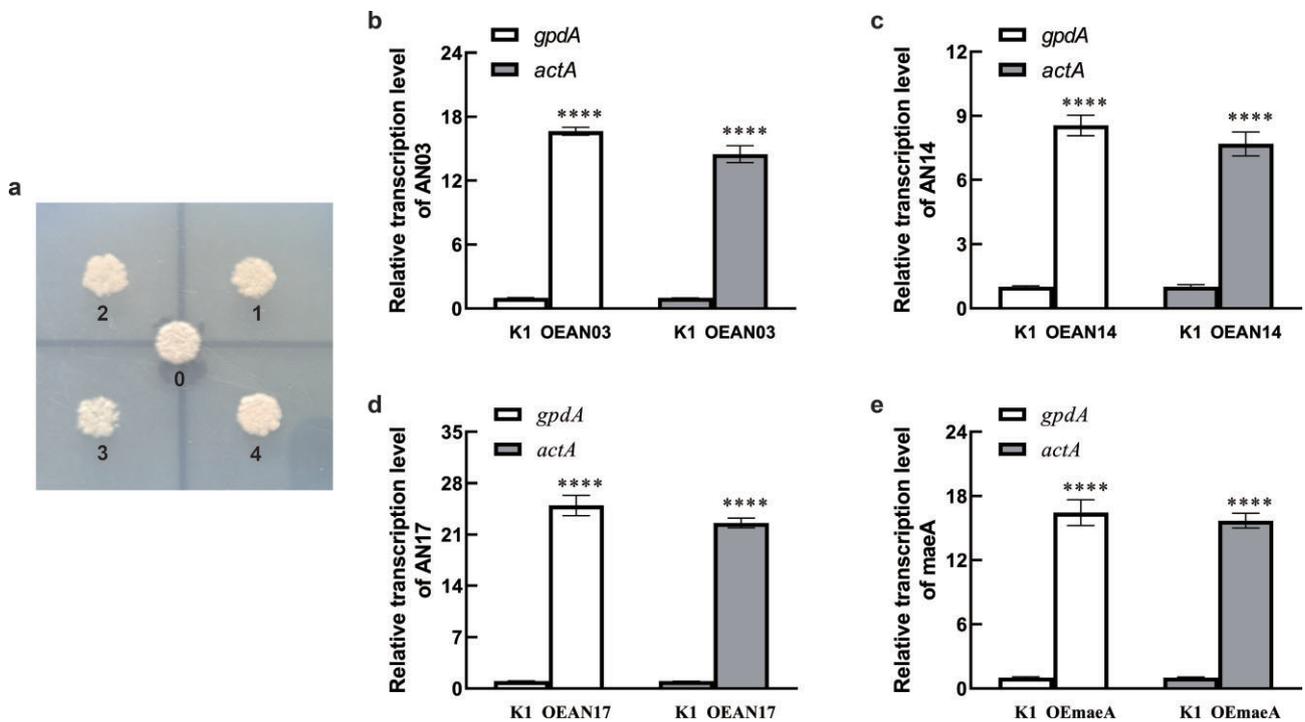
Fig. 2. Growth phenotype and transcription level analysis of recombinant and control strains. (a) Growth phenotype, 0 means control strain K1, 1 means OEAN03, 2 means OEAN04, 3 means OEAN17, and 4 means OEmaeA; (b) transcription level analysis of *AN03*; (c) transcription level analysis of *AN14*; (d) transcription level analysis of *AN17*; and (e) transcription level analysis of *maeA*.

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.m) (*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.m (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.
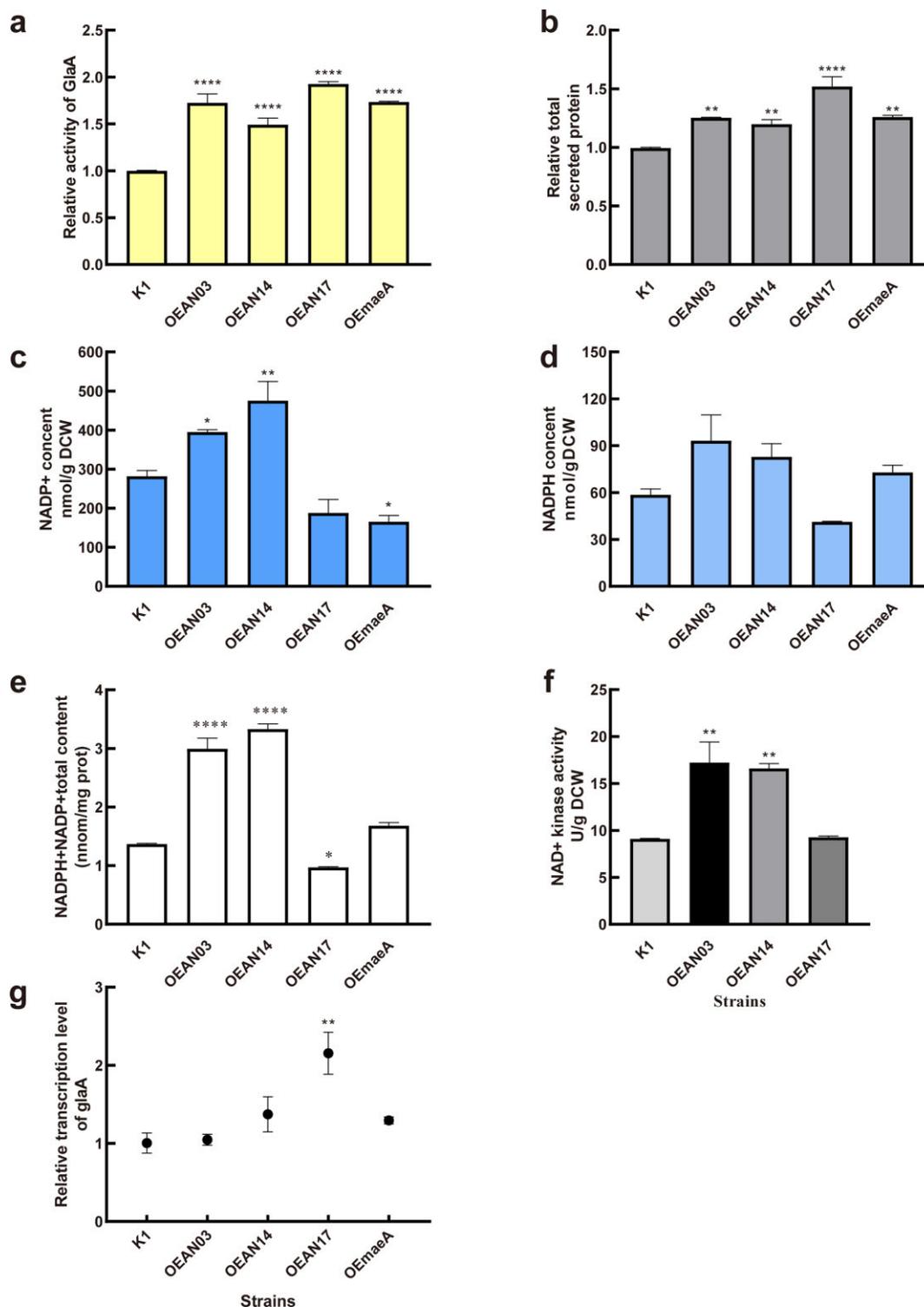
Fig. 3. Comparison of glucoamylase activity, total protein level, NADP+ and NADPH content between recombinant strains and control strains. (a) Relative levels of glucoamylase activity, (b) relative levels of total secreted proteins, (c) NADP+ content, (d) NADPH content, (e) total NADPH and NADP+ content, (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

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

Strains	NADPH/NADP+[a]
K1	0.2078
OEAN03	0.2361
OEAN14	0.1744
OEAN17	0.2199
OEmaeA	0.4409

[a]Values are the mean from two independent cultures.

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 P_{tef}-AN03, P_{tef}-AN17, P_{tef}-maeA, and P_{tef}-ANpyrG plasmids were co-transformed with pFC332 sgRNA

into OEmaeA strain to obtain the secondary recombinant strains OEm03, OEm17, OEmm, and control strain OEmp (Table 1).

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 OEmm 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
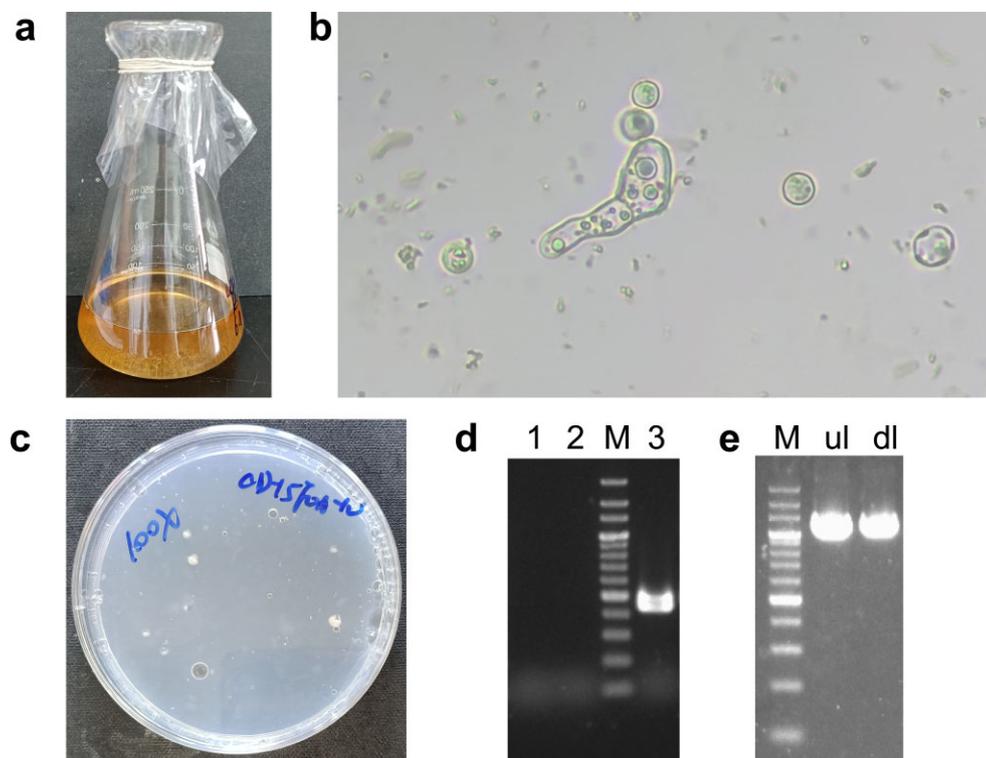

Fig. 4. Process of recycle of episomal pFC330 sgRNA plasmid in OEmaeA strain. (a) Phenotype of OEmaeA strain in shake flasks after 3 days of N-acetylglucosamine induction, (b) microscopic results of spore-like propagules, (c) positive strains grown on screening plates containing 1 mg/mL 5-FOA and 10 μM uridine, (d) PCR amplification of the *AfpyrG* 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.
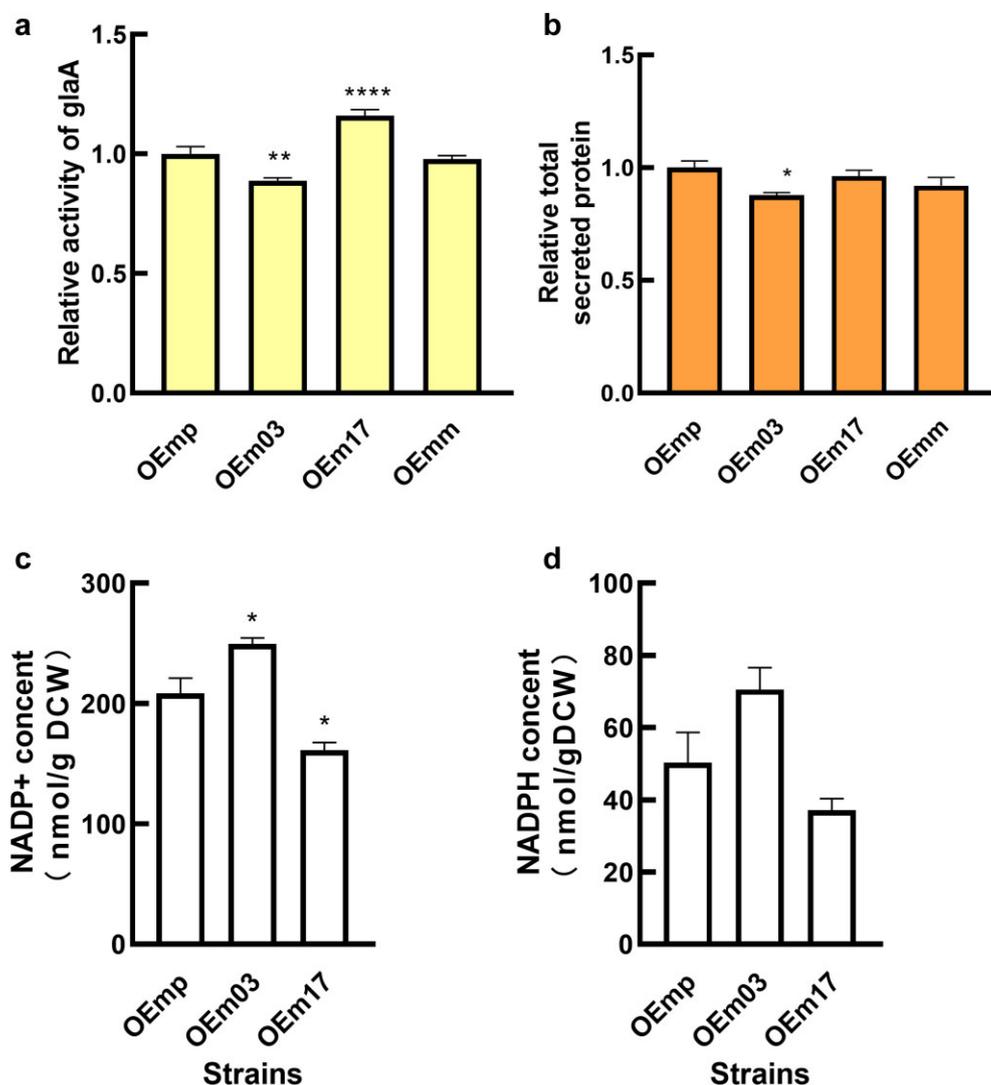
Fig. 5. Analysis of glucoamylase activity, total protein level, NADP+ and NADPH content in OEmp, OEm03, and OE17 strains. (a) Comparison of glucoamylase activity, (b) analysis of total protein level, (c) detection of intracellular NADP+ content, and (d) detection of intracellular NADPH content. OEmp, OEm03, OEm17, and OEmm, respectively represent the complementation 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 *($p < .05$).

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

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 (pntAB) 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 (pntAB) 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. nidulans 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 OEmp, 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 (Fig. 4) can rapidly achieve gene marker-free integration events. Repeated use of this method allows overexpression of multiple genes in a host to study the cumulative effect of multiple genetic manipulations on certain traits. Moreover, the induction of aconidial A. niger using N-acetyl-D-glucosamine in our previous study could rapidly and efficiently achieve heterozygote purification and rapid removal of the pyrG recycle structure (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 (An16g05570) 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 *JIMB* (*www.academic.oup.com/jimb*).

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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