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Characterization and optimization of *mnn11*Δ-mediated enhancement in heterologous protein production in *Kluyveromyces marxianus*

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Abstract

Background N-glycosylation is a prevalent post-translational modification in eukaryotes, essential for regulating protein secretion. In *Saccharomyces cerevisiae*, glycosylation mutants have been shown to enhance the secretion of heterologous glycosylated proteins. However, whether these mutants can also increase the secretion of non-glycosylated proteins and whether the growth defects associated with glycosylation mutations can be mitigated remains unclear. This study aimed to characterize and optimize enhanced secretory expression in the promising yeast host *Kluyveromyces marxianus* by deleting *MNN11*, which encodes a subunit of the mannose polymerase II complex responsible for elongating α-1,6-linked mannose chains.

Results Compared to wild-type cells, the *mnn11*Δ cells significantly increased the secretion activities of four glycosylated enzymes and three non-glycosylated enzymes in flasks, with increases ranging from 29 to 668%. Transcriptomic analysis of *mnn11*Δ mutant revealed upregulation of genes related to essential protein secretion processes, including vesicle coating and tethering, protein folding, translocation, and glycosylation. Additionally, genes involved in vacuolar amino acid transport and amino acid biosynthesis were upregulated, suggesting an amino acid shortage, which might contribute to the observed severe growth defect of the *mnn11*Δ mutant in a synthetic medium with inorganic nitrogen. Supplementation of the synthetic medium with amino acids or low concentrations of yeast extract alleviated this growth defect, reducing the specific growth rate difference between wild-type strain and *mnn11*Δ cells from 65% to as little as 2%. During high-density fermentation, the addition of 0.5% yeast extract substantially reduced the lag phase of *mnn11*Δ mutants and increased the secretory activities of α-galactosidase, endoxylanase, and β-glucanase, by 11%, 18%, and 36%, respectively, compared to *mnn11*Δ mutant grown without yeast extract.

Conclusion In *K. marxianus*, deletion of *MNN11* enhances the secretion of both glycosylated and non-glycosylated proteins by improving key protein secretion processes. The growth defect in the *mnn11*Δ mutant is closely tied to

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insufficient amino acid supply. Supplementing the synthetic medium with low concentrations of organic nitrogen sources effectively alleviates this growth defect and enhances secretory expression. This strategy could be applied to optimize the expression of other glycosylation mutants.

Keywords *MNN11*, Glycosylation, *Kluyveromyces marxianus*, Amino acid shortage, Heterologous protein expression

Background

N-glycosylation is a prevalent post-translational modification in eukaryotes [1]. In both yeasts and mammals, N-glycosylation begins in the endoplasmic reticulum (ER), where a pre-assembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) is attached to specific asparagine residues on nascent peptides. Glucosidase I, glucosidase II, and an ER-resident α-1,2-mannosidase sequentially remove three glucose moieties and a terminal α-1,2-mannose [2]. The resulting $Man_8GlcNAc_2$-containing glycoprotein is transported to the Golgi apparatus, where glycosylation differs significantly between yeasts and mammals. In *Saccharomyces cerevisiae*, α-1,2-, α-1,3-, and α-1,6-mannosyltransferases, along with mannosylphosphate transferases, create N-glycan structures that are mannosylated and hypermannosylated to varying extents. This hyper-mannose modification is unique to yeast and absent in mammals [3]. About 50% of yeast proteins are N-glycosylated [4], and this modification is essential for protein folding, transport, and maturation [5].

Like native proteins, heterologous proteins expressed in yeast undergoes N-glycosylation [6]. Several studies have shown that deleting non-essential N-glycosylation genes in *S. cerevisiae* enhances the secretion of heterologous proteins. For example, deletion of genes responsible for oligosaccharide synthesis in the ER, such as *ALG3*, *ALG5*, *ALG6*, *ALG8*, *ALG9*, *ALG10*, or *ALG12*, promotes α-amylase secretion [7]. Deleting genes encoding glucosidase I (*CWH41*) and glucosidase II's catalytic subunit (*ROT2*) enhances the secretion of cellulosomal enzymes [8]. Additionally, knocking out Golgi mannosyltransferase genes *OCH1* and *MNN9* improves the secretion of various cellulases [9], and deleting other mannosyltransferases genes *MNN10* and *MNN11* boosts amylase secretion [10]. Glycosylation mutants may enhance secretion through multiple mechanisms. First, glycosylation mutations might impair the function of cell wall-associated glycoproteins, increasing cell wall porosity and thereby facilitating protein secretion [9]. Second, these mutations reduce glycan moieties on heterologous proteins, potentially improving their permeability across membranes and the cell wall [11]. Third, glycosylation defects can activate stress response pathways such as the unfolded protein response (UPR), which may enhance heterologous protein secretion [9]. However, gaps remain in understanding N-glycosylation's impact on heterologous protein production. First, studies have focused on glycosylated heterologous proteins; it is unclear if glycosylation mutants also boost the secretion of non-glycosylated proteins. Second, N-glycosylation mutants often exhibit growth defects [9, 12], reducing cell productivity. Whether these growth defects can be mitigated while maintaining improved heterologous protein production remains unknown. Third, there are limited studies on the impact of glycosylation defects in non-conventional yeasts.

Kluyveromyces marxianus and *S. cerevisiae* both belong to the *Saccharomycetaceae* family. *K. marxianus*, the most common yeast found in dairy products, has a long history of safe human consumption, which has led to its recognition as a food-safe species [13]. Its rapid growth, thermotolerance, and broad substrate range make it a promising cell factory for the production of bioethanol, chemicals, and heterologous proteins. Over 50 proteins [14], including industrial enzymes [15, 16], virus-like particles [17,18], and edible proteins [19], have been efficiently expressed in *K. marxianus*, with the highest yield reaching 16.8 g/L [20]. Various strategies have improved protein expression in *K. marxianus*, such as weakening autophagy [15], optimizing UPR and disulfide bond formation pathways [20, 21], and reducing cAMP cyclase activity [22]. However, studies on enhancing protein secretion through glycosylation gene mutations in *K. marxianus* are still lacking.

In this study, we screened *K. marxianus* mutants for enhanced heterologous protein expression, with a deletion mutant of the glycosylation gene *MNN11* emerging as the top performer. The *mnn11*Δ mutant not only improved the secretion of glycosylated proteins but also non-glycosylated proteins. It showed upregulation in key protein secretion pathways. Additionally, the *mnn11*Δ mutant exhibited a potential amino acid shortage, which may contribute to the severe growth defect observed in synthetic medium with inorganic nitrogen. Supplementing the medium with individual amino acids or low concentrations of yeast extract effectively alleviated the growth defect. In high-density fermentation, the addition of 0.5% yeast extract further enhanced heterologous protein expression. This strategy may be applied to optimize the expression of other glycosylation mutants.

Materials and methods

Plasmids and strains

Plasmids used in this study are listed in Table S1. To construct plasmids for expressing heterologous proteins,

the open reading frames (ORFs) of *MEL1* (Uniprot ID: P04824, encoding α-galactosidase from *S. cerevisiae*) and *SpChi1* (Uniprot ID: A0A8D4BAQ1, encoding chitosanase from *Streptomyces pratensis*) were inserted between the *Sma* I and *Not* I sites of pUKDN132. This resulted in the plasmids LHZ1626 and LHZ1627, respectively. Plasmids expressing Xyn-CDBFV (LHZ443/pZP46), RuCelA (LHZ442/pZP52), MAN330 (LHZ444/pZP42), AnFaeA (LHZ766) and BadGLA (LHZ1020), were described previously [16, 20, 21]. To construct CRISPR plasmids (LHZ1608-1625, 1645-1649), primers containing 20 bp target sequence were annealed in pairs and inserted into *Sap* I sites of LHZ531 [21]. Primers used in the construction are listed in Table S2.

All yeast strains used in the study are listed in Table S3. FIM-1ΔU was used as a wild-type strain in this study [16] and cultured in YPD medium (1% yeast extract, 2% hipolypepton, 2% glucose, 2% agar for plates). To delete *MNN11* (gene ID: FIM-1_496), 500 bp sequence upstream and downstream of *MNN11* ORF were amplified and ligated together as the donor sequence. CRISPR plasmid, LHZ1608 was co-transformed with the donor sequence into FIM-1ΔU by a lithium acetate method [23]. The transformants were selected on the synthetic complete minus uracil (SC-Ura) plate. A positive *mnn11*Δ clone was identified by PCR and named LHP1126. Using the same strategy, *CWH41* (gene ID: FIM-1_900), *FLC1* (gene ID: FIM-1_3021), *NVJ3* (gene ID: FIM-1_3640), *UBX2* (gene ID: FIM-1_3063), *KEX1* (gene ID: FIM-1_1117), *OPT2* (gene ID: FIM-1_3542), *GDS1* (gene ID: FIM-1_5027), *ASE1* (gene ID: FIM-1_1956), *HUB1* (gene ID: FIM-1_4502), *OST5* (gene ID: FIM-1_2393), *PPM1* (gene ID: FIM-1_3059), *CAP1* (gene ID: FIM-1_1767), *TRM1* (gene ID: FIM-1_2607), *ANK1* (gene ID: FIM-1_2414), *GAG1* (gene ID: FIM-1_2904), *YBL081W* (gene ID: FIM-1_5156), *MNN10* (gene ID: FIM-1_2211), *ANP1* (gene ID: FIM-1_133) and *HOC1* (gene ID: FIM-1_1731) were individually deleted in FIM-1ΔU to obtain LHP1126-1143, 1343 and 1344. *MNN11* was deleted in LHP1143 to obtain LHP1157.To construct strains overexpressing *MNN11*, the ORF of *MNN11* was inserted between the *Spe* I and *Sac* I sites of LHZ424. Both sites are located between the upstream and downstream sequences of the *INU1* ORF (gene ID: FIM-1_326) [21]. The donor sequence was amplified from the resultant plasmid (LHZ1628) and co-transformed with a CRISPR plasmid targeting *INU1* (LHZ759). The transformants were selected on the SC-Ura plates and the positive clone was named LHP1248. Strains overexpressing other genes were constructed using the same strategy. Primers used in the construction are listed in Table S2.

To express heterologous proteins, plasmids LHZ442-444, LHZ766, LHZ1020, LHZ1626 and LHZ1627 were transformed into FIM-1ΔU and mutants. The transformants were selected on SC-Ura plates and then grown at 30 °C in either YD medium (2% yeast extract, 4% glucose) or synthetic mineral (SM) medium, which includes synthetic mineral seed (SMS) medium and synthetic mineral fed-batch (SMF) medium [17]. Furthermore, 0.1% of individual amino acid and various amounts of yeast extract were added into the SMS and SMF medium as specified.

Enzymatic assays and SDS-PAGE

Transformants expressing heterologous proteins were cultivated in 50 mL of YD medium for 72 h. The cells were pelleted, and the supernatant was collected. A total of 16 μL of supernatant was mixed with 4 μL of 5×SDS-PAGE loading buffer, boiled, and then subjected to SDS-PAGE analysis. The activities of Mel1, AnFaeA, MAN330, RuCelA, BadGLA and Xyn-CDBFV in the supernatant were measured as described previously [16, 20, 24]. To measure the activity of chitosanase, 100 μL of supernatant was mixed with 100 μL of a 1% (w/v) chitosan solution in 50 mM NaAc buffer (pH 6.5). The sample was incubated at 55℃ for 15 min, after which 200 μL of DNS solution (D7800, Solarbio) was added. The mixture was then incubated at 100℃ for 10 min, and the released N-acetylglucosamine was determined by measuring the optical density at 540 nm (OD_{540}). One unit of chitosanase activity was defined as the amount of enzyme required to release 1 μmol of N-acetylglucosamine per minute.

RNA-seq and data analysis

FIM-1ΔU and LHP1126 were grown in YPD liquid medium overnight and then transferred into 50 mL of fresh YD medium, starting at an OD_{600} of 0.01. The cells were collected after growth periods of 24 or 48 h. At each time point, three biological replicate samples were collected. RNA extraction and RNA-seq were performed by Biozeron (Shanghai, China). All gene expression levels were listed in Table S4. To identify differentially expressed genes (DEGs), the expression level for each gene was calculated using the fragments per kilobase of exon per million mapped reads (FRKM) method. R statistical package edgeR (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html/) was used for differential expression analysis. Genes with$|\log_2$ Fold Change$|\geq 1$ and p-value < 0.05 are defined as significantly differentially expressed. GO functional enrichment and KEGG pathway analysis were carried out by Goatools (https://github.com/tanghaibao/Goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do). DEGs were significantly enriched in GO terms and metabolic pathways when their p-value was less than 0.05. Results of GO and KEGG enrichment analysis were listed in Table S5 and Table S6, respectively.

Cell growth profiling

FIM-1ΔU and LHP1126 were grown in YPD liquid medium overnight and then transferred into 50 mL of fresh YD, SMS, or SMS medium supplemented with different amino acids or various amounts of yeast extract. The OD_{600} of the culture was adjusted to 0.01. Cells were cultured for 72 h, and the OD_{600} was measured at intervals of 8 or 12 h. Each test was performed in biological triplicate.

Spot assay

FIM-1ΔU and LHP1126 were grown in YPD liquid medium overnight. Cells were collected and adjusted to an OD_{600} of 1.0. Then, fivefold serial dilutions were performed and dilutions were spotted by a pinpad onto YPD and SMS plates containing hygromycin B (H8080, Solarbio). Plates were incubated at 30 °C for 24 h before imaging.

High cell-density fermentation

High cell-density fermentation was conducted in a 5-L fermenter (BXBIO, Shanghai, China) as previously described [17]. Cells expressing Mel1, Xyn-CDBFV or RuCelA were inoculated into 150 mL of SMS medium and grown for 16-18 h. The seed culture was then transferred into a fermenter containing 1.5 L of SMF medium, with or without 0.5% yeast extract. During fermentation, the dissolved oxygen level was maintained at approximately 10%, and the temperature was controlled at 30℃. The pH was adjusted to approximately 5.5 using ammonium hydroxide. At specified intervals, 50 mL of culture was taken to determine the OD_{600} and enzyme activities.

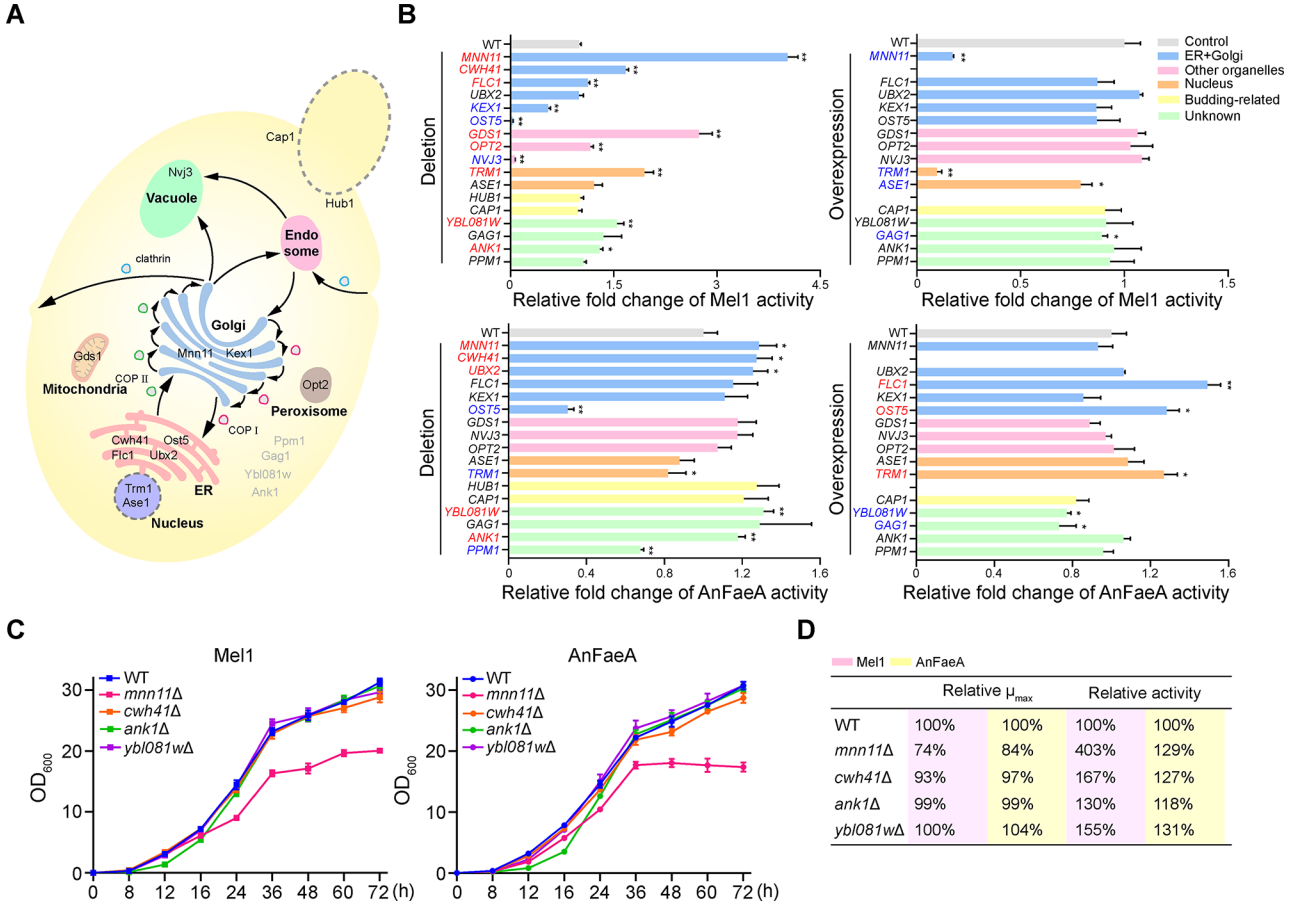
Results and discussions

Screening genes to enhance secretory expression in *K. marxianus*

To enhance the secretion capacity of *K. marxianus*, we selected 17 candidate genes from the literature. In *S. cerevisiae*, deletion of homologs of these candidate genes has been shown to improve secretion expression [7–10, 25, 26]. Predicted locations of the proteins encoded by the candidate genes include the ER (Cwh41, Ost5, Flc1, Ubx2), Golgi apparatus (Mnn11, Kex1), mitochondria (Gds1), nucleus (Trm1, Ase1), peroxisome (Opt2), bud neck (Cap1, Hub1), and vacuole (Nvj3) (Fig. 1A). We evaluated the effects of deleting or overexpressing the candidate genes on the secretion activities of two glycosylated enzymes: α-galactosidase Mel1 from *S. cerevisiae* and feruloyl esterase AnFaeA from *Aspergillus niger*. Eight deletion mutants (*mnn11*Δ, *cwh41*Δ, *flc1*Δ, *gds1*Δ, *trm1*Δ, *ybl081w*Δ, *gag1*Δ, *ank1*Δ) displayed significantly increased Mel1 activity. None of the overexpression significantly increased Mel1 secretion (Fig. 1B). Five deletion mutants (*mnn11*Δ, *cwh41*Δ, *ybl081w*Δ, *ank1*Δ, *ubx2*Δ) exhibited improved AnFaeA activity. Overexpression of *FLC1*, *TRM1*, or *OST5* increased AnFaeA activity (Fig. 1B). Interestingly, deletion, but not overexpression of *FLC1* and *TRM1* promoted Mel1 secretion. *FLC1* encodes a flavin adenine dinucleotide transporter [27], and *TRM1* encodes a tRNA methyltransferase [28]. The opposite effects of deletion and overexpression of *FLC1* and *TRM1* on the secretory activities of Mel1 and AnFaeA suggest these genes play pleiotropic roles in controlling the secretion of different proteins.

A total of four deletion mutants (*mnn11*Δ, *cwh41*Δ, *ybl081w*Δ, *ank1*Δ) simultaneously enhanced the secretory activities of both Mel1 and AnFaeA. The growth curves of these strains were investigated during the expression of Mel1 or AnFaeA (Fig. 1C). Compared with the wild-type strain, deletion of *CWH41* and *YBL081W* had little negative effect on growth. The *ank1*Δ mutant exhibited a prolonged lag phase, but its biomass and maximum specific growth rate (μ_{max}) were barely affected (Fig. 1C, D). The *mnn11*Δ mutant displayed substantially reduced biomass and μ_{max} (Fig. 1C, D). Meanwhile, compared with other mutants, the *mnn11*Δ mutant exhibited the highest average improvement in Mel1 and AnFaeA activities, suggesting a trade-off between cell growth and improved protein production in the *mnn11*Δ mutant (Fig. 1D).

MNN11, *OST5* and *CWH41*, are the three glycosylation genes included in this screen. Ost5 is located in the ER and functions as a zeta subunit of the oligosaccharyltransferase (OST) complex, which transfers 14-sugar branched oligosaccharides from dolichyl pyrophosphate to asparagine residues on nascent peptides [29]. Cwh41 possesses α-glucosidase I activity and is an integral membrane protein of the ER, responsible for removing the terminal glucose from core oligosaccharides immediately after they are transferred to proteins [30]. Mnn11 is a subunit of the Mannose polymerase II complex (M-Pol II) and contributes to the extension of α-1,6-linked mannose polymers in the Golgi [31]. Contrary to the improved activities observed in the *mnn11*Δ and *cwh41*Δ mutants, the *ost5*Δ mutant displayed significantly reduced secretory activities of Mel1 and AnFaeA. Overexpression of *OST5* even increased AnFaeA activity. Several key proteins involved in protein secretion, such as Ero1 and Pdi1, contain conserved glycosylation sites (Asn-Xaa-Ser/Thr). The deletion of *OST5* affects oligosaccharide addition, leading to significant changes in glycosylation [29, 32], which might impair the function of key proteins involved in secretion and reduce secretory activity. In contrast, the deletion of *CWH41* affects the removal of terminal glucose but does not disrupt the addition of the oligosaccharide backbone [33]. Deletion of *MNN11* reduces the length of the outside chain [34]. Neither deletion caused dramatic changes to the oligosaccharide backbone.



	Relative μ_{max}		Relative activity	
WT	100%	100%	100%	100%
mnn11Δ	74%	84%	403%	129%
cwh41Δ	93%	97%	167%	127%
ank1Δ	99%	99%	130%	118%
ybl081wΔ	100%	104%	155%	131%

Fig. 1 Effects of deletion or overexpression of candidate genes on the secretory activities of Mel1 and AnFaeA. (**A**) Schematic view of the protein secretion pathway. Directions of protein secretion are indicated by arrows, and the subcellular locations of proteins encoded by candidate genes are shown, based on SGD annotation. (**B**) Effects of deletion or overexpression of candidate genes on the secretory activities of Mel1 and AnFaeA. Cells were grown in YD medium for 72 h at 30 °C, and enzyme activities in the supernatant were measured. Activities expressed by the wild-type (WT) cells were set as unit 1. Relative activities of cells harbouring deletions (left) or overexpression (right) of candidate genes are shown. Increases or decreases in activities are indicated in red and blue, respectively. Values are expressed as mean ± SD ($n = 3$) from biological replicates. * $p < 0.05$, ** $p < 0.01$. We failed to obtain strains overexpressing *CWH41* or *HUB1*, probably because overexpressing either gene causes toxicity to the cells. Therefore, the effects of overexpressing *CWH41* or *HUB1* on the activities of Mel1 and AnFaeA were not measured. (**C**) Growth curves of wild-type strain and selected mutants during expression of Mel1 and AnFaeA. (**D**) Comparison between the maximum specific growth rate (μ_{max}) and improvement in the enzymatic activities in the selected mutants

Therefore, deletions of genes involved in late-stage glycosylation are more likely to maintain the activity of glycosylated proteins essential for secretion while promoting the secretion of heterologous proteins through other pathways.

Deletion of *MNN11* enhances the secretory expression of both glycosylated and non-glycosylated proteins

To determine the universality of the *mnn11*Δ mutant in promoting secretory expression, we tested the secretion of five different enzymes. These enzymes include glucoamylase BadGLA, endo-1,4-β-endoxylanase Xyn-CDBFV, endo-1,4-β-mannanase MAN330, chitosanase SpChi1, and endo-1,4-β-glucanase RuCelA. BadGLA and Xyn-CDBFV are hypermannosylated [16, 20], while the other three proteins are not glycosylated. As shown in Fig. 2A-E, compared to wild-type strain, the *mnn11*Δ mutant increased the secretion activities of BadGLA, Xyn-CDBFV, MAN330, SpChi1, and RuCelA by 176%, 43%, 130%, 668%, and 62%, respectively, indicating that the *mnn11*Δ mutant promotes the secretion of both glycosylated and non-glycosylated proteins.

SDS-PAGE showed that the glycosylated forms of BadGLA and Xyn-CDBFV expressed by wild-type cells appeared as smears above their theoretical molecular weights (Fig. 2F, G), consistent with previous studies [16, 20]. In the *mnn11*Δ mutant, the smears migrated faster (Fig. 2F, G). The differences in the glycosylation smears were more obvious in the SDS-PAGE using more samples (Fig S1). The results indicate that the deletion of *MNN11* affects M-Pol II activity, leading to defects in outside chain extension and reduced molecular weight. Compared to the wild-type cells, the intensity of the smear increased significantly in the *mnn11*Δ cells, suggesting an

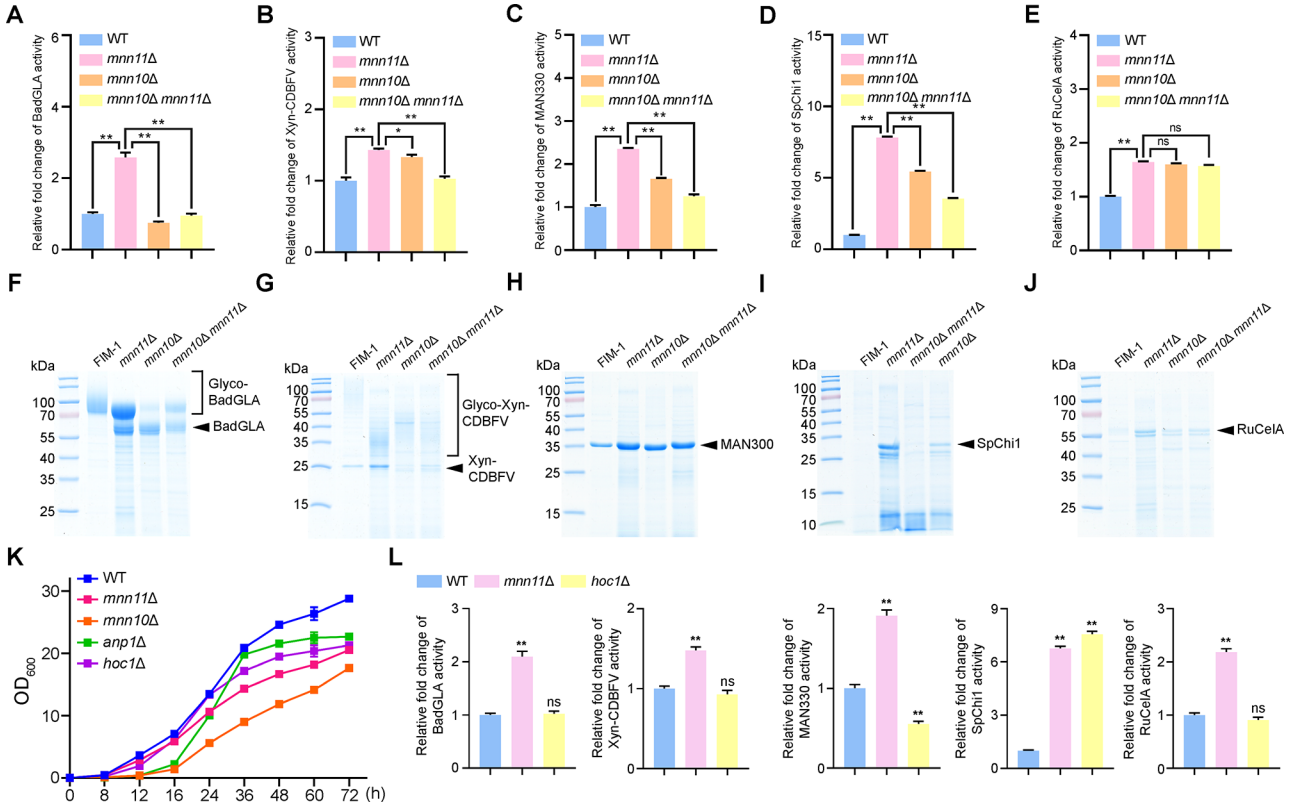


Fig. 2 Effect of *MNN11* and *MNN10* deletion on the secretory expression of glycosylated and non-glycosylated proteins. (**A-E**) Secretory activities of BadGLA (**A**), Xyn-CDBFV (**B**), MAN330 (**C**), SpChi1 (**D**), and RuCelA (**E**) in *mnn11*Δ, *mnn10*Δ and *mnn10*Δ *mnn11*Δ mutants. Cells were grown in 50 mL YD medium for 72 h at 30 °C, and enzyme activities in the supernatant were measured. Activities expressed by a wild-type cell (WT) were set as unit 1. Relative activities of *mnn11*Δ, *mnn10*Δ and *mnn10*Δ, *mnn11*Δ mutants are shown. Values are expressed as mean ± SD ($n = 3$) from biological replicates. * $p < 0.05$, ** $p < 0.01$, ns no significant. (**F-J**) SDS-PAGE of the supernatant. Arrows indicate the theoretical positions of the heterologous proteins. A smear that contained glycosylated proteins is indicated by the bracket. (**K**) Growth curves of mutants of M-Pol II complex subunits. Cells were inoculated into 50 mL YD medium at an initial OD_{600} of 0.01 and grown at 30 °C for 72 h. Values were calculated as mean ± SD ($n = 3$) from biological replicates. (**L**) Secretory activities of heterologous enzymes in the *mnn11*Δ and *hoc1*Δ mutants. Activities were measured as described in (**A**)

increase in the yield of BadGLA and Xyn-CDBFV. Similarly, *MNN11* deletion significantly increased the band intensity of MAN330, SpChi1, and RuCelA (Fig. 2H-J), indicating that the improved secretory activities in the *mnn11*Δ mutant are due to increased amounts of secreted proteins.

In addition to Mnn11, the M-Pol II subunits include Anp1, Mnn9, Hoc1, and Mnn10 [31]. Mnn10 is distantly related to Mnn11 (15.9% identity). Deletion of *MNN10* also affects M-Pol II activity [10]. Studies in *S. cerevisiae* suggest a positive genetic interaction between *MNN11* and *MNN10* [35]. Therefore, we investigated the effect of deletion of *MNN10* on secretion. Compared to the wild-type cells, the *mnn10*Δ cells did not significantly increase BadGLA activity (Fig. 2A). Although the *mnn10*Δ mutant improved the secretion activities of Xyn-CDBFV, MAN330, SpChi1, and RuCelA, the activities of three enzymes were significantly lower than those in the *mnn11*Δ mutant (Fig. 2A, C and D). Overall, the positive effects of *MNN10* deletion on secretion were weaker than those of *MNN11* deletion. The positions of the glycosylation smears of BadGLA and XynCDBFV in the *mnn10*Δ mutant differed from those in the *mnn11*Δ mutant (Fig. 2F, G). In *S. cerevisiae*, *mnn10*Δ and *mnn11*Δ mutants also displayed different migration speeds of glycosylated invertase [10]. These results suggest that the deletions of *MNN10* and *MNN11* affect M-Pol II activity to different extents, leading to varying degrees of defects in outside chain extension, which may explain the differential effects of *MNN10* and *MNN11* deletions on protein secretion.

To further investigate the relationship between *MNN10* and *MNN11* in promoting secretion, we constructed the *mnn10*Δ *mnn11*Δ double-deletion mutant. There was no positive synergistic effect of the double deletion on secretory activities (Fig. 2A-E). In contrast, the secretory activities of Xyn-CDBFV, MAN330, SpChi1, and RuCelA in the *mnn10*Δ *mnn11*Δ mutant were even lower than those in the *mnn10*Δ or *mnn11*Δ single mutant (Fig. 2B-E). These results suggest that a moderate defect (as seen in the *mnn10*Δ or *mnn11*Δ mutants), rather than a dramatic defect in M-Pol II complex (as seen in the *mnn10*Δ

*mnn11*Δ mutant), can achieve better improvement in protein secretion.

We also constructed mutants for two other subunits of the M-Pol II complex: Anp1 and Hoc1. However, we encountered difficulty deleting the third subunit, Mnn9. Compared to the transformation plate for deleting *HOC1*, far fewer colonies formed on the plate for deleting *MNN9*, and no positive *mnn9*Δ clones were identified afterward (Fig. S2). The results suggest that deleting *MNN9* causes a severe growth defect or even lethality in *K. marxianus*, reflecting the importance of the M-Pol II complex for cell growth. Consistent with this idea, mutants of other M-Pol II subunits (*mnn11*Δ, *mnn10*Δ, *anp1*Δ, and *hoc1*Δ) all exhibited growth defects to varying extents. The growth of the *anp1*Δ mutant was poor in overnight YD culture (12 h) (Fig. 2K). When transformed with a plasmid expressing a heterologous protein, the *anp1*Δ mutant obtained far fewer and slower-growing transformants compared to the wild-type and *hoc1*Δ cells (Fig. S3). Therefore, we focused on the effects of the *hoc1*Δ mutant. Results showed that the *hoc1*Δ mutant displayed improved expression of SpChi1 to a comparable level as the *mnn11*Δ mutant. However, deleting *HOC1* had no effect on the activities of BadGLA, XynCDBFV, and RuCelA, and even significantly reduced the activity of MAN330 (Fig. 2L). Consequently, *mnn11*Δ mutant stood out as the best performer in promoting protein expression among the M-Pol II subunit mutants.

mnn11Δ mutant upregulates key processes in protein secretion and exhibits a high demand for amino acids

To investigate the mechanism by which *MNN11* deletion enhances heterologous protein expression, we compared the transcriptomes of wild-type and *mnn11*Δ cells after 24 and 48 h of growth. At 24 h, there were 1,428 differentially expressed genes (DEGs) between wild-type and *mnn11*Δ cells, accounting for 27% of the total gene count, with 1,169 genes significantly upregulated and 259 genes significantly downregulated. At 48 h, there were 1,744 DEGs, accounting for 33% of the total, with 1,492 genes significantly upregulated and 252 genes significantly downregulated. Notably, 826 genes were consistently upregulated at both 24 h and 48 h, while 141 genes were consistently downregulated at both time points (Fig S4). The deletion of *MNN11* caused significant changes in the transcriptome.

We performed gene ontology (GO) term and KEGG enrichment analyses of the DEGs. Among the top 10 enriched GO terms present at both 24 h and 48 h were mitochondrial translation and carbohydrate metabolism, closely related to energy supply, as well as processes related to protein synthesis, including organic nitrogen component biosynthesis, peptide metabolism, and ribosome biogenesis. In the KEGG analysis, processes related to energy supply, such as the TCA cycle and oxidative phosphorylation, and those related to protein secretion, such as protein export and SNARE interaction, were enriched at 24 h and 48 h (Fig. 3B, C). These enriched processes are closely related to protein secretion, demonstrating the reliability of RNA-seq and DEGs analysis. Notably, the pyrimidine metabolism appeared in both the enriched GO and KEGG terms. Genes involved in pyrimidine metabolism generally upregulated in *mnn11*Δ mutant, which may be related to the enhanced gene transcription.

We selected 238 DEGs from enriched terms closely related to protein secretion and analyzed their upregulation and downregulation. In a previous study, we introduced heterologous or native disulfide bond formation modules into *K. marxianus* using artificial chromosomes, resulting in the P7 and K5 strains. Similar to the *mnn11*Δ mutant, the P7 and K5 strains exhibited broad-spectrum promotion of heterologous protein expression [20]. Therefore, we compared the regulation status of 238 DEGs with that in the P7 and K5 strains to identify shared regulatory characteristics in high-yield strains.

As shown in Fig. 3D, six glucose transporter genes, *ILV2* (involved in amino acid synthesis), *PDB1*, *PYC2*, and *CIT3* (involved in TCA cycles), and *TLG1* (involved in vesicle transport) were downregulated in the *mnn11*Δ mutant. All other DEGs in the *mnn11*Δ mutant were upregulated. The proportions of synchronously upregulated genes in *mnn11*Δ, P7, and K5 were 88%, 69%, 60%, and 60% for vesicle coating, protein translocation, protein glycosylation substrates, and vesicle tethering, respectively. This suggests that the upregulation of these processes is a common feature in high-secretion strains. Notably, even though P7 and K5 do not have glycosylation gene mutations, both strains, like *mnn11*Δ mutant, upregulated glycosylation genes and glycosylation substrate genes (43% and 60% synchronously upregulated, respectively). This suggests that the upregulation of glycosylation-related genes is not a compensatory response unique to glycosylation mutants but may be a general strategy used by *K. marxianus* to enhance secretory expression. All the processes containing high proportions of synchronously upregulated genes are essential for the protein secretion pathway. Enhancement of these processes in *mnn11*Δ mutant is expected to promote the secretory expression of various types of proteins, including both glycosylated and non-glycosylated proteins.

In addition to the shared characteristics with P7 and K5, *mnn11*Δ mutant exhibited unique gene expression features. The yeast vacuole is the primary intracellular degradation organelle and plays a key role in balancing amino acid concentrations [36]. In *mnn11*Δ mutant, amino acid transporters and V-ATPase on the vacuolar membrane were significantly upregulated, a trend

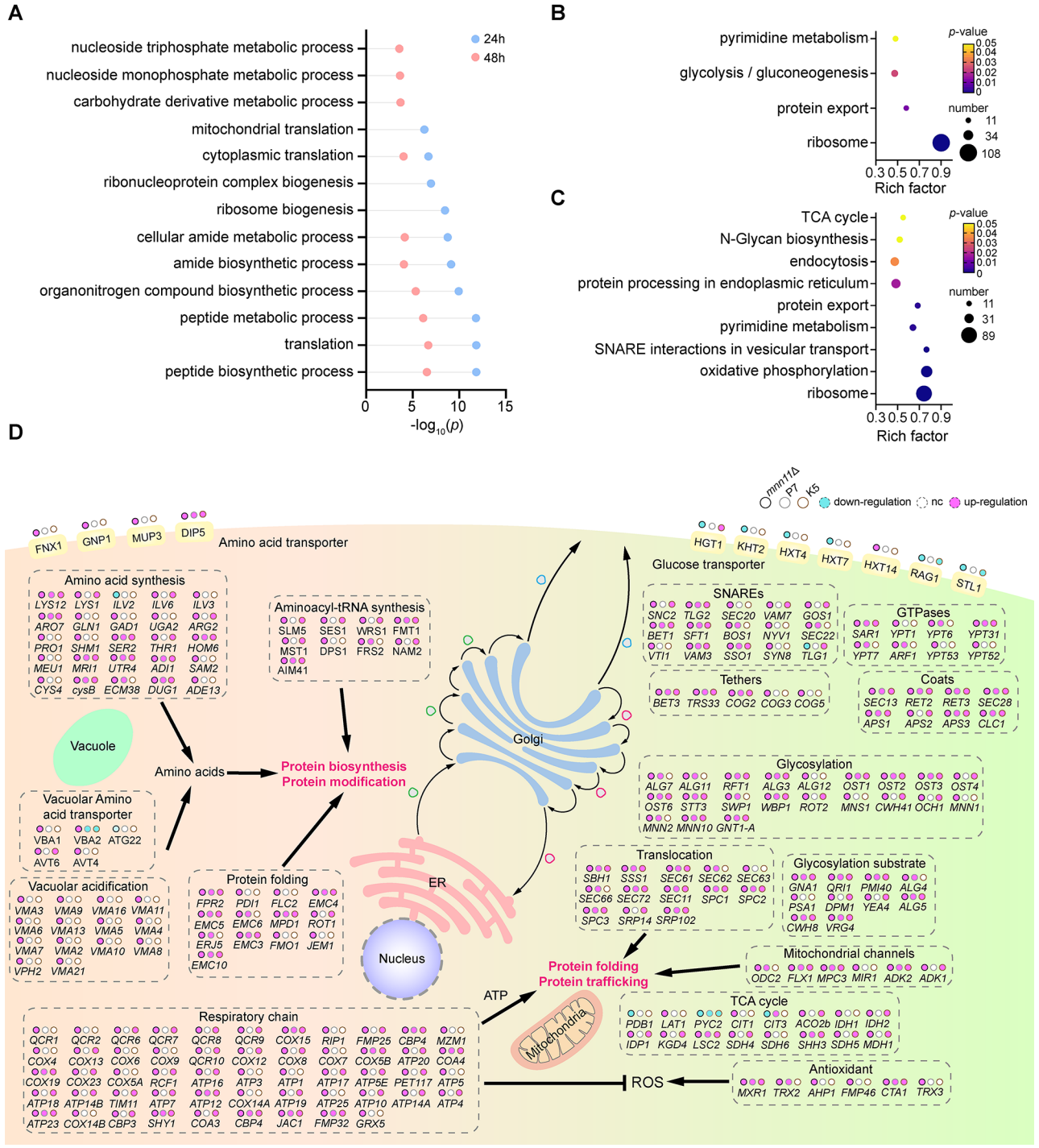


Fig. 3 *mnn11*Δ mutant upregulates key processes in protein secretion. (**A**) GO term enrichment analysis of DEGs. Wild-type and *mnn11*Δ cells were grown in YD liquid medium for 24 or 48 h. Cells were collected and subjected to transcriptomic analysis. Genes with a *p*-value < 0.05 and|$\log_2$ fold change| ≥ 1 were defined as DEGs. GO enrichment was performed based on biological processes (BP). The top 10 BP terms at both time points with a *p*-value < 0.05 are shown. (**B-C**) KEGG enrichment analysis of DEGs. Terms with *p*-value < 0.05 are listed in (**B**) (24 h) and (**C**) (48 h). (**D**) Comparison of DEG regulation among *mnn11*Δ, P7, and K5 strains. A total of 238 DEGs at 48 h were extracted from enriched terms, and the DEGs were categorized based on their functional annotation. The regulation status of these DEGs in P7 and K5 at 48 h was extracted from a previous study [19]. Circles filled with pink indicate upregulation, and those filled with blue indicate downregulation

not observed in P7 or K5 (Fig. 3D). The upregulation of V-ATPase may increase H^+ pumping, enhancing proteolytic activity to break down short peptides into amino acids. Upregulated transporters may release more amino acids to maintain intracellular levels, suggesting that *mnn11*Δ mutant has a higher amino acid demand. Supporting this, genes involved in amino acid biosynthesis and amino acid transportation across the plasma membrane were also significantly upregulated in *mnn11*Δ mutant (Fig. 3D).

We conducted a detailed analysis of amino acid biosynthesis gene expression (Fig. 4). For the synthesis of 16 amino acids, at least one gene in one step was upregulated at 24 or 48 h. In contrast, only one gene involved in the fourth step of leucine synthesis was significantly downregulated in *mnn11*Δ mutant at 24 h. The general upregulation of amino acid synthesis in *mnn11*Δ mutant suggests an insufficient intracellular amino acid supply. Mutations in M-Pol II complex subunits affect cell wall function [9, 10], such as altering porosity. Cell wall defects can impair nutrient uptake, including amino acids [37], leading to intracellular amino acid shortages, which may stimulate the upregulation of amino acid synthesis and enhance amino acid flux between the vacuole and cytoplasm.

Supplementation with organic nitrogen sources alleviated the growth defect of *mnn11*Δ mutant

The capacity of *mnn11*Δ mutant to promote the secretion of various heterologous proteins suggests that it could serve as a promising host for industrial protein production. In industrial production, growth rate and media composition are key factors for reducing costs. YD is a common fermentation medium for *K.*

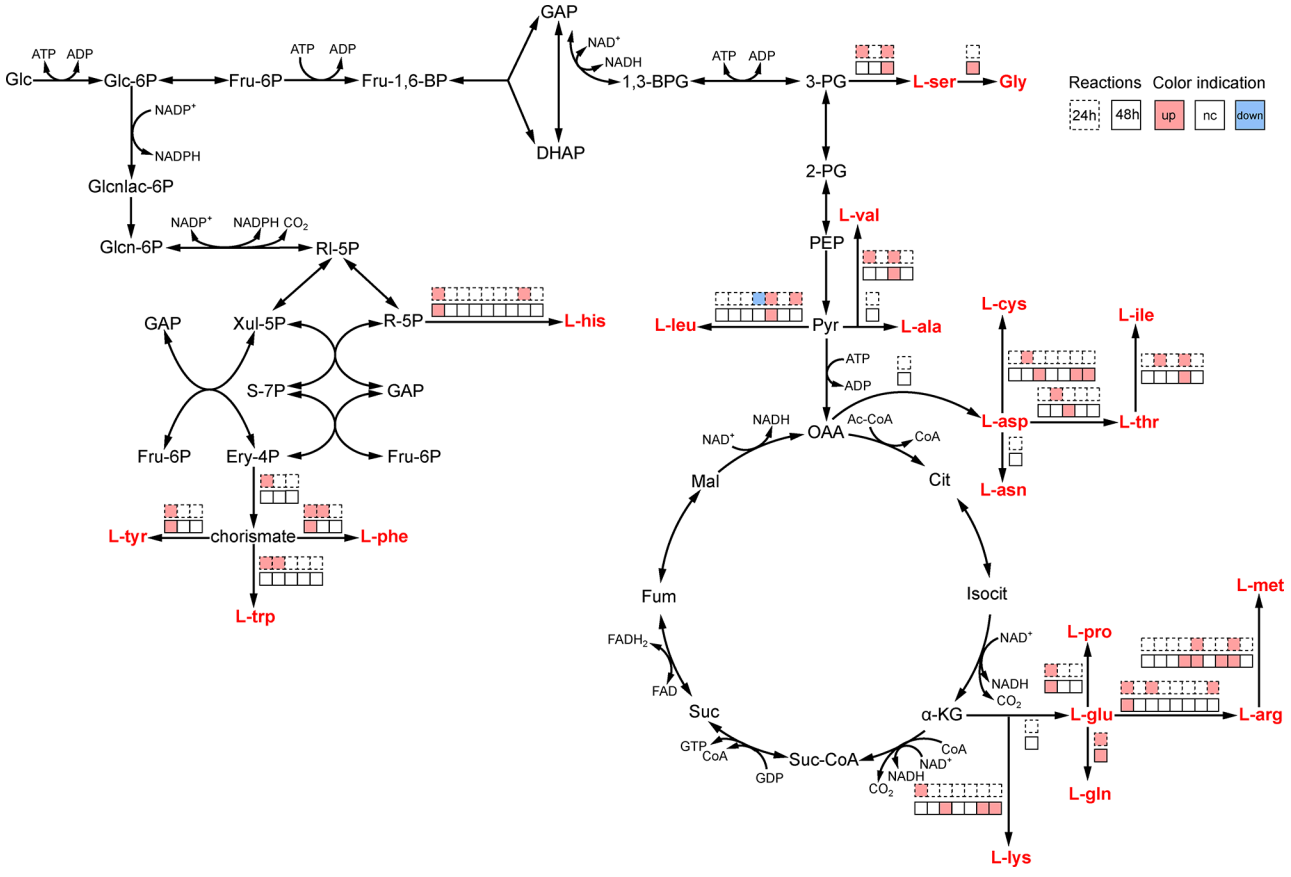


Fig. 4 *mnn11*Δ mutant upregulates amino acids biosynthesis. The biosynthesis pathways of 20 amino acids were depicted according to SGD YeastPathways (https://pathway.yeastgenome.org/). Boxes next to each amino acid represent steps in its biosynthesis. A solid or dashed line around each box indicates steps at 24 h and 48 h, respectively. One step can involve more than one gene. The expression levels of genes in *mnn11*Δ cells were compared to wild-type cells. If any gene in a step was significantly upregulated or downregulated, the step is labelled accordingly. There is no contradictory regulation within each step. Red indicates upregulation, and blue indicates downregulation. Relative gene levels in each step are shown in Table S7. Abbreviation: Glcnlac-6P, 6-phosphogluconolactone; Glcn-6P, 6-phosphogluconate; Rl-5P, ribulose 5-phosphate; R-5P, ribose 5-phosphate; Xul-5P, xylulose 5-phosphate; S-7P, sedoheptulose 7-phosphate; GAP, glyceraldehyde-3-phosphate; Ery-4P, erythrose 4-phosphate; Fru-6P, fructose-6-phosphate; Glc-6P, glucose-6-phosphate; Fru-1,6-BP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; 1,3-BPG,1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; OAA, oxaloacetate; Cit, citrate; Isocit, isocitrate; α-KG, α-ketoglutarate; Suc-CoA, succinyl-CoA; Suc, succinate; Fum, fumarate; Mal, malate

marxianus, containing 2% yeast extract and 4% glucose. Synthetic mineral (SM) medium is a most commonly used medium for *K. marxianus* in high-density fermentation [38], and it includes seed medium (SMS) and feed-batch medium (SMF). The main components of SMS and SMF are $(NH_4)_2SO_4$, glucose, KH_2PO_4, $MgSO_4$, and trace amounts of vitamins and minerals. SMS and SMF use inorganic nitrogen sources, reducing their costs to 13% and 40% of YD, respectively, making them more suitable for the industrial production of bulk protein products. SM medium has already been successfully used for high-density fermentation of various heterologous proteins, including industrial enzymes, virus-like particles, and edible proteins [16–19, 21, 39]. Therefore, we compared the growth of wild-type and *mnn11*Δ cells in both YD and SMS. In YD, the maximum specific growth rate (μ_{max}) of the *mnn11*Δ cells decreased by 17%, and the OD_{600} decreased by 26%, compared to wild-type cell (Fig. 5A). In SMS, the growth defect of the *mnn11*Δ cells was more pronounced, with μ_{max} and OD_{600} both decreasing by 65% compared to wild-type cells (Fig. 5A).

Transcriptomic analysis of the *mnn11*Δ mutant suggested an insufficient intracellular amino acid supply (Figs. 3D and 4). Amino acids are essential for cell

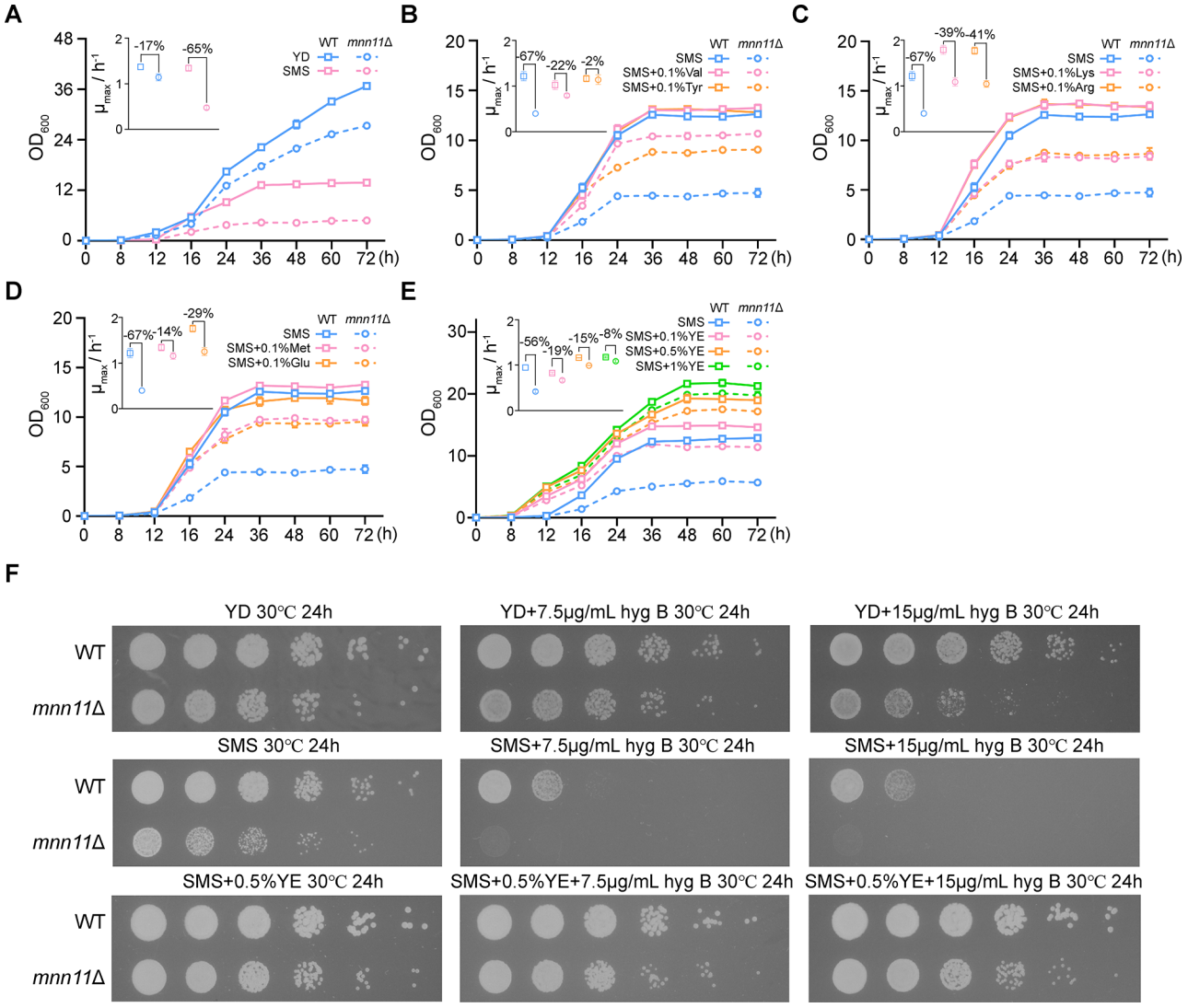


Fig. 5 Supplementation with organic nitrogen sources alleviated the growth defect of *mnn11*Δ mutant. (**A-E**) Growth curves of wild-type and *mnn11*Δ cells with different nitrogen sources. Cells were inoculated into 50 mL medium at an initial OD_{600} of 0.01 and grown at 30 °C for 72 h. Growth curves were obtained by monitoring OD_{600} in YD and SMS medium (**A**), SMS medium supplemented with 0.1% valine or tyrosine (**B**), 0.1% lysine or arginine (**C**), 0.1% methionine or glutamate (**D**), and different concentrations of yeast extract (YE) (**E**). Growth curves of wild-type cells in (**B**, **C**, **D**) were from the same sample. Values were calculated as mean ± SD ($n = 3$) from biological replicates. The maximum specific growth rate (μ_{max}) for each curve is shown in the inset. (**F**) Spot assay of wild-type and *mnn11*Δ cells. Overnight cultures were diluted 5-fold and spotted onto YD, SMS, and SMS plates supplemented with 0.5% YE. To test cell wall integrity, the medium was supplemented with hygromycin B. Plates were incubated at 30 °C for 24 h

growth, and their shortage can cause growth defects [40]. Since SMS uses ammonium sulfate as its nitrogen source, all amino acids must be synthesized de novo, exacerbating the deficiency in *mnn11*Δ mutant, and leading to its severe growth defect. If this hypothesis is correct, supplementing SMS with amino acids should alleviate this defect. Therefore, we tested the effect of adding 0.1% of individual amino acid to SMS on the growth of wild-type and *mnn11*Δ cells. In wild-type cells, no amino acid significantly increased OD_{600} at 72 h (Fig. 5B-D). However, adding glutamate, arginine, and lysine increased μ_{max} of wild-type cells by 48%, 44%, and 44%, respectively (Fig. 5C, D). Valine, tyrosine, and methionine had minimal impact on μ_{max} (Fig. 5B, D), likely due to yeast's preference for amino acid assimilation. Glutamate and arginine are preferred nitrogen sources, valine is intermediate, and tyrosine and methionine are non-preferred nitrogen sources [41]. As a result, glutamate and arginine are more efficiently assimilated, improving μ_{max}. While lysine cannot be utilized by *S. cerevisiae* or other yeast species in the post-whole-genome-duplication (WGD) clade [42], *K. marxianus*, a pre-WGD species, harbours potential lysine assimilation pathways [43]. Our results suggest lysine can serve as a preferred nitrogen source in *K. marxianus*.

On the other hand, adding any amino acid substantially improved the growth of *mnn11*Δ cells. The addition of arginine, lysine, glutamate, valine, methionine, and tyrosine reduced the gap in μ_{max} between wild-type and *mnn11*Δ cells from 67% to 41%, 39%, 29%, 22%, 14%, and 2%, respectively. Similarly, the addition of these amino acids reduced the OD_{600} gap between wild-type and *mnn11*Δ cells at 72 h from 63% to 35%, 38%, 18%, 19%, 27%, and 29%, respectively. These results suggest that the growth defect of *mnn11*Δ mutant in SMS is closely related to insufficient amino acid supply, and supplementation with amino acids effectively alleviates this defect. Since supplementing individual amino acids is costly for industrial production, we investigated whether low concentrations of yeast extract (YE) could achieve similar effects. The results showed that 0.1%, 0.5%, and 1% YE reduced the μ_{max} gap between wild-type and *mnn11*Δ cells from 56% to 19%, 15%, and 8%, respectively (Fig. 5E), and reduced the OD_{600} gap at 72 h from 56% to 22%, 9%, and 7%. These results suggest that YE, as a complex organic nitrogen source, can alleviate the growth defect of *mnn11*Δ mutant in a dose-dependent manner.

As suggested in the previous section, the cell wall defect caused by *MNN11* deletion may lead to insufficient amino acid supply. As shown in Fig. 5B-E, supplementing SMS with YE effectively alleviated the growth defect likely caused by an amino acid shortage. To confirm the connection between the cell wall defect and amino acid deficiency, we investigated the impact of YE supplementation on the cell wall defect phenotype. The spot assay revealed that *mnn11*Δ cells grew slightly worse than wild-type cells in YD (Fig. 5F). As the hygromycin B concentration increased, the growth difference between wild-type and *mnn11*Δ cells became more pronounced, indicating greater sensitivity to hygromycin B and a cell wall defect in *mnn11*Δ cells, consistent with findings in *S. cerevisiae* [10]. On SMS plates, *mnn11*Δ cells grew substantially worse than wild-type cells, consistent with the growth curve results in Fig. 5A. When hygromycin B was added to SMS, *mnn11*Δ cells exhibited a pronounced synthetic growth defect, suggesting a link between the amino acid shortage and the cell wall defect. Supplementing SMS + hygromycin B with 0.5% YE rescued the severe growth defect of *mnn11*Δ cells, restoring the growth difference between wild-type and *mnn11*Δ cells to the level observed in YD (Fig. 5F). This result indicates that supplementing SMS with low concentrations of organic nitrogen not only alleviates the growth defect induced by amino acid shortage but also mitigates the cell wall defect.

Supplementation with yeast extract improves the yield of *mnn11*Δ mutant in high-density fermentation

To verify whether supplementing organic nitrogen sources in SM could be applied in industrial production, we conducted high-density fermentation in a 5-L fermenter. At the flask scale, the μ_{max} of *mnn11*Δ mutant in SMS supplemented with 0.5% YE was close to that in SMS with 1% YE (Fig. 5E). To reduce costs, we used SMS and SMF containing 0.5% YE for high-density fermentation, which cost 32% and 59% of YD containing 2% YE, respectively. In the initial stage of fermentation, YE supplementation increased the $\mu_{0-12\ h}$ of wild-type cells expressing Mel1, Xyn-CDBFV, and RuCelA by 68%, 102%, and 69%, respectively. In contrast, the *mnn11*Δ cells exhibited a long lag phase in SMS without YE, leading to an extremely low $\mu_{0-12\ h}$. However, with YE supplementation, $\mu_{0-12\ h}$ of *mnn11*Δ cells expressing Mel1, Xyn-CDBFV, and RuCelA increased by 1318%, 1143%, and 1037%, respectively (Fig. 6A-C). Thus, YE supplementation effectively shortened the lag phase of the *mnn11*Δ mutant, allowing it to enter the exponential phase more rapidly. This improvement is valuable for industrial production, as a shorter lag phase reduces contamination risk.

During Mel1 expression, the OD_{600} of the *mnn11*Δ cells with YE supplementation remained consistently higher than that of the *mnn11*Δ cells without YE (Fig. 6A). However, during Xyn-CDBFV and RuCelA expression, the OD_{600} of the YE-supplemented *mnn11*Δ mutant fell below that of the mutant without supplementation after 48 h, likely due to the negative impact of overexpressed proteins on cell growth. Secretory activities in the YE-supplemented *mnn11*Δ mutant surpassed those in the

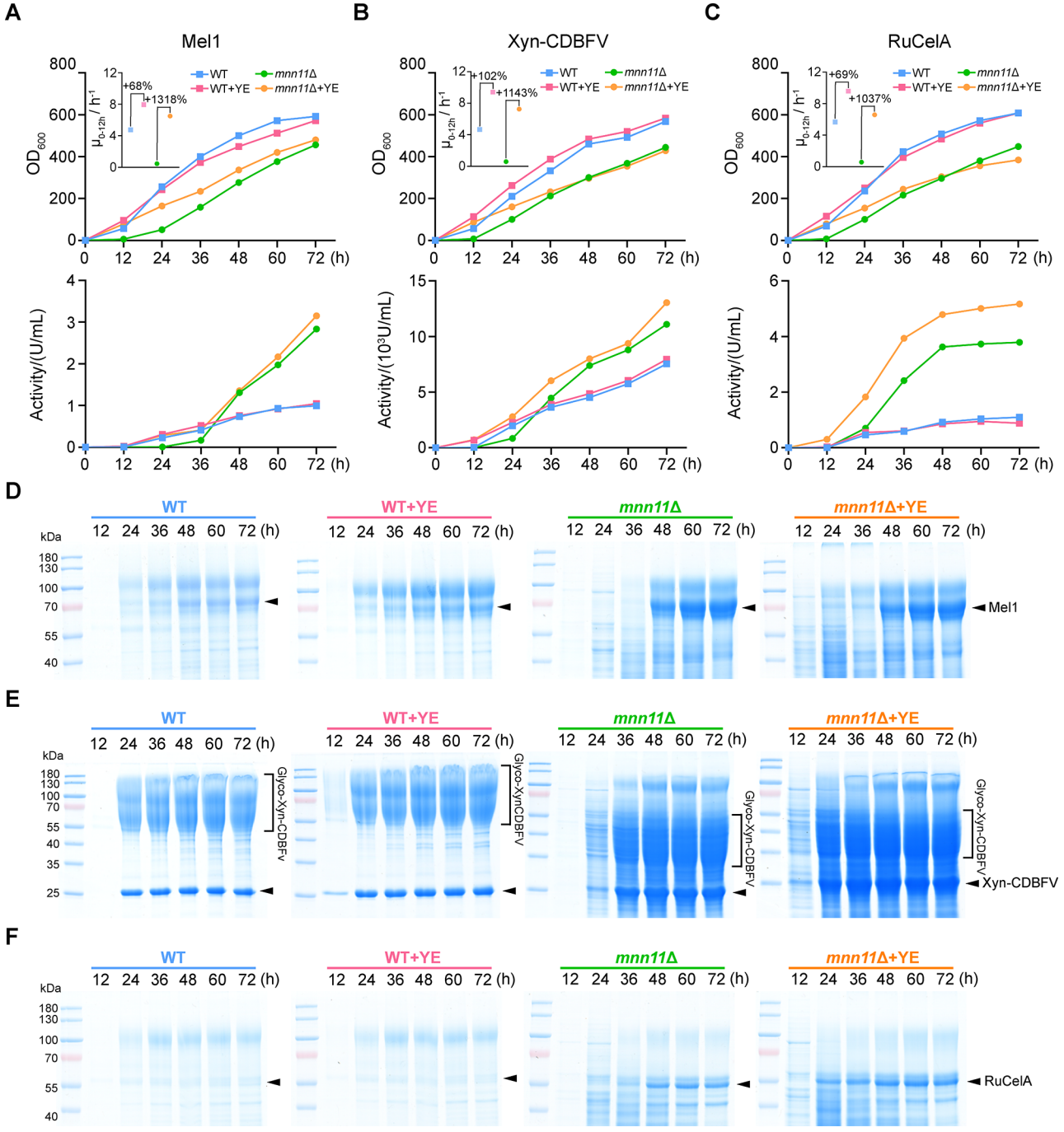


Fig. 6 Supplementation of low concentrations of yeast extract improves the yield of *mnn11*Δ mutant in high-density fermentation. (**A-C**) Growth curves and secretory activities during high-density fermentation of wild-type and *mnn11*Δ cells. Cells expressing Mel1 (**A**), Xyn-CDBFV (**B**), or RuCelA (**C**) were grown in SMS and SMF with or without 0.5% YE supplementation. Specific growth rates during the early stages ($\mu_{0-12\ h}$) are shown in the inset. (**D-F**) SDS-PAGE of the supernatant at 72 h. Arrows indicate the theoretical positions of heterologous proteins. A smear that contained glycosylated proteins is indicated by a bracket

mutant without supplementation after 12 h, and by 72 h, the secretory activities of Mel1, Xyn-CDBFV, and RuCelA were 11%, 18%, and 36% higher, respectively. Notably, YE did not affect the wild-type cells' secretory activity, suggesting that YE specifically enhances the secretion advantage of the *mnn11*Δ cells. In terms of proportion, without YE supplementation, the activities of Mel1, Xyn-CDBFV, and RuCelA expressed by the *mnn11*Δ cells were 186%, 47%, and 245% of those expressed by wild-type cells, respectively. With YE supplementation, these

proportions increased to 316%, 173%, and 470%, respectively. SDS-PAGE analysis of the supernatant was consistent with the secretory activities, as the amounts of heterologous proteins expressed by the YE-supplemented *mnn11*Δ mutant were significantly higher than those of the mutant without supplementation (Fig. 6D-F). Therefore, supplementing low concentrations of YE in SMS and SMF during high-density fermentation can shorten the lag phase of the *mnn11*Δ mutant and enhance its ability to express both glycosylated and non-glycosylated proteins.

Yet, it is worth noting that the addition of YE increases costs and causes issues in downstream protein purification. *K. marxianus* is recognized as a food-safe yeast and has been approved as a feed additive in China [44]. Therefore, in the large-scale expression of feed enzymes, such as Mel1, Xyn-CDBFV, and RuCelA, crude culture might be dried and used as feed additives without purification. When expressing high-value recombinant proteins, improved yields in *mnn11*Δ mutants might compensate for the cost of adding extra YE and further purification. In some cases, YE can be replaced by single amino acids, such as glutamate, in the fermentation of *mnn11*Δ strains, since single amino acids can be easily separated from the secreted proteins. A similar strategy has been applied in the fermentation of *Escherichia coli* [45], *Bacillus subtilis* [46], *S. cerevisiae* [47], *Pichia pastoris* [48], and CHO cells [49].

The effect of different amino acids on high-yield strains varies. For example, adding arginine or lysine, rather than isoleucine, to the medium of K5 can further increase BadGLA expression [20]. In a synthetic medium, adding tyrosine increased the yield of *Plasmodium falciparum* merozoite surface protein 3 in *P. pastoris* by 3.5-fold [50]. Among the six amino acids tested in this experiment, glutamate, arginine, and lysine provided better support for the growth of the *mnn11*Δ cells. Glutamic acid, arginine, and lysine are among the top six most abundant amino acids in YE [51], which explains the effectiveness of YE in alleviating the growth defect of *mnn11*Δ mutant and boosting recombinant protein expression. Soy peptone and tryptone, also commonly used organic nitrogen sources, have different proportions of preferred and non-preferred nitrogen compared to YE [52]. For different high-yield strains, selecting a nitrogen source that matches specific amino acid requirements may result in better expression enhancement at a lower cost.

Conclusions

This study demonstrates that glycosylation mutation offers a novel strategy to improve the secretory expression of heterologous proteins in *K. marxianus*. The *mnn11*Δ mutant enhances the secretion of both glycosylated and non-glycosylated proteins by upregulating key secretion processes. The *mnn11*Δ mutant exhibits a significant growth defect in the synthetic medium due to amino acid shortage. Supplementing the medium with low concentrations of organic nitrogen alleviates this defect and improves heterologous protein expression. The amino acid shortage in *mnn11*Δ mutant is linked to a cell wall defect, and most glycosylation mutants exhibit similar defects. Thus, this strategy of supplementing organic nitrogen sources might be applied to optimize the expression of other glycosylation mutants.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02676-2.

Supplementary Material 1: Additional file 1 – Figure S1 - Wild-type and ***mnn11***D cells were transformed with LHZ443 (Xyn-CDBFV) or LHZ1020 (BadGLA). Transformants were selected on SC-Ura plates and then grown in 50 mL YD medium for 72 h at 30 °C. The cells were pelleted, and the supernatant was collected. A total of 30 µL of supernatant was mixed with 6 µL of 5× SDS-PAGE loading buffer and boiled. Subsequently, 35 µL of the sample was subjected to SDS-PAGE analysis.

Supplementary Material 2: Additional file 2 – Figure S2 - Transformation plates of deleting ***HOC1*** and ***MNN9***.gRNA targeting ***HOC1*** was inserted into the CRISPR vector (LHZ531) to obtain LHZ1646. Three different gRNAs targeting ***MNN9*** were individually inserted into the CRISPR vector to obtain LHZ1647-1649. Primers containing gRNA sequences are listed in Table S2. A 500 bp sequence upstream and downstream of the ***HOC1*** or ***MNN9*** ORF was amplified and ligated together to form the donor sequence. LHZ1646 was co-transformed with the donor sequence into FIM-1ΔU to delete ***HOC1***. Similarly, LHZ1647-1649 were co-transformed with the donor sequence into FIM-1ΔU to delete ***MNN9***. Cells were selected on SC-Ura plates, and the plates were incubated at 30°C for 2 days before imaging

Supplementary Material 3: Additional file 3 – Figure S3 - Plates of WT, ***hoc1***Δ and ***anp1***Δ cells transformed with LHZ1627. A total of 10 OD_{600} cells from the overnight culture were transformed with 1 µg of LHZ1627 (SpChi1). Transformants were selected on SC-Ura plates, and the plates were incubated at 30°C for 3 days before imaging

Supplementary Material 4: Additional file 4 – Figure S4: Proportion of DEGs in the mnn11Δ mutant. (A) Proportion of upregulated and downregulated DEGs at 24 h and 48 h. (B) Overlap of DEGs between the two time points

Supplementary Material 5: Additional file 5 – Table S1: List of plasmids used in this study.

Supplementary Material 6: Additional file 6 – Table S2: List of primers used in this study

Supplementary Material 7: Additional file 7 – Table S3: List of strains used in this study

Supplementary Material 8: Additional file 8 – Table S4: Global transcriptional analysis

Supplementary Material 9: Additional file 9 – Table S5: GO term enrichment analysis

Supplementary Material 10: Additional file 10 – Table S6: KEGG enrichment analysis

Supplementary Material 11: Additional file 11 – Table S7: Relative gene expression levels of amino acids biosynthesis

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Author contributions
Yao Yu and Hong Lu planned the research design and supervised the experimental work. Shihao Zhou and Jungang Zhou performed the experimental work. Shihao Zhou, Pingping Wu and Haiyan Ren analyzed the data. Yao Yu and Shihao Zhou wrote the manuscript. All authors reviewed the manuscript.

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

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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