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Promotion of Extracellular Activity of Cellobiohydrolase I from *Trichoderma reesei* by Protein Glycosylation Engineering in *Saccharomyces cerevisiae*

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Abstract

The N-glycosylation in *Saccharomyces cerevisiae* is of the high-mannose type, which affects the activity of the secreted heterologous glycoproteins. Cellobiohydrolase I (*Tr*-Cel7A) from *Trichoderma reesei*, is thus hyperglycosylated when expressed in *S. cerevisiae*. In the present work, three genes encoding the endogenous mannosyltransferases, Och1p, Mnn9p and Mnn1p, involved in glycoprotein processing in the *S. cerevisiae* Golgi apparatus, were individually or combinatorially disrupted to investigate the effect of the glycosylation extent on the activity of the secreted *Tr*-Cel7A. The glycosylation of the recombinant *Tr*-Cel7A was decreased and its extracellular activity was increased in all the deletion mutants. The simultaneous deletion of *och1* and *mnn1* has the most improvement on extracellular *Tr*-Cel7A activity. After expressed the α-1,2-mannosidase (*Tr*-Mds1p) from *T. reesei* in *mnn1Δ/och1Δ* strain, the *Tr*-Cel7A activity was further increased up to 320 ± 8% higher than that of the wild type strain. Such activity improvement was due not only to the higher secretion yield but also to the increased specific activity resulted from the changes in glycosylation. The results thus indicated that protein glycosylation engineering in *S. cerevisiae* was an effective approach to improve the extracellular activity of *Tr*-Cel7A.

Keywords: Cel7A; Mannosyltransferase; α-1,2-Mannosidase; Glycosylation; Protein secretion; Budding yeast

Introduction

The suitable glycosylation in secretory pathway is crucial for the correct folding, stability, bioactivity and extracellular activity of secreted heterologous proteins [1]. One of the advantages of using budding yeast expression system for the production of heterologous glycoproteins is their capability to perform glycosylation, a process that does not occur in *Escherichia coli* [2]. In *Saccharomyces cerevisiae*, glycoprotein are normally modified by the core structure ($Man_9GlcNAc_2$) which contains three α-1,2 mannosyl linkages in the endoplasmic reticulum (ER). One of α-1,2 mannosyl linkages is hydrolyzed by the site-specific *α*-1,2-mannosidase Mns1p just before the glycoprotein leaves for the Golgi apparatus. The extension and embranchment of N-linked glycan chains on the newly formed $Man_8GlcNAc_2$ are performed by the α-1,6-mannosyltransferases such as Och1p, Mnn9p and Van1p, and the α-1,2 or α-1,3-mannosyltransferases such as Alg11p and Mnn1p [3] in Golgi apparatus. The mannose residues can reach to about 200 to form a complex mannan [4], which has a potential to affect the activity of N-glycosylated glycoprotein including the heterologous proteins expressed in *S. cerevisiae* [5]. Wang et al. [6] screened *S. cerevisiae* gene-knockout strains of glycosylation-related genes for improved extracellular activity of a heterologous exocellulase (PCX) from the cellulose digesting fungus *Phanerochaete chrysosporium*. They found that the degree of N-glycosylation plays an important role in heterologous cellulase activity and both over- and under-glycosylation may alter the enzyme activity of cellulases in *S. cerevisiae*. Expression of cellulases in the traditional ethanol production strain *S. cerevisiae* is one research aspect of the ethanol production from lignocelluloses using the consolidated bioprocessing (CBP) strategy [7,8]. Among the cellulase system in *Trichoderma reesei*, cellobiohydrolase I (Tr-Cel7A) is recognized as one of the effective enzymes for lignocellulose degradation. However, the *Tr*-Cel7A was generally hyperglycosylated with lower secretion in *S. cerevisiae* compared with it in *T. reesei* [9-11].

The native *Tr*-Cel7A in *T. reesei* is a glycoprotein with molecular mass about 70 kDa [10], which is larger than its molecular mass (~45.97 kDa) from the amino acid [12]. A glycosyl chain unit modified on *Tr*-Cel7A is $Man_{5\text{-}8}GlcNAc_2$ formed from the $Man_9GlcNAc_2$, a same core structure as it in *S. cerevisiae* [13,14]. But during its secretion process, unlike in *S. cerevisiae*, all of the three α-1, 2-mannosyl residues are digested by α-1,2-mannosidase (*Tr*-Mds1p) in the ER and/or outside of the cell in *T. reesei* [15-17].

In the present work, the glycosylation engineering including the disruption of *S. cerevisiae* endogenous mannosyltransferases and the expression of the *T. reesei* α-1,2-mannosidase, was performed. The *Tr*-Cel7A specific activity and protein secretion yield in yeast were detected to investigate the effect of the glycosylation close to the natural state on its extracellular activity.

Materials and Methods

Strain construction:

Three disruption cassettes containing homologous recombinant arms, MNN1F-*loxP-KanMX4-loxP*-MNN1R, MNN9F-*loxP-KanMX4-loxP*-MNN9R and OCH1F-*loxP-KanMX4-loxP*-OCH1R, were transformed independently or as two successively into the *S. cerevisiae* strain CEN.PK102-3A [18]. The *KanMX4* marker was removed using

the *Cre-loxP* system as previously described [19], resulting in the single-deletion strains *mnn1Δ, mnn9Δ* and *och1Δ*, and the double-deletion strains *mnn1Δ/mnn9Δ* and *mnn1Δ/och1Δ*.

The open reading frame (ORF) with its native signal sequence of *T. reesei* α-1,2-mannosidase gene (*Tr*-MDS1, GenBank accession no. AF212153.1) was amplified from pAJ401mds1 [15] (BCCM/LMBP Plasmid Collection) and ligated into the plasmid pYX242 (Novagen, Madison, WI), which contains the *TPI* promoter and HA tag coding sequence in the 3' end. The resulting plasmid pYX242MH (Figure 1) was then transformed respectively into the wild type and the gene deletion strains. The *Tr*-cel7A gene with a FLAG tag expressing plasmid pJCF [11] was introduced into all modified strains. All *S. cerevisiae* strains and plasmids used in the present work are listed in Table 1, and the primers used for PCR amplification in the present work are listed in Table S1.

Figure 1: The physical map of plasmid pYX242MH.

Culture conditions:

SC-Ura (1.7 g l^{-1} yeast nitrogen base, 5 g l^{-1} ammonium sulfate, and 0.77 g l^{-1} amino acid mixture with omitted uracil) and SC-Leu-Ura (1.7 g l^{-1} yeast nitrogen base, 5 g l^{-1} ammonium sulfate, 0.65 g l^{-1} amino acid mixture with omitted uracil, leucine and histidine, and 20 mg l^{-1} histidine) media were used for culture of the recombinant strains. Cultures were maintained at 30 °C for 48 h with shaking at the agitation speed of 200 rpm.

Purification of *Tr*-Cel7A from recombinant *S. cerevisiae*:

The *S. cerevisiae* strain expressing *Tr*-Cel7A was cultured in SC-Ura medium in 3 L at 30°C. After 48 h, the cells were separated from the growth medium by centrifugation (15 min at 10,000 × g) and filtration over a 0.22 μm pore size. The cell-free culture supernatants were concentrated approximately 100-fold using an ultrafiltration device ÄKTAcross flow automated filtration system (GE HealthCare, USA), equipped with 30 kDa cutoff membranes. This was also used for the purification of Tr-Cel7A.

Anion exchange chromatography using a Q Sepharose column (HiTrapTM 5 ml QHP, GE HealthCare, USA) and phosphate buffered saline (PBS) at pH 5.5 was used for purification. A linear NaCl gradient from 0 M to 1 M NaCl at 2 ml min^{-1} flow rate was used for eluting the protein. The purified *Tr*-Cel7A was used for further analysis.

Assay for *Tr*-Cel7A activity:

The *p*-nitrophenyl-β-D-cellobioside (pNPC) (Sigma, USA) was used as the substrate to assay the *Tr*-Cel7A activity [20]. The specific activity and the extracellular activity of *Tr*-Cel7A were determined by incubating the purified *Tr*-Cel7A protein and the supernatants collected from cell culture via centrifugation (15 min at 10,000 × g), respectively, following the described method [11]. One unit of *Tr*-Cel7A activity was defined as the amount of enzyme required to release 1 μmol of pNP from the pNPC in 1 min at 50°C and pH 5.0. The protein concentration of purified *Tr*-Cel7A was determined using the BCA Protein Assay Kit (Beyotime biotechnology, China). And the dry weight of yeast cells was determined as previously described [11].

Strain and plasmid	Relevant genotype	Source
Saccharomyces cerevisiae		
CEN.PK102-3A	MATα ura3-52 leu2-112	[18]
BSX000 (Control)	CEN.PK102-3A derivative; pJFE3	[11]
cel7AF (Wild type)	CEN.PK102-3A derivative; {pJCF}/(Tr-cel7A-FLAG)	[11]
mnn1Δ/cel7AF	CEN.PK102-3A derivative; *mnn1::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG)*	This work
mnn9Δ/cel7AF	CEN.PK102-3A derivative; *mnn9::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG)*	This work
och1Δ/cel7AF	CEN.PK102-3A derivative; *och1::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG)*	This work
mnn1Δ/mnn9Δ/ cel7AF	CEN.PK102-3A derivative; *mnn1::loxP-KanMX-loxP; mnn9::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG)*	This work
mnn1Δ/och1Δ/ cel7AF	CEN.PK102-3A derivative; *mnn1::loxP-KanMX-loxP; och1::loxP-KanMX-loxP; {pJCF}/(Tr-cel7A-FLAG)*	This work
MDS1/cel7AF	cel7AF derivative; *{pYX242MH}/(Tr-MDS1-HA)*	This work
mnn1Δ/och1Δ/ MDS1/cel7AF	*mnn1Δ/och1Δ/cel7AF* derivative; *{pYX242MH}/(Tr-MDS1-HA)*	This work
Plasmids		
pJFE3	*TEF1p-PGK1t*	[35]
pJCF	pJFE3; *TEF1p-Tr-cel7A-FLAG-PGK1t*	[11]
pAJ401mds1	*Tr-MDS1*	[15]
pYX242	*TPI1p-polyA*	[36]
pYX242MH	*TPI1p-Tr-MDS1-HA-polyA*	This work

Table 1: Strains and plasmids used in this work.

Western blot analysis:

Western blot analysis of total secreted proteins at 48 h was performed using the anti-FLAG monoclonal antibody OctA-Probe (Santa Cruz, USA) as the primary antibody to detect *Tr*-Cel7A. Horseradish peroxidase-conjugated affinipure goat anti-rabbit IgG antiserum (Zhongshan Goldenbridge Biotechnology Co., Ltd, China)

was used as the secondary antibody. The immunoreactive proteins were visualized using ECL (Thermo Scientific Pierce, USA) and detected using an ImageQuant 400 imaging system (GE Healthcare, USA).

Determination of *Tr*-Cel7A concentration by ELISA system:

The cells were incubated in 100 mL SC-Ura and SC-Leu-Ura medium. The culture supernatants were concentrated approximately 100-fold using Amicon Ultra-15 centrifugal filter units (Millipore, USA) equipped with 30 kDa cutoff membranes. *Tr*-Cel7A quantitation was performed following the procedure described previously [11] using the ELISA method [21].

Statistical analysis of data:

A two-tailed *t*-test, assuming equal variances, was used to determine whether the differences were statistically significant.

Results

The deletion of the endogenous mannosyltransferases decreased the glycosylation of *Tr*-Cel7A:

The secreted *Tr*-Cel7A from *S. cerevisiae* was proved to be N-hyperglycosylated [9-11]. Our previous result showed that the N-glycosidase F (PNGase F) removed almost all types of the N-linked glycosylation of recombinant *Tr*-Cel7A protein secreted by *S. cerevisiae* and lessened the molecular masses from over 100 kDa to two small types, which were ~90 and ~45 kDa [11]. To decrease the elongation of the outer chain of the N-linked oligosaccharides, the yeast α-1,6-mannosyltransferases, Och1p and Mnn9p, and the α-1,3-mannosyltransferase, Mnn1p, which were involved in the N-glycosylation process, were disrupted singly or doubly. As expected, all the single deletion *mnn1Δ*, *mnn9Δ* and *och1Δ* increased extracellular *Tr*-Cel7A activities (Figure 2a). The double deletion showed an additive effect on its extracellular activities (Figure 2b). At 48 h, *mnn1Δ/och1Δ* increased its extracellular activity by 272 ±2%, compared with the wild type strain *cel7AF*. Western blot analysis showed that all these modifications decreased the molecular masses of *Tr*-Cel7A. The absence of the Och1p or Mnn9p reduced the molecular masses of *Tr*-Cel7A more significantly and showed two bands at about 75 kDa and 45 kDa both in single- and double-modified strains (Figure 2c). This indicated that glycosylation of *Tr*-Cel7A decreased by deleting the endogenous mannosyltransferases thus improved the extracellular activity. Meanwhile the specific activity with purified proteins by the Q Sepharose column and the secretion yield of *Tr*-Cel7A from *mnn1Δ/och1Δ* were determined respectively. The specific activity of *Tr*-Cel7A proteins from *mnn1Δ/och1Δ* was increased by 175 ± 1%, and its secretion yield detected by ELISA assay was also increased by 86 ± 6%, compared with that of the wild type strain *cel7AF* (Figure 3a and 3b, column 1 and 2). This indicated that reduction in the glycosylation on *Tr*-Cel7A improved not only the specific activity but also the secretion yield.

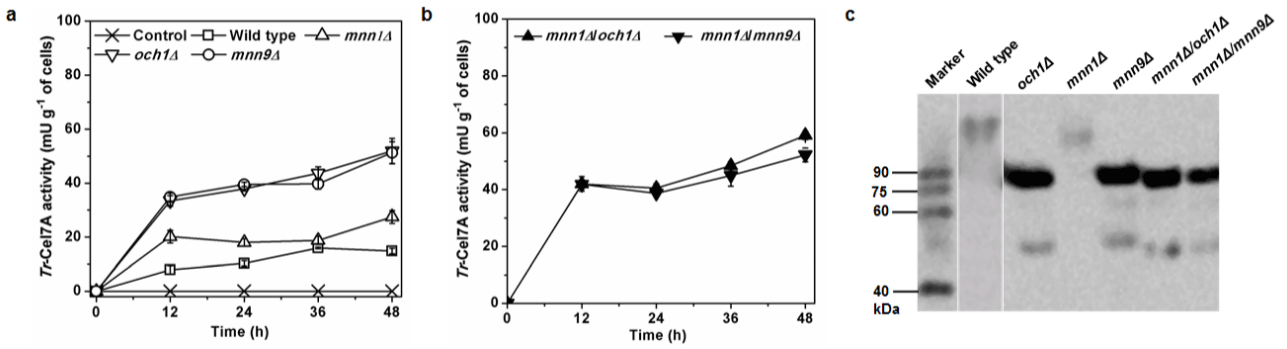

Figure 2: Deletion of yeast mannosyltransferases increased the extracellular activity (a, b) and decreased the glycosylation (c) of *Tr*-Cel7A. (a, b) The extracellular activity was assayed with culture supernatants using pNPC as the substrate. The definitions of the "Control" and " Wild type" were shown in Table 1. Data shown are the mean values (± standard error) obtained from three independent experiments. The differences between the modified strains and wild type were all significant (P0.05), even more significant (P<0.01) at 48h. (c) The secreted proteins were precipitated from culture supernatants at 48 h. The *Tr*-Cel7A proteins were detect by western blot with anti-Flag monoclonal antibody.

The expression of α-1,2-mannosidase Tr-Mds1p in S. cerevisiae ulteriorly decreased the molecular weight of Tr-Cel7A: The core structure $(Man_9GlcNAc_2)$ of modified glycoprotein in endoplasmic reticulum is same in both *T. reesei* and *S. cerevisiae*. In the following secretion process, only one of *α*-1,2 mannosyl linkages was digested in *S. cerevisiae* [22], but in *T. reesei* all three *α*-1,2 mannosyl linkages were cleaved by *Tr*-Mds1p inside and outside of the cells [15,17]. Therefore, we expressed the *Tr*-Mds1p with its native signal peptide in *S. cerevisiae* strains. The molecular masses of recombinant *Tr*-Cel7A became visibly smaller (Figure 4a, Line 1 and 2) with extracellular activity improved by 26 ± 1% at 48 h (Figure 4b) in the wild type background. When *Tr*-Mds1p was expressed in the mnn1Δ/och1Δ strain, more *Tr*-Cel7A proteins gathered at the status around 75 and 45 kDa (Figure 4a, Line 4), and the extracellular activity of *Tr*-Cel7A was further improved to 66.5 ± 2.1 mU g^{-1} dry weight of cells (P<0.01) at 48 h, which was 320 ± 8% higher than that in wild type strain (Figure 4b). This indicated that expression of α-1,2-mannosidase *Tr*-Mds1p in *S. cerevisiae* ulteriorly decreased the glycosylation of *Tr*-Cel7A, and thereby affected its extracellular activity. Meanwhile, about 45 ± 8% and 16 ± 3% improvements in the specific activity and the secretion yield of *Tr*-Cel7A were obtained when Tr-Mds1p was expressed in *mnn1Δ/och1Δ* (Figure 3b and 3c,

column 3). As it in *T. reesei* [12], the recombinant *Tr*-Mds1p was detected both inside and outside cells of *S. cerevisiae* by western blot analysis (data not shown). This revealed that *T. reesei* source α-1,2-mannosidase *Tr*-Mds1p reduced the glycosylation extent of the *Tr*-Cel7A in *S. cerevisiae*. Such glycosylation state change improved not only the specific activity but also the secretion yield.

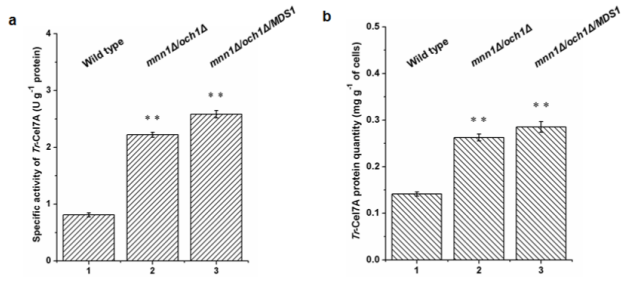

Figure 3: Deletion of yeast mannosyltransferases increased the specific activity and secretion yield of *Tr*-Cel7A. (a) The purified *Tr*-Cel7A protein by the Q Sepharose column was used for tested its specific activity with pNPC as the substrate. (b) The secretion yield was detected by ELISA assay with anti-Flag antibody. The samples were secreted protein precipitated from culture supernatants at 48 h. All data shown are the mean values (± standard error) obtained from three independent experiments. The differences between the modified strains and wild type were all significant P<0.01 (**).

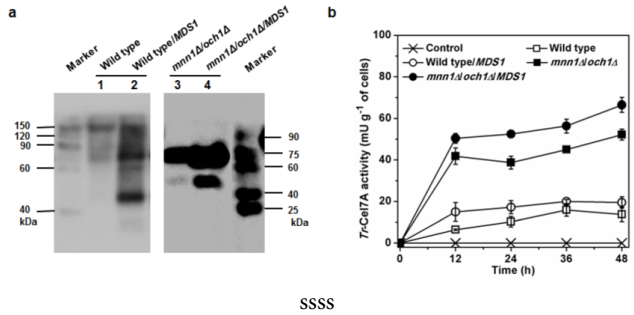

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Figure 4: The heterogenous α-1,2-mannosidase *Tr*-Mds1p decreased the glycosylation (a) and increased the extracellular activity (b) of *Tr*-Cel7A. (a) The secreted proteins were precipitated from culture supernatants at 48 h. The *Tr*-Cel7A proteins were detect by western blot with anti-Flag monoclonal antibody. (b) The extracellular *Tr*-Cel7A activity was assayed with culture supernatants using pNPC as the substrate. The definitions of the "Control" and " Wild type" were shown in Table 1. All data shown are the mean values (± standard error) obtained from three independent experiments. The differences between the modified strains and wild type were all significant (P<0.05), even more significant (P<0.01) at 48h.

Discussion

Hyperglycosylation of *S. cerevisiae* inhibited the activity of recombiant *Tr*-Cel7A enzyme, a glycoprotein from *T. reesei*, significantly [11,23]. In order to investegate if the glycosylation in *S. cerevisiae* close somewhat to its native state could bring benefit to the extracellular *Tr*-Cel7A activity, the protein glycosylation engineering including disruption of the yeast mannosyltransferases Och1p, Mnn9p and Mnn1p, and heterogeneous expression of *T. reesei* mannosidase *Tr*-Mds1p, was performed. The speculative structures of N-glycans on *Tr*-Cel7A proteins after modifications were compared with that of the wild type strain in Figure 5. All modifications increased extracellular activities of *Tr*-Cel7A and decreased its glycosylation. Based on *mnn1Δ/och1Δ*, expression of *Tr*-Mds1p enhanced the extracellular *Tr*-Cel7A activity up to 320 ± 8% higher than the wild type strain (Figure 4b). The glycosylation reduction improved not only the specific activity but also the secretion yield of the *Tr*-Cel7A.

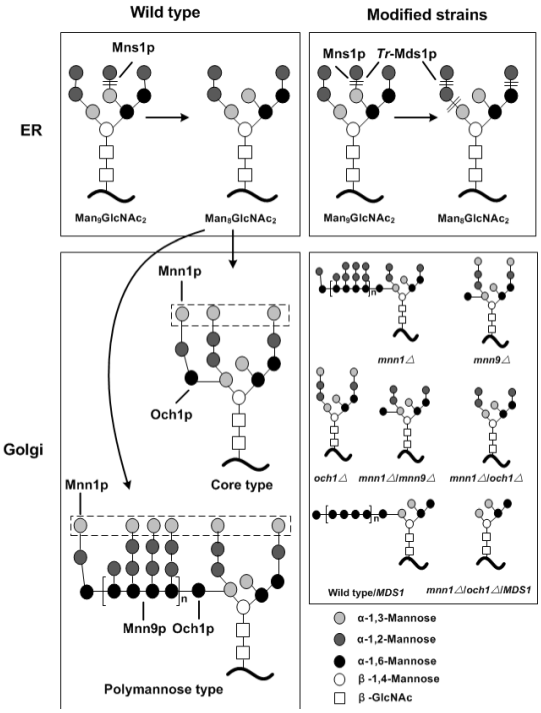

Figure 5: The speculative structures of N-glycans on *Tr*-Cel7A proteins in wild type and modified *S. cerevisiae* strains

The molecular mass of core *Tr*-Cel7A deduced from amino acid residues with no glycosylation is about 46 kDa. However, because of the changes in the glycosylation extent, *Tr*-Cel7A native status with different molecular masses (from ~57 to ~70 kDa) were detected in *T. reesei* [12]. Same as in *Penicillium decumbens*, the native *Pd*-Cel7A also showed different molecular masses (from ~57 to ~74 kDa) [24]. For same reason, in *S. cerevisiae*, the secreted *Tr*-Cel7A with the same amino acid residues sequence also showed different molecular masses even over 100 kDa, which meant it was hypeglycosylated. However, protein secretory pathway reconstruction including glycosylation engineering can significantly decrease the molecular mass of *Tr*-Cel7A produced by *S. cerevisiae,* declining the glycosylation degree close to its core molecular weight [11] (Figure 2c and Figure 4a). Such

phenomenon was also detected when the *Te*-Cel7A from *Talaromyces emersonii* was expressed in a modified *S. cerevisiae* strain [25].

Glycoprotein with appropriate glycosylation was favorable for its activity. Glycosylation might affect the protein secondary structure such as folding, disulfide bonds formation and so on [23,24,26]. For Cel7A and other enzymes with similar function, N-glycan in the catalytic domain might also affect its binding with the cellulose substrate and its stability [27-29]. These further impacted mainly on the specific activity. However, when the *Tr*-Cel7A was heterologous expressed in the glycosylation engineering *S. cerevisiae* strains, not only its secondary structure but also secretion might be affected by the modification. The three kinds of mannosyl transferases Och1p, Mnn9p and Mnn1p take charge in adding mannose on some secreted proteins and mainly on cell wall mannoproteins [30,31]. Disruption of Och1p and Mnn1p weakened the outer layer of the cell wall and disrupted the integrity of the cell wall architecture [32], which might be benefit for *Tr*-Cel7A secretion. It was detected that disruption of them improved both the specific activity and secretion of the *Tr*-Cel7A in the present work (Figure 3). Meanwhile its glycosylation decreased visibly (Figure 2c). In *S. cerevisiae*, the first α-1,6-linked mannose is transferred to the N-linked glycans ($Man_8GlcNAc_2$) by Och1p to initiate the elongation of the oligosaccharides chain. Based on the α-1,6-chain, Mnn9p extend the mannose chains, which lead mainly to hypermannosylation. This means that Mnn9p cannot execute its function without *Och1p*. Therefore, the double deletion of och1 and *mnn9* was not mentioned in the present work.

The α-1,2-mannosidase from *T. reesei Tr*-Mds1p, which functioned in the N-deglycosylation, affected the glycosylation of *Tr*-Cel7A in *S. cerevisiae* (Figure 4a, Line 2). Based on *mnn1Δ/och1Δ*, expression of *Tr*-Mds1p increased the extracellular activity of *Tr*-Cel7A (Figure 4b) and made more *Tr*-Cel7A proteins gather at the status around 75 kDa and 45 kDa (Figure 4a, Line 4). Furthermore, this modification also resulted in 45 ± 8% and 16 ± 3% improvement in the specific activity and the secretion yield of *Tr*-Cel7A. The specific activity of *Tr*-Cel7A contributed more to the improvement of the extracellular activity than its secretion yield.

The humanization of glycosylation pathways in yeast was used to keep the half-life and therapeutic potency of some glycoproteins [33,34]. In the present work, the *Tr*-Cel7A from *T. reesei* suffered from hyper-glycosylation and poor secretion in *S. cerevisiae*. In order to decrease the hyper-glycosylation of *Tr*-Cel7A close to its natural state, the yeast glycosylation pathway was engineered to reduce its glycosylation and to strengthen its secretion ability. The modifications on the protein glycosylation pathway increased *Tr*-Cel7A extracellular activity in *S. cerevisiae*. Although both protein secretion yield and specific activity were affected, the improvement in extracellular activity was mainly contributed by the increase of specific activity due to glycosylation changes. The deficiency of the endogenous mannosyl transferases, Mnn1p and Och1p, and the expression of the *T. reesei* α-1,2-mannosidase *Tr*-Mds1p increased the extracellular activity of Tr-Cel7A by 320 ± 8%. It was presumed that the modifications on glycosylation in *S. cerevisiae* to more closely resemble Tr-Cel7A native state in *T. reesei* would be benefit for its extracellular activity. Even though, the extracellular activity produced by *S. cerevisiae* is still quite low. Meanwhile these mannosyltransferases are related to cell wall synthesis [31], to some extent, lacking of them will affect the cells growth and their robustness. Therefore, there are still some limitations for using CBP yeast in industrial settings.

Conflict of Interests

The authors declare that they have no conflicts of interest.

Acknowledgments

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