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Novel helper factors influencing recombinant protein production in *Pichia pastoris* based on proteomic analysis under simulated microgravity

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Abstract Microgravity and simulated microgravity (SMG) have quite significant effects on numerous microbial cellular processes. The effects of SMG on the production of recombinant proteins and transcription profiling in prokaryotic and eukaryotic expression host have been investigated. The present study showed that SMG significantly enhanced the specific productivities and activities of the reporter enzymes PGUS and AtXYN that were expressed in recombinant *Pichia pastoris*. Proteomic profiling revealed that 21 proteins were significantly up-regulated and 35 proteins were drastically down-regulated at the stationary phase, when the recombinant *P. pastoris* responded to SMG. Six strongly up-regulated genes, *TPX*, *FBA*, *PGAM*, *ENO*, *SBA1*, and *AKR-E*, involved in the oxidative stress response, methanol metabolism, glycolytic pathway, and protein folding, were selected to analyze their impacts on recombinant protein production by co-overexpression in the shaker flask fermentation. The co-overexpressed strains, particularly *TPX*, *FBA*, and *PGAM*, demonstrated promising results with approximately 2.46-fold, 1.58-fold, and 1.33-fold increases in the specific yields of PGUS compared to the control after 48 h of methanol induction, respectively. In the meantime, the corresponding PGUS specific activities were increased by 2.33-fold, 2.09-fold, and 1.32-fold, respectively. Thiol peroxidase (*TPX*), which is involved in the oxidative stress response, significantly influenced the transcriptional levels of the reporter gene *PGUS*. The present study provides valuable information for further exploration of the molecular mechanism of *P. pastoris* response to SMG and facilitates simulated microgravity for finding novel helper factors to rationally engineer the strains in normal fermentation by using proteomic studies.

Keywords Recombinant protein · Strengthen production · Proteomics analysis · Simulate microgravity (SMG) · *Pichia pastoris*

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Introduction

Over the past several decades, *Pichia pastoris* has become an attractive eukaryotic host for the cost-efficient production of heterologous proteins due to the availability of the strong inducible and constitutive promoters and high cell density production protocols. Nevertheless, there is evidence of major bottlenecks to heterologous protein production (Thomas et al. 2013). It has been demonstrated that correct protein folding and secretion were highly interrelated with environmental stress factors. Scientists have investigated the impact of environmental perturbations, such as temperature, osmolality, and oxygen availability, on recombinant protein production by "omics" data mining and re-engineering yeast strains (Baumann et al. 2010). Dragosits et al. investigated the effects of temperature and osmolality on the transcriptome, proteome, and fluoxome of recombinant *P. pastoris* (Dragosits et al. 2009, 2010). Gasser et al. selected a range of significantly regulated genes based on the transcriptional profile under stress conditions and tested their *Saccharomyces*

cerevisiae homologues for co-expression in a recombinant *P. pastoris* strain (Gasser et al. 2007). These studies demonstrated that the environmental stress on protein production conditions could provide important platforms to identify helper targets for cell engineering.

Simulated microgravity (SMG) is an exceptional environmental condition that can be modeled by special bioreactors in ground-based experiments. One such bioreactor is Rotary Cell Culture System (RCCS, Synthecon Inc., NASA), using High Aspect Ratio Vessel (HARV) to model the environment of microgravity on the ground. The RCCS randomizes the unidirectional gravitational force, minimizes turbulence of the fluid shear levels on the cell surface, and creates the net effect of "functional weightlessness" (Sheehan et al. 2007). The suspension culture system also provides efficient gas exchange, which is necessary for rapidly dividing cells, through a permeable membrane at the back of the HARV. The SMG and normal gravity (NG) control environment are created when the HARV is rotated horizontally (with the axis of the vessel perpendicular to the gravitational force) and vertically (with the axis of the vessel parallel to the gravitational force), respectively. In SMG, sedimentation, which leads to the different dispersion of nutrients and wastes within the vessel, is prevented. Previous studies showed the response of a variety of microorganisms to the reduced gravity in the form of variations in physiological characteristics and perturbations at the molecular levels (Nickerson et al. 2003, 2004). Furthermore, multiple research approaches reported microgravity effects in the fields of materials (Vailati et al. 2011) and space medico-engineering (Sanderson 2011).

Microbial glycoside hydrolases (GH) are industrial enzymes for biocatalysis and biotransformation of medicinal products. GH also occupies an important place in biomass utilization in biofuel applications. β-Glucuronidase from *Penicillium purpurogenum* Li-3 (PGUS) could directly biosynthesize glycyrrhetinic acid monoglucuronide (GAMG) from glycyrrhizin (GL) (Feng et al. 2006). GAMG is useful in the clinical treatment of inflammatory diseases; it is safer, more effective, and more absorbable than GL. In the xylan degradation process, endo-β-1, 4-xylanase plays an important role in efficient hydrolysis of this renewable source and abundant agricultural waste. It randomly cleaves the β-1, 4-glycosidic linkages of xylan into short xylo-oligosaccharides. Therefore, for sustainable and economical biotechnology processes, large amounts of hemicelluloses with low costs are highly desirable (Guo et al. 2013). *P. pastoris* has been considered as a good expression system for the heterologous production of the glycoside hydrolases due to its potential for higher protein productions and increasing specific activities that consequently reduced the cost. However, not all of the enzymes and biopharmaceutical proteins can be efficiently produced by *P. pastoris*. Developing new methodologies to improve industrial enzyme production has become a new research area.

In our latest published work, the beneficial impact of SMG on the secretion of the recombinant PGUS from *P. pastoris* in RCCS has been reported. The efficiency of PGUS secretion was enhanced up to 30 % under SMG. Comparative analysis of the transcriptomic data was performed to assess the behavior of *P. pastoris* in response to SMG (Qi et al. 2011a, b). The results indicated that the up-regulated genes of methanol metabolism, protein transportation, and secretion mainly contributed to enhanced production and secretion of the recombinant protein under SMG. However, a more detailed analysis of the mechanism of *P. pastoris* sensing microgravity and how the cells convert these mechanical signals into molecular responses to impact recombinant protein production needs to be further investigated. Conversely, little effective progress has been achieved regarding the utilization of reduced gravity effects in the fermentation industry. Therefore, it is imperative to unravel the global mechanism connecting protein production to environmental conditions and engineer the *P. pastoris* strain by introducing the new helper genes. In this study, for the first time, we examined the proteomic profiling of the SMG cultured recombinant *P. pastoris* and then identified potential helper genes by gene co-expression.

Materials and methods

Strains cultured under SMG condition

The two recombinant *P. pastoris* strains *P. pastoris*-PGUS (*pgus*/pPIC9k GS115) expressing the β-glucuronidase (*PGUS*) derived from *P. purpurogenum* Li-3 (GenBank accession no. EU095019) and *P. pastoris*-AtXYN (*atxyn*/pPIC9k GS115) expressing the endo-β-1, 4-xylanase (*AtXYN*) derived from *At. terreus* Li-20 (GenBank accession No.JQ087496) were previously described (Qi et al. 2011a; Guo et al. 2013). The reporter genes were both ligated into pPIC9K and integrated into the AOX1 loci of the yeast genome. The production of the recombinant proteins was tightly regulated by the methanol inducible promoter AOX1. Conditions referred to as SMG were created by rotating the HARV in a horizontal direction perpendicular to the gravitational vector on rotating cell culture systems (RCCS-4H, 50 ml; Synthecon, USA). The NG control environment was created by vertical rotation. The rotation speed was kept at 15 rpm for efficient production of the recombinant protein. A single colony of recombinant *P. pastoris* was isolated from the YPD (1 % yeast extract, 2 % peptone, 2 % glucose, and 2 % agar) plate and inoculated with 200 ml of YPD medium in a 250-ml shaker flask at 28 °C, and 220 rpm for 24 h to OD_{600}= 13–16. The seed liquid cells were diluted (1:100) and resuspended in 100 ml YPD medium (OD600=0.5–0.8). Then, aliquots of the YPD cultures were loaded into two sterile HARV vessels that were completely filled with 55 ml

of medium for SMG and NG, respectively. Both HARV vessels were incubated at the speed of 15 rpm and 28 °C for 48 h. The cells were then harvested by centrifugation at 5,000 rpm for 5 min, washed twice with phosphate buffer (100 mM, pH 6.0), and re-suspended in 55 ml of BMMY medium (1 % yeast extract, 2 % peptone, 100 mM phosphate buffer saline, pH 6.0, 1.34 % YNB, 1.61 μM biotin, 1 % methanol). The BMMY medium was loaded into two HARV vessels for SMG and NG cultivation, respectively. All bubbles were removed to reduce shear using a sterile disposable syringe. Methanol was added every 12 h to a final concentration of 1 %. One milliliter of culture medium was sampled every 24 h, centrifuged at 5,000 rpm for 5 min at 4 °C, and the supernatant was analyzed for the specific productivities of reporter enzymes. The cultured processes were carried out in triplicate.

Cell density, protein production analysis, and enzyme assay

Cell density was tested periodically by measuring the OD_{600} using an ultraviolet spectrophotometer (Hitachi) and the dry cell weight was determined through the standard curve. The total crude protein concentration was determined using a BCA Protein Assay Kit (Applygen, China). Semi-quantitative determination of recombinant protein concentration was analyzed by SDS–PAGE and quantified by densitometric analysis with a ProExpress Imaging System (PerkinElmer, USA). TotalLab100 version 2006 software was used after the staining with Coomassie brilliant blue R250 (Qi et al. 2011a). Bovine serum albumin was used as an internal standard. The β-glucuronidase activities and the xylanase activities were assayed using HPLC (Shimadzu, Japan) and micro-plate reader (Biotek, USA), respectively. All the experiments from the biological samples were carried out in triplicate.

The β-glucuronidase activities were determined by the hydrolysis of GL (China). The assay mixture consisted of 100 μl of enzyme solution and 400 μl of 2 g/l GL (pH 5.0). After incubation at 55 °C for 30 min, the reaction was stopped by heating in boiling water for 5 min and then centrifuged at 10,000 rpm for 5 min. The supernatant was used for enzyme activity analysis using HPLC. The reaction mixture (10 μl) was subjected to reverse-phase HPLC with a C_{18} column (4.6×250 mm, 5 μm particle size; Kromasil, Sweden) at 40 °C. Separation was achieved with a mobile phase consisting of a mixture of methanol–0.6 % acetic acid in water (81:19 v/v) at 25 min. Elution was monitored with UV detection at 254 nm. The amount of GAMG could be read from the standard curve between the peak area and concentration of GAMG. One unit (U) of activity was defined as the amount of enzyme that released 1 μmol of biosynthesized GAMG in the reaction mixture per minute (Feng et al. 2006). The xylanase activities were assayed using birchwood xylan (Sigma, USA) as the substrate (Bailey et al. 1992). The amount of reducing sugars released was determined by the standard dinitrosalicylic acid method. One unit of xylanase activity was defined as the amount of enzyme producing 1 μmol of reducing sugar (xylose) from the substrate solution per minute under the assay conditions.

Proteomic analysis

Samples for proteomic analysis were harvested at the steady phase and mid-exponential growth phase of *P. pastoris*-PGUS in SMG and NG conditions. Continuous dilutions were performed after every 48 h of methanol induction to guarantee all the cells were maintained in the steady phase by monitoring the optical density. After four repeated processes, the culture medium was sampled and centrifuged at 8,000 rpm for 15 min at 4 °C, and then pellets were washed with PBS (100 mM, pH 7.0), frozen, and stored at −80 °C for further proteomic analysis. To obtain the cells in the mid-exponential phase, four repeated 12 h of methanol induction processes were carried out. Cell extraction was performed with the lysing buffer: 8 M urea, 65 mM DTT, 0.1 % Triton X-100, 100 mM NaCl, 50 mM Tris–HCl, 1 mM PMSF, 1 mM EGTA, and 1 mM EDTA (pH 7.4). The supernatant was kept to determine the protein content, and then precipitated by chloroform/methanol treatment, followed by re-dissolution in 0.2 ml buffer containing 8 M urea and 50 mM Tris–HCl (pH 8.2). The protein samples were treated with DTT at 37 °C for 2 h, followed by iodoacetamide (IAA) at room temperature in the dark for 40 min. Then, the solutions were diluted 8-fold using 50 mM Tris–HCl (pH 8.2) and subjected to trypsin digestion (trypsin/protein, 1:50, w/w) at 37 °C for 20 h. The tryptic digests were desalted with C_{18} solid-phase cartridges and lyophilized. Protein analysis technology was used by a 2D nano-LC–MS/MS system (Thermo, USA). The acquired MS/MS spectra were searched against the uniProt database (http://www.uniprot.org/) and the *Pichia* database (http://www.pichiagenome.org/) using the TurboSEQUEST (version 3.2) and XTandem software. The results were filtered using the SFOER software with optimized criteria, and the corresponding FDR was below 1 %. Proteins with a fold change >2, and a P value <0.05 for the t test of each of the two samples were assigned as differentially expressed.

Construction of engineered vectors for co-overexpression

Six host-specific regulated helper genes based on proteomic profiling data analysis were amplified from *P. pastoris* GS115 (Invitrogen, USA) genomic DNA. The list of the helper genes of interest and the corresponding primers of the restriction sites (*Eco*RI, *Xba*I, and *Kpn*I) are given in Table S1. The helper genes were cloned into the vector pGAPZB (Invitrogen, USA), which confers resistance to zeocin in bacteria and in yeast. The verified plasmids containing the genes

of interest were linearized with *Avr*II and transformed by a Gene PulserXcell™ Electroporation System (Bio-Rad, USA). The transformed DNA fragments were integrated into the GAP loci of the recombinant *P. pastoris*-PGUS and the recombinant *P. pastoris*-AtXYN genome. Large colonies of reconstructed yeast strains on zeocin (Invitrogen, USA, 50 μg/ml) YPD agar plates were picked after 2–3 days, and the genomic DNA was extracted and checked for correct integration by PCR with the pGAPZB primers. The copy numbers of obtained transformants were quantitatively determined by real-time quantitative PCR. Single clone was inoculated in 50 ml BMGY medium in a 250-ml baffled shaker flask at 28 °C and 170 rpm for nearly 24 h. The cells were then harvested and suspended in 50 ml BMMY medium. Methanol was added every 12 h to a final concentration of 1 % for the reporter proteins PGUS and AtXYN production. After 48–96 h of induction, the culture medium was sampled and centrifuged at 5,000 rpm for 5 min at 4 °C to determine the specific productivities of the reporter enzymes. Each engineered strain was cultured through triplicate independent experiments. The cell density, protein production analysis, and enzyme assay were calculated as triplicate. The strain having the empty pGAPZB vector was set as control.

RNA extraction and qRT-PCR analysis

Yeast cells were harvested during the exponential growth phase. Approximately 1×10^7 cells were used for the total RNA extraction using a Yeast RNA Kit (Omega, USA). Genomic DNA contamination was eliminated by *DNase*I (TaKaRa, Japan). RNA concentration was quantified by measuring the absorbance at 260 nm using a NanoDrop 2000c (Thermo, USA). The readings of extracted RNA samples between 1.5 and 2 were selected as the template for the Transcript First Strand cDNA Synthesis Kit (Roche, Swiss) and library construction. Single-stranded cDNA was used as a template in the real-time PCR analysis. The reaction conditions had been established as recommended by SYBR *Premix Ex Taq*™ manual (TaKaRa, Japan). All real-time qPCR reaction assays were performed in biological replicates to allow for statistical confidence in differential gene expression. Triplicate samples of each template were run on LightCycler 480 real-time System (Roche, Swiss) using fast 96-well plates. The data was analyzed using LightCycler Software (v.1.5). The housekeeping gene *GADPH* was used as a reference gene. The data was normalized using *GADPH* as the endogenous control. The reaction without reverse transcriptase was used as the negative control. The genes *TPX* and *GUS* were used as target genes. The obtained relative mRNA levels indicated the relative comparisons of transcription levels between the target genes and reference genes. The mRNA levels of *TPX* and *GUS* in each strain were calculated and converted into relative ratios following the comparative CT method. (The data from the real-time PCR was converted to $2^{-\Delta\Delta iCT}[\Delta\Delta C_T = C_T(\text{target}) - C_T(\text{ref})]$ where C_T represented the threshold cycle.)

GenBank accession numbers

The GenBank accession number of *PGUS* gene is EU095019, and the GenBank accession number of *AtXYN* gene is JQ087496. The GenBank accession numbers of *TPX*, *FBA*, *PGAM*, *ENO*, *SBA1*, and *AKR-E* gene for co-overexpression were XM_002491758.1, XM_002489668.1, XM_002493014.1, XM_002492248.1, XM_002490110.1, and XM_002493818.1, respectively.

Results

Yields and activities of reporter enzymes in *P. pastoris* under SMG

Two recombinant *P. pastoris* strains, producing the β-glucuronidase (PGUS) and the endo-β-1, 4-xylanase (AtXYN), were grown in the HARVs (RCCS-4H) to access the effect of the simulated microgravity culture environment on the cell growth and recombinant protein production. Glucose and methanol were used in a two-phase feeding to produce heterologous proteins in *P. pastoris*.

In the *P. pastoris* system, the expression of foreign genes is usually driven by the outstanding promoter of the alcohol oxidase I (*AOX1*) gene, which encodes the first enzyme in the methanol utilization pathway. The *AOX1* promoter is transcribed only in response to methanol and repressed by other carbon sources, such as glucose, fructose, or glycerol. However, *P. pastoris* grows slowly when using methanol as the sole source. Therefore, the general cultivation of methanol-inducible *P. pastoris* is by two-step fermentation conditions: (1) firstly, glucose, glycerol, or sorbitol is used as the main initial carbon source for increasing the cells concentration until it is exhausted or removed by washing with PBS buffer; (2) secondly, the inducer methanol is added for recombinant protein production. In this study, we used glucose instead of the traditional source of glycerol in the first stage. The interpretation was that the recombinant protein was not induced under SMG when glycerol was used. The catabolic repression from glycerol was not completely solved under SMG.

The recombinant *P. pastoris* was cultured in the first stage for 48 h, in which glucose was used as the sole carbon source. Then, the strains were harvested and methanol instead of glucose was used in the second stage for 72 h to induce the recombinant proteins continuously. The growth curves of *P. pastoris* strains in SMG were different from NG, showing shortened lag phase and prolonged exponential phase. After

48 h of methanol feeding, the strains reached the steady-state phase under SMG and NG. After methanol feeding for 12 h, the strains were at the mid-exponential growth phase under SMG and NG (Fig. 1). The physiological parameters of two recombinant strains are shown in Table 1. The recombinant protein concentration was analyzed by SDS–PAGE (Fig. S1) and semi-quantitative measuring methods. The AtXYN and PGUS yields were 0.31±0.04 and 0.39±0.05 mg/ml after methanol feeding of 48 h under SMG, which were about 2.81-fold and 2.43-fold increases in comparison to the NG control, respectively. Compared to the NG control, the SMG environment increased the specific productivities of both enzymes.

Proteomic analysis

In this study, we first examined the physiological changes of recombinant *P. pastoris* grown in SMG, and then we explored the proteomic profiling to identify the significant genes with changes in protein expression in response to modeled microgravity. According to a previous study, the efficiency of recombinant protein production at the steady-state phase, after methanol feeding for 48 h under SMG, was significantly higher than NG. The steady-state phase seemed to be a better time point to investigate microgravity effects on the heterologous protein production in *P. pastoris*. Furthermore, the cells at the mid-exponential growth phase of methanol feeding for 12 h was chosen as another time point. Of total proteins, 218 differentially expressed proteins in the steady stationary phase and 72 proteins in mid-exponential growth phase were identified (FDR<1 %, RSD<0.5) (Table S2, Table S3). Twenty-one proteins were significantly up-regulated (ratio fold change >2) and 35 proteins were drastically down-regulated (ratio fold change <0.5) at the stationary phase (S). In addition, seven proteins were up-regulated at the mid-exponential growth phase (L). It was observed that SMG affected the gene expression of *P. pastoris* (Fig. 2). These differentially expressed proteins were classified into four functional categories: (1) methanol metabolism and carbohydrate metabolic process, (2) cell redox homeostasis and oxidative stress, (3) translation and protein folding-related, and (4) other metabolism and uncharacterized protein (Table 2).

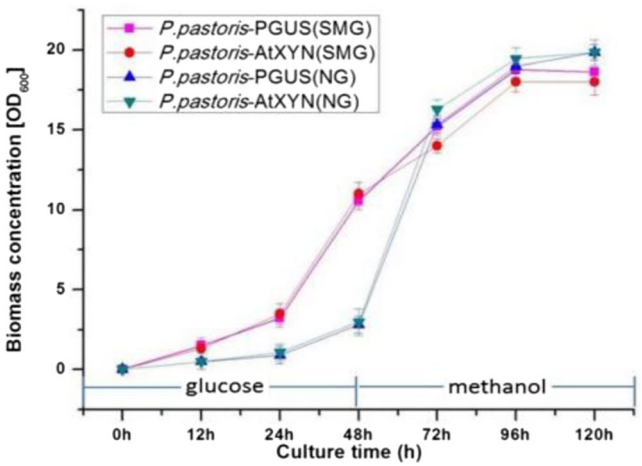

Fig. 1 Growth kinetics of the recombinant *P. pastoris* strains under SMG and NG. Data are represented as means±standard deviation (SD)

Table 1 Recombinant protein specific yields and specific productivities of PGUS and AtXYN

Strain cultivation	Specific protein yield (mg/ml)	Specific productivity (U/mg)
PGUS-SMG	0.39±0.04	15.83±0.50
PGUS-NG	0.16±0.01	6.85±0.50
AtXYN-SMG	0.31±0.05	24.48±0.51
AtXYN-NG	0.11±0.01	10.56±0.51

Values represent the mean±standard error from three biological replicas

The enzymes involved in the carbohydrate metabolic process, including FBA, PGAM, PEPCK, ICL1, and ENO, were identified to be strongly induced at the stationary phase and mid-exponential growth phase under SMG. However, the alcohol oxidases (AOX1, AOX2) that were the major source of methanol-oxidizing activity in *P. pastoris* showed decreased levels at the stationary phase. On the other hand, seven proteins including GLTA, CTA, PYK2, SFA1, ATP1, FDH, and DAK involved in methanol metabolism were down-regulated at the stationary phase under SMG. At the expression levels, all of the six identified proteins involved in the response to cell redox homeostasis and oxidative stress, including COX12, TPX, QCR, LIA1, AKR-E, and GLO1, were significantly up-regulated under SMG compared to the NG control.

Notably, co-chaperones AHA1 showed strongly increased abundances at the stationary phase, and SBA1 was significantly up-regulated at the mid-exponential growth phase. Co-chaperone SIS1 was down-regulated at the stationary phase. The components of protein folding and secretion machinery were also differentially regulated by SMG cultivation. ARF1 and PSME4 were up-regulated at the stationary phase, whereas SHP1 was down-regulated at the stationary phase. The expression levels of GLY1, MET17, YEF3, and elongation factor TUF1 involved in amino acid metabolism were decreased during SMG cultivation at the stationary phase. Translation initiation factor HYP2 and TAD1 were up-regulated at the mid-exponential growth phase and the stationary phase. The levels of ribosomal proteins RPL5, RPS7, RPS12, RPS21, and RPS29 were up-regulated at the mid-exponential growth phase. RPL8, RPL9, RPL11, RPL32, RPS4, RPS7, RPS10, RPS15, RPS17, RPS24, and RPS25 were down-regulated at the stationary phase. There were also other cellular mechanisms that seemed to be affected by SMG at the proteomic levels, like 6, 7-dimethyl-8-ribityllumazine

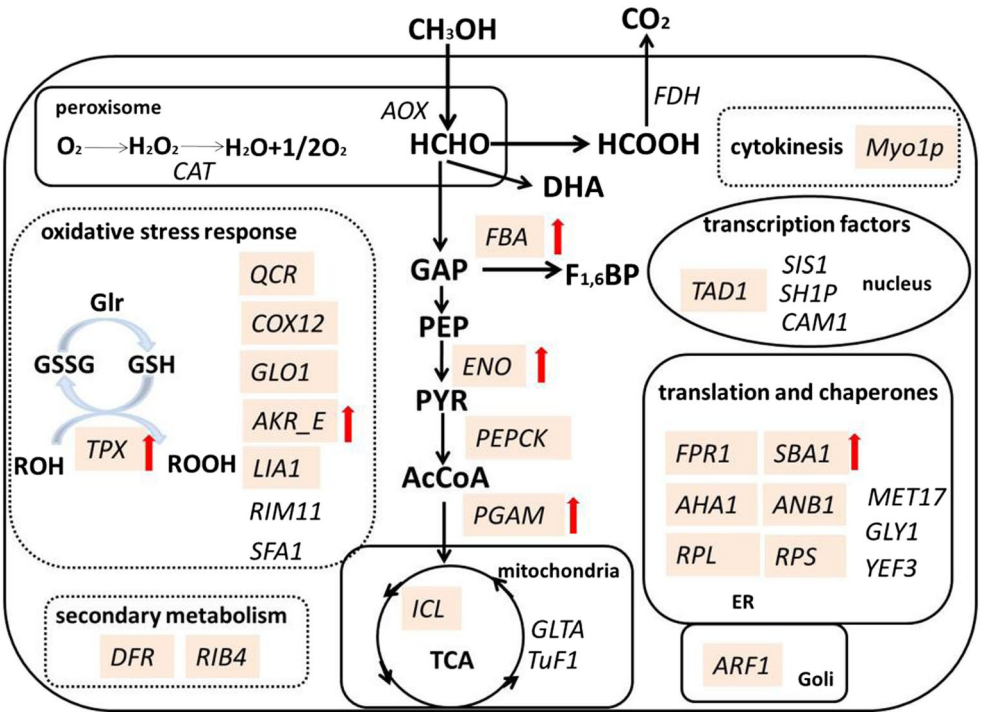

Fig. 2 Schematic representing some significant differences of proteins in the recombinant *P. pastoris*-PGUS on the proteomic levels under SMG and NG cultivation during the steady phase and the mid-exponential growth phase. *Italics* indicate the lower abundance under SMG. *Italics in shadows* indicate the higher abundance under SMG. *Upward arrows* indicate the genes chosen for co-expression

synthase (RIB4) involved in riboflavin production and dihydrokaempferol 4-reductase (DFR) involved in flavonoid biosynthesis metabolism. The protein FPR1 involved in DNA mechanisms was up-regulated at the mid-exponential growth phase.

The detailed analysis of proteomic data mining could provide a library of potential helper genes for the improvement of yeast *P. pastoris*. Focusing on up-regulated genes, which have potential functions in the carbohydrate metabolic process, secretion machinery, and stress regulation, six potentially superior up-regulated genes (*TPX*, *FBA*, *PGAM*, *ENO*, *SBA1*, and *AKR-E*) were selected for further analysis of effects on the recombinant protein production by co-overexpression methods.

The effects of co-overexpressing helper genes on recombinant PGUS production in *P. pastoris*

The strains harboring a single copy of the helper genes were screened for testing protein expression changes. The strains were obtained via integration of the GAP loci of a single copy of the helper genes. The helper genes were expressed under the control of a constitutive promoter of GAP in vivo and to detect their functions on the production and secretion of reporter enzymes which were expressed using the methanol-inducible promoter AOX. The PGUS secretion capacities of verified clones were performed in methanol batch cultivation using baffled shaker flasks. The strains overexpressing *TPX*, *FBA*, and *PGAM* genes demonstrated improved recombinant protein production after 48 h of methanol induction. Approximately 2.46-fold, 1.58-fold, and 1.33-fold increases in the PGUS specific yields were achieved compared to the control without co-overexpressing genes. However, the clones of co-overexpressing *AKR-E* and *ENO* showed the disadvantageous rather than beneficial effects. Overexpressing the molecular chaperone *SBA1* significantly increased the PGUS up to 0.25-fold (Fig. 3a). The protein production capacities of positively impacted genes were measured by SDS–PAGE stained by Coomassie brilliant blue R250 and semi-quantitative determination (Fig. 3b). *TPX*, *FBA*, and *PGAM* enhanced PGUS specific activities to about 2.33-fold (49.16 U/mg), 2.09-fold (44.14 U/mg), and 1.32-fold (27.93 U/mg) compared to the control (21.09 U/mg) (Fig. 4). Overall, the *TPX* overexpressing mutant clone seemed to favor PGUS secretion more significantly than other target genes.

Transcriptional effects on the PGUS specific yields by different *TPX* gene copies

To further understand the *TPX* gene dosage effects on recombinant protein production, four recombinant *P. pastoris* clones Tpx-1, Tpx-2, Tpx-3, and Tpx-4 harboring disparate copies of *TPX* gene were cultured after methanol induction for 48 h in baffled shaker flasks (Fig. 5a). Compared to the control (strain without overexpressing helper gene cultured in normal fermentation condition), the strain (Tpx-1, two copies) showed the highest PGUS specific activity, while the strain (Tpx-4, 10 copies) exhibited the lowest PGUS specific activity (Fig. 5b). The higher transcriptional levels of the helper gene could not

Table 2 Differentially expressed proteins of the *P. pastoris*-PGUS under SMG compared with NG

Spot no.	Majority protein ID.	Protein MP	Sequence coverage (%)	Mol. weight (kDa)	Fold SMG/NG	RSD	Accession no.	Protein description	Gene name	Up- or down-regulated
Methanol metabolism and carbohydrate metabolic process										
22	C4QW09	8	31.3	39.732	2.002	0.032	PAS_chr1-1_0072	Fructose 1,6-bisphosphate aldolase, required for glycolysis and gluconeogenesis	FBA	Up (S)
266	C4R5P4	7	29.4	28	2.319	0.210	PAS_chr3_0826	Phosphoglycerate mutase	PGAM	Up (S)
77	C4QY57	2	7.1	61.533	3.163	0.010	PAS_chr1-4_0338	Isocitrate lyase, catalyzes the formation of succinate and glyoxylate from isocitrate	ICL1	Up (S)
111	C4QZB8	7	16.2	62.073	4.378	0.014	PAS_FragB_0061	Phosphoenolpyruvate carboxykinase	PEPCK	Up (S)
63	C4R3H8	3	12.2	46.527	3.189	0.254	PAS_chr3_0082	Enolase	ENO	Up (L)
204	C4R2S1	3	13.9	57.864	0.074	0.158	PAS_chr2-2_0131	Catalase	CTA	Down (S)
270	C4R5Q6	2	5.1	65.311	0.043	0.400	PAS_chr3_0841	Dihydroxyacetone kinase, required for detoxification of dihydroxyacetone	DAK	Down (S)
27	C4QW60	4	8.5	51.905	0.292	0.179	PAS_chr1-1_0475	Citrate synthase	GLTA	Down (S)
275	C4R606	12	44.9	40.309	0.037	0.309	PAS_chr3_0932	NAD(+)-dependent formate dehydrogenase, may protect cells from exogenous formate	FDH	Down (S)
349	C4R917; C4R702	5	16	73.897	0.018	0.142	PAS_chr4_0821, PAS_chr4_0152	Alcohol oxidase	AOX1 AOX2	Down (S)
175	C4R1P9	2	8.3	55.572	0.477	0.030	PAS_chr2-1_0769	Pyruvate kinase	PYK2	Down (S)
282	C4R6A5	1	2.6	40.559	0.094	0.009	PAS_chr3_1028	S-(Hydroxymethyl)glutathione dehydrogenase	SFA1	Down (S)
248	C4R4Y8	15	31.3	58.767	0.453	0.132	PAS_chr3_0576	ATP synthase subunit alpha	ATP1	Down (S)
Cell redox homeostasis and oxidative stress										
324	C4R7U6	1	13.8	9.4526	2.424	0.182	PAS_chr4_0422	Subunit VIb of cytochrome *c* oxidase	COX12	Up (S)
180	C4R1Z9	2	14.9	18.233	2.594	0.286	PAS_chr2-2_0382	Glutathione peroxidase	TPX	Up (S)
141	C4R0S5	1	6.1	23.448	3.260	0.008	PAS_chr2-1_0850	Ubiquinol–cytochrome *c* reductase iron–sulfur subunit	QCR	Up (S)
157	C4R113	2	9.8	35.251	4.810	0.183	PAS_chr2-1_0553	Deoxyhypusine hydroxylase, a heat-repeat containing metalloenzyme that catalyzes hypusine formation	LIA1	Up (S)
106	C4R936	1	6.9	36.669	4.238	0.082	PAS_chr4_0842	Monomeric glyoxalase I	GLO1	Up (L)
98	C4R7V9	1	8	33.711	5.689	0.198	PAS_chr4_0433	NADPH-dependent alpha-keto amide reductase	AKR-E	Up (L)
Translation and protein folding-related										
262	C4R5J2	1	0.8	241.33	2.162	0.031	PAS_chr3_0776	Proteosome activator subunit	PSME4	Up (S)
209	C4R2W7	2	12.2	20.599	2.696	0.045	PAS_chr2-2_0087	ADP-ribosylation factor, GTPase of the Ras superfamily	ARF1	Up (S)
226	C4R3R2	1	8.7	17.115	2.794	0.371	PAS_chr3_0170	Co-chaperone that binds to Hsp82p and activates its ATPase activity	AHA1	Up (S)
124	C4QZV3	1	2.7	41.261	3.656	0.046	PAS_chr2-1_0170	tRNA-specific adenosine deaminase, deaminates adenosine-37 to inosine in tRNA-Ala	TAD1	Up (S)
17	C4QXA1	1	7.5	22.472	3.949	0.308	PAS_chr1-4_0043	Co-chaperone that binds to and regulates Hsp90 family chaperones	SBA1	Up (L)
82	C4R6C4	2	25.8	17.019	5.418	0.122	PAS_chr3_1048	Translation initiation factor eIF-5A, promotes formation of the first peptide bond	HYP2	Up (L)
14	C4QWU8	1	8.1	34.137	2.521	0.091	PAS_chr1-1_0345	60S ribosomal protein L5	RPL5	Up (L)

Table 2 (continued)

Spot no.	Majority protein ID.	Protein MP	Sequence coverage (%)	Mol. weight (kDa)	Fold SMG/NG	RSD	Accession no.	Protein description	Gene name	Up- or down-regulated
250	C4R4Z8	6	54.9	15.597	2.057	0.139	PAS_chr3_1200	40S ribosomal protein S12	RPS12	Up (L)
252	C4R507	4	52.9	9.6816	2.093	0.036	PAS_chr3_0596	40S ribosomal protein S21	RPS21	Up (L)
97	C4QYN4	2	32.1	6.6696	9.200	0.304	PAS_chr1-4_0504	40S ribosomal protein S29	RPS29	Up (L)
76	C4QY12	3	14.7	29.72	0.461	0.039	PAS_chr1-4_0297	Suppressor protein STM1	TIF3	Down (S)
296	C4R6W2	1	5.4	41.358	0.175	0.107	PAS_chr4_0112	Threonine aldolase	GLY1	Down (S)
246	C4R4X4	5	11.5	46.817	0.282	0.290	PAS_chr3_0562	Elongation factor Tu	TUF1	Down (S)
201	C4R2Q1	2	7.2	37.021	0.306	0.320	PAS_chr2-2_0151	Type II HSP40 co-chaperone that interacts with the HSP70 protein Ssa1p	SIS1	Down (S)
288	C4R6E8	1	4.6	24.343	0.020	0.314	PAS_chr3_1071	Translation elongation factor EF-1 gamma	YEF3	Down (S)
122	C4QZU1	2	9.6	36.7	0.426	0.149	PAS_chr2-1_0159	UBX (ubiquitin regulatory X) domain-containing protein that regulates Glc7p phosphatase activity	SHP1	Down (S)
318	C4R7J7	2	6.1	48.195	0.451	0.083	PAS_chr4_0330	Methionine and cysteine synthase (*O*-acetyl homoserine-*O*-acetyl serine sulfhydrylase)	MET17	Down (S)
35	C4QWG6	2	15.4	27.209	0.182	0.228	PAS_chr1-1_0219	60S ribosomal protein L8	RPL8	Down (S)
286	C4R6D3	1	17.4	15.233	0.277	0.017	PAS_chr3_1057	60S ribosomal protein L32	RPL32	Down (S)
37	C4QWI2	4	22	21.691	0.375	0.214	PAS_chr1-1_0236;	60S ribosomal protein L9	RPL9	Down (S)
45	C4QWU8	2	15.8	34.137	0.496	0.121	PAS_chr1-1_0345	60S ribosomal protein L6	RPL6	Down (S)
335	C4R8K3	3	19	19.882	0.497	0.136	PAS_chr4_0669	60S ribosomal protein L11	RPL11	Down (S)
311	C4R7A1	2	4.6	31.75	0.408	0.029	PAS_chr4_0246	40S ribosomal protein S4	RPS4	Down (S)
307	C4R762	4	36.7	21.333	0.390	0.070	PAS_chr4_0211	40S ribosomal protein S7	RPS7	Down (S)
176	C4R1R2	4	39.7	15.689	0.273	0.117	PAS_chr2-1_0779	40S ribosomal protein S17	RPS17	Down (S)
315	C4R7F4	1	8.9	15.409	0.287	0.116	PAS_chr4_0292	40S ribosomal protein S24	RPS24	Down (S)
356	C4R9C0	4	36	13.215	0.450	0.006	PAS_FragD_0013	40S ribosomal protein S10	RPS10	Down (S)
96	C4QYM1	1	6.2	16.361	0.282	0.062	PAS_chr1-4_0491	40S ribosomal protein S15	RPS15	Down (S)
185	C4R256	1	13	12.022	0.310	0.110	PAS_chr2-2_0326	40S ribosomal protein S25	RPS25	Down (S)
48	C4QX43	2	15	22.474	0.458	0.059	PAS_chr1-1_0439	40S ribosomal protein S8	RPS8	Down (S)
261	C4R5H6	3	23.5	24.618	0.493	0.044	PAS_chr3_0762	40S ribosomal protein S5	RPS5	Down (S)
Other metabolism and uncharacterized protein										
240	C4R4L0	7	28.5	39.243	2.314	0.114	PAS_chr3_0449	Putative dihydrokaempferol 4-reductase	DFR	Up (L)
110	C4R9G1	1	2.2	85.595	2.008	0.212	PAS_c034_0039	Peptidyl-prolyl isomerase	FPR1	Up (L)
283	C4R6B4	2	15.5	17.734	2.788	0.110	PAS_chr3_1037	6,7-dimethyl-8-ribityllumazine synthase	RIB4	Up (L)
167	C4R1K9	1	13.1	16.101	43.155	0.164	PAS_chr2-1_0733	Essential light chain for Myo1p, light chain for Myo2p	MYO1	Up (L)
316	C4R7G2	2	26	14.49	2.131	0.044	PAS_chr4_0299	Putative uncharacterized protein		Up (L)
155	C4R0Z8	2	19.7	23.657	2.455	0.194	PAS_chr2-1_0539	Putative uncharacterized protein		Up (L)
151	C4R0V6	2	5.1	29.431	2.919	0.053	PAS_chr2-1_0852	Putative uncharacterized protein		Up (L)
219	C4R3D8	1	1.5	61.878	0.007	0.206	PAS_chr3_0045	Putative uncharacterized protein		Down (S)

Table 2 (continued)

Spot no.	Majority protein ID.	Protein MP	Sequence coverage (%)	Mol. weight (kDa)	Fold SMG/NG	RSD	Accession no.	Protein description	Gene name	Up- or down-regulated
117	C4QZN3	1	9.7	29.025	0.310	0.013	PAS_chr2-1_0809	Putative uncharacterized protein		Down (S)
108	C4QZ90	1	8.4	24.957	0.409	0.093	PAS_FragB_0034	Putative uncharacterized protein		Down (S)
120	C4QZQ5	2	3.2	93.468	0.467	0.095	PAS_chr2-1_0812	Putative uncharacterized protein		Down (S)
81	C4QY88	2	10.1	45.371	0.014	0.166	PAS_chr1-4_0368	Protein kinase required for signal transduction during entry into meiosis	RIM11	Down (S)
295	C4R6V3	2	7.8	47.109	0.225	0.252	PAS_chr4_0102	Nuclear protein required for transcription of MXR1	CAM1	Down (S)

Up-regulated (fold change ratio >2) and down-regulated (fold change ratio <0.5) (FDR < 1 %, RSD<0.5)
MP total number of identified peptides, *S* stationary phase, *L* mid-exponential growth phase

increase the specific protein yields. Notably, the transcriptional levels of the reporter gene *PGUS* seemed to be inhibited in the strains co-expressing the helper gene. In order to better evaluate the transcriptional effects, the strains without overexpression of the helper gene were cultured under SMG and NG environments as another two controls, respectively. The transcriptional level of *TPX* under SMG was much higher than NG, and the reporter gene *PGUS* under SMG was also higher than NG. The transcriptional level of the reporter gene *PGUS* in baffled shaker flasks control was higher than SMG control. However, the PGUS specific yields showed no significant difference under the two culture conditions.

Verification of *TPX* transcription effects on reporter enzyme AtXYN production

Another reporter enzyme endo-β-1, 4-xylanase (AtXYN) was chosen to evaluate the potential benefit of the helper gene *TPX* in order to preclude a reporter protein-specific effect. PGAPZB-*TPX* plasmid was transformed into the *P. pastoris*-AtXYN strain. Eight verified clones co-overexpressing *TPX* were randomly chosen and cultured in baffled shaker flasks. After 48 h of methanol feeding, the xylanase specific activities were detected (Fig. 6). Only two *TPX* mutants (clone #1, clone #3) seemed to favor AtXYN specific activities, while the other five mutants showed a slight increase or negative impacts on AtXYN specific activities. The relative transcriptional levels of *TPX* were determined by qRT-PCR for the differentially AtXYN expressed clones. However, the differences of *TPX* mRNA levels were no quite significant (data not shown).

Discussion

As a unique environment, microgravity and simulated microgravity have significant effects on numerous microbial cellular processes. Changes in cellular functions include cell clumping, shortened lag phase and prolonged exponential phase in the gene expression, and protein folding to alter the productivities of the secondary metabolites in bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) and yeast (*S. cerevisiae*) (Benoit et al. 2006; Baker et al. 2004; Crabbé et al. 2011; Sheehan et al. 2007). Only a few studies have shown that microgravity can impact the heterologous proteins production, such as enhanced expression of the recombinant β-glucuronidase in *E. coli*, and β-galactosidase and glycodelin in human cells (Xiang et al. 2010; Stephen 2007). In contrast, some other studies indicated that SMG could not always promote protein expression (Boyle et al. 1995). In the present study, our results showed that SMG could facilitate the specific productivities of β-glucuronidase and endo-β-1, 4-xylanase. We hypothesized that the important reason for the enhancement of protein expression was the

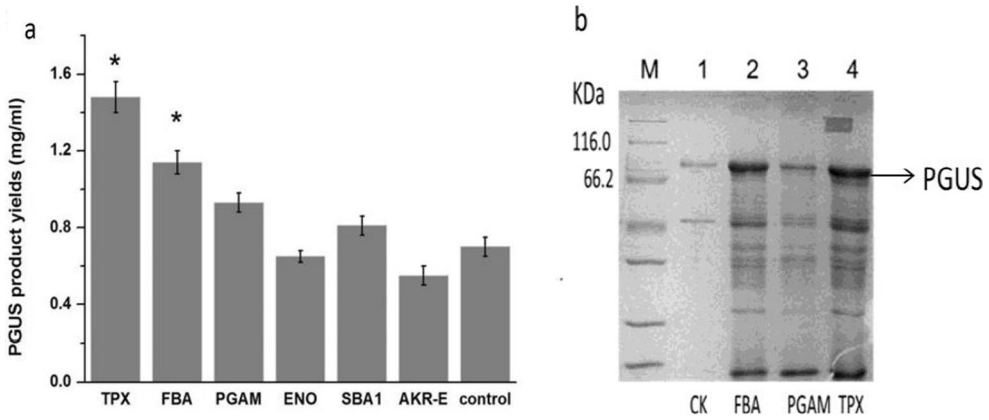

Fig. 3 The PGUS specific yields of the strains co-expressing different target genes. **a** The protein concentration of PGUS after 48 h of methanol induction. The yield ratios were relative to the control strain without co-expressing any target genes (value=1). Data are represented as means± standard deviation (SD), two-sided paired *t* test, $P<0.05$ (∗). **b** Coomassie-stained SDS–PAGE (12 %) examination of the effective helper genes on the PGUS production (*CK* the control strain)

genomic response of yeast to microgravity. Our previous study revealed differentially regulated genes of the recombinant *P. pastoris*-PGUS under SMG by next-generation sequencing (NGS) technologies (Qi et al. 2011b). We summarized four categories of significantly changed genes. Most of the positive genes were related to methanol metabolism and protein transportation and were up-regulated in both the exponential and stationary phase. The capabilities of recombinant protein production were not just relative to the multiple metabolic processes but also subject to specific environmental disturbances and different protein properties (size, number of disulfide bonds and glycan). The low-shear fluid environment of SMG may alter the genes transcription, which modulates cell growth and correct protein folding.

Using the 2D nano-LC–MS/MS approach and sequence similarity search strategy, we first assessed and quantified the global protein expression patterns in the recombinant *P. pastoris*-PGUS cultured under SMG condition. The proteins involved in the glycolytic pathway showed higher expression levels under SMG, which indicated that SMG could increase the recombinant protein yields. Interestingly, the proteomic levels of alcohol oxidases were down-regulated at the stationary phase. However, the transcriptional levels of alcohol oxidase were 3.6-fold up-regulated at the logarithmic phase and 1.4-fold up-regulated at the stationary phase in our previous study (Qi et al. 2011b). The discrepant results were also found in some proteins involved in methanol metabolism according to the present proteomic profiling data. Our previous study suggested that the significant transcriptional changes of the methanol metabolism genes would cause faster oxygen and methanol uptake, which promotes transcription of the recombinant gene. Apparently, the proteomic research findings could not support the hypothesis. There must be other reasons responsible for the enhanced protein expression of *P. pastoris* in a SMG environment.

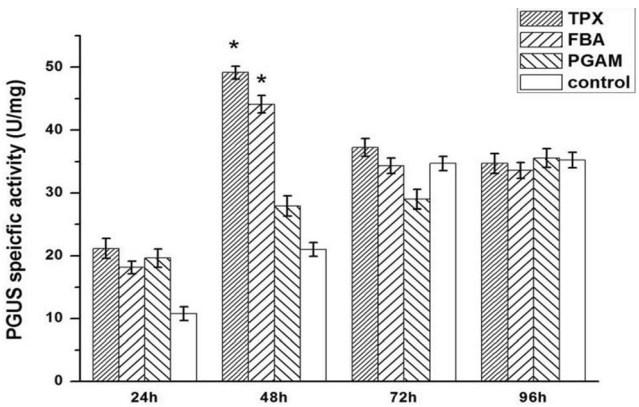

Fig. 4 Time courses of the PGUS specific activities of the strains co-expressing the effective helper genes. Methanol concentration was maintained at 1 % at 12-h intervals. The every group of samples was compared with control strain without co-expressing any helper genes. Data are represented as means±standard deviation (SD), two-sided paired *t* test, $P<0.05$ (∗)

In the methanol fed-batch phase, the cells were exposed to dual oxidative stress resulting from methanol degradation and recombinant protein production. Previous studies described that the protein folding process was also related to oxidative stress (Malhotra et al. 2008; Vanz et al. 2012). The proteins involved in oxidative stress had a higher abundance under SMG according to the proteomic data, implying that microgravity increased the oxidative stress response in the cell processing. The transcriptome profiling data was in accordance with the proteomic data. The transcriptional levels of thiol peroxidase, which exhibited a major role in weakening the oxidative stress, were 1.69-fold up-regulated at the logarithmic phase and 1.93-fold up-regulated at the stationary phase. The proteomic level of thiol peroxidase was highly up-regulated to 2.59-fold at the stationary phase. The chaperones with higher transcriptional and proteomic levels indicated that the SMG environmental stresses could easily provoke overload of the endoplasmic reticulum (ER) folding machine, resulting in the aggregations of unprocessed proteins and

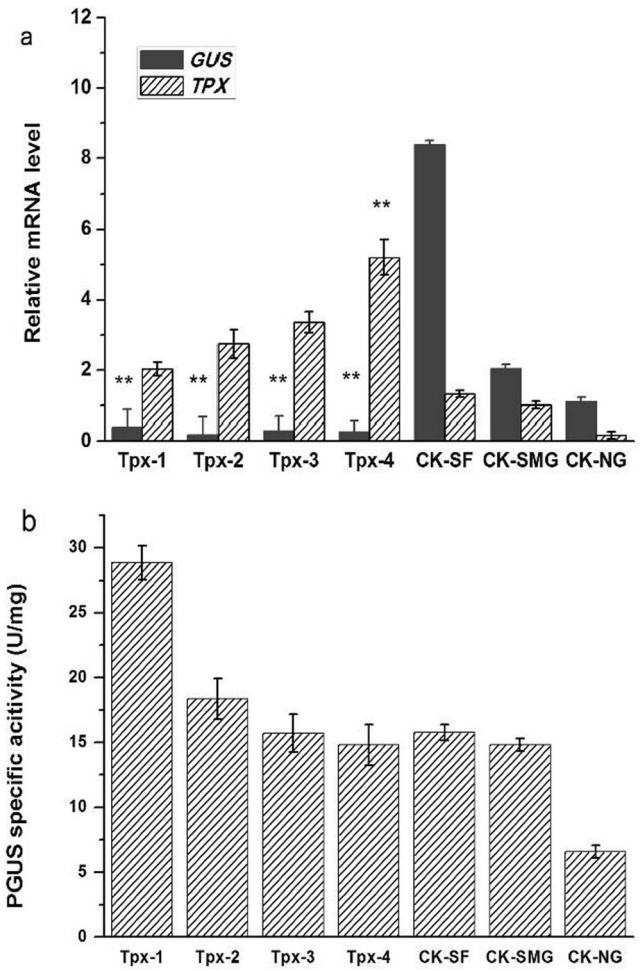

Fig. 5 Transcriptional effects on the PGUS specific activities by co-expressing different *TPX* gene copies. All the strains were harvested and their mRNA levels were tested by qRT-PCR after 48 h of methanol induction. **a** Comparison of the relative transcriptional levels of *PGUS* and *TPX*. **b** The PGUS specific activities of the strains harboring different *TPX* gene copies. (Tpx-1, Tpx-2, Tpx-3, and Tpx-4—four recombinant *P. pastoris* strains harboring 2, 4, 8, and 10 copies of *TPX* gene, respectively; *CK-SF* the control strain cultured in a baffled shaker flask; *CK-SMG* the control strain cultured under SMG; *CK-NG* the control strain cultured under NG. Methanol concentration was maintained around 1 % at 12-h intervals. Results are expressed relative to mean for the every control group samples for each gene. Data are represented as means± standard deviation (SD). Paired comparing Tpx-1, -2, -3, -4 and CK-SF, two-sided paired *t* test, $P<0.01$ (**)

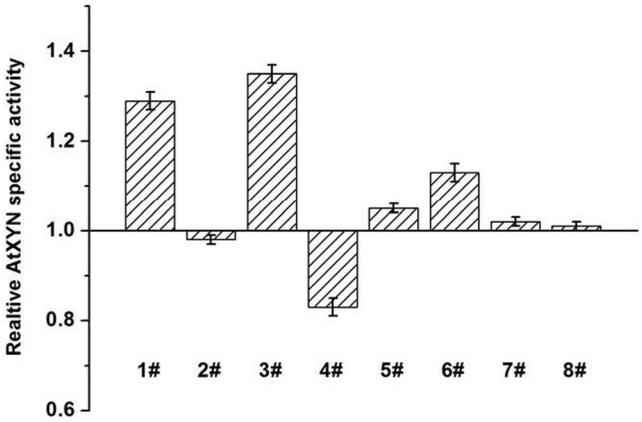

Fig. 6 Identification of the *TPX* effects on AtXYN expression. Eight individual clones co-expressing *TPX* were used for a small-scale screening in baffled shaker flasks. The AtXYN specific activities were calculated after 48 h of methanol induction (U/mg). The specific activities were relative to the control strain without co-expressing any target genes (value=1). Data are represented as means±standard deviation (SD). There were no significant differences of the samples compared with the control strain ($P<0.05$)

consequently the onset of the unfolded protein response. The high abundance of ribosome-associated proteins indicated that the changes on the cellular protein synthesis machinery contributed to the enhanced production of the recombinant protein under SMG.

A systematic approach for finding novel targets could facilitate the subsequent rational strain engineering strategies. We compared the proteomic data and selected six significantly regulated genes and tested their functions in recombinant protein production. Thiol peroxidases were involved in transferring oxidative signals and regulating transcription by direct oxidation of numerous cellular proteins. Fructose 1, 6-bisphosphate aldolase, an important enzyme for cofactor regeneration, catalyzed the formation of fructose 1, 6-bisphosphate from dihydroxyacetone and glyceradehyde-3-phosphate and replenished the xylulose-5-phosphate to the downstream reactions of the methanol metabolism. In methylotrophic yeasts, the first step in the metabolism of methanol was the oxidation of formaldehyde by the alcohol oxidase, which generated high level of H_2O_2 and caused the oxidative stress response (Fomenko et al. 2011). The refolding of unfolded or misfolded proteins in the ER had a chain of oxidative folding processes. Improving the transcription of two helper genes could significantly enhance the heterologous PGUS production. Research showed that overexpressing helper genes supporting folding and secretion like *PDI* and *HAC1* turned out to be valuable strategies to improve recombinant protein production (Guerfal et al. 2010). Thus, *SBA1*, an up-regulated co-chaperone, was selected. However, the secretion of the heterologous PGUS was slightly improved by *SBA1*. In addition, *AKR-E* was selected in order to determine whether other genes involved in the oxidative response would get the similar improvement like *TPX*. However, the result did not support the hypothesis. The relation of oxidative response and protein production were still not fully understood even though the whole genome sequence of the organism had been available. Apparently, the supporting factor *TPX* seemed to play a positive role to raise the correct protein yields at the initial period of the fermentation process.

Several cases showed that increased copy numbers of a helper gene can affect normal metabolism in *P. pastoris* (Zhu et al. 2011). To understand the regulation mechanism of a helper gene, the transcriptional levels were investigated in this study. Clones harboring the low copy numbers of the helper

gene *TPX* had positive effects on the specific PGUS yields. Improvement of target protein production was normally thought to be due to the increasing level of transcription. However, co-expression of the helper gene could also compete with the target gene for transcription. Baumann et al. reported that the reporter gene mRNA levels among the recombinant protein production improved strains of overexpressing helper genes were similar to the reference strain (Baumann et al. 2011). In our research, the transcriptional level of *PGUS* was decreased when the helper gene *TPX* was overexpressed. Inversely, the PGUS specific activities were increased. The helper gene here seemed to facilitate the enhancement of the ability of the protein to fold correctly and be secreted but not help the transcriptional regulation. To validate the discrepant results, we also have investigated the PGUS secretion of *TPX* overexpressing strains (Tpx-1, 2, 3, 4 and CK-SF) by Coomassie brilliant stained SDS–PAGE. The results showed that the PGUS productions in *TPX* overexpressing strains were enhanced (data not shown).

Poorly folded proteins in the ER generally involve a chain of oxidative folding processes. Thiol peroxidases were reported as global regulators of gene expression for providing an antioxidant defense by reducing hydroperoxides. Therefore, overexpression of *TPX* might efficiently counteract oxidative stress arising from heterologous protein production. We compared the transcription levels of genes involved in folding stress and oxidative stress and in the low copy of the *TPX* gene strain (Tpx-1) and the control strain without co-expressing the *TPX* gene (CK-SF) by real-time PCR after 24 h of methanol induction. The relative transcription level of *GLR1* (glutathione reductase, responsible for converting oxidized glutathione to reduced glutathione) was up-regulated by 10 % in Tpx-1, indicating that the strain of co-expression was undergoing oxidative stress owing to the folding of *TPX*. The relative transcription level of *PDI1* (disulfide isomerase, a chaperone that catalyzes the formation of disulfide bonds) was increased by 15 % in Tpx-1, indicating the process of correct folding of PGUS (data not shown). The results indicated that the strain overexpressing *TPX* was undergoing oxidative stress owing to the process of recombinant proteins correctly folding.

Alteration of the expression levels of the helper gene seemed to impact the protein production process. There were significant differences in specific yields among clonal variations of AtXYN, whereas transcriptional levels of *TPX* gene were similar. The transcriptional levels of AtXYN in the improved clones were also decreased when *TPX* was overexpressed (data not shown). This result suggested that other factors may be involved in the clonal variation. These conflicting results indicated that the ways of controlling helper gene expression levels to get stable improved protein expression needed further and deeper investigation.

Therefore, additional studies should further deeply analyze the omic data and investigate the potential synergetic actions of the individual helper factors in the future. Studies concerning the influence of the microgravity environment on microbial cells may receive much attention, and such information could provide insights into the ways that microgravity affects the yeast cells. Utilizing the microgravity effect to find novel targets related with protein production would be a new and more robust tool for subsequent strain improvement in normal fermentation.

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Conflict of interest The authors declare that no competing financial interests exist.

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