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ORIGINAL PAPER

Transcriptional investigation of the effect of mixed feeding to identify the main cellular stresses on recombinant *Pichia pastoris*

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Abstract Heterologous protein expression using *Pichia pastoris* causes metabolic stress on the physiology of host cells, which may compromise the yields of secreted foreign proteins. Thus, understanding these metabolic stresses during secretory expression allows us to circumvent these undesirable effects. We investigated the effect of co-feeding two alternative carbon resources, sorbitol and yeast extract (YE), on the physiology of A3, a *P. pastoris* strain carrying 18 copies of the porcine insulin precursor (*PIP*) gene. Comparative transcriptional analysis was performed on 13 selected genes involved in important cellular processes. Results showed that co-feeding of either sorbitol or YE along with methanol improved the performance of A3. The co-feeding of YE enhanced the specific growth rate of A3 and the specific PIP productivity. However, the oxidative stress in the yeast cells increased. The co-feeding of methanol and sorbitol increased the specific growth rate of A3 but did not affect the specific PIP productivity. The transcriptional results suggested that sorbitol may have repressed the expression of foreign proteins. These observations will not only guide the application of mixed feeding strategies but also give a deeper understanding of the metabolic burden in the secretory expression of foreign proteins.

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Introduction

Over the past 30 years, *Pichia pastoris* has been increasingly popular in the production of pharmaceutical proteins and industrial enzymes [3, 4, 6]. To achieve the maximum production level, researchers have exploited the expression potential of *P. pastoris* by using stronger promoters [7, 13], increasing the copy number of foreign genes [18, 20], and optimizing the cultivation media and protocols [1], among others. Although these methods effectively elevated foreign protein production levels, overexpression-mediated metabolic stress in the physiology of recombinant *P. pastoris* is commonly observed [12]. This overexpression eventually affects the yields of foreign proteins. In the case of secretory expression, protein overexpression leads to a reduced methanol consumption capacity, specific growth rate, and/or viability of the yeast cells [2, 10, 20].

To circumvent these problems better, we need a deeper understanding of the metabolic burden brought by overexpression. In secretory expression, the protein folding stress caused by a large flux of heterologous nascent polypeptides is generally regarded as the main contributor to the metabolic burden [5]. Folding stress also causes oxidative stress because of the repetitive oxidative folding of foldases such as protein disulfide isomerase (PDI), which may readily cause cellular damage or even cell death [5, 8]. Aside from these two types of stress, our previous report suggested that in the context of secretory expression in the *P. pastoris* system, protein overexpression makes host cells unable to assimilate carbon effectively when methanol is utilized as

the sole carbon source [21]. In that work, the physiological conditions of *P. pastoris* strains carrying 6 and 18 copies of the porcine insulin precursor (*PIP*) gene were compared using the transcriptional analysis of key genes. The results revealed a major redistribution of C_1 metabolism in the 18-copy strain. Consequently, the C_1 carbon flow directed toward the assimilation pathway was significantly reduced. We therefore hypothesized that, except for oxidative stress, the weakened methanol assimilation capability of *P. pastoris* may be one of the main factors affecting cell growth and, hence, foreign protein expression.

To test this hypothesis and identify the main cause of cellular stress during *PIP* overexpression, in this study, we investigated the effect of the co-feeding of alternative carbon resources with methanol on the physiological performance of *P. pastoris*. Two carbon sources, sorbitol and yeast extract (YE), were considered because they were found to be less repressive to the *AOX1* promoter compared to other carbon sources such as glucose and glycerol [1]. More importantly, this study provides a better understanding of cellular stress during the secretory expression of *PIP* and the reduction of stress via co-feeding. A comparative transcriptional analysis was also performed, with 13 selected key genes used as indicators of the physiological status of *P. pastoris* hosts. To our knowledge, this article is the first report on the influences of co-feeding on the physiology of *P. pastoris* hosts on the transcriptional level.

Materials and methods

Strains, media, and growth conditions

A *P. pastoris* GS115-derived strain (Invitrogen, Carlsbad, CA, USA) having 18 copies of the *PIP* gene (designated as A3) was used in this study. Its detailed construction history was described in our previous work [20].

A single colony of *P. pastoris* grown on a yeast peptone dextrose (YPD) plate was inoculated into a 500-ml shake flask containing 50 ml of YPD medium (per liter: 10 g of yeast extract, 20 g of peptone, and 20 g of glucose) and then cultured at 30 °C at 300 rpm. After 24 h of incubation, the cells were harvested via centrifugation at 12,000×*g* and re-suspended in basic salt medium [per liter: 5.0 g of $(NH_4)_2SO_4$, 0.465 g of $CaSO_4{\cdot}2H_2O$, 9.1 g of K_2SO_4, 7.5 g of $MgSO_4{\cdot}7H_2O$, 6.8 g of KH_2PO_4, and 20 g of glycerol] with 4.4 ml/l of *Pichia* trace metal (PTM1) salt stock solution (per liter: 6 g of $CuSO_4{\cdot}5H_2O$, 0.09 g of KI, 3 g of $MnSO_4{\cdot}H_2O$, 0.02 g of H_3BO_3, 0.2 g of $MoNa_2O_4{\cdot}2H_2O$, 0.5 g of $CoCl_2$, 20 g of $ZnCl_2$, 65 g of $FeSO_4{\cdot}7H_2O$, 0.2 g of biotin, and 5.0 ml of H_2SO_4) before induction.

Three induction strategies, namely, the standard procedure, sorbitol co-feeding, and YE co-feeding, were applied. The standard protocol (also referred to as the control) for induction was performed as follows: The induction cultivation was started by adding 500 µl of pure methanol to each 500-ml flask containing 50 ml media. The residue methanol concentration was assayed every 12 h, and then pure methanol was added to maintain the final methanol concentration at around 1 %. The pH of the medium was adjusted and controlled at 5.0 every 24 h by supplementing 1 M of KOH. Sorbitol and YE co-feeding was the same as the standard procedure except that sorbitol and YE were fed together with methanol (the mass ratio of methanol to sorbitol or YE was 10:1). The cell density and PIP production were determined after 72 h of induction. For the transcriptional analysis, all samples were collected after 24 h of induction and then frozen at −80 °C.

RNA extraction and cDNA synthesis

The total RNA was prepared using an RNAprep pure kit (Tiangen Biotech, Beijing). The isolated RNA was treated with DNAse I (Tiangen Biotech, Beijing). RNA integrity was tested in 1.2 % agarose gels, and its concentration was measured via densitometry with a 260/280 nm absorbance ratio. After heating at 85 °C for 10 min to denature the RNases, 400 ng of total RNA was subjected to reverse transcription using the PrimeScript™ real-time polymerase chain reaction (RT-PCR) kit (TaKaRa Bio Co., Ltd., Dalian). The reaction was terminated by heating at 70 °C for 10 min.

Design of primer sets for RT-PCR

The PCR primer design was conducted using Primer3 software (http://www.genome.wi.mit.edu/ftp/distribution/software/). All primers used in this work are listed in Table 1.

RT-PCR

Genomic DNA or synthesized single-strand cDNA was used as a template in the RT quantitative PCR (qPCR) analysis. The reaction conditions were established as recommended by the SYBR *Premix Ex Taq*™ manual (TaKaRa Bio Co., Ltd). Each 20-µl reaction contained 10 µl of 2× SYBR *Premix Ex Taq*™, 0.4 µl of 50 µM of forward and reverse primers, respectively, 2.0 µl of sample genomic DNA, and 7.2 µl of sterile deionized water. All RT-qPCRs were run in triplicate in an FC-2000 PCR Cycler (Funglyn Biotech Inc., Shanghai) using the following program: 95 °C for 3 min, 45 cycles of 95 °C for 5 s, and 60 °C for 20 s. The specificity of amplicons was verified via melting curve analysis after 40 cycles and agarose gel electrophoresis.

Table 1 Sequences of primers used for real-time quantitative PCR (qPCR)

Gene	Accession no.	Primer	Amplicon size (bp)	Sequence 5′–3′
PIP	Synthetic gene	pp1	165	TGTTTTGCCATTTTCCAACA
		pp2		TCACCGCAAACCAAGTACAA
AOX1	U96967	aox1	171	GTGCTAACGTCTTGGGTGGT
		aox2		CGTGAATGTCAGGGTTGTTG
GAP	U62648	gap1	98	ATGACCGCCACTCAAAAGAC
		gap2		GCACCAGTGGAAGATGGAAT
ACT1	AF216956	act1	181	AGTGTTCCCATCGGTCGTAG
		act2		GGTGTGGTGCCAGATCTTTT
KAR2	AY965684	kar1	132	CACTTGGGTGGTGAGGACTT
		kar2		GGCCTTTTCGACCTCTCTCT
PDI1	AJ302014	pdi1	145	GCCGTTAAATTCGGTAAGCA
		pdi2		TCAGCTCGGTCACATCTTTG
DAK	AF019198	dak1	175	TCTTTGCCTCGCCATCTACT
		dak2		TCGTCCTGAACAATCAGCAG
FLD1	AF066054	fld1	128	GGTGTATGCCACACTGATGC
		fld2		ACCTTCACGGACTCAACACC
PDH	DQ337111	pdh1	153	ATGGGTTTCACTGGTCTTGC
		pdh2		ACAGGGTTGCTTACCACCAG
CIT1[a]	RPPA11375	cit1	175	TTCAAAGACGGAAAGGTTGG
		cit2		CAACCAAGTGAGTGGTGTGG
TRR1[a]	RPPA06201	trr1	185	CTTTGGCAGTTGTTGGAGGT
		trr2		CTTTGCCTCCTTGGAAACAG
GLR1[a]	RPPA07699	glr1	152	ATGGTGCAAAGACCCTTTTG
		glr2		ATCGCCTGCCACATTAAATC
PYK2[a]	RPPA12008	pyk1	191	CAAGTGCAATCTGGCAGGTA
		pyk2		GCATCATAGCAACAGCCTCA
ZWF1[a]	RPPA11829	zwf1	140	CGACTTGCAGTCAGCAGAAG
		zwf2		TGATGAATGCGTTTCCAAAA

[a] Sequence information was provided by Prof. James Gregg from Keck Graduate Institute of Applied Life Sciences

RT-qPCR data were normalized using the *ACT1* gene as the endogenous control (housekeeping gene). To analyze each gene of the different strains, we treated the data using the $2^{-\Delta\Delta}$ method [11]. In this case, the transcriptional level of A3 with the standard induction protocol was set as control to normalize the data (assigning the expression level value to one). The qRT-PCRs were run in triplicate with biological replicates to allow for statistical confidence in differential gene expression. The qRT-PCR expression values obtained for each gene differ by less than 30 %.

Results

Fermentation profiles of A3 with three induction strategies

After 72 h of induction, the sorbitol and YE co-feeding with methanol significantly improved the PIP production level and cell growth (Fig. 1a–c). Sorbitol co-feeding resulted in a 40 and 50 % increase in optical density (OD) and PIP production, respectively, compared with pure methanol feeding (control). YE co-feeding led to a 30 and 50 % increase in OD and PIP production, respectively. The average specific PIP productivity of A3 was not significantly affected by sorbitol co-feeding but was increased by 30 % by YE co-feeding (Fig. 1d).

Transcription of foreign gene *PIP* in cultures with three induction strategies

The relative transcriptional levels of the *PIP* gene with three strategies after 24 h of induction are shown in Fig. 2a. Compared with that obtained by methanol feeding, the *PIP* transcription was decreased by almost 50 % by sorbitol co-feeding but was not affected by YE co-feeding.

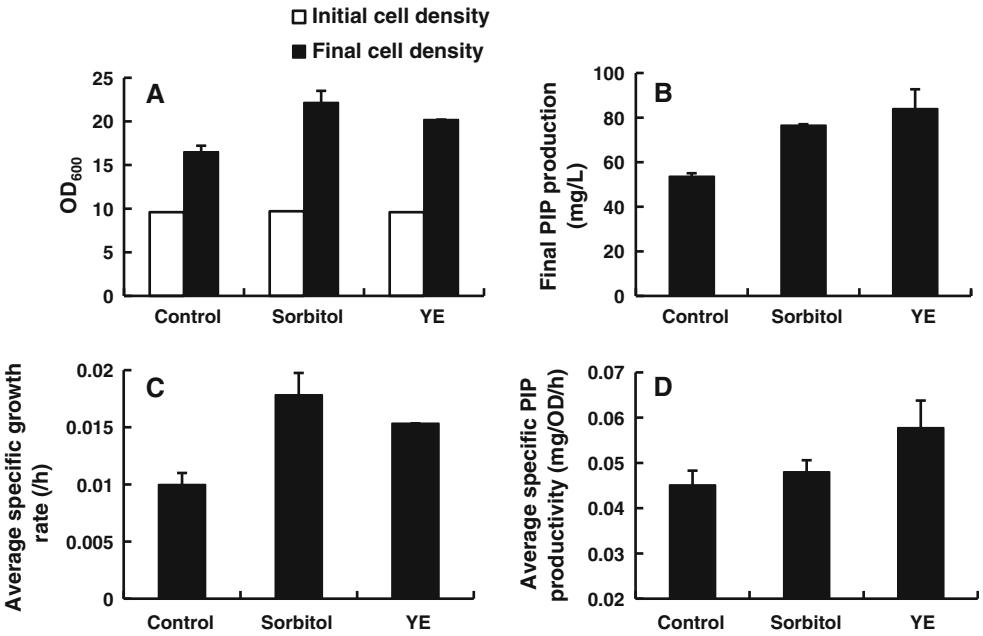

Fig. 1 Fermentation profiles of A3 in shake flasks after 72 h of induction with the three fermentation strategies. **a** Cell density, **b** final PIP production, **c** average specific growth rate, **d** average specific PIP productivity. Pure methanol feeding was used as the control strategy. Three *parallel* flasks were tested for each strategy

Transcription of methanol metabolism genes *AOX1*, *FLD1*, and *DAK* in cultures with three induction strategies

The relative transcriptional levels of alcohol oxidase I (the first enzyme in the methanol utilization pathway, *AOX1*), formaldehyde dehydrogenase (the first enzyme in the methanol dissimilatory pathway, *FLD1*), and dihydroxyacetone kinase (a key enzyme implied in the methanol assimilatory pathway, *DAK*) are shown in Fig. 2b. The transcriptional levels of *AOX1* with the three strategies were almost the same. Compared with the control, the transcriptional levels of *FLD1* was decreased to 40 % by sorbitol co-feeding but was increased by 50 % by YE co-feeding. As for *DAK*, the transcriptional level was down-regulated (decreased to 35 %) with sorbitol but remained constant with YE.

Transcription of central metabolic pathway genes *GAP*, *PYR2*, *ZWF1*, *PDH*, and *CIT1* in cultures with three induction strategies

Figure 2c shows the relative transcriptional levels of the key enzyme representing the central metabolic pathway with three feeding strategies. These enzymes include glyceraldehyde-3-phosphate dehydrogenase (*GAP*) and pyruvate kinase (*PYR2*) (two key enzymes in glycolysis), pyruvate dehydrogenase (an acetyl-CoA producing enzyme, *PDH*), citrate synthase (a key enzyme in TCA cycle, *CIT1*), and glucose-6-phosphate dehydrogenase (a key enzyme in pentose phosphate pathway, *ZWF1*). A significant increase was observed in the transcriptional levels of *GAP*, *PYR2*, *CIT1*, and *ZWF1* in cultures with sorbitol and YE co-feeding compared with the reference cultures. However, the transcriptional levels of the *PDH* gene in the reference and co-feeding cultures were not significantly different.

Transcription of chaperone genes *PDI1* and *KAR2* in cultures with three induction strategies

The relative transcriptional levels of two important chaperones located in the endoplasmic reticulum (ER), namely, BiP protein (a chaperone of the HSP70 class that plays an important role in the unfolded protein response, *KAR2*) and protein disulfide isomerase (a chaperone that catalyzes the formation of the disulphide bond, *PDI1*) were determined. These proteins are shown in Fig. 2d. The transcriptional levels of *KAR2* in both sorbitol and YE cultures were not significantly changed. However, *PDI1* was increased by 40 and 110 % in the sorbitol and YE cultures, respectively.

Transcription of antioxidative genes *GLR1* and *TRR1* in cultures with three induction strategies

The relative transcriptional levels of two antioxidative proteins, namely, glutathione reductase (responsible for converting oxidized glutathione to reduced glutathione, *GLR1*) and thioredoxin reductase (responsible for converting oxidized thioredoxin to reduced thioredoxin, *TRR1*), in three cultures are shown in Fig. 2e. *GLR1* was basically transcribed at the same level in reference and sorbitol cultures but was upregulated by 330 % in YE co-feeding. Compared with the control, the transcription of

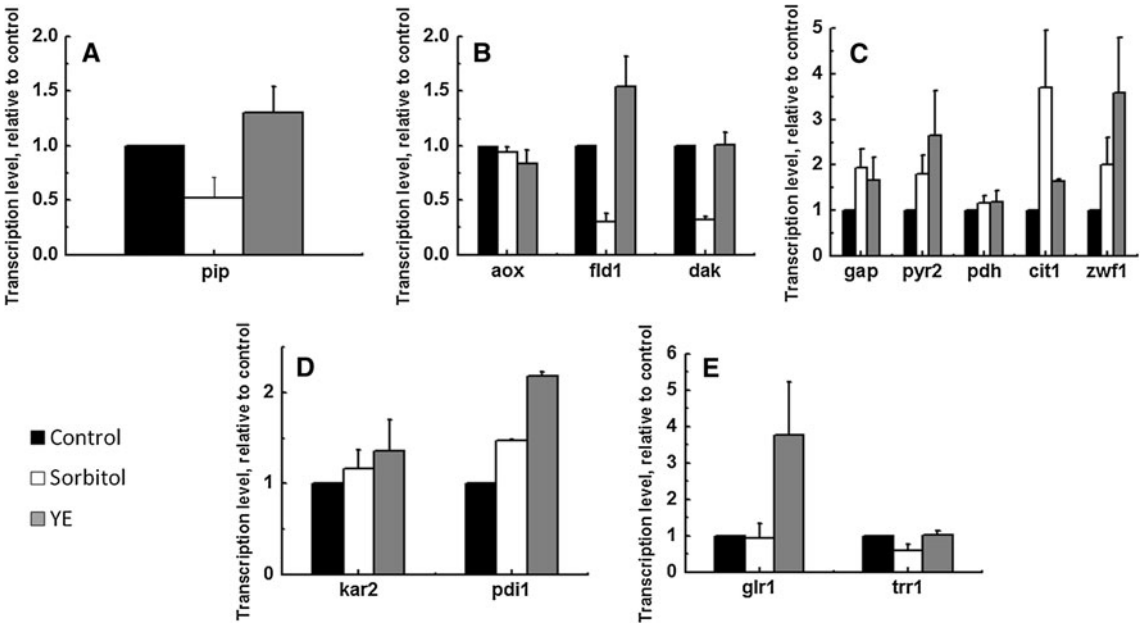

Fig. 2 Transcriptional levels of selected genes representing the physiological status of *P. pastoris*. **a** Foreign gene, **b** genes involved in the methanol metabolic pathway, **c** genes involved in the central metabolic pathway, **d** genes involved in folding stress, **e** genes involved in oxidative stress. All three strains were harvested, and their mRNA levels were tested via qRT-PCR after 24 h of induction in shake flasks. Values were normalized relative to control (standard protocol)

TRR1 was slightly decreased by 40 % with sorbitol and remained almost constant with YE.

Discussion

The mixed feeding strategy has been a common practice for improving the performances of recombinant *P. pastoris* [1]. It can effectively enhance the cell growth and hence foreign protein production particularly for Mut^s (methanol utilization slow phenotype, because of the *AOX1* gene knockout) strains or high-copy strains, where the specific growth rate of recombinant *P. pastoris* is slow. Therefore, this strategy was thought to play a key role in reducing the metabolic burden of the production host [14, 19]. However, the underlying cause for the reduction of the metabolic burden on *P. pastoris* by co-fed carbon sources (e.g., sorbitol, glycerol, and YE) has rarely been studied. In this study, with the help of transcriptional analysis, we tried to gain some insight into the effect of mixed feeding on the performance of a high-copy *P. pastoris* strain carrying 18 copies of the *PIP* gene (Fig. 3).

The comparison of the fermentation profiles of A3 with three different strategies showed that both sorbitol and YE co-feeding strategies significantly enhanced the cell growth. However, when the key genes in the methanol metabolic pathway were examined, neither the reduced *DAK* transcription with sorbitol nor the increased *FLD1* transcription with YE indicated that the C_1 assimilation is strengthened by co-feeding. Instead, the results seemed to suggest that C_1 assimilation was repressed with either sorbitol or YE co-feeding. Meanwhile, four of the five central metabolic genes were apparently upregulated, suggesting that the central metabolic pathway was highly active. Therefore, during co-feeding, sorbitol or YE appeared to be the main substrate supplier and energy source.

For the foreign protein production, the co-feeding of both sorbitol and YE increased the final PIP yield. However, a careful examination of specific PIP productivity suggested that YE worked more effectively than sorbitol in terms of enhancing PIP production, because average specific PIP productivity did not increase significantly with sorbitol but increased by 30 % with YE. This conclusion can be drawn from the transcriptional analysis of *PIP* gene. Sorbitol appeared to affect the transcription of the *PIP* gene negatively, whereas YE did not affect the *PIP* transcription. Interestingly, sorbitol has been regarded as a nonrepressive carbon source with respect to the *AOX1* promoter [9, 16, 17]. Our work suggested that sorbitol may have repressed the foreign gene transcription. In fact, sorbitol appeared to have a general repressive effect on the methanol metabolic pathway because both *DAK* and *FLD1* were significantly downregulated. Although sorbitol co-feeding led to a lower *PIP* mRNA level as compared with the control, the specific PIP productivity was not decreased, indicating that PIP production was compensated at the translational level owing to improved availability of precursors or energy.

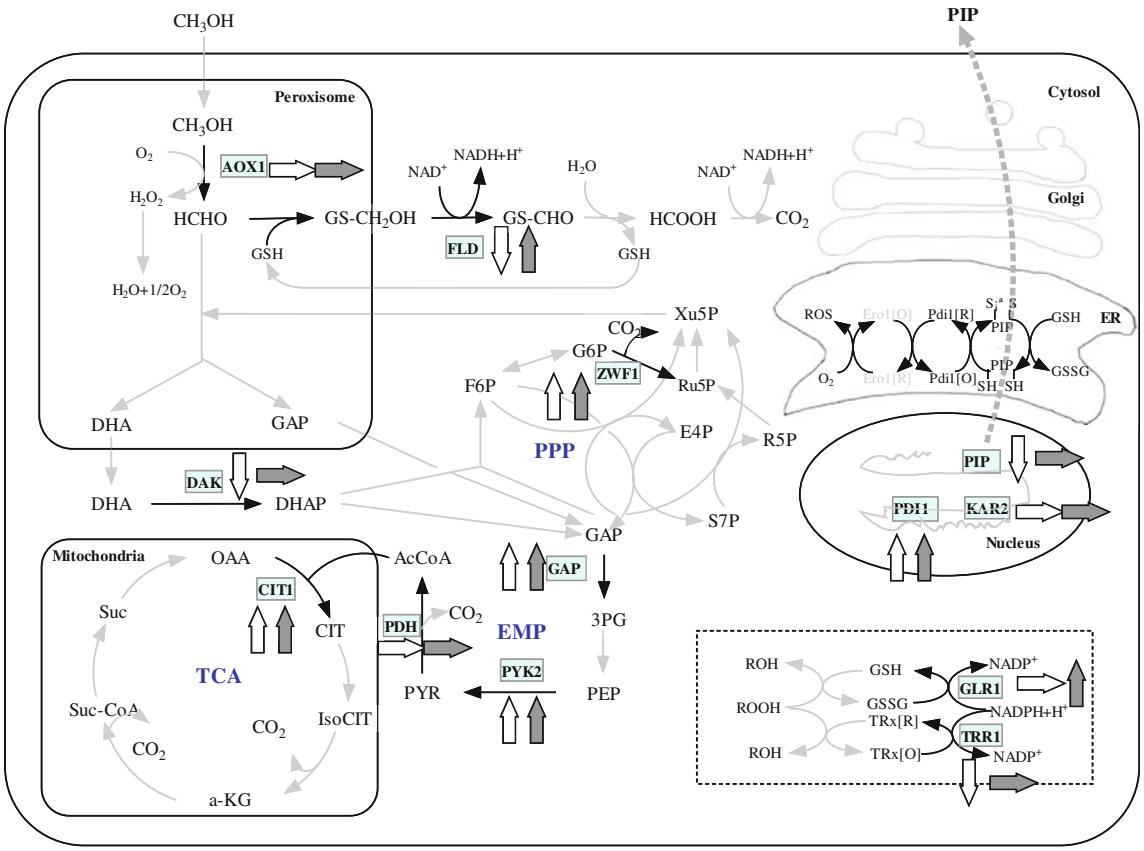

Fig. 3 Main physiological changes in the transcriptional levels with two co-feeding strategies. The first of the two *arrows* for each gene indicates the sorbitol co-feeding strategy, and the second shows the YE co-feeding strategy. *Downward*, *upward*, and *right arrows* indicate the reduction, increase, and absence of significant change in the mRNA levels, respectively

The transcriptions of chaperone genes *KAR2* and *PDI1* were recognized as indicators of ER stress [15]. The *PDI* increase indicated the higher ER stress during mixed feeding and implied that the cells synthesized more PIP with more substrate when mixed feeding is applied. Furthermore, the increase in *PDI1* was higher with YE than with sorbitol, suggesting that the synthesis flux was higher with YE.

Oxidative stress, a common feature of secretory expression, is due to the oxidative folding of polypeptides to form a disulfide bond. Our previous studies observed increased ROS levels and decreased glutathione-SH (GSH) pools in high-copy strains (PIP contains three pairs of disulfide bridge), which resulted in the upregulation of antioxidation-related genes, such as *GSH1* and *TRR1*, and the *FLD1* gene in *P. pastoris* [21]. When YE was co-fed with methanol, *FLD1* and *GSH1* increased, indicating that the oxidative stress on A3 is higher with YE than with pure methanol. In addition, YE co-feeding significantly enhanced cell growth and PIP production, implying that the weakened C_1 assimilation, instead of oxidative stress, is the main metabolic burden for the recombinant *P. pastoris* in PIP expression. With sorbitol co-feeding, although the protein synthesis flux was significantly increased, no indicators of elevated (possibly lowered as indicated by the downregulation of *TRR1*) oxidative stress were observed. Sorbitol possibly repressed the methanol utilization pathway as aforementioned. Thus, a great portion of the GSH pool used to dissimilate formaldehyde was spared from coping with oxidative stress [21].

In this article, we investigated the effects of the YE and sorbitol co-feeding strategies on the physiology of *P. pastoris* through the transcriptional analysis of 13 genes involving important cellular processes. The results suggested that both strategies improved the performance of A3. YE co-feeding increased the specific growth rate of A3 and the specific production of PIP. However, it also led to the increase in the oxidative stress in yeast cells. This observation indicates that the weakened C_1 assimilation, instead of oxidative stress, is the main metabolic burden for recombinant *P. pastoris* in *PIP* expression. On the other hand, sorbitol co-feeding increased the specific growth rate of A3 but not the specific production of PIP. The transcriptional results suggested that sorbitol may have repressed the transcription of foreign proteins, whose expression level could largely be compensated by increased availability of precursors or energy. These

observations will not only guide the application of mixed feeding strategies but also provide a deeper understanding of the metabolic burden in the secretory expression of foreign proteins in the *P. pastoris* system.

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