Biotechnology and Bioprocess Engineering 22: 767-773 (2017)
DOI 10.1007/s12257-017-0011-9

pISSN 1226-8372
eISSN 1976-3816

RESEARCH PAPER

Enhanced Protein Production by Sorbitol Co-feeding with Methanol in Recombinant *Pichia pastoris* Strains

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Received: 9 January 2017 / Revised: 8 May 2017 / Accepted: 29 August 2017

Abstract *Pichia pastoris* strains carrying 1, 6, 12, and 18 copies of the porcine insulin precursor (*PIP*) gene, were employed to investigate the effects of sorbitol co-feeding with methanol on the physiology of the strains. Multicopy clones of the methylotrophic yeast were generated to vary the *PIP* gene dosage and recombinant proteins. Elevated gene dosage increased levels of the recombinant PIP protein when methanol served as the sole carbon and energy source *i.e*., an increase of 1.9% for a strain carrying 1 copy, 42.6% for a strain carrying 6 copies, 34.7% for a strain carrying 12 copies and 80.9% for a strain carrying 18 copies, respectively (using sorbitol co-feeding with methanol during the induction phase). However, it had no significant influence on a lower gene dosage strain (1 copy), but this approach affirmed enhancement in cell growth and PIP production for higher gene dosage strain (6, 12, and 18 copies) *via* using sorbitol co-feeding with methanol. Additionally, the co-feeding strategy could hold vital importance for recombinant protein production by a multi-copy *P. pastoris* system.

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Keywords: sorbitol, co-feeding, multi-copy, *Pichia pastoris*, porcine insulin precursor

1. Introduction

The methylotrophic yeast, *Pichia pastoris*, has been developed to be a highly successful system for large-scale production of functionally active recombinant proteins [1,2]. As a yeast, it is a unique system which has many advantages *e.g.,* its ability to produce foreign proteins at high levels using one of the strongest and highly regulated eukaryotic promoters, alcohol oxidase I (*AOX*1). This facility can be used in performing many post-translational modifications [3,4]. Thus far, many proteins have been expressed in this system, with an expression level ranging from micrograms to grams per liter.

Researchers have exploited the expression potential of *P. pastoris* to increase processing productivity and cell growth. They have exploited the expression potential of the yeast by using stronger promoters [5,6], increasing the copy number of foreign genes [7,8], and optimizing the cultivation media and protocols [9,10]. The effect of copy number on recombinant protein expression levels is unpredictable. It has been demonstrated that increasing the copy number of the expression cassettes could have both positive and negative effects on different proteins [11]. The production of the heterologous *Rhizopus oryzae* lipase (ROL) has been shown to have a negative effect on *P. pastoris* growth [12]. Following introduction of 11 copies of genes, the expression of an insulin precursor was increased by 13-fold in *P. pastoris* [13]. Although increasing the gene copy number has been shown to effectively elevate recombinant protein levels in *P. pastoris,* over-expression of these compounds are known to stress yeast

metabolism and affect the physiology of a strain [14,15]. Overexpression of a foreign protein leads to a reduction in methanol consumption capacity, specific growth rate, and/or viability of the yeast cells [16].

Use of methanol as the sole carbon source produces a high yield of recombinant protein, but result in a slower growth rate of the strains [17]. Compared to glycerol, sorbitol is a non-repressing carbon source for the *AOX*1 gene promoter. Thus sorbitol accumulation during the induction phase does not affect the expression level of recombinant protein [18,19]. Sorbitol co-feeding with methanol also presents other advantages, including a lower heat production rate and lower oxygen consumption rate for growth on sorbitol than for growth on glycerol and methanol, or methanol alone, since the occurrence of the combustion of sorbitol is much less than for glycerol and methanol [20,21]. Thus,co-feeding of the strains with sorbitol and methanol can increase recombinant protein yield.

Prior studies mainly focused on the mixed-feed strategy or copy number of genes in *P. pastoris* strains, respectively, but none of these studies simultaneously focused on both variable. We conducted a literature search that confirmed the absence of any study experimentally assessing the sorbitol co-feeding with methanol [22,23]. Therefore, in this work we have compiled a comprehensive study on the effects of sorbitol co-feeding with methanol on the physiological performance of *P. pastoris* carrying different copy numbers of porcine insulin precursor (PIP) (1, 6, 12, and 18 copies). The metabolic response of *P. pastoris* to methanol feeding in the presence of sorbitol was investigated in detail, considering the production of cells, cell viability, recombinant protein, as well as a specific oxygen uptake rate (SOUR) of *P. pastoris* during the induction stage in a 5 L fermenter culture.

2. Materials and Methods

2.1. Strains

A set of *P. pastoris* GS115-derived strains (Invitrogen, Carlsbad, CA, USA) containing 1, 6, 12 and 18 copies of porcine insulin precursor (*PIP*) gene, respectively, was used in this study. Their detailed genetic construction was described in our previous work [24].

2.2. Media

The BMGY medium for inoculum culture contained (per liter): yeast extract (Oxoid, UK), 10 g; polypeptone (Daigo Eiyo, Tokyo, Japan), 20 g; KH_2PO_4/K_2HPO_4 buffer (pH 6.0), 100 mmol; yeast nitrogen base without amino acid (Difco, Sparks, MD, USA), 13.4 g; $(NH_4)_2SO_4$, 5 g; biotin, 400 μg; and glycerol, 10 mL. The basic salt medium (BSM) fermentation medium contained (per liter):glycerol, 40 g; 85% (w/w) H_3PO_4, 26.7 mL; $CaSO_4$, 0.93 g; K_2SO_4, 18.2 g; $MgSO_4 \cdot 7H_2O$, 14.9 g; KOH, 4.13 g; trace salts (PTM1, see below), 4.35 mL; and pH 5.0 adjusted with 28% (w/w) NH_4OH. This medium except for trace salts was sterilized at 120°C for 30 min. *Pichia* trace metal (PTM1) salt stock solution contained (per liter): $CuSO_4$, 6.0 g; KI, 0.08 g; $MnSO_4$, 3.0 g; Na_2MoO_4, 0.2 g; H_3BO_3, 0.02 g; $CoCl_2$, 0.5 g; $ZnCl_2$, 20.0 g; $FeSO_4 \cdot 7H_2O$, 65.0 g; biotin, 0.2 g; and 98% (w/w) H_2SO_4, 5 mL. This solution was filter sterilized and stored at 4°C.

2.3. Fed-batch cultivation set-up and operational conditions

The primary inoculum culture was prepared by transfer of 0.7 mL glycerol stock to 25 mL BMGY medium in a 250 mL flask and at 30°C and 250 rpm grown overnight. The secondary inoculum was obtained by distributing the primary inoculum culture to two 500 mL flasks each containing 50 mL BMGY and incubated at 30°C and 250 rpm for 7.5 h. All the secondary cultures were combined and inoculated to 2 L BSM fermentation medium in a 5 L fermenter (Model RIBE-5, ECUST, China).

The 5 L fermenter was controlled by a personal computer with a software program (Tophawk Fermentation Control System, National Center for Biochemical Engineering Research, Shanghai, China), and the online and off-line data were collected. The cultivation conditions were: 700 rpm, 30°C, pH controlled at 5.0 by adding 28% (v/v) NH_4OH, dissolved O_2 (DO) tension controlled above 20% with aeration at 5 L/min.

The fermentation experiments began with a batch-growth phase on glycerol for approximately 24 h when the initial glycerol was exhausted, followed by a fed-batch growth phase in which a solution of 50% (w/w) glycerol supplemented with PTM1 (12 mL/L) was continuously added. Fed-batch phases for all strains ended when OD_{600} reached 120. After a carbon-source starvation period of 30 min, 0.25% methanol was pulsed to induce the methanol metabolism. Finally, the induction phase was carried out, and 50 g sorbitol was added, and the methanol addition rate was manually modified attempting to maintain methanol concentration between 1 and 2 g/L. The cell density and PIP production were determined after 72 h of induction.

2.4. Absorbance measurements (OD_{600})

The OD_{600} was measured after dilution, and deionized water was used as the control for the colorimetric determination (OD_{600} = OD reading × dilution).

2.5. Cell viability

Cell viability was studied by fluorescein isothiocyanate (FITC) labeling technique. Fermentation samples were

taken at regular intervals, 1 mL of culture broth was centrifuged at 10,000 g for 1 min, and the cells were re-suspended by Tris–HCl (pH 9.0). The samples mixed with an appropriate volume of FITC dye were incubated at 30°C in darkness for 30 min. Then centrifuge the tubes to remove the unbound FITC and resuspend the cells with Tris-HCl. After that, the samples were mounted on a hemocytometer to count the percentage of live cells in the total population. Cells that could take up FITC dye and appeared bright green under a fluorescence microscope (Nikon Digital Camera DXM 1200C) were considered dead compared to live cells appeared dark [25].

2.6. PIP concentration assay

Porcine insulin precursor (PIP) was measured by HPLC (HP1100 series, Agilent, USA) with a MacroSep C8 column with 5 μ 300 Å (15 cm × 4.60 mm) (ES, West Berlin, NJ, USA). Elution was performed at a flow rate of 1 mL/min and with a linear gradient of two solvents, A and B, composed of 20% (v/v) acetonitrile, 0.1% (v/v) trifluoracetic acid (TFA) and 90% (v/v) acetonitrile, 0.1% (v/v) TFA in HPLC-grade water, respectively. The HPLC analysis was started with 100% solvent A, and solvent B was increased from 0 to 30% within 14 min. PIP was detected by UV absorbance at 214 nm and quantified using the standard curve [24].

3. Results

3.1. Effect of sorbitol co-feeding with methanol on cell growth by multi-copy *P. pastoris* strains

As shown in Fig. 1, the optical density profile of multi-copy *P. pastoris* cells in methanol group was compared with that of the methanol/sorbitol co-feeding group. After induction (72 h), the optical density of high-copy cells (6, 12, and 18 copies) in methanol/sorbitol co-feeding group was significantly ($P < 0.01$) increased when compared with methanol group, although low-gene-copy-number cells (1 copy) in methanol/sorbitol co-feeding and methanol groups had similar optical densities. The higher copy cells in methanol group reached the stationary phase at 40 h after induction, whereas high-copy cells in methanol/sorbitol co-feeding group continued to grow until 60 h. The specific growth rates (μ) of 1- and 6-copy cells in methanol/sorbitol co-feeding group (0.02 and 0.019, respectively) were comparable to that in methanol group (0.021 and 0.020, respectively). The μ in 12- and 18-copy cells exhibited similar growth rate within 40 h after methanol/sorbitol co-feeding and methanol induction. The μ in 12- and 18-copy cells in methanol/sorbitol co-feeding group (0.0074 and 0.0058, respectively) was significantly higher compared with methanol group (0.0029 and 0.0024, respectively, $P < 0.01$) from 40 h to final fermentation in the induction phase.

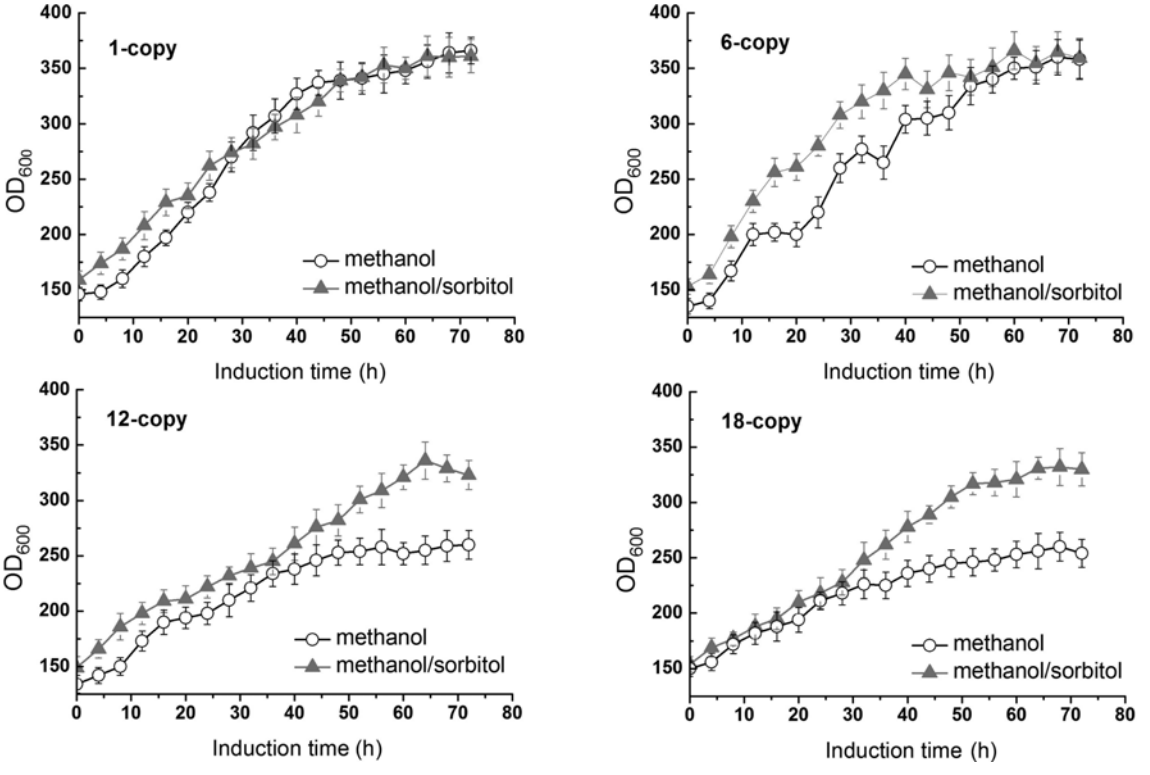

Fig. 1. The optical density profiles of multi-copy *P. pastoris* cells with methanol as sole carbon source and sorbitol co-feeding with methanol. Data are mean ± SD of three parallel measurements (*$P < 0.05$, **$P < 0.01$).

3.2. Effect of sorbitol co-feeding with methanol on recombinant protein production by multi-copy *P. pastoris* Strains

In methanol/sorbitol co-feeding group, the expression of PIP in 6-, 12- and 18-copy *P. pastoris* cells reached a peak of approximately 900 mg/L, and was 42.6, 34.7, and 80.9% higher compared with cells cultivated with methanol as the sole carbon source (Fig. 2), although there was no obvious difference in PIP expression between 1-copy cells cultivated in the two conditions ($P > 0.05$), suggesting that the addition of sorbitol in culture medium induced high PIP expression in *P. pastoris* cells, especially 18-copy cells.

3.3. Effect of sorbitol co-feeding with methanol on specific oxygen uptake rate by multi-copy *P. pastoris* strains

The specific oxygen uptake rate (SOUR) is a quantitative measure of oxygen consumed by microorganisms and is a relative reflection of the rate of biological activities including the carbon source reduction and other intracellular metabolism. The SOUR profiles of multi-copy *P. pastoris* cells in methanol and methanol/sorbitol co-feeding groups are shown in Fig. 3. The highest SOUR of 1-, 6-, 12- and 18-copy strain in methanol/sorbitol co-feeding group (2.23, 2.06, 2.3, and 1.9 mmol/g/h, respectively) was significantly ($P < 0.05$) lower compared with methanol group (3.27, 2.58, 2.7, and 2.2 mmol/g/h, respectively), suggesting that the addition of sorbitol in culture medium greatly reduced the oxygen consumption and heat production.

3.4. Effect of sorbitol co-feeding with methanol on cell viability by multi-copy *Pichia pastoris* strains

Live and dead cells during the fermentation course were differentiated using the fluorescence isothiocyanate (FITC) dye exclusion technique. Under mercury light, dead cells absorb the dye and appear as florescence green, whereas live cells appear as a normal green smear. As shown in Fig. 4, cell viability fell slowly as gene dosage increased by methanol/sorbitol co-feeding. The cell viability of 12- and 18-copy cells in methanol/sorbitol co-feeding group (78 and 75%, respectively) was significantly increased compared with cells in methanol group (87 and 85%, respectively, $P < 0.01$).

4. Discussion

The methylotrophic yeast *P. pastoris* has become a well-established host system for heterologous protein production. However, the cell growth of *P. pastoris* was lower, or the productivity of the process was limited under few

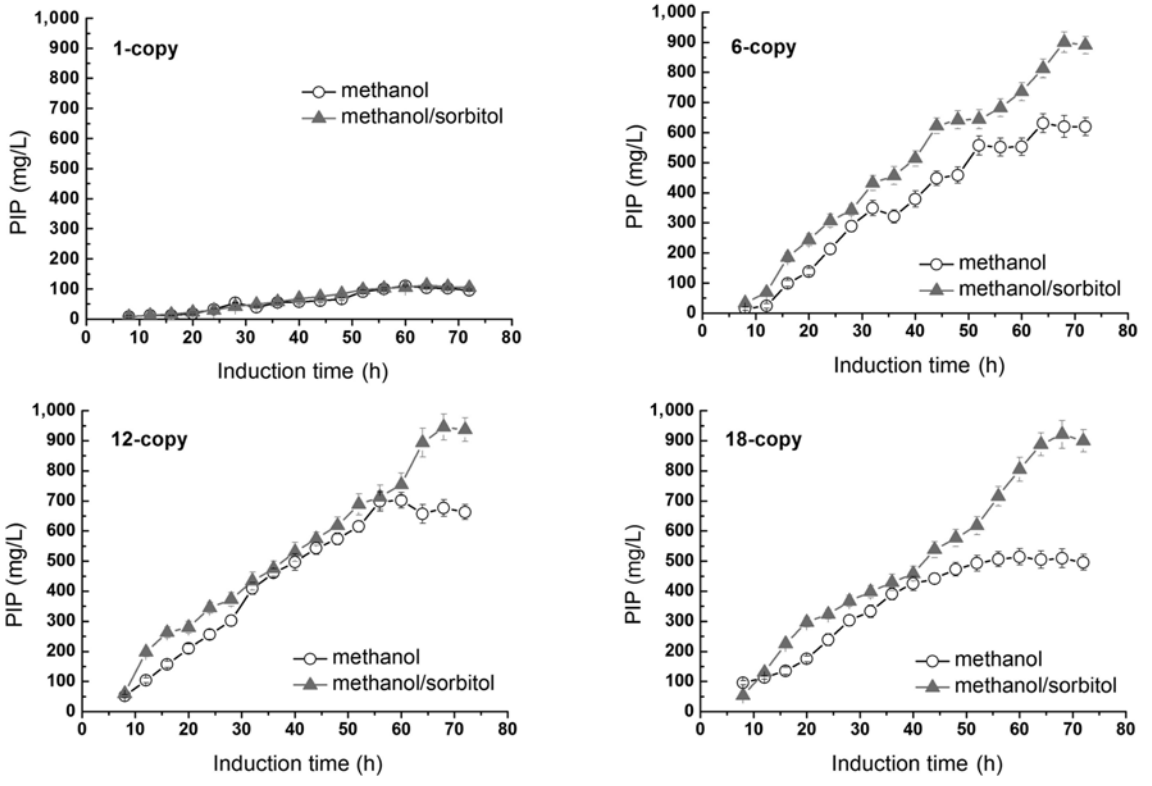

Fig. 2. The PIP profiles of multi-copy *P. pastoris* cells with methanol as sole carbon source and sorbitol co-feeding with methanol. Data are mean ± SD of three parallel measurements (*$P < 0.05$, **$P < 0.01$).

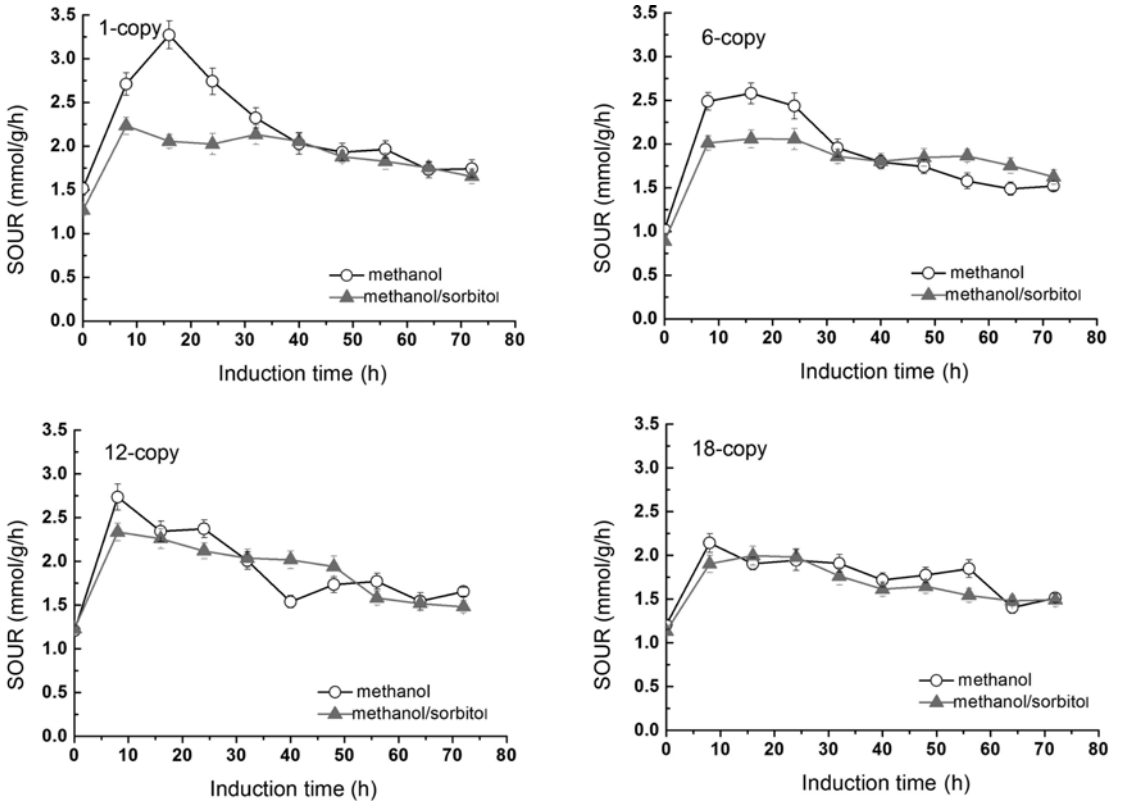

Fig. 3. SOUR profiles of multi-copy *P. pastoris* cells with methanol as sole carbon source and sorbitol co-feeding with methanol. Data are mean ± SD of three parallel measurements (*$P < 0.05$, **$P < 0.01$).

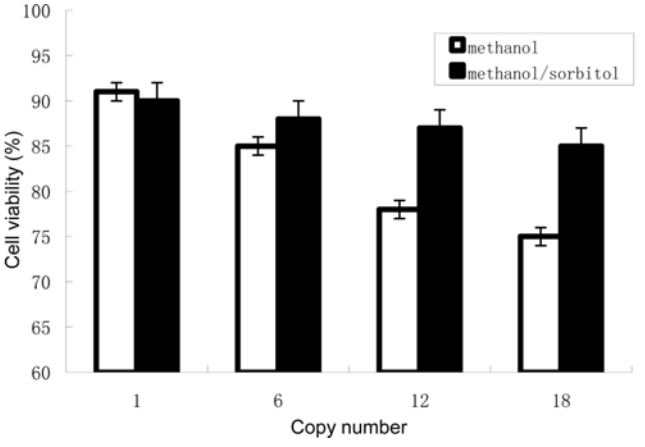

Fig. 4. The cell viability profiles of multi-copy *P. pastoris* cells with methanol as sole carbon source and sorbitol co-feeding with methanol. Data are mean ± SD of three parallel measurements (*$P < 0.05$, **$P < 0.01$).

circumstances such as type Mut^s and higher copy number strains [26]. To increase cell density and process productivity as well as to reduce the induction time, a typical approach is the use of a multi-carbon substrate in addition to methanol [27].

Brierley *et al*. (1990) where the first group attempting the fed-batch strategy using mixed substrates of glycerol and methanol for *P. pastoris*, to increase productivity and cell density and to reduce the induction time. However, the optimal level of protein expression is not achievable with mixtures of glycerol and methanol, due to a partial repression of the *AOX*1 promoter by glycerol, which may result in lower specific productivities of recombinant protein [28]. Thus, to use carbon sources that support growth but do not repress the *AOX1* promoter is essential to control and scale-up fermentation process. On the other hand, sorbitol is a widely accepted non-repressive carbon source for *P. pastoris*, which can produce a higher level of foreign protein compared with glycerol [29,30]. Arnau *et al.* [31] made a systematic study of the influence of methanol set-point and sorbitol feeding rate in fed-batch operation with a *P. pastoris* strain producing *Rhizopus oryzae lipase* (ROL), and ROL production and $Y_{P/X}$ were 1.25 fold higher and volumetric and specific productivity were 1.35 fold higher using mixed substrates comparing with the methanol as sole carbon source. Jungo *et al.* [32] optimized the sorbitol content in the feed in a continuous bioreactor experiment, and performed two fed-batch bioreactor experiments, at $\mu = 0.03$/h and $\mu = 0.05$/h, using a 43% methanol: 57% sorbitol (C-mol: C-mol) feeding ratio. The main conclusions

from this study concerned the simultaneous consumption of methanol and sorbitol; it has been being shown that at $\mu = 0.05$/h accumulation of sorbitol did not affect the specific productivity.

Other mixed carbon sources were used. Xie *et al.* [33] reported the angiostatin concentration was 141 mg/L as sorbitol was used to compare with 108 mg/L glycerol added, while only 52 mg/L were obtained on acetate. The highest angiostatin production of 191 mg/L was achieved as the lactic acid was used. And the accumulation of lactic acid did not interfere with angiostatin production, indicating that lactic acid to be a non-repressive carbon source for expression of foreign genes in *P. pastoris*.

The following were effects of sorbitol co-feeding with methanol on recombinant protein production by multi-copy *P. pastoris* strains. Firstly, overexpression of heterologous proteins caused physiologic burden and triggered the unfolded protein response (UPR) in *P. pastoris*. Sorbitol co-feeding could reduce metabolic burden which affects the secretion and the energetic state of cells, and alleviate methanol toxicity, because of enhanced cell viability. The growth and PIP expression in 1-copy cells in methanol/sorbitol co-feeding group were comparable to those in methanol group, suggesting that methanol/sorbitol co-feeding had little effect on 1-copy cells, which might be because of the low PIP expression and metabolic burden in these cells. In contrast, the high PIP expression in 6-, 12- and 18-copy cells induced high metabolic burden when cultured with methanol as the sole carbon source, and thus, the co-feeding of sorbitol markedly improved the growth and PIP expression of these cells. Secondly, Sorbitol co-feeding could enhance cell viability and reduce the level of proteases, thereby decrease the proteolytic degradation of recombinant proteins. It is most likely that more carbon and energy source w proved when sorbitol was used [34]. Although cell viability in both methanol/sorbitol co-feeding and methanol groups gradually decreased with increasing number of target gene copies, the decrease rate in methanol/sorbitol co-feeding group was much lower than that in methanol group. The cell viability of all strains in methanol/sorbitol co-feeding group was higher than 85% at the end of fermentation. Furthermore, methanol/sorbitol co-feeding greatly reduced the oxygen consumption and heat production, which will undoubtedly improve the large-scale industrial fermentation based on microbes with high rates of oxygen consumption, such as *P. pastoris.* Thirdly, based on the analysis ofe the transcriptional level of main genes of *Pichia*, sorbitol co-feeding could repress methanol assimilation; meanwhile, the central metabolic pathway was highly active, indicating that sorbitol appeared to be the main substrate during the co-feeding period [21].

5. Conclusion

Here we investigated the effects of sorbitol co-feeding with methanol on the physiology of *P. pastoris* with different copy number (1, 6, 12, and 18 copies). The results suggested that the μ in 12- and 18-copy cells in methanol/sorbitol co-feeding group (0.0074 and 0.0058, respectively) was significantly higher compared with methanol group (0.0029 and 0.0024, respectively) from 40 h to final fermentation. The expression of PIP in 6-, 12- and 18-copy *P. pastoris* cells were 42.6, 34.7, and 80.9% higher compared with cells cultivated with methanol as the sole carbon source. The results of SOUR showed the highest SOUR multi-copy strains was lower in methanol/sorbitol co-feeding group compared with methanol group, which suggested that the addition of sorbitol in culture medium greatly reduced the oxygen consumption and heat production. And the cell viability of 12- and 18-copy cells in methanol/sorbitol co-feeding group was significantly increased compared with cells in methanol group. Sorbitol co-feeding strategy which has been explored in the present study would be promising in a multi-copy *P. pastoris* system.

Acknowledgements

This research was supported by Special Fund for Agro-scientific Research in the Public Interest (No. 201303046), Independent Innovation of Agricultural Sciences Program of Jiangsu Province (No. CX (11)4037), and National High Technology Research and Development Program of China (No. 2014AA021705).

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