Systematic assessment of *Pichia pastoris* system for optimized β-galactosidase production

Hongbing Sun b,1, Olufemi Emmanuel Bankefa a,1, Ijeoma Oninyechi Ijeoma a,c, Liangtian Miao a, Taicheng Zhu a,*, Yin Li a,**

a CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, China
b Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin, 300308, China
c Department of Microbiology, University of Port Harcourt, Port Harcourt, Nigeria

**ARTICLE INFO**

Article history:
Received 15 February 2017
Received in revised form 23 March 2017
Accepted 12 April 2017

1. Introduction

β-Galactosidase (commonly known as β-lactase; EC 3.2.1.23) is a multifunctional enzyme that can catalyze the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides or transfer the galactosyl residue to saccharide acceptors to yield galactooligosaccharides (GOS). β-Galactosidase has a variety of applications in foods and medical industries such as hydrolysis of lactose in milk, manufacture of galactooligosaccharides (GOS) and treatment of lactose malabsorption [1]. Although β-galactosidase is an ubiquitous enzyme existing in plants, animals and microorganisms, only a few β-galactosidases from *Kluyveromyces lactis*, *Aspergillus niger* and *Aspergillus oryzae* are regarded as safe for food related industry applications.

To achieve commercial scale production of β-galactosidase, heterologous expression systems were applied including *Saccharomyces cerevisiae* and *Pichia pastoris* [1]. *P. pastoris* is a methylo-trophic yeast with great protein expression potential, and has been used as host for expression of many proteins both experimentally and industrially. *P. pastoris* has also been used for the extracellular expression of β-galactosidase from *Paecilomyces aeruginus* [2], *Lactobacillus crispatus* [3] and strains belonging to *Aspergillus* spp [4]. Despite its advantage in expression of proteins, *P. pastoris* system usually needs to be optimized to achieve maximum possible production level for a given protein. In achieving this, potential expression bottlenecks are analyzed and alleviated through perturbing and engineering of *P. pastoris* at different levels. And this process is often performed in a protein-specific manner, depending on the inherent nature and applications of target protein as well as its interaction with *P. pastoris* host. Take β-galactosidase for example, while some previous works have reported its successful expression in *P. pastoris* with reasonably high level, there are still several concerns needing to be addressed before further optimization, for instance: 1) Which kind of promoters are suitable to express β-galactosidase, the inducible or constitutive promoters? The strong AOX1 (alcohol oxidase I) promoter has been the most frequently used one. Nevertheless, the adoption of constitutive promoters has been appreciated in recent years because it does not need methanol to induce the expression, and therefore is safer (especially for food-grade β-galactosidase production) and eases the process control during the fermentation. 2) Unexpected N-glycosylation of foreign proteins are very commonly observed in *P. pastoris* system and its effects on the activity of expressed proteins remain unpredictable. In some cases, glycosylation is essential for maintaining the activities of expressed enzymes [5–7], while in other cases, glycosylation can negatively affect the enzyme activity [8–10]. Although β-galactosidase possesses multiple potential N-glycosylation sites, the effects of N-glycosylation on β-galactosidase activity were rarely investigated. 3) Of thousands of proteins that have been expressed using *P. pastoris*, the protein expression levels can range from tens of milligrams to tens of grams per liter. β-galactosidase can easily reach several grams per liter of production, which is obviously near the high end of this range. This raises the concern that the high enzyme yield may saturate the protein secretion ability of *P. pastoris* and thus limit the further improvement of its production level.

In order to address the above concerns and systematically assess *P. pastoris* system for optimized β-galactosidase, we expressed β-galactosidase from *K. lactis* and *A. oryzae* in *P. pastoris*, evaluated...
different constitutive promoters in addition to AOX1 promoter and examined co-expression of different chaperones in hope of enhancing the secretion of β-galactosidase in P. pastoris. We equally assess the effect of glycosylation on β-galactosidase activity using OCH1 disrupted strain. Combining these strategies, the production level of β-galactosidase from A. oryzae reached 1434.75 U/mL in 1 L fermentor, which therefore provided a basis for further optimization and industrial scale production of β-galactosidase in future works.

2. Materials and methods

2.1. Strains and plasmids

P. pastoris GS115, Escherichia coli DH5α and GS-OCH1 were stored in our lab, K. lactis and A. oryzae were all purchased from China General Microbiological Culture Collection Center (CGMCC, Beijing, China). Plasmids pPICZαA, pGAPZα and pGAPZβ were purchased from Invitrogen (Carlsbad, CA, USA). Information on the strains and plasmids used in this study were reported in Table 1, all primers synthesized by Invitrogen (Beijing, China) were also listed in Table 2 and the construction of recombinant plasmids were detailed in Fig. 1.

2.2. Construction of recombinant plasmids

The β-galactosidase gene of K. lactis was amplified by PCR from genomic DNA of K. lactis (Klglag) CGMCC 2.1494 using Kla-F/Kla-R primers. The β-galactosidase gene from A. oryzae (Aorgal) were cloned from the cDNA of its native strain. Total RNA was extracted using RNA pure prep Kit (Tiangen Biotech, Beijing, China) and subjected to reverse transcription to get the single-strand cDNA, followed by PCR amplification using Aor-F/Aor-R primer pairs. The PCR products were inserted into Xhol and NotI site of inducible vector pAO9sMH [11] to produce pAO9sMH-Kla and pAO9sMH-Aor respectively. The constitutive vector pGAPZα was double digested by XhoI and NotI for subsequent integration of DNA fragment containing Aorgal gene to produce pGAPZα-Aor. Promoter SDH AND TEF1 were cloned from genomic DNA of P. pastoris GS115 using SDH-F/SDH-R and Tef1-F/Tef1-R respectively and then double digested by BglII and BstII prior to insertion into the same sites of pGAPZα to replace Gap promoter to obtain pSDHZα and pTef1Zα.

Table 1

| Plasmids or strains | Short descriptions | Reference or source |
|---------------------|--------------------|---------------------|
| pPICZαA             | Vector for extracellular expression | Invitrogen |
| pAO815              | Vector for extracellular expression | Invitrogen |
| pAO9sMH             | Vector for extracellular expression; derived from pPICZαA and pAO815, containing 6 × His tag | This study |
| pAO9sMH-Aor         | pAO9sMH based vector, carryinggα-lactosidase gene from A. oryzae; His4, Amp* | This study |
| pAO9sMH-Kla         | pAO9sMH based vector, carryinggα-lactosidase gene from K. lactis; His4, Amp* | This study |
| pGAPZα              | Vector for extracellular expression; Zeo* | Invitrogen |
| pSDHZα              | Vector for extracellular expression; Zeo* | This study |
| pTef1Zα             | Vector for extracellular expression; HBS, Zeo* | This study |
| pAO9sMH-His        | pAO9sMH based vector, carrying His4 tag; HBS, Zeo* | This study |
| pSDHZα-His         | pSDHZα based vector, carrying His4 tag; HBS, Zeo* | This study |
| pGAPZα-Aor         | pGAPZα based vector, carryingα-galactosidase gene from A. oryzae; Zeo* | This study |
| pSDHZα-Aor         | pSDHZα based vector, carryingα-galactosidase gene from A. oryzae; His4, Zeo* | This study |
| pTef1Zα-Aor        | pTef1Zα based vector, carryingα-galactosidase gene from A. oryzae; His4, Zeo* | This study |
| pGAPZβ             | Vector for intracellular expression; Zeo* | Invitrogen |
| pGAPZ-FDI          | pGAPZ based vector, carrying DFI gene; Zeo* | This study |
| pGAPZ-KAR          | pGAPZ based vector, carrying KAR2 gene; Zeo* | This study |
| pGAPZ-SSO          | pGAPZ based vector, carrying SS1 gene; Zeo* | This study |

| Strains | Strain Name | Source |
|---------|-------------|--------|
| E. coli DH5α | Commercial transformation host for cloning | Takara |
| A. oryzae | CGMCC Number 3.05232 | CGMCC |
| K. lactis | CGMCC Number 2.1494 | CGMCC |
| P. pastoris GS115 | Commercial transformation host for Cloning; his4α, Mut+ | Invitrogen |
| GS-OCH1 | GS115 with its OCH1 gene disrupted | Our lab |
| GSC-Aor | GS115 integrated with linearized pGAPZα-Aor | This study |
| GS-Aor | GS115 integrated with linearized pSDHZα-His-Aor | This study |
| GST-Aor | GS115 integrated with linearized pTef1Zα-His-Aor | This study |
| GSA-Aor | GS115 integrated with inducible β-galactosidase from A. oryzae borne vector | This study |
| GSA-Kla | GS115 integrated with linearized pAO9sMH-KLA | This study |
| GSA-Aor-FDI | GSA-Aor integrated with linearized pGAPZβ-FDI | This study |
| GSA-Aor-KAR | GSA-Aor integrated with linearized pGAPZβ-KAR | This study |
| GSA-Aor-SSO | GSA-Aor integrated with linearized pGAPZβ-SSO | This study |
| GSA-OCH1 | OCH1 disrupted strain integrated with inducible vector β-galactosidase gene from A. oryzae borne vector | This study |

Note: the italic fonts indicate restriction enzyme sites.

Table 2

| All primers used in this study. |
|-------------------------------|
| Name | Sequence (5′→3′) |
|---|---|
| 5-GAP | gcggccgcgtccatcatataaacgtacac |
| 5-AOX | gactggtgccactgacagc |
| 3-AOX | gcgaagttgcatgtcactec |
| SDH-F | gtagctagctactatatattatataatgcgggg |
| SDH-R | gatccgctgtgatagaggtagtaag |
| Tef1-F | gtagctagctactagctgctttaattacttgc |
| Tef1-R | gtacgcctgtgatagaggtagtaag |
| His-F | gtagctagctactatatattatataatgcgggg |
| His-R | gatccgctgtgatagaggtagtaag |
| Aor-F | cttggcagagtagcatagtctgctgcttgaag |
| Aor-R | gtagcctgtgatagaggtagtaag |
| Kla-F | Cttgacgccagagtagcatagtctgctgcttgaag |
| Kla-R | cttggcagagtagcatagtctgctgcttgaag |
| PDI-F | ggtggctacgatcatcaactgagttgaat |
| PDI-R | ggtggctacgatcatcaactgagttgaat |
| KAR-F | gttggcagagtagcatagtctgctgcttgaag |
| KAR-R | gttggcagagtagcatagtctgctgcttgaag |
| SSO-F | ggtggctacgatcatcaactgagttgaat |
| SSO-R | gttggcagagtagcatagtctgctgcttgaag |

Note: the italic fonts indicate restriction enzyme sites.

Fig. 1. Plasmids or strains Short descriptions Reference or source
His fragment was double digested by BglII and BamHI and inserted into BamHI site of pSDHZα or pTef1Zα for integration into GS115 to produce pSDHZαH or pTef1ZαH. Aorgal gene was subsequently inserted into XhoI and NotI sites of pSDHZαH and pTef1ZαH to generate pSDHZαH-Aor and pTef1ZαH-Aor. The chaperone genes of PDI1, KAR2 and SSO1 were cloned from genomic DNA of P. pastoris GS115 using PDI-F/PDI-R, KAR-F/KAR-R and SSO-F/SSO-R respectively and double digested by EcoRI and NotI for subsequent insertion into intracellular expression vector pGAPZB thus generating the recombinant plasmids pGAPZ-PDI, pGAPZ-KAR and pGAPZ-SSO.

2.3. The generation of recombinant P. pastoris

All transformation with P. pastoris GS115 was performed by electroporation according to Invitrogen protocol. The recombinant vectors pAOαMH-Kla, pSDHZαH-Aor and pTef1ZαH-Aor were linearized by BspEI while pAOαMH-Kla by Stul. Transformants were screened on minimal plates (MD per liter: YNB 13.4 g, biotin 0.4 mg, glucose $\cdot$H2O 20 g, and agar 20 g) and designated as GSA-Aor, GS-S-Aor, GST- Aor and GSA-Kla respectively. The glycosylated P. pastoris GS-OCH1 [11] was transformed with BspEI linearized pAOαMH-Aor and transformants were designated as GSA-Aor-OCH1 upon screening on MD plates. For constitutive recombinant plasmid, GS115 was transformed with pGAPZ-PDI, pGAPZ-KAR and pGAPZ-SSO and transformants were selected on YPD (per liter: yeast extract 10 g, peptone 20 g, glucose $\cdot$H2O 20 g, agar 20 g and Zeocin 40 mg) and designated as GSG-Aor, GSA-Aor-PDI, GSA-Aor-KAR and GSA-Aor-SSO respectively.

2.4. Shake-flask fermentation

For assessing β-galactosidase production, the constructed P. pastoris strains were pre-incubated on YPD at 30 °C until a stationary phase is reached. 1 mL of constitutive expression strains were re-inoculated into 25 mL BMGY (per liter: mono-potassium phosphate 8.7 g, YNB 13.4 g, biotin 0.4 mg, peptone 20 g, yeast extract 10 g, glucose $\cdot$H2O 20 g; pH 6.0) while inducible expression strains were inoculated into BMMY (same as BMGY without glucose $\cdot$H2O) in 250 mL shake flask and cultured at 30 °C. The induction phase was initiated by adding 200 μL absolute methanol to each flask following subsequent methanol feeding at 12 h interval for 96 h.

2.5. High density fermentation

A 1-L stirred tank reactor (Infors, Switzerland) was used in the fermentation of GSA-Aor-KAR with 0.8 L of medium contained (per liter): 23.7 mL H3PO4, 0.6 g CaSO4 2H2O, 9.5 g K2SO4, 7.8 g MgSO4 7H2O, 2.6 g KOH, 40 g glycerol supplemented with 4.2 g histidine and 4.4 mL of trace salts. The following culture conditions were applied: 30 °C, pH 6.0 controlled by NH3 (25%) and dissolved oxygen (DO) controlled between 10 and 30% by stirrer (500–1000 rpm) with air flow rate at 2 L/min. A conventional P. pastoris fermentation protocol containing four phases was adopted: starting with a batch growth phase (phase I) lasting between 18 and 22 h followed by a glycerol (85% w/v) fed-batch phase (phase II) until OD600 reached 200. A transition phase (phase III) preceded by 30–60 min of starvation, began with induction of (0.2%) methanol until cells adapted to methanol metabolism followed by methanol feeding phase (phase IV) for 96 h. Cell growth was determined at OD600 and samples stored at −20 °C at 12 h interval.
3. Results

3.1. Expression of different sources of β-galactosidase genes in P. pastoris GS115

β-galactosidase genes were amplified from K. lactis and A. oryzae using genomic DNA and cDNA respectively. Both genes were placed under AOX1 promoter by inserting them into the secretory expression vector pAOxMH and then transformed into P. pastoris GS115. The generated recombinant strains GSA-Kla and GSA-Aor were then evaluated for their enzyme expression in shake flasks. After 96 h of induction, a final activity of 76.06 U/mL was achieved for Aorgal (Fig. 2A) and the time course accumulation of proteins was also observed during the induction process (Fig. 2B). In contrast, neither protein expression nor β-galactosidase activity could be detected for GSA-Kla revealing unsuccessful expression of Klaagal in P. pastoris. Therefore, only β-galactosidase derived from A. oryzae was used for the rest of the studies.

Despite observed remarkable expression, the Aorgal showed extensive diffusion and smearing on the SDS-PAGE and the average molecular mass of the enzyme was larger than the calculated value (108.1 kDa) thereby suggesting a high degree of glycosylation of the recombinant enzyme. Further Endo H treatment of the samples showed that the band heterogeneity was considerably reduced and the average molecular mass of the β-galactosidase was closer to the theoretical size confirming the glycosylation of β-galactosidase in P. pastoris (Fig. 2C).

3.2. Comparison of constitutive promoters for expression of β-galactosidase

The effect of constitutive promoter was also assessed by placing Aorgal under the control of some of the strongest constitutive promoters reported to date which include GAP (glyceraldehyde 3-phosphate dehydrogenase), SDH (sorbitol dehydrogenase) and TEF1 (elongation factor 1-alpha) promoters. The generated recombinant expression vectors pGAP2-Aor, pSDH7zH-Aor and pTef12zH-Aor were then transformed into P. pastoris GS115, resulting in the positive recombinant strains GSG-Aor, GSS-Aor and GST-Aor respectively. A 96 h cultural system in BMGY medium revealed that neither GSG-Aor nor GST-Aor exhibited detectable enzyme activity in the fermentation broth and only 2.74 U/mL of β-galactosidase was detected for GSS-Aor, which was significantly lower than that obtained using AOX1 promoter (Fig. 3A). SDS-PAGE results indicated that the protein expression levels of GSS-Aor were also very low compared with GSA-Aor (Fig. 3B).

3.3. The effects of OCH1 knock-out strain on the β-galactosidase expression

In vitro experiment using Endo H treatment confirmed that β-galactosidase was expressed as a glycoprotein in P. pastoris. To investigate the effects of glycosylation on β-galactosidase expression, we attempted to produce the enzyme in a P. pastoris strain with disrupted OCH1 gene (denoted as GS-OCH1) which encodes α,1,6-mannosyltransferases that initiates the first step of out-chain elongation of high mannose type N-glycan in P. pastoris since previous reports showed that disruption of OCH1 could significantly reduce the hyper-glycosylation and increase the homogeneity of expressed proteins in P. pastoris.

The expression plasmid pAOxMH-Aor was transformed into the GS-OCH1 and generated the recombinant strain GSA-Aor-OCH1 which was then compared with GSA-Aor for β-galactosidase expression. As expected, SDS-PAGE analysis showed that the heterogeneity of glycoproteins was remarkably reduced in GSA-Aor-OCH1 as only one single band could be observed. However, GSA-Aor-OCH1 only reached a production level of 23.58 U/mL, a 31% value of the control strain (76.06 U/mL) (Fig. 4B, Table 3). The protein level of GSA-Aor-OCH1 (94 mg/L) was also 44% lower than that of GSA-Aor (168 mg/L) which could probably be attributed to its relatively slow growth (Fig. 4A and C). The specific enzyme activity of GSA-Aor-OCH1 was estimated to be 250.85 U/mg, 44.6% lower than the value of 452.76 U/mg achieved for GSA-Aor, showing that deglycosylated protein was less active than the glycosylated counterparts.

3.4. Effect of co-expression of chaperone genes on the β-galactosidase expression level

Optimization of β-galactosidase expression by co-expressing chaperones PDI1, KAR2 and SS01 from P. pastoris GS115 genome to generate GSA-Aor-PDI, GSA-Aor-KAR and GSA-Aor-SSO revealed no significant difference in cell growth between the three strains and the control GSA-Aor (Fig. 5A) while the β-galactosidase expression of GSA-Aor-SSO remained almost the same as that of control (Fig. 5B). The enzyme expression levels of GSA-Aor-PDI and GSA-Aor-KAR reached 98.88 and 119.81 U/mL, 30% and 57.51% higher than that of GSA-Aor (76.06 U/mL) respectively, while the SDS-PAGE results also confirmed that co-expression of chaperone genes of KAR2 or PDI1 could improve the secretion of β-galactosidase (Fig. 4C).

3.5. High level expression of β-galactosidase by high density fermentation

The β-galactosidase producing potential of GSA-Aor-KAR was further investigated by high density fermentation in a 1-L fermentor. The cell growth of GSA-Aor-KAR increased steadily during the entire 94 h fermentations and a final OD600 value of 550 (estimated to be 138 gDCW/L) was obtained (Fig. 6). The β-galactosidase activity kept increasing after induction, which was also confirmed by building up of secreted proteins on SDS-PAGE (data not shown). The maximum volumetric β-galactosidase productivity reached 1434.75 U/mL at 94 h of induction, which was 17.9-fold of that in shake-flask cultivation (Fig. 6).

4. Discussion

In this study, P. pastoris was used as host to express β-galactosidase from K. lactis and A. oryzae both of which are regarded safe
for food related industrial applications. The *K. lactis* β-galactosidase is produced industrially by intracellular expression in its native host [1]. Due to the high cost associated with its extraction and following downstream process, secretory expression of *K. lactis* β-galactosidase was explored in *K. lactis* and *S. cerevisiae* [13,14], but only trace amount of enzyme activities could be detected in these works, suggesting that *Kla* gal may not be suitable for extracellular expression due to its inherent nature. On the contrary, the *Aor* gal is a native extracellular protein and have shown successful expression in *P. pastoris* in this work as well as previous reports [4].

Despite the significant expression level of β-galactosidase on inducible strong AOX1 promoter, different reports has raised concern on the use of AOX1 promoter ranging from drawbacks during process scale up, sophisticated operation and longer fermentation period to safety issues raised as a result of large amount of methanol used during the process [15,16]. Constitutive
promoters were therefore applied as alternatives to overcome these problems [17]. While the GAP promoter was the most commonly used constitutive promoter in P. pastoris system [18], other promoters like TEF1 [19] and SDH [20] were also reported to have promoting strength comparable to GAP. These three promoters were thereby evaluated and compared. Unfortunately, GAP and TEF1 promoters exhibited no expression while only trace expression was noticed on SDH promoter. The reason for this is still not clear yet.

β-galactosidase contains multiple potential glycosylation sites and are expressed in gram-per-liter level which might cause a severe folding stress upon P. pastoris and subsequently impaired cell growth and even decreased stability of Aor gal gene [21]. The dissociation of cell growth with induction phase as applied in inducible expression under AOX1 promoter would help to minimize this adverse effects and thus remarkably improve the protein expression level.

N-glycosylation is ubiquitous in eukaryotic systems, where the asparagine residues within the N-X-S/T (X is any amino acid except proline) sequence is glycosylated by glycotransferases. Although not as hyper-mannosylated as S. cerevisiae (adding up to 50 mannoses), the N-glycans of P. pastoris are also of the high mannose type (8–14 mannoses) [22,23]. The effect of glycosylation on expressed proteins in P. pastoris are unpredictable and vary on a case-by-case basis. For β-galactosidase, a previous work showed that removal of glycans would decrease the specific activity of the β-galactosidase from P. aeruginus by treating the enzyme with Endo H in vitro [2]. In this work, we investigated the effect of glycosylation on β-galactosidase with an in vivo strategy through the use of an OCH1 disrupted strain. OCH1 encoding the α-1, 6-mannosyltransferase is responsible for triggering the afterwards outer-chain elongation of N-glycans [24,25] and disruption of OCH1 would thus eliminate hyper-mannosylation of glycoproteins. As shown in this work, knockout of OCH1 successfully generate a more
unified β-galactosidase protein band. Unfortunately, the prevention of hyper-glycosylation of β-galactosidase seems to decrease the specific activity of β-galactosidase, which is in accordance with previous report [24,25], thereby suggesting certain degree of glycosylation was necessary for maintaining the activity of this enzyme.

Previous work showed β-galactosidase can easily reach gram/liter production. This large protein synthesis flux was assumed to cause the overloading on the secretion capacity of *P. pastoris*. We therefore confirm this hypothesis and overcome the limitation by co-expressing chaperone genes. Three chaperone genes relating to protein folding, disulfide bond formation and protein transporting which includes KAR2 (or BiP, a ER-resident chaperone of the HSP70 class that mediate protein folding in the ER), PDI1 (a chaperone that is responsible for the correct formation of disulfide bonds during oxidative folding) and SSO1 (involved in fusion of secretory vesicles at the plasma membrane) were chosen because they are closely related to the folding and formation of disulfide bond. Previous literature has reported that the three chaperones could significantly improve the secretion of heterologous proteins in some cases [24,25]. Overexpression of PDI1 and KAR2 resulted in 30% and 57.51% increase in the β-galactosidase production due to the increase in protein expression level (data not shown). The positive effects of PDI1 and KAR2 confirmed the secretion bottleneck hypothesis and provided solutions for further improvement of β-galactosidase which has not been reported before.

Finally, the evaluation of the optimized strain on a 1-L fermentor with final production level of 1434.75 U/mL (approximately 2.5 g/L of protein) in 96 h of induction. *P. pastoris* has been shown to be the ideal host for β-galactosidase expression, and very high enzyme titers were achieved in some cases, e.g. 3.5 g/L of protein for *Aspergillus niger* β-galactosidase [4], an enzyme level of 22 g/L or 9500 U/ml for *P. aeruginosa* β-galactosidase [2]. This work systematically examined some of the major concerns regarding to high expression of β-galactosidase in *P. pastoris* and successfully identified enzyme secretion as a potential limiting factor, which will help to guide further improvement of β-galactosidase in *P. pastoris* system.

### Acknowledgments

This work was supported by Key International Cooperation Project from Chinese Academy of Sciences (155112KYSB20160010), Beijing Municipal Natural Science Foundation (5132024) and National Natural Science Foundation of China (31000026).

### References

1. Oliveira C, Guimaraes PM, Domingues L. Recombinant microbial systems for improved β-galactosidase production and biotechnological applications. Bio-technol Adv 2011; 29:600–9.
2. Katrolia P, Yan Q, Jia H, Li Y, Jiang Z, Song C. Molecular cloning and high-level expression of a β-galactosidase gene from *Pseudomonas aeruginosa* in *Pichia pastoris*. J Mol Catal B-Enzym 2011; 69:112–9.
3. Nie C, Liu B, Zhang Y, Zhao G, Fan X, Ning X, et al. Production and secretion of *Lactobacillus crispatus* β-galactosidase in *Pichia pastoris*. Protein Expr Purif 2013; 92:88–93.
4. Dragosits M, Pflugl S, Kurz S, Razzazi-Fazeli E, Wilson IB, Rendic D. Recombinant *Aspergillus* β-galactosidases as a robust glycomic and biotechnological tool. Appl Microbiol Biotechnol 2014; 98:3553–67.
5. Perez DLSA, Cayetano-Cruz M, Gutierrez-Anton M, Santiago-Hernandez A, Plascencia-Espinosa M, Farres A, et al. Improvement of catalytical properties of two invertases highly tolerant to sucrose after expression in *Pichia pastoris*. Effect of glycosylation on enzyme properties. Enzyme Microb Technol 2016; 83:48–56.
6. Capone S, Corajevic L, Bonifert G, Murth P, Maresch D, Altman F, et al. Engineering for the production of glyco-engineered horseradish peroxidase C1A.

---

### Table 3

| Strains       | Enzyme production (U/mL) | Protein production (mg/mL) | Crude specific activity (U/mg) |
|---------------|--------------------------|----------------------------|--------------------------------|
| GSA-Aor       | 76.06                    | 0.168                      | 452.76                        |
| GSA-Aor-OCH1  | 23.58                    | 0.094                      | 230.85                        |

---

### Fig. 5.

Effects of co-expression of chaperone genes on β-galactosidase expression. The chaperone genes PDI1, KAR2 and SSO1 were all driven by GAP promoter, and the GSA-Aor was used as a control. (A) Growth curves of co-expression strains. (B) β-galactosidase expression levels of co-expression strains. β-Galactosidase activities were assayed at the end of fermentation for 96 h. Three parallel flasks are tested for each strain.

### Fig. 6.

High-density culture of GSA-Aor-KAR in 1 L scale fermentor. Data shown are mean values from experiments performed in triplicate. Closed square, cell growth; closed diamond, β-galactosidase activity.
in Pichia pastoris. Int J Mol Sci 2015;16:23127–42.

[7] Maestre-Reyna M, Liu WC, Jeng WT, Lee CC, Hsu CA, Wen TN, et al. Structural and functional roles of glycosylation in fungal laccase from Lentinus sp. PLoS One 2015;10:e120601.

[8] Ranaei SS, Mollasalehi HHeydarzadeh N. Substrate affinity and catalytic efficiency are improved by decreasing glycosylation sites in Trichoderma reesei cellobiohydrolase I expressed in Pichia pastoris. Biotechnol Lett 2016;38:483–8.

[9] Yang YL, Chang SH, Gong X, Wu J, Liu B. Expression, purification and characterization of low-glycosylation influenza neuraminidase in \( \alpha1,6 \)-mannosyltransferase defective Pichia pastoris. Mol Biol Rep 2012;39:837–64.

[10] Yurimoto H, Yamane M, Kikuchi Y, Matsu H, Kato N, Sakai Y. The pro-peptide of Streptomyces mobaraensis transglutaminase functions in cis and in trans to mediate efficient secretion of active enzyme from methylophilic yeasts. Biosci Biotechnol Biochem 2004;68:2058–69.

[11] Zhu T, You L, Gong F, Xie M, Xue Y, Li Y, et al. Combinatorial strategy of sorbitol feeding and low-temperature induction leads to high-level production of alkaline \( \beta \)-mannanase in Pichia pastoris. Enzyme Microb Technol 2011;49:407–12.

[12] Becerra M, Prado SD, Siso MI, Cerdan ME. New secretory strategies for Kluyveromyces lactis \( \beta \)-galactosidase. Protein Eng 2001;14:379–86.

[13] Becerra M, Díaz Prado S, Cerdan E, González Siso MI. Heterologous Kluyveromyces lactis \( \beta \)-galactosidase secretion by Saccharomyces cerevisiae super-secretion mutants. Biotechnol Lett 2001;23:33–40.

[14] Zhu T, Sun H, Li P, Xue Y, Li Y, Ma Y. Constitutive expression of alkaline \( \beta \)-mannanase in recombinant Pichia pastoris. Process Biochem 2014;49:2025–9.

[15] Cos G, Ramon R, Montesinos J, Valero F. Operational strategies, monitoring and control of heterologous protein production in the methylophilic yeast Pichia pastoris under different promoters: a review. Microb Cell Fact 2006;5:17.

[16] Vogl TGlieeder A. Regulation of Pichia pastoris promoters and its consequences for protein production. N Biotechnol 2013;30:385–404.

[17] Zhang AL, Luo JX, Zhang TY, Pan YW, Tan YH, Fu CY, et al. Recent advances on the GAP promoter derived expression system of Pichia pastoris. Mol Biol Rep 2009;36:1611–9.

[18] Ahn J, Hong J, Lee H, Park M, Lee E, Kim C, et al. Translation elongation factor 1-\( \alpha \) gene from Pichia pastoris: molecular cloning, sequence, and use of its promoter. Appl Microbiol Biotechnol 2007;74:601–8.

[19] Periyasamy S, Govindappa N, Sreenivas S, Sastry K. Isolation, characterization and evaluation of the Pichia pastoris sorbitol dehydrogenase promoter for expression of heterologous proteins. Protein Expr Purif 2013;92:128–33.

[20] Curvers S, Linnemann J, Klauser T, Wandrey C, Takors R. Recombinant protein production with Pichia pastoris in continuous fermentation – kinetic analysis of growth and product formation. Eng Life Sci 2002;2:229–35.

[21] Krayner FW, Gmeiner C, Neutsch L, Windwarder M, Pletzenauer R, Herwig C, et al. Knockout of an endogenous mannosyltransferase increases the homogeneity of glycoproteins produced in Pichia pastoris. Sci Rep 2013;3:3279.

[22] Grinna LSTschopp JF. Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, Pichia pastoris. Yeast 1989;5:107–15.

[23] De Pourcq K, De Schutter KCallewaert N. Engineering of glycosylation in yeast and other fungi: current state and perspectives. Appl Microbiol Biotechnol 2010;87:1617–31.

[24] Bobrowicz P, Davidson RC, Li H, Potgieter TJ, Nett JH, Hamilton SR, et al. Engineering of an artificial glycosylation pathway blocked in core oligosaccharide assembly in the yeast Pichia pastoris: production of complex humanized glycoproteins with terminal galactose. Glycobiology 2004;14:757–66.