The *Pichia pastoris* transmembrane protein GT1 is a glycerol transporter and relieves the repression of glycerol on AOX1 expression

Chunjun Zhan[1, 2, 3], Songwei Wang[1, 2, 3], Yang Sun[1, 2, 3], Xiaofeng Dai[1, 2, 3], Xiuxia Liu[1, 2, 3], Linda Harvey[4], Brian McNeil[4], Yankun Yang[1, 2, 3*], Zhonghu Bai[1, 2, 3*]

[1] National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University，Wuxi 214122，China

[2] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

[3] The Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

[4] Fermentation Centre, Institute of Pharmacy and Biomedical Science, Strathclyde University, Glasgow G1 1XQ, Scotland, UK

[*]Corresponding authors: Yankun Yang: yangyankun@jiangnan.edu.cn; Zhonghu Bai: baizhonghu@jiangnan.edu.cn. Both contributed equally to this work.

Abstract:

Promoter of alcohol oxidase I (P_{AOX1}) is the most efficient promoter involved in the regulation of recombinant protein expression in *Pichia pastoris* (*P. pastoris*). P_{AOX1} is tightly repressed by the presence of glycerol in the culture medium; thus, glycerol must be exhausted before methanol can be taken up by *P. pastoris* and the expression of the heterologous protein can be induced. In the present study, a candidate glycerol transporter (GT1, GeneID: 8197545) was identified, and its role was confirmed by further studies (e.g., bioinformatics analysis, heterologous complementation in *Schizosaccharomyces pombe* (*S. pombe*)). When GT1 is co-expressed with enhanced green fluorescent protein (EGFP) it localizes to the membrane and *S. pombe* carrying *gt1* but not the wild type strain can grow on medium containing glycerol as the sole carbon source. The present study is the first report that AOX1 in the X-33 Δ*gt1* mutant can achieve constitutive expression in medium containing glycerol; thus, knocking down *gt1* can eliminate the glycerol repression of P_{AOX1} in *P. pastoris*. These results suggest that the glycerol transporter may participate in the process of P_{AOX1} inhibition in glycerol medium.

Key words: Glycerol transporter, *P. pastoris*, P_{AOX1}, repression, constitutive expression, release

1. Introduction:

In the 1960s, Ogata *et al.* found that methanol could be used as a carbon source in some yeasts. Due to the superiorities of methylotrophic yeast over other hosts, *P. pastoris* became one of the most attractive hosts for heterologous protein expression(Koutz, et al. 1989: 167-77). These advantages include: (i)

The strong promoter (P_{AOX1}) could promote the expression of foreign protein; (ii) the whole genome of *P. pastoris*(De Schutter, et al. 2009: 561-6) has been sequenced, which is great benefit for molecular genetic manipulation; (iii) compared with the mammalian cells cultured medium, the growth medium and culture conditions for *P. pastoris* is relatively simpler; (iv) the high-density fermentation could be achieved, and which is benefit for obtaining high levels of foreign protein expression; (v) the target protein (product) can be secreted into the culture broth, and compared with other eukaryotic or prokaryotic expression systems its background proteins are lower, which is benefit for the target protein purification; (vi) the ability to perform higher eukaryotic protein modifications, such as glycosylation, disulphide bond formation, and proteolytic processing.

Over the past decades, *P. pastoris* has been developed into a successful and popular expression host due to these remarkable advantages. Over 1000 proteins, including epidermal augmentum factor (EGF), human serum albumin(HAS), hepatitis B surface antigen (HBsAg), have been expressed in *P. pastoris* to date (Staley, et al. 2012: 118-27). However, the critical reason for the great success of *P. pastoris* as a favourable microbial eukaryotic host is its unique strong promoter P_{AOX1}.

P. pastoris naturally possesses two different genes encoding alcohol oxidases (*aox1* and *aox2*). *aox1* is more frequently transcribed than *aox2 (Ozimek, et al. 2005: 975-*

83, Vogl and Glieder 2013: 385-404). *aox1* is expressed at a high level when *P. pastoris* is grown on medium with methanol as the sole carbon source, and the *aox1* promoter is utilized more often to regulate the production of foreign proteins(Cregg, et al. 1989: 1316-23). However, a large amount of enzyme is needed to sustain cell growth on methanol during the process of foreign gene expression because the oligomeric flavoenzyme alcohol oxidase has a low affinity for its substrates (methanol and oxygen)(Ozimek, et al. 2005: 975-83, R. and J. 1980: 2279-89). Thus, AOX usually accounts for approximately 5% of the total mRNA and 30% of the total intracellular soluble proteins under culture conditions when methanol is present(Xuan, et al. 2009: 1271-82). A major consequence of this phenomenon is that there is a significant diversion of both amino acids and energy towards AOX synthesis, which greatly reduces the resources available for the production of the target protein.

There are a few drawbacks involved in the use of methanol as the culture carbon source during the development of a *P. pastoris* process. First, methanol is derived mainly from petrochemical sources and is unsuitable for the production of certain food products and additives. Second, hydrogen peroxide (H_2O_2) is a by-product produced during methanol metabolism that causes oxidative stress to host cells and results in undesirable proteolytic degradation (Hilt and Wolf 1992: 2437-42, Tian, et al. 2013: 507-13, Xiao, et al. 2006: 837-44). Third, methanol is potentially hazardous due to its toxicity and flammability in large scale fermentation(Gao, et al. 2011: 1412-9). Fourth, the amount of oxygen required for biomass production by methanol metabolism is 3-4 times higher than the amount required for glucose or glycerol(Mateles 1971: 581-2), which potentially results in a severe oxygen limitation on the expression of foreign genes (Cereghino and Cregg 2000: 45-66). Fifth, considerable heat is produced due to the consumption of large amounts of oxygen,

causing extra difficulty in controlling the culture within the bioreactor (Lin, et al. 2000: 31-5). Consequently, the high demands of oxygen transfer and heat removal increase the manufacturing costs of the *P. pastoris* process.

Because the problems associated with the current *aox* promoter-based *P. pastoris* processes have been realized, some efforts have been made to resolve them. However, these efforts have mainly focused on P_{AOX1} reform(Vogl and Glieder 2013: 385-404) to depress the glycerol inhibition, and research into the glycerol transporter has rarely been reported. This lack has most likely occurred because the underlying mechanisms are unclear. *aox1* was reported to achieve constitutive expression in glucose medium when one or two glucose transporters (i.e., *hxt1 and gss1*) were knocked out (Stasyk, et al. 2004: 8116-25, Zhang, et al. 2010: 6108-18). However, this type of observation has not been reported to date in *P. pastoris* cultures with glycerol used as the carbon source, although glycerol is a more favourable carbon source than glucose in *P. pastoris* cultures, e.g. the reducing power caused by glycerol is more higher than that caused by glucose, which is benefit for the growth of cells (Hu, et al. 2008: 44-9). The explanation for this lack may be the lack of knowledge concerning the glycerol transporters of *P. pastoris*.

In the present study, possible glycerol transporters were analysed using bioinformatics tools such as HMMTOP, Tmrpres2D and homologous modelling(Thompson, et al. 1997: 4876-82). After identifying a candidate glycerol transporter, a series of experiments was performed, including subcellular localization and evolutionary tree analysis. Finally, a twelve-transmembrane protein was identified as a potential glycerol transporter of *P. pastoris*. The present study showed that knocking-out *gt1* eliminated the glycerol inhibition on AOX1 expression in the presence of glycerol;

however, the underlying mechanism was not uncovered. Our ongoing studies will address this issue.

2. Materials and Methods:

2.1 Media, strains, and microbial techniques.

The strains listed in Table 1 were used in this study. *P. pastoris* was cultured with shaking at 30 °C in 1% yeast extract, 2% tryptone and 2% glucose (YPD), BMGY medium (1 L: 10 g/L yeast extract, 20 g/L tryptone, 3 g/L K_2HPO_4, 11.8 g/L KH_2PO_4, 13.4 g/L YNB, 4×10^{-4} g/L biotin, and 10 ml of glycerol), BMMY medium (1 L: 10 g/L yeast extract, 20 g/L tryptone, 3 g/L K_2HPO_4, 11.8 g/L KH_2PO_4, 13.4 g/L YNB, 4×10^{-4} g/L biotin, and 5 ml of methanol 5), and BMGMY (BMGY supplemented with methanol) with 1% glycerol, 0.5% methanol, or 1% glycerol plus 0.5% methanol added throughout the study as a carbon source. *S. pombe* were cultured in YPD medium (1% yeast extract, 2% tryptone, and 2% glycerol). The *Escherichia coli* strain DH5α, Trans110 (TransGen) was used as a host for the construction of plasmids and was grown at 37 °C in LB medium (0.5% yeast extract, 1% tryptone, and 1% NaCl). For solid medium, 2% powdered agar was added. EMM medium (Edinburgh minimal medium) contained potassium hydrogen phthalate (3 g/l), Na_2HPO_4 (2.2 g/l), NH_4Cl (5 g/l), glucose (20 g/l), salt stock (1 ml/l), and the vitamin (0.1 ml/l) and mineral stock (0.01 ml/l). The salt stock (50x) contained $CaCl_2.2H_2O$ (0.735 g/l), $MgCl_26H_2O$ (52.5 g/l), KCl (50 g/l), and Na_2SO_4 (2 g/l).The vitamin stock (1000x) contained pantothenic acid (1 g/l), nicotinic acid (10 g/l), inositol (10 g/l), and biotin (10 mg/l). The mineral stock (10,000x) contained boric acid (5 g/l), $MgSO_4$ (4 g/l), $ZnSO_4.7H_2O$ (4 g/l), $FeCl_2.6H_2O$ (2 g/l), molybdic acid (0.4 g/l), KI (1 g/l), $CuSO_4$-$5H_2O$ (0.4 g/l), and citric acid (10 g/l). EMMG indicated EMM supplemented with glycerol (2%) instead of glucose (2%). *P. pastoris* and *S. pombe* were transformed by

electroporation and the lithium acetate method, respectively. Transformation and other standard recombinant DNA operations used in this study for *E.coli* were performed. Ampicillin or kanamycin was added to the LB medium at a final concentration of 50 μg/ml, and G418 and Zeocin were added to the YPD medium at final concentrations of 0.3 and 0.1 mg/ml, respectively.

2.2 Plasmids and strain construction.

Plasmids were generated by standard techniques, and the strains, primers and plasmids used in this study were listed in Tables 1 and 2 and Table 3 respectively.

2.2.1 △*GT1* strain.

A *P. pastoris* strain with the *P.pgt1* gene deleted was constructed by homologous recombination with the G418 resistance gene *kan* as the marker. First, the upstream region of the *P.pgt1* gene was amplified by PCR using the *pfu* DNA polymerase (Thermo Scientific) and the genomic DNA as the template. The primers for this PCR (*gt1*s-1 and *gt1*s-2) carried restriction sites for *Eco*RI and *Bam*HI, respectively. The 0.5-kb PCR-amplified fragment was inserted into the *Eco*RI/*Bam*HI-digested pMD™19-T vector plasmid (TAKARA) to yield pGT1UP. The downstream region of the *P.pgt1* gene was amplified with the primers *gt1*x-1 and *gt1*x-2 carrying the restriction sites for *Xba*I and *Hind*III, respectively, to yield a 0.4-kb PCR fragment. After insertion into the pMD™19-T vector (TAKARA), pGT1Down was obtained. Afterward, the G418 resistance gene sequence together with its own promoter and terminator (1556 bp) were amplified by PCR using the plasmid pFA6a-KanMX6 as the template and the primers *kan*-1 and *kan*-2 (containing the restriction sites for *Bam*HI and *Xba*I, respectively). The fragment was cloned into the *Eco*RI/*Bam*HI-

digested pGT1UP vector to construct the pGT1UP-Kan vector. This plasmid was digested with *Xba*I/*Hind*III to generate a 2.1-kb fragment that was inserted into the *Xba*I/*Hind*III-digested pGT1Down vector to obtain the *P.p*GT1 deletion vector pMD™19-T-GT1-del. The deletion cassette was released from pMD™19-T-GT1-del as a 2.5-kb *Eco*RI/*Hind*III-digested fragment and transformed by electroporation into the wild-type *P. pastoris* strain X-33. G418-resistant transformants were isolated on YPD medium supplemented with 0.3 mg/ml G418. The correct integration of the deletion cassette into the genome and the replacement of the *P.pgt* open reading frame (ORF) in the transformants were confirmed by PCR and DNA sequencing (data not shown).

2.2.2 Construction of the EGFP expression strains.

The EGFP coding sequence was amplified from the pEGFP-N1 plasmid using the *pfu* DNA polymerase and the primers EGFP-1 and EGFP-2 (carrying restriction sites for *Eco*RI/*Xho*I, respectively). The PCR product was inserted into *Eco*RI/*Xho*I-digested pPICZ B (Invitrogen) to generate the pPICZ B-EGFP plasmid (a vector capable of expressing a green fluorescent protein under the control of P_{AOX1}). pPICZ B-EGFP was linearized with *sac*I and transformed into X-33 and X-33Δ*gt1* by electroporation. Transformants expressing EGFP were selected on YPD medium by zeocin in the dark. The correct integration into the genome was confirmed by PCR and DNA sequencing, and the x-33 and x-33Δ*gt1* strains harbouring a single copy of the *egfp* expression cassette were selected according to the data reported in other studies(Xuan, et al. 2009: 1271-82).

2.2.3 Construction of membrane localization plasmid.

First, the *kan* region was amplified by PCR using pFA6a-KanMX6 as the template and the *pfu* polymerase (Thermo Scientific). The primers used for this PCR carried restriction sites for *Kpn*I/*Kpn*I. The fragment and pRS424 were digested with *Kpn*I to generate the recombinant plasmid pRS424-*kan*. Second, the genomic DNA was extracted from the wild-type X-33 strain. *P.pGT1* was amplified using the genomic DNA as the template and *P.pGT1*-rong-1 and *P.pGT1*-rong-2 as the primers. The 1.6 kb PCR-amplified fragment was inserted into the digested pRS424-kan plasmid to yield the recombinant plasmid pRS424-*kan-gt1*. Third, the last fragment was amplified using EGFP-rong-1 and EGFP-rong-2 as the primers and the pEGFP-N1 plasmid as the template and then digested with *Sma*I and *Spe*I. The recombinant plasmid pRS424-*kan-gt1* was treated similarly to the amplified PCR fragment to insert the *egfp* region into the digested pRS424-*kan-gt1* plasmid to yield a new recombinant plasmid pRS424-*kan-gt1-egfp*. The new recombinant plasmid was transformed into *S. pombe* by electroporation and the lithium acetate method.

2.2.4 Heterologous complementation.

To identify the function of gt1, the recombinant plasmid pRS424-*kan-gt1-egfp* was used to express *P.pGT1* in *S. pombe* (which cannot grow on glycerol medium)(Klein, et al. 2013: 5013-26) with G418 as the selection marker. The recombinant plasmid was transformed into *S. pombe* by electroporation.

2.2.5 Fluorescence microscopy.

Fluorescence microscopy was used to observe the subcellular localization of *P.pGT1*. The *P. pastoris* strains X-33, X-33-*egfp*, and X-33∆*gt1-egfp* were precultured in YPD medium to log phase and then washed twice with PBS (pH 7.4). Subsequently, the yeast were shifted into YNB medium containing 1% glycerol, 0.5% methanol, or 1%

glycerol plus 0.5% methanol at an initial OD_{600} of 0.05. Ten microliters of the culture was visualized by fluorescence microscopy (Olympus X53, excitation wavelength 488nm, emission wavelength 505 nm).

2.2.6 Cell extract preparation, SDS-PAGE, enzyme assays, Real Time-PCR and western blotting.

To prepare the total cellular proteins, the cells were cultured in YPD for 16 hours and then transformed into 2×YPD. Six hours later, 20 to 30 OD_{600} units of cells were harvested by centrifugation at 4,000×g for 5 min and washed twice with ice-cold 50 mM potassium phosphate buffer (pH 7.0). After adding 200 μl of ice-cold PEBF (0.7882% Tris-HCl, 0.0585% EDTA, and 2 μl of 100 mM PMSF), the cells were mixed with glass beads. The EP tube was placed in an oscillator for 30 s (10 cycles with the tube in ice). The cells were centrifuged at 12,000 rpm for 1 min, and the supernatant was prepared for SDS-PAGE.

The AOX activity was assayed using peroxidase and OPD(Verduyn, et al. 1984: 15-25). One unit of alcohol oxidase represented 1 μmol of product/min/mg of protein at 30 °C. Samples with the methanol omitted were run as blanks.

Total RNAs were prepared by a standard procedure according to the RNA kit (TAKARA).RT-PCR was carried out with the Quantscrip TR kit (TAKARA) as manufacturer's instructions, According to the protocol the RT-PCR mixtures contains 5×gDNA Eraser Buffer (2μl), gDNA Eraser 1.0μl, Total RNA 4μl (500ng) and RNase Free dH_2O 3μl were incubated at 42 °C for 2 min and then the cDNA was subjected to real-time PCR according to protocol. qRT-PCR was carried out by performing three independent experiments, the mixture contains SYBR Premix Ex Taq II (2×) 5ul, PCR forward primer (10μM) 0.5μl, PCR reverse primer (10μM)

0.5 ul, cDNA 1 ul, dH_2O 3 ul. Transcription level were normalized to an endogenous reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the datas were processed by the $\Delta\Delta$CT method(Livak and Schmittgen 2001: 402-8).

The total proteins obtained by the above methods were transformed onto a polyvinylidene difluoride (PVDF) membrane according to the electrophoretic transfer method. Anti-AOX (Acris) was used as the primary antibody and peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG, Thermo Fisher) was used as the secondary antibody.

2.3 The specific glycerol consumption rate.

To examine the relationship between gt1 and AOX1 depression and the function of *gt1* in *P. pastoris*, the dry cell weight of the wt and mutants in different media were collected from 24 h to 48 h. The specific consumption rate of glycerol was obtained according to a previously reported analytical method(Kastner, et al. 2003: 96-100).

3. Results:

3.1 Sequence analysis of the *P. pastoris* glycerol transporter.

Using the *Saccharomyces* genome database (SGD), we identified a *P. pastoris* gene with high sequence similarity with *stl1* of *Saccharomyces cerevisiae*. According to HMMTOP (http://www.enzim.hu/hmmtop) and Tmrpres 2D (Fig. 1), both *S.c*STL1 and *P.pGT1* contained twelve transmembrane regions; additionally, several transmembrane regions of the two proteins shared identical primary sequence structures. Further study indicated that both proteins fell into the major facilitator superfamily (MFS), which is one of the largest protein families and contains more than 10,000 members (www.tcdb.org). Some MFS members are known transmembrane proteins (e.g., HXT and Gss1 (glucose transporter))

(http://www.rcsb.org/pdb/home/home.do). However, only nine of the transmembrane proteins' 3D structures were reported (Booth, et al. 1979: 687-96, Geertsma, et al. 2008: 256-66, Kraulis 1991: 946-50, Leive 1965: 290-6, Merritt and Bacon 1997: 505-24, Murshudov, et al. 2011: 355-67, Robertson, et al. 1980: 5692-702, Salton 1971: 161-97, Short, et al. 1975: 4291-6). According to NCBI, both *S.c*STL1 and *P.p*GT1 share the sugar transporter conserved domain. Additionally, the phylogenetic analysis (Fig. 2) revealed that both *S.c*STL1 and *P.p*GT1 were transmembrane proteins. The branch containing *S.c*STL1 and *P.p*GT1 belonged to the same subgroup, although the relatedness to the other reported transporters was very weak (Fig. 2). These results indicated that *S.c*STL1 and *P.p*GT1 might share the same function. STL1 was reported to be a glycerol transporter in *S. cerevisiae* in a previous study(Ferreira, et al. 2005: 2068-76). Thus, we could infer that *P.pGT1* might also function as a glycerol transporter. The 3D-structure of *P.p*GT1 was obtained by homology modelling from the glycerol-3-phosphate transporter using SWISS-MODEL (Fig. 3), and both of them shared twelve transmembrane-domain (α spin), thus *P.p*GT1 maybe a transmembrane protein too.

3.2 Subcellular localization of GT1.

To determine whether GT1 was a transmembrane protein, EGFP was co-expressed with GT1. The subcellular localization of GT1 showed that *P.p*GT1 was located in the membranes of *P. pastoris* (Fig. 4) and *S. pombe* (Fig. 4), whereas in the strain with EGFP but without P.pGT1, the fluorescence was distributed within cytoplasma.

(Fig. 4). This result suggested that *P.p*GT1 was a membrane protein.

3.3 The *P.p*GT1 protein is a glycerol transporter.

The high similarities between *P.p*GT1 with *S.c*STL1 suggest its potential role as a glycerol transporter. To prove this hypothesis, we transferred *P.pgt1* into *S. pombe* to investigate whether it could utilize glycerol as its sole carbon source. A recombinant

plasmid containing *P.pgt1* was constructed and transformed into *S. pombe* (Fig. 5). The *S. pombe* (WT) and *S. pombe* (pRS424-P*gtl1*) strains both grew in EMM+A (adenylate)+H (L-histidine)+L (L-leucine)+NaCl medium after 72 h, and *S. pombe* (pRS424-P*gtl1*) grew much better than the *S. pombe* (WT) in the EMMG+A+H+L+B1+NaCl medium. The expression of *P.p*GT1 allowed the *S. pombe* (pRS424-P*gtl1*) strain to grow on glycerol medium, whereas *S. pombe* (WT) could not grow on glycerol medium (Fig. 5). Thus, *P.p*GT1 may play a role in glycerol uptake or metabolism.

3.4 *P.p*GT1 plays an important role in the catabolic repression of AOX1 expression.

P_{AOX1} was tightly repressed by glycerol and glucose in *P. pastoris*. Normally, *aox1* is repressed when glycerol or glucose is added to methanol medium. To quantify the expression level and determine the enzyme activity of AOX1, cells were collected from different media (i.e., 0.5% glycerol, 0.5% glycerol plus 0.5% methanol and 0.5% methanol) at different time points. Total proteins were extracted and quantified by Bradford's method as described in the "Materials and Methods". We found that *aox1* achieved constitutive expression when X-33Δ*gt1* was cultured in different media, including 0.5% glycerol, 0.5% glycerol plus 0.5% methanol; in contrast, X-33 did not achieve constitutive expression (Fig. 6, Fig. 7). Additionally, the growth of X-33Δ*gt1* and x-33 were comparable in the same medium (Fig. 6). X-33 and X-33Δ*gt1* grew better in glycerol or glycerol plus methanol medium than in methanol alone. The AOX1 activity in X-33Δ*gt1* in glycerol medium was comparable with that in X-33 cultured in methanol (Fig. 6A, Fig. 7). However, the activity of AOX1 was sharply reduced when glycerol was added to the medium (Fig. 6). Besides, according to RT-PCR, it could be found that the transcriptional level of *aox*1was higher in X-33Δ*gt1*

mutant than in x-33 cultured in glycerol or glycerol plus methanol medium. Based on these results, we concluded that *gt1* most likely played a role in carbon repression, inhibit the expression of aox1 at transcriptional level (Fig. 8).

3.5 The specific consumption rate of glycerol in different strains.

To confirm the role of *gt*1 in *P. pastoris*, we examined the specific consumption rate of glycerol in different strains. According to the results shown in Fig. 9, the specific consumption rate of glycerol in the mutant grown in glycerol medium was lower than the rate in the wild type strain regardless of whether the medium contained glycerol or glycerol plus methanol. In contrast, the specific consumption rate of glycerol was faster than glycerol plus methanol in both the wild type and the mutant strains, the result suggested that there may be some other factor participant in glycerol uptake and metabolism.

4. Discussion:

In *S. cerevisiae*, *stl1* was reported to participate in the osmoregulation of components such as high osmolarity glycerol (HOG) (Causton, et al. 2001: 323-37, Clotet, et al. 2006: 2338-46). The HOG pathway is the best understood and most intensively studied MAPK system. Sugar Transporter-Like 1 (*stl1*) has been reported as an important glycerol transporter in *S. cerevisiae* (Ferreira, et al. 2005: 2068-76, Kayingo, et al. 2009: 1547-57). HOG interacts with different transport factors in the nucleus, such as SKO1, Hot and probably also Smp1, to control different sets of genes(Alepuz, et al. 2003: 2433-42). Moreover, *stl1* is induced by salt and osmotic shock in a Hog1p- and Hot1p-dependent manner Thus, when the cells were transferred from BMGY into BMMY, glycerol was absorbed by the same system to adapt to the osmotic pressure. P_{AOX1} is tightly repressed, and some relationships may

exist among HOG, *stl1* and P_{AOX1}. Mxr1p was recently reported to play an important role in the induction of P_{AOX1}, and Mxr1p seems to be post-translation regulated by its subcellular localization(Lin-Cereghino, et al. 2006: 883-97, Parua, et al. 2012: 282-98) because it has been reported to be constitutively transcribed at low levels, to localize to the cytosol in cells grown on glucose and to migrate to the nucleus upon the shift to methanol or oleic acid. However, Mxr1p is localized in the nucleus in cells grown on glycerol. The repression of *aox1* expression suggests the involvement of other factors involved in glycerol repression. P_{AOX1} and P_{gt1}, share the Mxr1p binding site(Lin-Cereghino, et al. 2006: 883-97) CYCCNY, indicating the different roles played by Mxr1p when the cells are grown in glycerol medium. These roles could facilitate P_{AOX1} and alternatively repress the expression of *gt1* or genes involved in glycerol metabolism that in turn enhance AOX1 expression. Our results together with previous reports suggest the presence of inter-connections among *gt1*, Mxr1p and methanol, although the underlying mechanisms require further investigation.

Many disadvantages arise when methanol is used as the sole carbon source. For instance, the targeted gene could not be expressed in glycerol or methanol mixed medium because P_{AOX1} was inhibited when the cells were grown in glycerol medium. In our study, the mutant could achieve constitutive expression in glycerol or glycerol plus methanol medium, and the growth of the mutant in the glycerol medium was similarity to the wild type. This result was inconsistent with the function of *gt1*; however, the reason maybe that glycerol could be taken up by the cells through passive diffusion. Carbon catabolite repression (CCR) was recently reported to exist in many microorganisms(Magasanik 1961: 249-56). CCR is an important global regulatory system in various bacteria that allows them to preferentially metabolize the

most energy-efficient carbon source in a mixture, such as the lactose operon. Similar to the lactose operon, P_{AOX1} is tightly induced by methanol and repressed by glycerol and glucose. Thus, it could be conjectured that a similar regulatory system exists in *P. pastoris*. In other words, glycerol is a better energy-efficient carbon source than methanol, and glycerol would be preferably metabolized while AOX1 would be repressed in methanol and glycerol medium. In this study, AOX1 was repressed in the WT strain in glycerol or glycerol plus methanol medium in contrast to the mutant, which achieved constitutive expression. Thus, $P_{AOX1}$is subject to CCR by glycerol. In this process, signalling pathways must exist to regulate AOX1 expression because methanol is located outside of the nucleus. In the mutant, methanol participates in the signalling pathway involving *gt1*, and *aox1* achieves constitutive expression in the medium containing glycerol or glycerol plus methanol and is finally repressed due to the presence of other transcriptional regulators.

5. Conclusions:

In this study, a glycerol transporter in *P. pastoris* (*P.p*GT*1*) was identified using a bioinformatics approach. Its transmembrane structure was predicted by homologous modelling and compared against *S.c*STL1 via HMMTOP and Tmrpres2D. *P.p*GT*1* was suggested as a glycerol transporter due to the presence of a shared 12-transmembrane region (Fig. 1). *P.p*GT1 is a transmembrane protein and shares transmembrane regions with several transmembrane proteins, such as the D-xylose-proton symporter, glucose transporter and glycerol-3-phosphate transporter. *P.p*GT1 is closely related to *S.c*STL1 based on the phylogenetic tree constructed by MEGA. Thus, we propose that *P.p*GT1 functions as a glycerol transporter. Importantly, *P.p*GT1 is located at the cell membrane (Fig. 4) and allows *S. pombe* (which does not

exhibit growth in glycerol medium) to grow in a glycerol medium which identified GT1 as a glycerol transporter.

P_{AOX1} is induced by methanol and repressed by glucose and glycerol. Several studies have been performed to reform P_{AOX1}. However, we demonstrated that AOX1 could also be expressed in X-33△*gt1* but not X-33 in glycerol medium. In glycerol and methanol mixed medium, *aox1* could also be expressed in X-33△*gt1* but not in X-33, although X-33 outgrew X-33△*gt1*. Additionally, *aox1* achieved constitutive expression in glycerol or glycerol and methanol mixed medium, whereas WT did not. When glycerol was added to the medium, the activity of AOX1 was sharply reduced. One explanation is that the reduction of AOX1 activity was caused by the reduction in the AOX1 expression level; alternatively, the addition of glycerol might have influenced the signalling pathway involving *aox1*. Otherwise, the quantity of glycerol in X-33△*gt1* might be lower than the quantity in the wild type strain, thereby weakening the inhibition of glycerol on P_{AOX1}. Thus, we concluded that *P.p*GT1 was a glycerol transporter and was involved in glycerol repression.

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Protein:*P.p*GT1

Length:530 aa
N-terminus:IN
Number of transmembrane helices:12
Transmembrane helices: 31-49 80-98 107-125 130-152 165-183 200-218 287-305 324-346 355-373 382-406 419-441 450-468

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Fig.8. Quantitative analyses of transcript level of aox1 in different strains response to different carbon substrates. Wide type x-33 and x-33 Δ gt1 mutant were cultured overnight in BMGY, BMGMY or BMMY medium at 30 °C 230 rpm/min. Cells were harvested at 8h, 16h, 20h and 24h, then total RNA from different strain and medium were prepared by the standard protocol according to the kit (TAKARA).

Fig.9. The specific consumption rate of glycerol in different strains (mutant: Δ gt1; wide type)and different medium (glycerol; glycerol + methanol).

TABLE 1. Strains used in this study

Strain	Genotype[a]	Source or reference
E.coli		
Trans5α	F-φ80d lacZΔM15 Δ(lacZYA-argF) U169 end A1 recA1 hsdR17 (rk-,mk+) supE44λ- thi-1 gyrA96 relA1 phoA	TransGen Biotech
Trans110	*rpsL* (StrR) *thr leu thi*-1 *lacY galK galT ara ton*A *tsx dam dcm sup*E44 Δ(lac-*proAB*) /*F'* [*traD*36 *proAB lacI*q *lacZ*ΔM15]	TransGen Biotech
Pichia pastoris		Invitrogen
X-33	Wide type Mut^+,His^+	Invitrogen
△*gt1*	X-33 *gt1*△::*Kan* Selection of Zeocin™-resistant expression vectors and G418	This study
WT-EGFP	Selection of Zeocin™-resistant expression vectors	This study
△*gt1*-EGFP	X-33*gt1*△::*Kan* Selection of Zeocin™-resistant expression vectors and G418	This study
Schizosaccharomyces pombe		
WT (yAS56)	h^-ura-D18, leu-32 trz2-A623V	Nanjing Normal University
S. pombe (pRS424-P*gtl1*)	h^-ura-D18, leu-32 trz2-A623V, transformed into pRS424-gt1 plasmid	This study

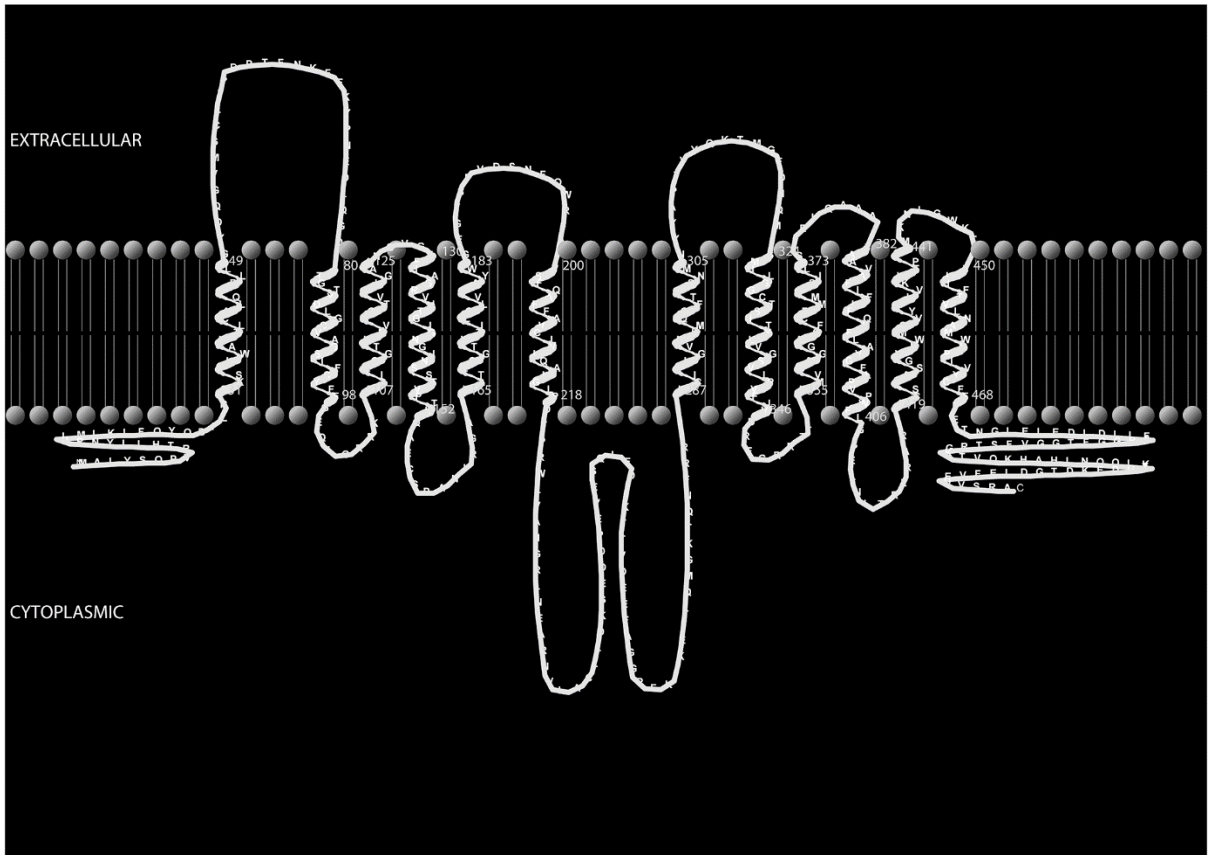
TABLE 2. Primers used in this study

Primer	Sequence
gt1s-1	5'-GCGAATTC CCGACAGAAGCAACCTCAGATCAACC -3'
gt1s-2	5'-AGGGATCC ATGGAGCGTTAATCCGGAGTGTAAGAG-3'
gt1x-1	5'-GCTCTAGA AACATCTCGTTTCGTGTGCTTGTGG -3'
gt1x-2	5'-GCTAAGCTT CTTGCATTCGCTCAGGGCTCATTAC -3'
kan-1	5'-GCGGATCCCCGGTTAATTAA-3'
kan-2	5'-GCTCTAGAGAGCTCGTTTAAAC-3'
GAPDH-1	5'- CACAATGGCTATCACTGTCG-3'
GAPDH-2	5'- GACACACTACAGCCCGCATT-3'

RT-GAPDH-1	5′- GGTATTAACGGTTTCGGACGTATTG-3′
RT-GAPDH-2	5′- GATGTTGACAGGGTCTCTCTCTTGG-3′
EGFP-1	5′- GCGAATTCATGTTGACAGGGTCTCTCTCTTGG-3′
EGFP-2	5′- GCCTCGAGTTACTTGTACAGCTCGTCCATGCC-3′
RT-EGFP-1	5′- CGACCACATGAAGCAGCACG-3′
RT-EGFP-2	5′- TGCTTGTCGGCCATGATATGACG-3′
EGFP-rong-1	5′- GGC ACTAGT ATGGTGAGCAAGGGCGAGGA-3′
EGFP-rong-2	5′-GCCCCGGGCTTGTACAGCTCGTCCATGCC-3′
P.pGT1-1	5′- GGCCTCGAG ATGGCAATCTATTCTCAACCCGTAAG-3′
P.pGT1-2	5′- GGCCCCGGGTTAGGCCCTTGACACGTCCTCTA-3′
P.pGT1-rong-1	5′-GGC CCCGGG ATGGCAATCTATTCTCAACCCGTAAG-3′
P.pGT1-rong-2	5′-GGC CTCGAG TTAGGCCCTTGACACGTCCTCTAC-3′
Kan-pRS424-1	5′- CGGGTACC GGGTTAATTAAGGCGCGCCAGATC-3′
Kan-pRS424-2	5′- CGCTCGAG CGTTTAAACTGGATGGCGGCGTTAG-3′
kan-3	5′-CGGGTACC GGGTTAATTAAGGCGCGCCAGATC-3′
kan-4	5′- CGCTCGAG CGTTTAAACTGGATGGCGGCGTTAG-3′

TABLE 3 Plasmids in the study

Plasmid	Genotypea	Source or reference
pRS424	Kan^R; *E. coli* and *P.pastoris* shuttle plasmid	This study
pPICZ B	$Zeocin^R$; P_{AOX1}-based expression vector	Invitrogen
pRS424-Pgt1	pRS424 connected *gt1* gene	This study
pRS424-egfp-Pgt1	pRS424 connected *gt1* and egfp gene	This study
pPICZ B-egfp-gt1	pPICZ B connected *gt1* gene	This study
pPICZ B-egfp	pPICZ B connected *egfp* gene	This study
pMD19-T Simple	$Ampicillin^R$; *E. coli* subcloning vector	This study
pEGFP-N1	Kan^R , $G418^R$ carrying the *egfp* gene	This study



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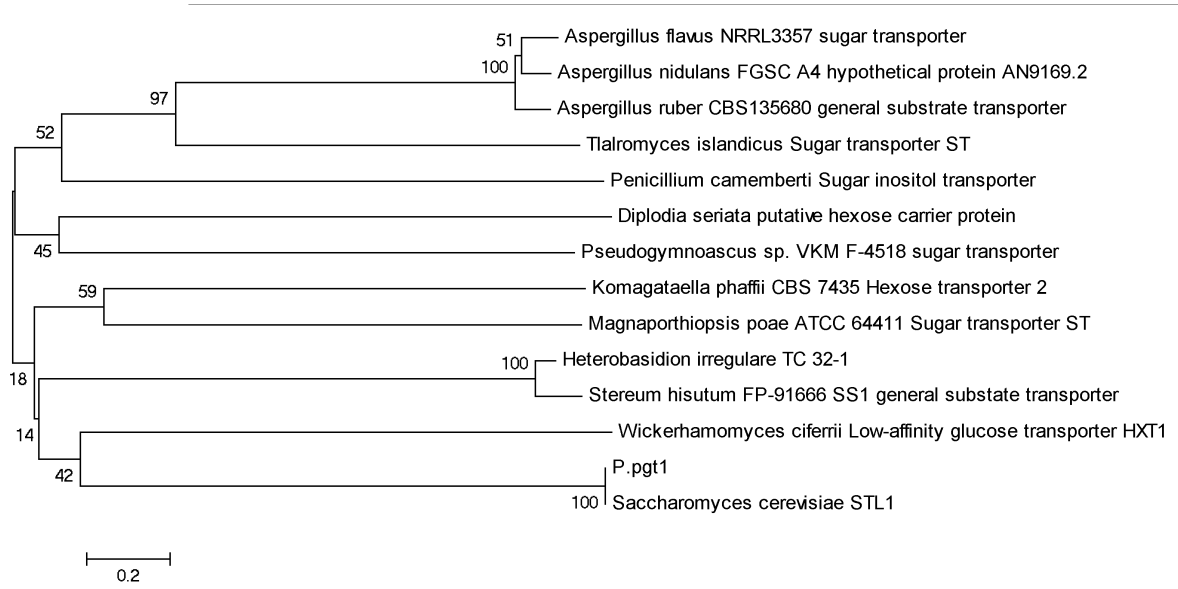

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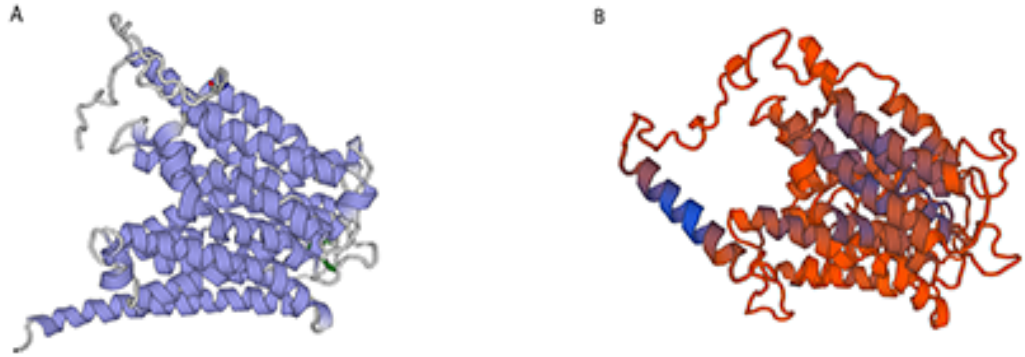

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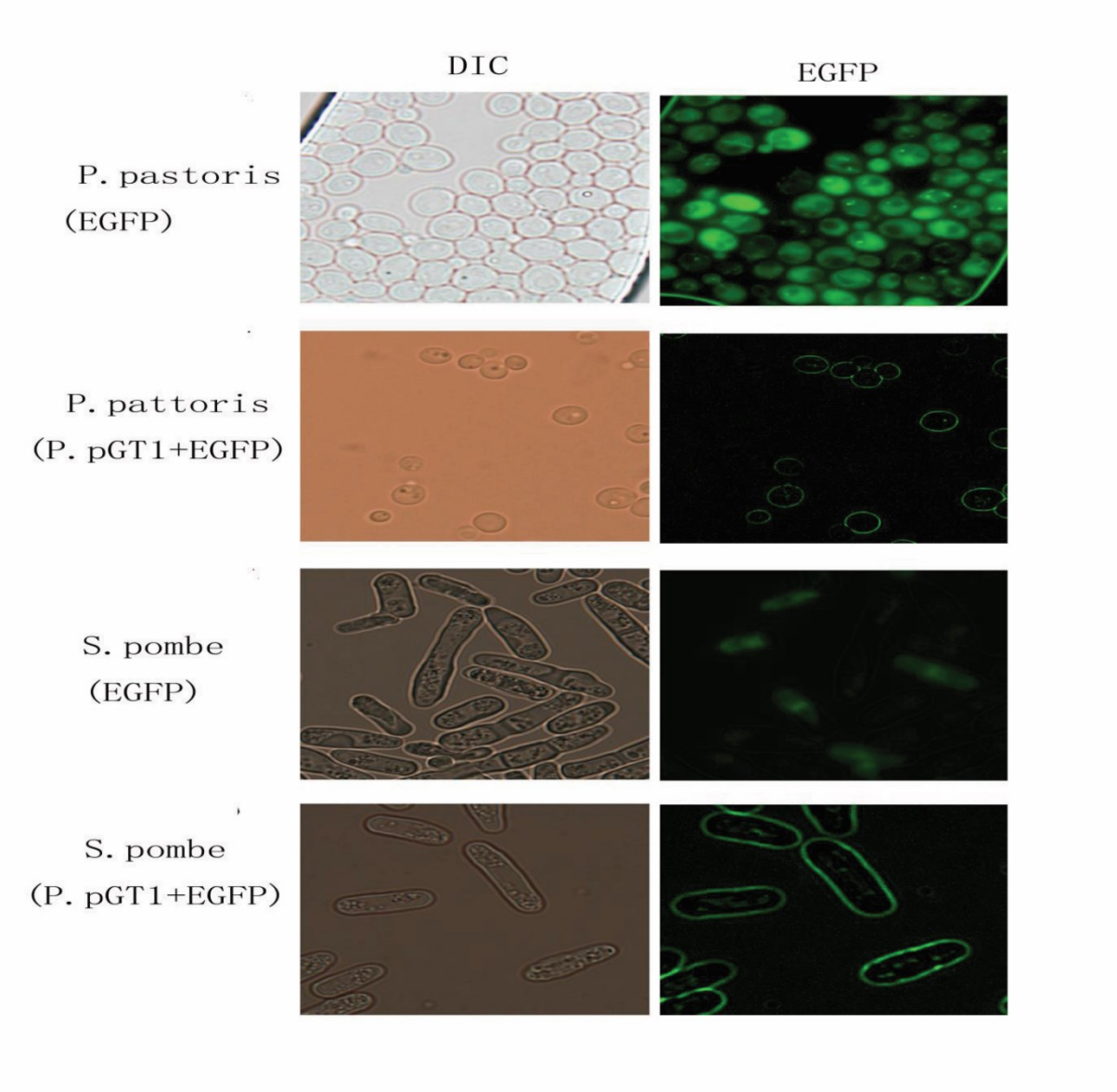

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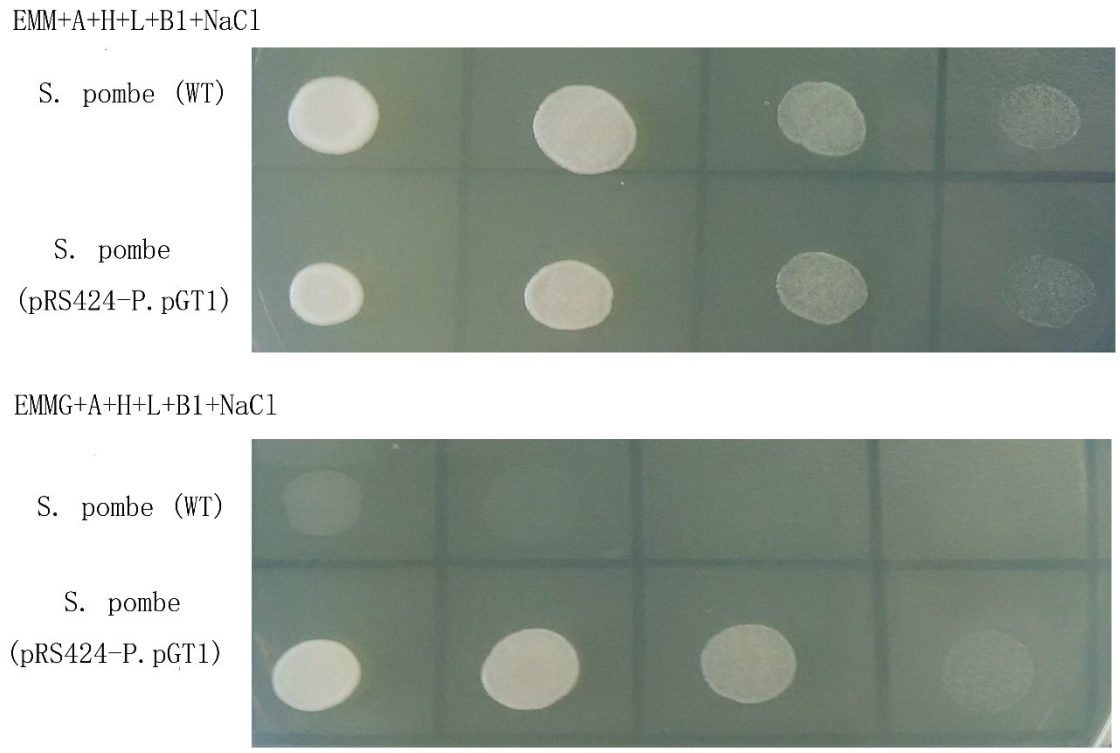

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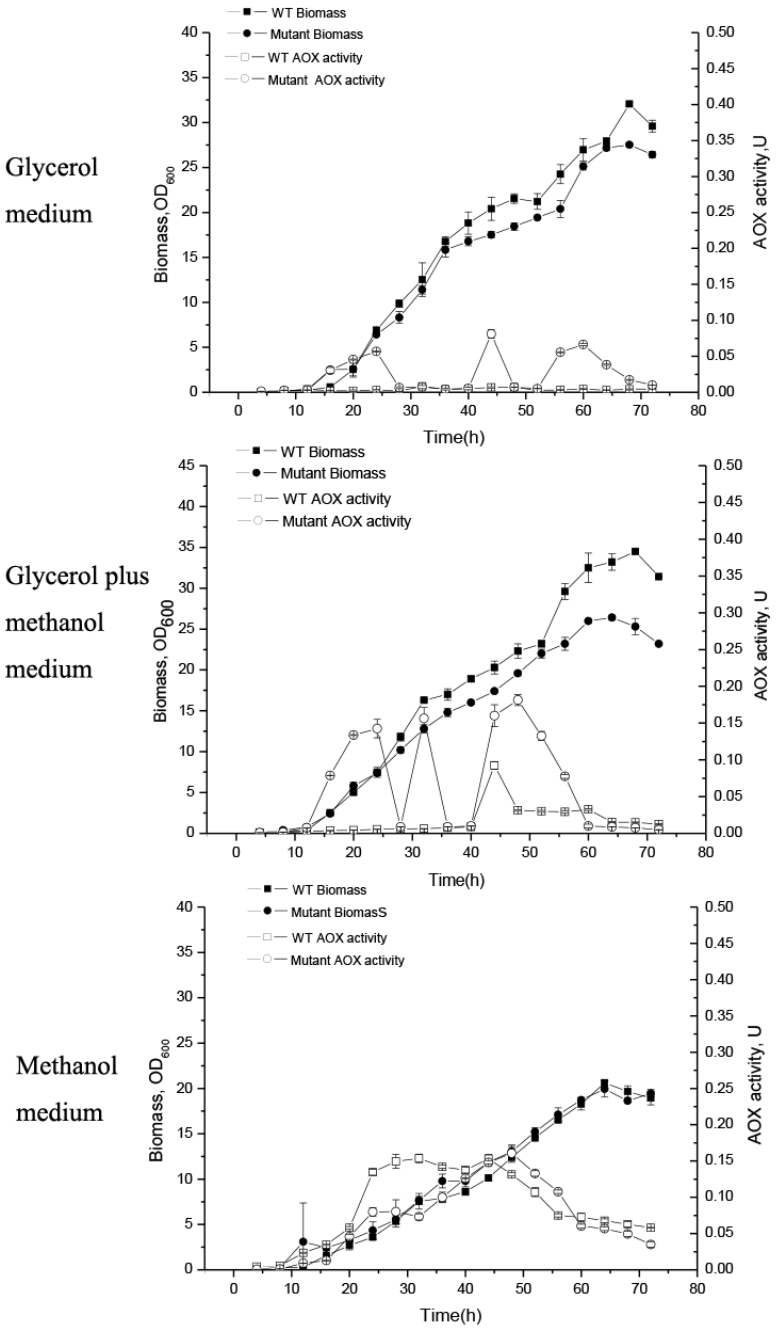

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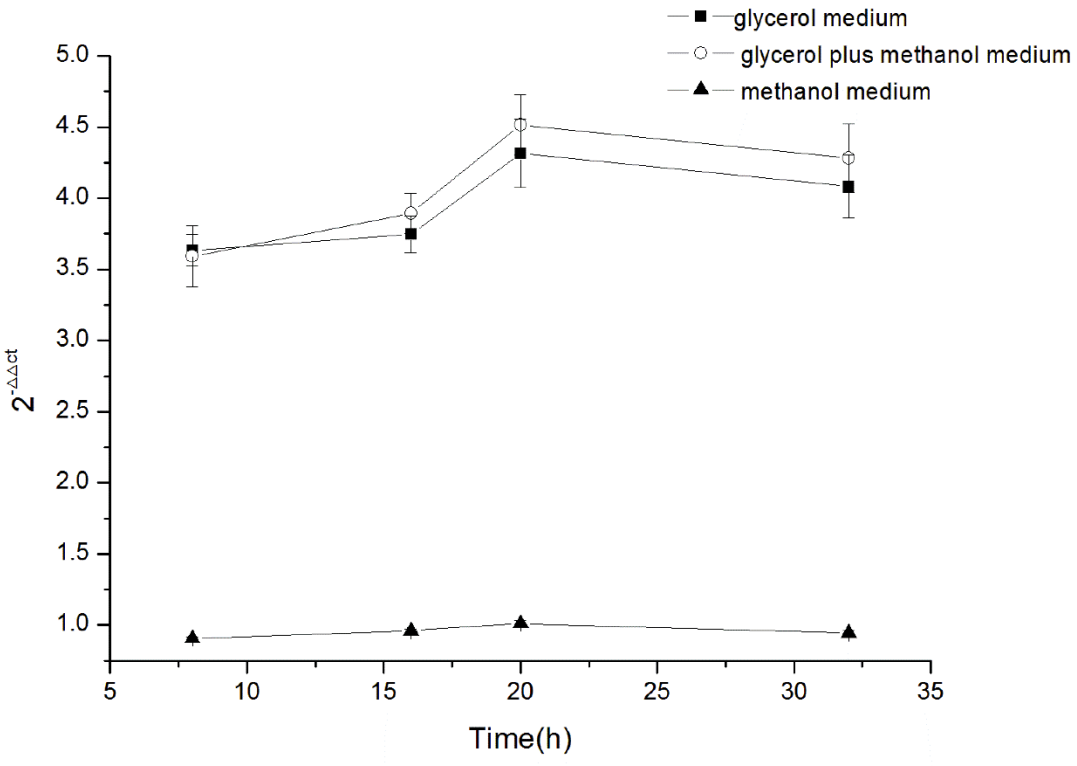

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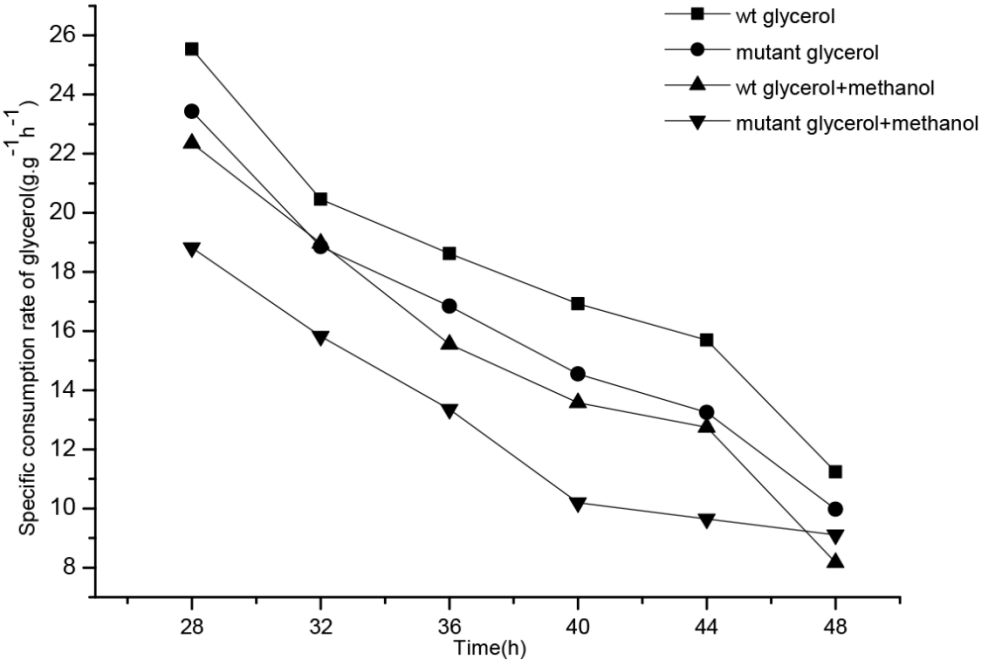

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