A novel methanol-free *Pichia pastoris* system for recombinant protein expression

Wei Shen1, Ying Xue1, Yiqi Liu1, Chuixing Kong1, Xiaolong Wang1, Mengmeng Huang1, Menghao Cai1, Xiangshan Zhou1, Yuanxing Zhang1,2 and Mian Zhou1*

**Abstract**

**Background:** As one of the most popular expression systems, recombinant protein expression in *Pichia pastoris* relies on the AOX1 promoter (P\textsubscript{AOX1}) which is strongly induced by methanol. However, the toxic and inflammatory nature of methanol restricts its application, especially in edible and medical products. Therefore, constructing a novel methanol-free system becomes necessary. The kinases involved in P\textsubscript{AOX1} activation or repression by different carbon sources may be promising targets.

**Results:** We identified two kinase mutants: Δgut1 and Δdak, both of which showed strong alcohol oxidase activity under non-methanol carbon sources. Based on these two kinases, we constructed two methanol-free expression systems: Δgut1-HpGCY1-glycerol (P\textsubscript{AOX1} induced by glycerol) and Δdak-DHA (P\textsubscript{AOX1} induced by DHA). By comparing their GFP expression efficiencies, the latter one showed better potential. To further test the Δdak-DHA system, three more recombinant proteins were expressed as examples. We found that the expression ability of our novel methanol-free Δdak-DHA system was generally better than the constitutive GAP promoter, and reached 50–60 % of the traditional methanol induced system.

**Conclusions:** We successfully constructed a novel methanol-free expression system Δdak-DHA. This modified expression platform preserved the favorable regulatable nature of P\textsubscript{AOX1}, providing a potential alternative to the traditional system.

**Keywords:** Recombination protein expression, AOX1 promoter, Dihydroxyacetone, GUT1, DAK, *Pichia pastoris*

**Background**

Methylotrophic yeast refers to a limited number of yeast species which are able to utilize methanol as the sole carbon and energy source for cell growth. *Pichia pastoris*, *Hansenula polymorpha*, *Candida boidinii* and *Pichia methanolica* are the most typical examples [1, 2]. In order to metabolize methanol, these yeast species express an alcohol oxidase, named Aox in *P. pastoris*, Mox in *H. polymorpha* and Aod in *C. boidinii* [3]. *Pichia pastoris* has two alcohol oxidase coding genes, AOX1 and AOX2. The strength of AOX1 promoter (P\textsubscript{AOX1}) is much stronger than P\textsubscript{AOX2} under methanol induction, therefore AOX1 is the major source of methanol-oxidizing activity [4]. Besides, in methanol cultured *P. pastoris* cells Aox protein level could reach 30 % of total soluble proteins [5]. *Pichia pastoris* has been exploited as an excellent heterologous protein expression system in 1980s [1]. So far, over 5000 recombinant proteins have been successfully expressed in *P. pastoris* including insulin, α-interferon and hepatitis B antigen [6] (http://www.pichia.com/). The increasing popularity of this particular expression system could be attributed to the following reasons [1, 7, 8]: (1) The *P. pastoris* genome has been completely sequenced and a lot of genetic manipulation tools are available; (2) The culture condition is simple, and cells can do high-density culture with high levels of protein expressed at the intra- or extra-cellular level; (3) As a eukaryote, *P. pastoris* is able to perform special modifications such as glycosylation.
In most cases, recombinant protein expression is driven by \( \text{P}_{\text{AOX1}} \) in \( \text{P. pastoris} \). \( \text{P}_{\text{AOX1}} \) is induced only by methanol and repressed by other carbon sources such as glucose, glycerol and ethanol [4]. This special induction and repression feature functions as a switch which turns recombinant protein expression on and off under different culture conditions. This is beneficial especially when expressing proteins that are toxic towards cell growth. However, this system also has limitations. Since \( \text{P}_{\text{AOX1}} \) induction requires methanol, this toxic and inflammable material needs special handling and is not suitable for producing edible and medical products [8, 9]. In addition, the by-product hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) of methanol metabolism brings oxidative stress, which may result in the degradation of recombinant proteins [10, 11].

One way to solve the problem is to develop a methanol-free expression system, which does not rely on methanol to induce the \( \text{AOX1} \) promoter. Since \( \text{P}_{\text{AOX1}} \) is activated by methanol and repressed by glucose and glycerol, interrupting the glucose/glycerol repression pathway, or activating the methanol activation pathway will be a good strategy. The activation or repression by carbon molecules towards \( \text{P}_{\text{AOX1}} \) is not direct, but rather through complicated signaling pathways which have not been fully understood yet. So far several protein factors have been reported to be involved in the alcohol oxidase gene promoter regulation. One example lies in the hexose transporter and sensor family. Gcr1 in \( \text{H. polymorpha} \) [9, 12] and Hxt1 in \( \text{P. pastoris} \) [8] are hexose transporters, and their mutation result in a de-repression of the alcohol oxidase promoter in glucose. As for hexose sensors, \( \text{H. polymorpha} \) Hxx1 [13] mutation and \( \text{P. pastoris} \) Gss1 mutation [14] cause de-repression of alcohol oxidase under glucose culture. Other existing studies focus on transcription factors. As shown in Table 1, a few transcription activators and repressors have been identified in methylotrophic yeasts. However, how the induction or repression signals are transduced from carbon molecules to these transcription factors is still largely unknown. Exploring these elements will be helpful to reveal more potential targets for constructing the methanol-free expression system.

Kinas always play an important role in cell signaling, since phosphorylation and de-phosphorylation processes are crucial for many biological activities. However, few kinases involved in \( \text{P}_{\text{AOX1}} \) activation/repression have been identified so far. Therefore we performed a kinase screening and identified two kinases named \( \text{GUT1} \) and \( \text{DAK} \). By analyzing the phenotypes of the knocked out strains under different carbon sources, we constructed two strains whose \( \text{AOX1} \) promoter could be activated by glycerol or dihydroxyacetone (DHA) as sole carbon source. Then we discussed and tested the possibility for each of these strains to become a novel methanol-free system by expressing several recombinant proteins as examples.

### Results

**The \( \Delta \text{gut1} \) and \( \Delta \text{dak} \) strains have abnormal Aox activity or growth rates under different carbon sources**

In the genome of \( \text{P. pastoris} \), 152 genes were annotated as kinase coding genes [15]. In order to screen for kinases involved in the \( \text{P}_{\text{AOX1}} \) activation/repression pathways, we knocked out 92 kinase genes separately and examined strain phenotypes under different carbon sources. For each knockout strain, a colorimetrical assay was used to measure the alcohol oxidase activity while cell growth was checked by the spotting assay. Among these 92 kinase genes, two members attracted our attention: \( \text{PAS}_{\text{chr4}}\_0783 \) and \( \text{PAS}_{\text{chr3}}\_0841 \). \( \text{PAS}_{\text{chr4}}\_0783 \) encodes a glycerol kinase which converts glycerol to glycerol-3-phosphate, and \( \text{PAS}_{\text{chr3}}\_0841 \) is the gene of dihydroxyacetone kinase which converts DHA to dihydroxyacetone phosphate (DHAP) (Fig. 1a). Therefore \( \text{PAS}_{\text{chr4}}\_0783 \) is named \( \text{PpGUT1} \) or \( \text{GUT1} \) here, and \( \text{PAS}_{\text{chr3}}\_0841 \) is named \( \text{PpDAK} \) or \( \text{DAK} \). As shown by Fig. 1b, Aox in the wild-type strain GS115 was induced by methanol and strictly repressed by glucose and glycerol. However, Aox expression was de-repressed in glycerol cultured \( \Delta \text{gut1} \) strain although cell growth was also largely restricted. The \( \Delta \text{dak} \) strain did not have any abnormality in Aox activity, but its growth under methanol was severely impaired (Fig. 1b).

**The \( \Delta \text{gut1} \)-HpGCY1 strain has the potential to be developed to a novel methanol-free expression system**

Usually glycerol could be metabolized through two pathways in yeast, including the glycerol kinase mediated phosphorylation pathway and the glycerol dehydrogenase mediated oxidation pathway (Fig. 1a). As the first

| Table 1 Summary of transcription factors of the alcohol oxidase promoter in three types of methylotrophic yeast |
|---------------------------------|-------------------------------|-------------------------------|
| **Organism**                    | **Factors**                   | **Classification**          |
| \( \text{H. polymorpha} \)      | Mig1,2                        | Repressor [31]              |
|                                 | Mut3                          | Activator [32]              |
|                                 | Mpp1                          | Activator [33]              |
| \( \text{P. pastoris} \)        | Nrg1                          | Repressor [20]              |
|                                 | Mit1                          | Activator [34]              |
|                                 | Prm1                          | Activator [34]              |
|                                 | Mxr1                          | Activator [35]              |
| \( \text{C. boidinii} \)        | Mig1                          | Repressor [36]              |
|                                 | Trm1                          | Activator [37]              |
|                                 | Trm2                          | Activator [38]              |
step of phosphorylation or oxidation pathway, glycerol is converted to glycerol 3-phosphate or DHA, respectively. Then both of them are converted to DHAP by different enzymes. Different methylotrophic yeasts prefer different pathways. For example, *C. boidinii* NO. 2201 utilizes the phosphorylation pathway and *Hansenula ofunaensis* prefers the oxidation pathway, while *H. polymorpha* has both [16]. As for *P. pastoris*, since Δ*gut1* showed significant Aox activity on glycerol but impaired cell growth (Fig. 1b), we considered that *P. pastoris* might preferentially use the phosphorylation pathway for the initial step of glycerol usage, and metabolites in this pathway may be repressing signals against Aox expression. These repressing signals are likely localized upstream of DHAP, since DHA and DHAP are common metabolites of both methanol and glycerol utilization pathways. Besides, DHA was an inducible carbon source and it supported Aox expression (Fig. 2a). Therefore, introducing the glycerol oxidation pathway into Δ*gut1* may be a good way to construct a methanol-free expression system. By converting glycerol directly to an inducible carbon source DHA, repressing signals generated by the phosphorylation pathway could be circumvented.

To test this idea, we introduced the glycerol dehydrogenases gene (*GCY1*) of *S. cerevisiae* and *H. polymorpha* into the Δ*gut1* strain separately, constructing the Δ*gut1*-Sc*GCY1* and Δ*gut1*-Hp*GCY1* strains. As shown by Fig. 2b, Hp*GCY1* was able to rescue cell growth on glycerol while Sc*GCY1* could not. Besides, the growth rate of the Δ*gut1*-Hp*GCY1* strain increased with the elevated initial glycerol concentration, with a heavier final cell biomass at the stationary phase than WT. These results suggested that Hp*GCY1* works in *P. pastoris* to metabolize glycerol through the oxidation pathway while Sc*GCY1* does not. The reason why Sc*GCY1* did not work in *P. pastoris* may be due to the difference in conserved domains (Additional file 1: Figure S1). Usually, Sc*GCY1* works better in extreme conditions, such as high osmolality and micro-aerobic conditions [17, 18] while Hp*GCY1* works in both common and high osmolality conditions [19].

Then we checked the Aox activity in glycerol cultured Δ*gut1*-Hp*GCY1* strain (Fig. 2c). Aox activity could be detected in different glycerol concentrations from 0.5 to 6 %, suggesting P*AOX1* was at least partially de-repressed in the Δ*gut1*-Hp*GCY1* strain. In addition, lower glycerol concentrations (0.5 and 1 %) supported longer Aox activity. Aox expression in the Δ*gut1*-Hp*GCY1* strain was still repressed by glucose (Fig. 2c, bottom). Therefore, this modified expression platform preserved the favorable regulatable nature of P*AOX1*.

The catabolism of methanol depends on both methanol utilization pathway (MUT pathway) and peroxisomes biogenesis [3]. Usually, the de-repression of Aox expression is accompanied by elevated activities of enzymes involved in MUT pathway and peroxisomes biogenesis [3, 20, 21]. These genes include *AOXI, CAT, DASI, DAS2, FDH* and *FLD* in MUT, and *PEX3, PEX5, PEX10, PEX14, PMP20* and *PMP47* in peroxisome biogenesis. Therefore we checked the transcriptional levels of these genes in glycerol cultured WT, Δ*gut1*, and Δ*gut1*-Hp*GCY1* strains (Fig. 2d). Compared with the glycerol
cultured WT strain, the transcriptional levels of these genes in \( \Delta\)gut1 and \( \Delta\)gut1-HpGCY1 strains were much higher, especially AOX1 and FDH. Significant amount of Aox protein could be detected in glycerol cultured \( \Delta\)gut1 and \( \Delta\)gut1-HpGCY1 strains (Fig. 2e).

Taken together, these results indicate that \( P_{\text{AOX1}} \) derepression under glycerol is at least partially achieved in the \( \Delta\)gut1-HpGCY1 strain, and it has the potential to be developed to a novel methanol-free expression system. Here we named this system \( \Delta\)gut1-HpGCY1-glycerol.
The Δdak strain has the potential to be developed to a novel methanol-free expression system as well

Another interesting target revealed from our kinase screening is DAK. The Δdak strain showed similar Aox activity profile as WT, but impaired cell growth under methanol (Fig. 1b). In 1998, Luers et al. deleted this gene in P. pastoris PPY4, and the strain growth was abolished by methanol but supported by DHA [22]. Since DHA is an inducible carbon source for P AOX1 in WT cell (Fig. 2a), we checked the effect of DHA on P. pastoris Δdak strain.

In agreement with previous studies, the Δdak strain recovered growth on DHA and its growth rate increased with elevated DHA concentration (Fig. 3a).

The Aox activity in the Δdak strain was then measured by the colorimetrical assay (Fig. 3b). The deep red color suggested that DHA was able to induce AOX1 expression, and lower DHA concentrations (0.2 and 0.5 %) showed better Aox activities than higher DHA concentration, especially at the later growth stage. Aox induction here could also be switched on and off easily by changing carbon sources since it was strictly repressed by glucose (Fig. 3b, bottom). Therefore, this modified expression platform also preserved the inducible nature of P AOX1.

Again we examined the transcriptional levels of genes involved in MUT pathway and peroxisome biogenesis in DHA cultured WT and Δdak strains. The transcriptional levels of these genes in DHA cultured Δdak strain were much higher than that in DHA cultured WT strain (Fig. 3c). Being consistent with the colorimetrical assay, significant amount of Aox protein could be detected in
DHA cultured Δdak strain, which were comparable with that in methanol culture WT strain (Fig. 3d).

Taken together, these results suggested that the Δdak strain also has the potential to be developed to a novel methanol-free expression system in which DHA functions as an inducible carbon source instead of methanol. This system is named Δdak-DHA system.

Compare the two potential novel methanol-free expression systems using GFP as a reporter

In order to test the abilities of the two potential systems in recombinant protein production, we expressed GFP under P_AOX1 in Δgut1-HpGCY1-glycerol system and Δdak-DHA system. Green fluorescence intensity was measured to represent the GFP expression level, and GFP intensity in methanol cultured WT strain was used as a reference here. As shown by Fig. 4a, the fluorescence intensity of the Δgut1-HpGCY1-glycerol system was only 20–25 % of that from WT strain grown on methanol, while the Δdak-DHA system showed 80–90 % (Fig. 4b). These distinct phenotypes could be traced to different growth rates between Δgut1-HpGCY1 and Δdak strains. Both glycerol and DHA are three-carbon molecules, however, the final cell density of glycerol cultured Δgut1-HpGCY1 strain was much higher than that of DHA cultured Δdak strain (Figs. 2b, 3a). Therefore, it is possible that Δgut1-HpGCY1 converted more carbon sources into biomass, while Δdak converted them into protein more efficiently. As a summary here, the Δdak-DHA expression system seemed to function better than the Δgut1-HpGCY1-glycerol system, thus the former one was then selected for further study.

Examine the recombinant protein production efficiencies in the Δdak-DHA system

In order to further elucidate the potential of the Δdak-DHA system, we expressed three more heterologous proteins and compared the expression levels with that in methanol induced WT strains. These heterologous proteins were amylase (Amy) from Geobacillus sp. 4j, glucose oxidase (God) from Aspergillus niger and hepatitis B small surface antigen (HBsAg) from human. Among them, Amy and God were secretory proteins while HBsAg was intracellular. Genes of these recombinant proteins were inserted after the AOX1 promoter. In order to eliminate the influence of gene copy number, single copied expression cassette was selected for all strains. The widely used constitutive promoter P_GAP was also examined here as another control.

As measured by enzyme activities, the expression levels of three recombinant proteins in the Δdak-DHA system reached 50–60 % of methanol induced WT system, and became comparable (Amy and God) or even higher (HbsAg) than the constitutive P_GAP system (Fig. 5; Table 2). As shown by enzyme activity to biomass (U/OD600), the Δdak-DHA system worked generally better than the constitutive P_GAP expression system, and showed about 50–60 % expression ability of the traditional methanol induced system.

Discussion

In this study we constructed and tested two modified P. pastoris expression systems on the basis of two kinase mutants. In both Δgut1-HpGCY1-glycerol and Δdak-DHA systems, P_AOX1 could be induced by non-methanol
carbon sources (glycerol or DHA) and repressed by glucose. Between them, the Δdak-DHA system showed better expression capacity. Exemplified by three typical recombinant proteins, its protein expression ability generally exceeded the constitutive P_GAP system, and reached 50–60% of the traditional methanol induced system. It should be noted that further optimization could be done to improve the protein expression efficiency in the Δdak-DHA system. Since several transcription activators and repressors for P_AOX1 are already identified, a combination strategy by overexpressing activators and knocking down repressors in the Δdak-DHA system is worth trying. Besides, optimization of expression conditions and parameters in the Δdak-DHA system will be likely to further increase the protein expression levels.

As the simplest ketose, DHA is always used as supplements in cosmetics, medicine and food industry [23]. DHA is non-toxic towards human and environment, and was added to FDA’s list of approved cosmetic ingredients in the 1970s. This novel methanol-free system will help to broaden the application of P. pastoris mediated recombinant protein expression, especially in producing medical and edible products.

Another interesting question attracted our attention is why the Δdak strain grows on DHA but not methanol, since DHA is an intermediate in methanol metabolism (Fig. 1a). As one of the steps in methanol assimilation pathway, formaldehyde and xylulose 5-phosphate (Xu-5-P) are converted to one molecule of DHA and one molecule of glyceraldehyde 3-phosphate (GAP) (Fig. 1a). If DHA cannot be phosphorylated to DHAP, DHA will accumulate and cannot contribute to the regeneration of the C1-acceptor molecule Xu-5-P, which will not be sufficient for a continued function of the Xu-5-P cycle.

Table 2 A summary of the protein expression levels in Fig. 5

| Strain              | Amy (U/mL) | God (U/mL) | HBsAg* (Abs/mL) | Amy (U/OD₆₀₀) | God (U/OD₆₀₀) | HBsAg* (Abs/OD₆₀₀) |
|---------------------|------------|------------|-----------------|--------------|--------------|-------------------|
| Δdak-DHA (P_AOX1)   | 67.18 ± 4.44 | 0.42 ± 0.03 | 4.51 ± 0.22     | 2.61 ± 0.12  | 0.012 ± 0.001  | 0.18 ± 0.01       |
| WT-Methanol (P_AOX1)| 130.02 ± 5.32 | 0.83 ± 0.05 | 7.52 ± 0.43     | 4.90 ± 0.20  | 0.029 ± 0.002  | 0.26 ± 0.02       |
| WT-Glucose (P_GAP)  | 73.46 ± 4.32 | 0.57 ± 0.03 | 2.43 ± 0.19     | 1.37 ± 0.09  | 0.010 ± 0.001  | 0.049 ± 0.003     |

Abs absorbance
* Represented by relative enzyme activity

Fig. 5 Expressional profiles of three heterologous proteins in the Δdak-DHA system. The WT (P_AOX1) system induced by methanol and the constitutive WT (P_GAP) system serve as controls here. a, b The expressional profiles of amylase (AMY). c, d The expressional profiles of glucose oxidase (GOD). e, f The expressional profiles of hepatitis B small surface antigen (HBsAg)
Since methanol and its first step product formaldehyde are toxic to cells, delayed dissimilation may cause growth arrest. We found that addition of xylose recovered cell growth in methanol cultured Δdak strain (Additional file 1: Figure S2A) and promoted methanol utilization (Additional file 1: Figure S2B). It is likely that Xu-5-P generated in xylose metabolism served as acceptor molecule for formaldehyde fixation and then made the cell recover the growth in methanol.

In order to examine why the Δdak strain growth was supported by DHA, we examined the activities of several related enzymes (Additional file 1: Table S2). Dak activity was totally abolished in the knockout, suggesting that P. pastoris does not have any additional isozymes. Both WT and DAK mutant showed significant DHA reductase (Dhar) activity (Additional file 1: Table S2, last column). These results indicate that in DHA cultured DAK mutants, DHA may be reduced to glycerol first and then be metabolized through the phosphorylation pathway. In order to test this, we constructed a double mutant Δgut1Δdak in which glycerol phosphorylation were blocked by GLUT1 mutation. As expected, this strain failed to grow on both glycerol and DHA (Additional file 1: Figure S2C).

Conclusions

Our results indicate that the Δdak-DHA system is a novel methanol-free P. pastoris system for recombinant protein expression. The AOX1 promoter in this system is induced by non-methanol carbon source DHA and repressed by glucose. The protein expression ability of this novel system generally exceeds the constitutive P_{GAP} system, and reaches 50–60 % of the traditional methanol induced system. Therefore, this modified expression platform has reached 50–60 % of the traditional methanol induced related enzymes (Additional file 1: Table S2). Dak activity was totally abolished in the knockout, suggesting that P. pastoris does not have any additional isozymes. Both WT and DAK mutant showed significant DHA reductase (Dhar) activity (Additional file 1: Table S2, last column). These results indicate that in DHA cultured DAK mutants, DHA may be reduced to glycerol first and then be metabolized through the phosphorylation pathway. In order to test this, we constructed a double mutant Δgut1Δdak in which glycerol phosphorylation were blocked by GLUT1 mutation. As expected, this strain failed to grow on both glycerol and DHA (Additional file 1: Figure S2C).

Methods

Strains and culture conditions

Pichia pastoris GS115 (invitrogen) was used as the wild-type (WT) strain. Unless indicated, P. pastoris strains were grown at 30 °C in YPD medium [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose] or minimal YNB medium [0.67 % (w/v) yeast nitrogen base without amino acids] supplemented with different carbon sources, e.g., 1 % (w/v) glucose (YND), 1 % (w/v) glycerol (YNG), or 0.5 % (v/v) methanol (YNM). For solid media, agar was added to 2 % (w/v). Cell density (OD_{600}) was determined spectrophotometrically at the wavelength 600 nm. Escherichia coli TOP 10 cells were used for plasmid propagation. Primers used in this study were listed in Additional file 1: Table S1.

Quantitative real-time RT-PCR (qPCR) analysis

The WT, Δgut1, Δgut1-HpGCY1 and Δdak cells were pre-grown in YPD to OD_{600} of 2–8 and washed three times with sterile water. The washed cell pellets were transferred to YNG and YNDHA media. After cultured at 30 °C for 2.5 h, cell pellets were harvested and subsequently used to mRNA isolation. Genomic DNA was removed and cDNA was synthesized using ReverTra Ace qPCR RT Kit (TOYOBO). qPCR was carried out as described previously [8] using primers (Additional file 1: Table S1) designed by Beacon designer 7.9.

Cell extract preparation and western blot analysis

To prepare cell extracts, 30–50 OD_{600} units of cells were harvested by centrifugation at 6000g for 3 min, washed twice with ice-cold 50 mM potassium phosphate buffer (pH 7.0), and then frozen at −20 °C. Cells were thawed and re-suspended in 1 ml lysis buffer [50 mM potassium phosphate buffer (pH 7.0), 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Aliquots of 1 ml were mixed with 1.8 g glass beads (Biospec Products, Bartlesville, OK, USA) in a 2.0 ml screw-cap tube followed by disruption with a bead disrouter (Mini-BeadBeater-8; Biospec Products) for 8 cycles (1 min vibrating and 1 min resting in ice for each cycle). The lysate was centrifuged at 20,000g for 30 min, the pellet was discarded, and the supernatant was utilized for western blotting. The protein concentration was determined with a Bradford protein assay kit (Tiangen, Shanghai, China).

Each lane was loaded 10 μg total proteins for SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane using the electrophoretic transfer method with rabbit anti-Aox antibody (a kind gift from Suresh Subramani, University of California, San Diego, USA) as the primary antibody and peroxidase-conjugated goat anti-rabbit immunoglobulin G as the secondary antibody.

Construction of Δgut1-HpGCY1 and Δgut1-ScGCY1 strains

As Δgut1 has used Sh ble selection marker, we need new marker to construct the GCY1 expression strains. We used primers Scal-GAP/GAP-BamHI to amplify the GAP promoter from pGAPZA. pPIC3.5K was digested by restriction enzyme Scal/BamHI to remove the AOX1 promoter and then ligated with the GAP promoter which was digested by the same restriction enzymes. Finally, we got a new plasmid which contains the GAP promoter and the geneticin selection marker. HpGCY1 was amplified from H. polymorpha genome by using primers BamHI-HpGcy1/HpGcy1-NotI. ScGCY1 was
amplified from *S. cerevisiae* genome using primers BamHI-ScGcy1/ScGcy1-NotI. After using restriction enzymes *BamHI*/NotI to digest the fragment HpGCY1, ScGcy1, and the above plasmid, the two fragments were ligated into the plasmid respectively. Then we obtained two plasmid P$_{GAP}$-HpGCY1 and P$_{GAP}$-ScGcy1. The two plasmids were linearized by *SalI* and transformed into Δgut1 strain by electroporation. The positive transformants Δgut1-HpGCY1 and Δgut1-ScGcy1 were selected with histidine self-synthesis ability.

**Construction of WT-GFP, Δgut1-HpGCY1-GFP, and Δdak-GFP strains**

The primers 5-PBR-AOXTT/PBR-AOXTT-3 were used to amplify the fragment PBR-AOXTT [including three parts: *E. coli* origin of replication pBR322, Ampicillin resistance gene, and green fluorescent protein (GFP) expression cassette] from the plasmid pBluescript. The primers 5-hph/hph-3 were used to amplify the hygromycin B phosphotransferase expression cassette from the plasmid pP-GFP. The three single copy strains of GFP expression cassette were screened according to the previously described method [24]. The single copy strains of GFP expression cassette were screened according to the previously described method [24].

**Assays of yeast growth, Aox activities and GFP expression**

The strains were pre-grown in YPD media to OD$_{600}$ of 2–8. The cells were harvested by centrifugation at 3000 g for 5 min, washed three times with sterile water, and resuspended with initial OD$_{600}$ of 1.0 in 50 mL YNB media supplemented with various carbon sources. At suitable intervals, OD$_{600}$ was measured for growth curve, 1 mL aliquot of culture media was removed, and cells were harvested by centrifugation and then stored at −80 °C for colorimetrical assay of Aox activities or measurement of GFP.

The reaction buffer of colorimetrical assay including 0.05 % (w/v) O-dianisidine, 0.15 % (w/v) CTAB, 1 % (v/v) methanol, 3 U/mL HRP, and 100 mmol/L potassium phosphate buffer (pH 7.5) [25]. When reacting, frozen cells were thawed and added 800 μL reaction buffer to incubate for about 20 min. Then 100 μL mixtures were transferred into 96-well plates and scanned into images by scanner.

For measuring GFP, frozen cells were thawed, washed twice with sterile water, and transferred into 96-well plates with diluting to about OD$_{600}$ = 1. OD$_{600}$ and GFP were measured by enzyme-labeled instrument (BioTek) with three biological replicates.

**Construction of three heterologous proteins expression strains**

The GOD ORF was amplified from plasmid RINA1297-GOD (kindly provided by Juan Zhang, Jiangnan University) with primers SnaBI-GOD/NotI and KpnI-GOD/NotI. The fragment was digested by *SnaBI*/NotI and ligated into vector pPIC9K opened with the same restriction enzymes to yield the expression vector pPIC9K-GOD. With *KpnI*/NotI, the GOD ORF was ligated into pGAPZαA to yield the expression vector pGAPZαA-GOD. The vector pPIC9K-GOD was linearized with *Pmel* and transformed by electroporation into WT and Δdak. The vector pGAPZαA-GOD was ligated with *BlnI* and transformed into WT. In order to measure the recombinant proteins expression ability of the three expression systems, and eliminate the influence of copy number, single copy strain of God expression cassette was screened according to the previously described method [24]. The three single copy God expression strains were called WT (P$_{GAP}$-GOD), Δdak (P$_{GAP}$-GOD) and WT (P$_{GAP}$-GOD), respectively.

The construction process of Amy expression strains was just similar to strains WT (P$_{AOX1}$-GOD), Δdak (P$_{AOX1}$-GOD) and WT (P$_{AOX1}$-GOD). The three single copy Amy expression strains were called WT (P$_{AOX1}$-AMY), Δdak (P$_{AOX1}$-AMY) and WT (P$_{AOX1}$-AMY), respectively.

The HBsAg sequence was synthesized by Suzhou GENEWIZ biotech Co., Ltd., China. Primer pairs BamHI-HBsAg/HBsAg-NotI were used to amplify the sequence and the 680 bp product was digested with *BamHI*/NotI. It was then ligated into pPIC3.5K opened with the same restriction enzymes to yield the expression vector pPIC3.5K-HBsAg. With *BspT104I*/NotI, the HBsAg was ligated into pGAPZαA to yield the expression vector pGAPZαA-HBsAg. By using *BspT104I*/NotI, the α-Factor secretion signal in pGAPZαA can be removed. The vector pPIC3.5K-HBsAg was linearized with *SalI* and transformed by electroporation into WT and Δdak. The vector pGAPZαA-HBsAg was linearized with *BlnI* and Zeocin was used to select the positive transformant. The three single copy HBsAg expression strains were called WT (P$_{AOX1}$-HBsAg), Δdak (P$_{AOX1}$-HBsAg) and WT (P$_{GAP}$-HBsAg), respectively.

**Production and activity assays of three recombinant proteins**

The strains WT (P$_{AOX1}$-GOD), Δdak (P$_{AOX1}$-GOD) and WT (P$_{GAP}$-GOD) were pre-grown in YPD media at 30 °C, 200 rpm. When OD$_{600}$ reached 2–8, cells were harvested by centrifugation at 3000 g for 5 min, washed three times...
with sterile water, and resuspended with initial OD_{600} 1.0 in 50 mL BMMY, BMDHAY and BMDY, respectively. The initial concentration of carbon source in three media was 0.5 % methanol, 0.2 % DHA, and 2.5 % glucose. Every 24 h after the shift, 1 mL aliquot of culture media was removed, and cells were separated by centrifugation (2 min at 8000g). PMSF was added to the culture supernatants to the final concentration of 1 mM to inactivate proteases, and samples were stored frozen at −20 °C for subsequent analysis. Cells biomass was also monitored during the course of cultivation. Methanol and DHA were fed every 24 h to keep the concentration around 0.5 and 0.2 %, respectively. The strains WT (P_{AOX1} GOD) and Δdak (P_{AOX1}-GOD) were induced for 120 h. The strain WT (P_{GAP2} GOD) was batch culture for 120 h. God activity was measured using the coupled o-dianisidine-peroxidase reaction. It was determined as in Bankar’s manuscript [26].

The culture condition of Amy expression strains followed the same procedure of God. Amy activity was measured using the DNS method [27].

The culture condition of HBSAg expression strains followed the same procedure of God. The difference is that cells were harvested instead of the culture supernatants. HBSAg relative concentration was measured by HBSAg ELISA kit (Kehua, Shanghai, China).

Enzyme activities assays for Dak, Gcy1 and Dhar
Assays were performed as described [28, 29]. Enzyme activities were examined by either NADH production or consumption. NADH level change was measured by UV absorbance at 340 nm.

**Additional file**

Additional file 1. Additional files and tables.

**Abbreviations**

Aox: alcohol oxidase; DHA: dihydroxyacetone; DHAP: dihydroxyacetone phosphate; MUT: methanol utilization pathway; Amy: amylase; God: glucose oxidase; HBSAg: hepatitis B small surface antigen; Dhar: DHA reductase.

**Authors’ contributions**

WS, XZ and MC designed the experiments and WS conducted most of the experiments. YX, YL and CK screened the kinase mutants. WS and MH assayed the recombinant protein expression. WS, MZ wrote this manuscript and YZ, MC helped to revise. All authors read and approved the final manuscript.

**Acknowledgements**

We thank Dr. Suresh Subramani (University of California, San Diego, USA) and Dr. Juan Zhang (Jiangnan University, Wuxi, China) for kindly providing the anti-Aox antibody, and pAG32 and RINA1297-GOD plasmids, respectively.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and material**

All datasets generated by this study are included within the manuscript and in the Additional files.

**Ethics approval and consent to participate**

This manuscript does not report data collected from humans or animals.

**Funding**

This work was sponsored by Chinese National High Technology Research and Development Program (2014AA093501), National Special Fund for State Key Laboratory of Bioreactor (2060204), and Shanghai Pujiang Program (15PJ1401600).

Received: 23 May 2016 Accepted: 13 October 2016
Published online: 21 October 2016

**References**

1. Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev. 2000;24:45–66.
2. Houard S, Heinderyckx M, Bollen A. Engineering of non-conventional yeasts for efficient synthesis of macromolecules: the methylotrophic genera. Biochimie. 2002;84:1089–93.
3. Hartner FS, Gleder A. Regulation of methanol utilisation pathway genes in yeasts. Microb Cell Fact. 2006;5:39.
4. Cregg JM, Maddon KR, Barrington KJ, Thill GP, Stillman CA. Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris*. Mol Cell Biol. 1989;9:1316–23.
5. Cregg JM, Cereghino JL, Shi J, Higgins DR. Recombinant protein expression in *Pichia pastoris*. Mol Biotechnol. 2000;16:23–52.
6. Pichia Technology From RCT. http://www.pichia.com/.
7. De Schutter K, Lin YC, Tiels P, Van Hecke A, Glinka S, Weber-Lehmann J, Rouze P, Van de Peer Y, Callewaert N. Genome sequence of the recombinant protein production host *Pichia pastoris*. Nat Biotechnol. 2009;27:561–6.
8. Zhang P, Zhang W, Zhou X, Bai P, Cregg JM, Zhang Y. Catabolite repression of *Aox* in *Pichia pastoris* is dependent on hexose transporter Hxt1 and peoxphagy. Appl Environ Microbiol. 2010;76:6108–18.
9. Krasovska OS, Stasyk OG, Nahorny VO, Stasyk OV, Granovski N, Kordium VA, Voziyanof OF, Sibirny AA. Glucose-induced production of recombinant proteins in *Hansenula polymorpha* mutants deficient in catabolite repression. Biotechnol Bioeng. 2007;97:858–70.
10. Hilt W, Wolf DH. Stress-induced proteolysis in yeast. Mol Biotechnol. 1993;6:2437–42.
11. Xiao A, Zhou X, Zhou L, Zhang Y. Improvement of cell viability and hispurin production by ascorbic acid in *Pichia pastoris* fermentation. Appl Microbiol Biotechnol. 2006;72:837–44.
12. Stasyk OG, Stasyk OG, Janet K, Marten V, Cregg JM, Sibirny AA. A hexose transporter homologue controls glucose repression in the methylotrophic yeast *Hansenula polymorpha*. J Biol Chem. 2004;279:1116–25.
13. Stasyk OG, Daind MM, Stasyk OV, Van Dijck P, Thevelein JM, Sibirny AA. Identification of hexose transporter-like sensor HXST and functional hexose transporter HXT1 in the methylotrophic yeast *Hansenula polymorpha*. Eukaryot Cell. 2008;7:735–46.
14. Polupanov AS, Nazarko YY, Sibirny AA. Gs1 protein of the methylotrophic yeast *Pichia pastoris* is involved in glucose sensing, peoxphagy and catabolite repression. Int J Biochem Cell B. 2012;44:1906–18.
15. *Pichia pastoris* GS115 (*Komagataella pastoris* GS115) chromosome assembly. http://www.ncbi.nlm.nih.gov/assembly/GCF_000027005.1/.
16. Tani Y, Yamada K. Diversity in glycerol metabolism of methylotrophic yeasts. FEMS Microbiol Lett. 1987;40:151–3.
17. Blomberg A. Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. FEMS Microbiol Lett. 2000;182:1–6.
18. Liang Z, Yan T, Guo Z, Shi G. Engineering of the glycerol decomposition pathway and cofactor regulation in an industrial yeast improves ethanol production. J Ind Microbiol Biotechnol. 2013;40:1153–60.
19. Nguyen HTT, Nevoigt E. Engineering of Saccharomyces cerevisiae for the production of dihydroxyacetone (DHA) from sugars: a proof of concept. Metab Eng. 2009;11:335–46.

20. Wang X, Cui M, Shi L, Wang Q, Zhu J, Wang J, Zhou M, Zhou X, Zhang Y. PpNrg1 is a transcriptional repressor for glucose and glycerol repression of AOX1 promoter in methylotrophic yeast Pichia pastoris. Biotechnol Lett. 2016;38:291–8.

21. Hristozova TML, Tuneva D, Gotcheva V, Angelov A, Roshkova Z. Mutant Hansenula polymorpha strain with constitutive alcohol oxidase and peroxisome biosynthesis. Z Naturforsch C. 2002;57:858–62.

22. Luers GH, Advani R, Wenzel T, Subramani S. The Pichia pastoris dihydroxyacetone kinase is a PTS1-containing, but cytosolic, protein that is essential for growth on methanol. Yeast. 1998;14:759–71.

23. Drysdale GS, Fleet GH. Acetic acid bacteria in winemaking: a review. Am J Enol Vitic. 1988;39:143–54.

24. Xuan Y, Zhou X, Zhang W, Zhang X, Song Z, Zhang Y. An upstream activation sequence controls the expression of AOX1 gene in Pichia pastoris. FEMS Yeast Res. 2009;9:1271–82.

25. Sibirny AA, Titorenko VI. A method of quantitative determination of alcohol oxidase and catalase in yeast colonies. Ukr Biokhim Zh. 1986;58:65–8.

26. Bankar SB, Bule MV, Singhal RS, Ananthanarayan L. Optimization of Aspergillus niger fermentation for the production of glucose oxidase. Food Bioprocess Tech. 2009;2:344–52.

27. Ghose TK. Measurement of cellulase activities. Pure Appl Chem. 1987;59:257–68.

28. Wang ZX, Zhuge J, Fang H, Prior BA. Glycerol production by microbial fermentation: a review. Biotechnol Adv. 2001;19:201–23.

29. de Koning W, Gleeson MAG, Harder W, Dijkhuizen L. Regulation of methanol metabolism in the yeast Hansenula polymorpha. Arch Microbiol. 1987;147:375–82.

30. Rußmayer H, Buchetics M, Gruber C, Vally M, Grillitsch K, Modarres G, Guerraño R, Klavins K, Neubauer S, Drexler H, Steiger M, Troyer C, Al Chalabi A, Krebiehl G, Sonntag D, Zelling G, Daum G, Graf AB, Altmann F, Koeiersperger G, Hann S, Sauer M, Mattanovich D, Gasser B. Systems-level organization of yeast methylotrophic lifestyle. BMC Biol. 2015;13:80.

31. Stasyk OG, Zutphen TV, Yang HA, Stasyk OV, Veenhuis M, Sibirny AA. The role of Hansenula polymorpha Mig1, homologues in catabolite repression and pexophagy. FEMS Yeast Res. 2007;7:1103–13.

32. Vallini V, Berardi E, Strabbioli R. Mutations affecting the expression of the MCR gene encoding peroxisomal methanol oxidase in Hansenula polymorpha. Curr Genet. 2000;38:163–70.

33. Leão-Helder AN, Kikkin AM, VDK, Kiel JA, Veenhuis M. Transcriptional down-regulation of peroxisome numbers affects selective peroxisome degradation in Hansenula polymorpha. J Biol Chem. 2003;278:40749–56.

34. Wang X, Wang Q, Wang J, Bai P, Shi L, Shen W, Zhou M, Zhou X, Zhang Y, Cai M. Mtr1 transcription factor mediates methanol signaling and regulates alcohol oxidase 1 (AOX1) promoter in Pichia pastoris. J Biol Chem. 2016;291:6245–61.

35. Lin-Cereghino GP, Godfrey L, da Cruz BJ, Johnson S, Khounsathiene S, Tolstorukov I, Yan M, Lin-Cereghino J, Veenhuis M, Subramani S, Cregg JM. Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in Pichia pastoris. Mol Cell Biol. 2006;26:883–97.

36. Zhai Z, Yurimoto H, Sakai Y. Molecular characterization of Candida boidinii MIG1 and its role in the regulation of methanol-inducible gene expression. Yeast. 2012;29:293–301.

37. Sasano Y, Yurimoto HM, Sakai Y. Trm1p, a Zn(II) 2 Cys6-type transcription factor, is a master regulator of methanol-specific gene activation in the methylotrophic yeast Candida boidinii. Eukaryot Cell. 2008;7:527–36.

38. Yu S, Yurimoto H, Kuroyama M, Sakai Y. Trm2p-dependent derepression is essential for methanol-specific gene activation in the methylotrophic yeast Candida boidinii. FEMS Yeast Res. 2010;10:535–44.