

# Methanol expression regulator 1 (Mxr1p) promotes xylulose 5-phosphate recycle via increasing transketolase activity in *Pichia pastoris*

**Chunjun Zhan**

Jiangnan University <https://orcid.org/0000-0001-9504-6439>

**Yingyue Pan**

Jiangnan University

**Xiuxia Liu**

Jiangnan University

**Chunli Liu**

Jiangnan University

**Jinling Zhan**

Jiangnan University

**Dinghua Xu**

Sinosbio Biomedical Technologies

**Zhonghu Bai**

Jiangnan University

**Yankun Yang** (✉ [yangyankun@jiangnan.edu.cn](mailto:yangyankun@jiangnan.edu.cn))

Jiangnan University <https://orcid.org/0000-0003-4815-6832>

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## Research

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# Abstract

**Background** Methanol expression regulator 1 (Mxr1p) is a key transcription factor that plays a vital role in the methanol utilization pathway in *Pichia pastoris* (*P. pastoris*). Most genes referred to the methanol utilization pathway were regulated by Mxr1p. However, some genes did not show a significant difference between methanol and glycerol even though they play an important role in the methanol utilization pathway. So far, the regulation mechanism about these genes and the relationship with Mxr1p are still unknown.

**Results** Methanol metabolic pathway analysis revealed that most of the methanol-induced genes were upregulated in transcriptional level when cultured in methanol. Whereas some genes like tk11 (transketolase 1) did not show significant up-regulation in methanol even though it plays a very important role in Xu5P recycle, the reason is still not clear. To clarify this point, firstly, pull-down and MS experiments were performed. The result shows that Tkl1p protein combined with Mxr1p in vitro. Subsequently, this result was further confirmed by yeast two-hybrid in vivo, and the binding region mainly located from 150AA to 400AA. Moreover, Ser215 phosphorylation did not affect this interaction. In addition, Mxr1p-400AA integration into  $\Delta$ mxr1 could rescue cell growth in methanol. All the above results proved that Mxr1p played a post-translational role in the methanol utilization pathway and Mxr1p-400AA may achieved most of Mxr1p functions. Secondly, the function of Mxr1p-Tkl1p complex was expounded by detecting formaldehyde consumption and xylulose production in cell-free systems. Results showed that Mxr1p-Tkl1p mixture could promote formaldehyde consumption and xylulose production in vitro.

**Conclusion** Mxr1p promotes methanol utilization via combining with Tkl1p to accelerate Xu5P recycle and this interaction was not affected by Ser215 phosphorylation.

# Background

As an important heterologous protein expression system, more than 1,000 proteins have been successfully expressed via *P. pastoris*, including angiostatin,  $\alpha$ -glucosidase [1] and *Anti-CEACAM5 nanobody* [2, 3]. Moreover, as the rare methylotrophic microbes, detailed methanol metabolic pathway especially important intermediates recycle pathway not only significantly promotes other methylotrophic microbes construction but also promotes autotrophic microorganism design [4]. Many results showed that the robust alcohol oxidase 1 promoter ( $P_{AOX1}$ ) contributes most to this successful expression system [5]. Data showed that methanol expression regulation 1 protein (Mxr1p) plays a key role in *aox1* and other methanol-induced genes transcription [6]. For example, *aox1* was repressed in *Dmxr1* mutant even though cells were cultured in methanol [7, 8]. The reason is that Mxr1p binds to methanol-induced promoter e.g., *aox1*, *das* and *pex8* [7, 9] to promote transcription. Our previous results also revealed that Mxr1p promotes *aox1* transcription via binding on  $P_{GT1}$  to inhibit *gt1* transcription [10, 11]. All of the above results proved that Mxr1p could regulate methanol metabolic pathway by transcription level. Recently, Parua *et al.* demonstrated that a 14-3-3 protein (GenBank accession no. CCA38880) regulates Mxr1p activity in a carbon source-dependent manner. The mechanism is that 14-3-3 protein binds on Mxr1p

between residues 212–225. Ser215 phosphorylation promotes the interaction between Mxr1p and the 14-3-3 protein and subsequently leads to methanol metabolic pathway repression [12, 13]. However, although phosphorylation of Ser215 is similar in methanol and glucose, cells showed a significant difference in methanol utilization ability. Thus, there may be other proteins interact with Mxr1p to regulate MUT pathway. Based on that, several transcription factors such as Trm1, Prm1 and Mit1 have been identified [14-17]. Disappointedly, there is no interaction among these transcription factors even though some of them, e.g., Mxr1 and Rop1, shared the same binding site in methanol-induced genes[18].

In this report, methanol-induced genes were identified by methanol metabolic pathway analysis based on transcription level. Furthermore, potential proteins (e.g., Tkl1p) interacting with Mxr1p *in vitro* were identified via pull-down analysis and Mass spectrometry (MS). Yeast two-hybrid screening showed that Tkl1p interacted with Mxr1p *in vivo*, the interaction region mainly located between 150AA to 400AA and Ser215 phosphorylation did not affect this interaction. Finally, formaldehyde consumption and xylulose production assay proved that the complex formed by Tkl1p and Mxr1p could promote methanol utilization by accelerating xylulose recycle. All of the above results not only be benefit for clarifying Xu5P recycle route but also would help enrich Mxr1p regulation system.

## Results

### Transcription level referred to methanol utilization pathway in *P. pastoris*

#### Fig. 1.

Transcriptional levels referred to MUT genes in different media (methanol and glycerol) were detected after 48h. Hierarchical clustering analysis provides a holistic view about MUT genes expression level in methanol or glycerol media (Fig. 1). Results showed that compared with glycerol, *aox1* and *das1/2* were significantly upregulated in methanol. Moreover, *das2* upregulation was more significant than *das1*, which indicated that the regulation mechanism between *das1* and *das2* might be different [19]. As the key intermediate, Xu5P plays a crucial role in formaldehyde fixation and degradation, which means genes related to Xu5P recycle could be promoted in methanol. Results showed that most genes referred to Xu5P recycle route, such as *rep1* and *tal*, were upregulated in methanol. Surprisingly, *tkl1* gene did not show significantly improved as other MUT genes, even though it plays a very important role in Xu5P recycle route (Fig.1).

### Detection *tkl1* expression levels in different cells

#### Fig. 2

According to transcriptome analysis, many genes related to MUT pathway and non-oxidative pentose phosphate pathway (NOP) were up-regulated in methanol medium. However, *tkl1* did not show significant up-regulated like *das* even though it plays a key role in the carbon rearrangement process, which indicates that maybe some other unrevealed regulation pathways participate in *tkl1* regulation. To clarify this

hypothesis, firstly, the transcriptional and translational level of *tkl1* were measured. Results showed that *tkl1* did not show a significant difference between methanol and glycerol medium (Fig.2a). It has been reported that many genes were promoted by Mxr1p in MUT and NOP pathway[9], which inspired us that Mxr1p may promote *tkl1* expression. To verify this hypothesis, *tkl1* expression level and methanol consumption in *Dmxr1* mutant and *mxr1* over-expression strains were detected. Unfortunately, results showed that Mxr1p did not show obvious improvement for *tkl1* both in RNA and protein level (Fig.2b) even though it promotes methanol utilization (Fig.2a).

### Excavation Mxr1p-interaction protein via pull-down assay and Yeast two-hybrid assay

#### Fig. 3

The above results indicated that although Tkl1p participates in Xu5P recycle, its transcriptional and translational level did not improve a lot in methanol, which indicated that Tkl1p might be involved in post-transcriptional modification. Based on that, M1 and M2 proteins were purified and pull-down assay was performed in this research to find some candidate proteins (Fig.3a,b), which may interact with Mxr1p *in vitro*. Results proved that some proteins could combine with Mxr1p *in vitro* (Fig.3). Each candidate band was collected and sent for MS analysis, results showed the interesting protein is Tkl1p. This result was further confirmed *in vivo* by yeast two-hybrid assay (Fig.3b). Moreover, we found the diploid containing M1 and Tkl1p did not grow on selection plate, whereas the diploid containing M2 and Tkl1p grew on selection plate, this result indicated that the region Mxr1p interacting with Tkl1p mainly located between 150AA to 400AA. Our previous results showed that M1 plays a crucial role in the process of binding on  $P_{GT1}$  and  $P_{AOX1}$ [10]. Some other researchers showed that N400 of Mxr1p play the main function between protein interaction [12, 13]. Considering all these results, Mxr1p was divided into two parts; the first part contains a DNA binding domain (mainly 1–150AA), which mainly regulates gene transcription. The second is protein interaction domain (mainly 150–400AA), which may be involved in different metabolic pathways via interacting with different proteins.

### Tkl1p interact with Mxr1p did not via phosphorylation of Ser215

#### Fig. 4

It has been proved that 14-3-3 protein interacted with Mxr1p via phosphorylation of Ser215. In order to examine whether Ser215 phosphorylation would affect the interaction between M2 and Tkl1p. M2-Ser215 was mutant to M2-Asp215 (M3). Tkl1p-His and M3-Flag protein were purified and used for pull-down assay. The targeted band was recognized by Western blotting (Fig.4a), which indicates that phosphorylation of Ser215 or not did not affect the interaction between Tkl1p and Mxr1p. Meanwhile, different strains growth curve were measured in methanol medium (Fig.4b). Results showed that *mxr1* deletion (*Dmxr1*) affect cell grew. Moreover, when M2 or M3 was integrated back into genome respectively, cell grew would be rescued. This indicated that Mxr1p N-400AA take main function of Mxr1p.

## Functions of the Mxr1-TKL1 complex

### Fig. 5

Based on Fig.1, it could be inferred that Tkl1p mainly promotes Xu5P recycle in the carbon rearrangement route. Considering all results, one hypothesis is that the complex formed by Mxr1p and Tkl1p may participate in Xu5P recycle route. In order to test this hypothesis, xylulose production was measured *in vitro* using crude total protein. Results (Fig.5) showed that xylulose production in *tkl1* overexpression strain (32.7 mM, 1% formaldehyde) is much higher ( $P < 0.01$ ) than that in wild type (12.6 mM, 1% formaldehyde), which confirmed that Tkl1p promote Xu5P production. Moreover, when the purified M2 protein was added into this reaction system, higher production of xylulose (36.2 mM, 1% formaldehyde) was detected ( $P = 0.0009 < 0.01$ ), the above results proved that the complex formed by Mxr1p and Tkl1p promotes xylulose production. In order to further confirm this result, formaldehyde consumption in different reaction systems were detected, results showed that (Fig.5a) more formaldehyde consumption was detected in Tkl1p-Mxr1p reaction system than others. Based on the above results, it concluded that Mxr1p could combine with Tkl1p to promote Xu5P recycle and further accelerate formaldehyde consumption.

Many intermediates, especially formaldehyde, in methanol utilization are toxic for cells, therefore, how to convert these toxic intermediates into useable substrates is very important for cell to survive. It has been proved that high efficient Xu5P recycle route would promote formaldehyde degradation and rescue cells[10]. However, no definite results illustrate Xu5P recycle route in methylotrophic microbes. Based on the above results, it showed that complex formed by Mxr1p and Tkl1p participate in Xu5P recycle route. It has been proved that xylulose production increased *in vitro* experiments. The reason may be that excessive Xu5P was converted into xylulose by some enzyme. To verify this hypothesis, xylulokinase (XK) expression level was measured *in vivo*, the result showed that XK expression level was promoted in Tkl1-Mxr1 over-expression cells, meanwhile, methanol consumption was also improved (Fig.5b).

## Discussion

As an important renewable carbon source, methanol has drawn lots of attention during the past few years due to its low energy cost, liquid form [20-22] and other advantages. Moreover, compared with other one-carbon sources like CO<sub>2</sub>, methanol could be high efficiency used by native methylotrophic microbes[23]. Besides, both CO<sub>2</sub> fixation pathway and formaldehyde degradation pathway share similar intermediate such as Xu5P and Ru5P [24, 25]. Based on the above reasons, lots of attention has been paid to engineer non-native methylotrophic microbes. Unfortunately, most of the recombinant cells did not grow well in methanol. Many reasons may lead to this problem, but inefficiency intermediates supplement may be an important reason. Therefore, Clarify intermediates recycle regulation mechanism in methanol utilization pathway maybe not only beneficial for other non-native methylotrophic microbes construction but also would promote other one-carbon source utilization [4, 26]. In *Saccharomyces cerevisiae*, Tkl1 has been shown as the rate-limiting factor in NOP pathway [27]. Moreover, in *P. pastoris*

Tkl1p showed similar conserved domain structure with Das1p and Das2p. Some results indicated that cells losing *das1* and *das2* are still able to grow in methanol [28], which indicated that Tkl1p may be involved in some other pathways. However, Tkl1 did not show significant difference between methanol and glycerol as DAS1/2, which also indicated that *tkl1* and *das1/2* regulation mechanism might be different [29].

In this paper, our results showed that Tkl1p interacted with M2, and the interaction region mainly located between 150AA to 400AA. In addition, the function of Tkl1p-Mxr1p was confirmed to promote Xu5P recycle. However, when we detected XK transcriptional level in different cells, we found that there is no significant difference between Tkl1p overexpression strain and Tkl1p-Mxr1p double overexpression strains (Fig.4), the reason may be that Xu5P could be highly recycled *in vivo* to support formaldehyde fixation. As a critical transcription factor, Mxr1p is inextricably linked with the methanol utilization pathway both in transcriptional and post-translational [8], which indicated that Mxr1p may be the core of methanol regulation system in *P. pastoris*. In this study, many efforts have been tried to purify integral Mxr1p protein, unfortunately, all of them were failed (Fig.S1/2). Some researchers have indicated that overexpressed Mxr1p is toxic to cells [30], but the reason is not clear now. Our results also indicated that Mxr1p overexpression would significantly inhibit cell growth (Fig.S4). Based on our results, it is inferred that Mxr1p might be involved in many different metabolic pathways besides MUT pathway. Therefore, Mxr1p overexpression may inhibit other important metabolic pathways, not 'toxic' for cells.

## Conclusion

In this paper, Tkl1p regulation mechanism was clarified via different experiments, results showed that Tkl1p could bind on Mxr1p specific region (mainly located from 150AA to 400AA), and the complex would promote Xu5P recycle then further promote methanol utilization.

## Materials And Methods

### Medium, strain and plasmid

*P. pastoris* was cultured in YPD broth and BMY medium along with its derivatives, BMMY, BMGY, BMGMY and BMMGY. *Saccharomyces cerevisiae* (*S. cerevisiae*) was incubated in YPD broth and YPDA medium, Y<sub>2</sub>HGold in SD/Try (TAKARA) and Y187 in SD/Leu medium (TAKARA). Constructed plasmids were transformed into *Escherichia coli* (*E. coli*) DH5 $\alpha$  and Trans110 (Trans Gen) cells, and these cells were cultured at 37 °C in Luria-Bertani (LB) medium. All formulas used are listed below. YPD (1% yeast extract, 2% tryptone, 2% glucose), BMY (1 L: 10 g yeast extract, 20 g tryptone, 3 g K<sub>2</sub>HPO<sub>4</sub>, 11.8 g KH<sub>2</sub>PO<sub>4</sub>, 13.4 g YNB, 4 × 10<sup>-4</sup> g biotin, 10 mL glycerol), BMMY (1 L: 10 g yeast extract, 20 g tryptone, 3 g K<sub>2</sub>HPO<sub>4</sub>, 11.8 g KH<sub>2</sub>PO<sub>4</sub>, 13.4 g YNB, 4 × 10<sup>-4</sup> g biotin, 5 mL methanol), BMGMY (BMGY plus 0.5% methanol) and BMMGY medium (BMGY plus 0.5% methanol). YPDA (YPD with the addition of 15 mL 0.2% adenine), LB (0.5% yeast extract, 1% tryptone, 1% NaCl), TB/SB (2.6 g peptone, 4.8 g yeast extract, 0.462 g KH<sub>2</sub>PO<sub>4</sub>,

2.51 g K<sub>2</sub>HPO<sub>4</sub>, 2 g glycerol). SD/Try and SD/Leu medium was purchased from TAKARA. The solid medium was prepared by adding 2 % agar powder.

Electroporation was used to transform *P. pastoris*. Transformation and recombinant DNA operations were performed as described previously[11]. Selection of marker-resistant colonies was performed using LB with 50 µg/mL ampicillin or kanamycin, and YPD with 0.3 mg/mL G418 or 0.1 mg/mL zeocin.

### **Strains, plasmids and primers**

See Tables 1, 2 and 3.

### **Plasmid and strain construction**

#### **M1/2 expression plasmid and strain construction**

Initially, the *P. pastoris* genome was extracted. The sequences of Mxr1-150AA (M1) and Mxr1-400AA (M2) were amplified by PCR using *Pfu* DNA polymerase (Thermo Scientific, Waltham, MA, USA) and the *P. pastoris* genome. These two sequences vary in the number of N-terminal residues: 150 amino acids (M1) and 400 amino acids (M2), respectively. Subsequently, the PCR products were digested with *Nde*I/*Xho*I, and the digested fragments were inserted into the pSVT7 plasmid. The recombinant plasmid was chemically transformed into *E. coli* BL21(DE3) cells.

#### **M1/2-pGBKT7 recombinant plasmid construction**

M1/2 were amplified with *Pfu* DNA polymerase and the *P. pastoris* genome was used as the template. The *Nde*I/*Xho*I-digested PCR products were ligated into the pGBKT7 plasmid. Recombinant plasmids M1-pGBKT7 and M2-pGBKT7 were transformed into *S. cerevisiae* following the protocol provided by TAKARA.

#### **AD-pGADT7 recombinant plasmid construction**

The general procedure was the same as above. Target genes (like *tk11*) were amplified and the PCR products were double-digested with the same restriction enzymes, and the digested fragments were ligated into the appropriate plasmid. Recombinant plasmid AD-pGADT7 was transformed into *S. cerevisiae* using the protocol provided by TAKARA.

#### **Gene expression studies in *E. coli* BL21 (DE3) cells**

*E. coli* BL21(DE3) cells were inoculated into LB medium and grown at 30 °C, 230 rpm for 16 h. The cells were then transferred to TB/SB and cultured at 30 °C, 230 rpm for 5 h. IPTG at a final concentration of 0.5 mmol/L was added to the culture to induce protein expression. Cells were harvested 20 h later by centrifugation at 4 °C and 4000 ´ g. Then recombinant protein was extracted from the cells.

#### **Protein extraction and purification**

One hundred microliters of culture ( $OD_{600} = 3$ ) was collected by centrifugation (4000  $\times$  g, 5 min, 4 °C) and resuspended in 400 mL lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 0.01% Tween-20, 1 mM PMSF, pH 8.0). Cell disruption was achieved by ultrasonic vibration with 20 % power (SONIC uibra cell). The supernatant containing the recombinant protein was collected following centrifugation (4000  $\times$  g, 5 min, 4 °C). The target protein was isolated using an ÄKTA purifier<sup>TM</sup> UPC10 system.

### **M1/2/3 pull-down assay**

His-M1/2 and His-M3 fusion proteins were immobilized to beads (Dynabeads<sup>®</sup> His-Tag Isolation) and washed four times with washing buffer (50 mM sodium phosphate, 300 mM NaCl, 0.01% Tween-20, pH 8.0). A total protein (or purified Tkl1p-His protein) from a yeast culture in BMMY or BMGY medium was added (Reaction solution). The mixture was incubated for 30 min and the beads washed four times with A solution (3.25 mM sodium phosphate, 70 mM NaCl, 0.01% Tween-20, pH 7.4) (Washing solution). Finally, 50 mL His elution buffer (300 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, 0.01% Tween-20, pH 7.4) was added to the samples and these were stirred for 2 min. The His-tagged protein was eluted (Elution solution), and proteins that interacted with the His-tag protein were transferred to sample tubes. All mixtures were subjected to SDS-PAGE and MS analysis.

### **M1/2 yeast two-hybrid**

The protocol from TAKARA was used. Y2HGold cells transformed with the M1 or M2-pGBKT7 recombinant plasmids (SD) were cultured in YPD medium. Y187 with the AD-pGADT7 recombinant plasmid (AD) were also cultured in YPD medium. Equal volumes of the cultures containing SD and AD were then mixed and added to 0.5 mL 2 $\times$  YPDA. The resuspended cells were mixed thoroughly. This mixture was incubated overnight (20–24 h) at 30 °C, 200 rpm. Finally, a 100 mL aliquot of the incubated mating culture was incubated in SD/Try/Leu/His/Ade medium at 30 °C for 3–5 d until visible single colonies appeared.

### ***Dmxr1* mutant construction**

The *P. pastoris* strain carrying the *mxr1* gene deletion was constructed by homologous recombination using *kan* as a marker. The upstream region of the *mxr1* gene was amplified initially by PCR using *Pfu* DNA polymerase (Thermo Scientific) and the *P. pastoris* genome as the template. The primers for this PCR, *mxr1s-1* and *mxr1s-2*, included *SphI* and *BamHI* restriction sites, respectively. The 0.6-kb PCR-amplified fragment was inserted into *SphI/BamHI*-digested pMD<sup>TM</sup>19-T plasmid (TAKARA) to create the pMXR1UP plasmid. The downstream region of the *mxr1* gene was also amplified with primers *mxr1x-1* and *mxr1x-2*, carrying restriction sites for *KpnI* and *EcoRI*, respectively. This 0.5-kb PCR fragment was inserted into pMD19-T to yield the pMXR1Down plasmid. Next, the G418 resistance gene with its own promoter and terminator (1556 bp) was amplified by PCR, using pFA6a-KanMX6 as the template and primers *kan-1* and *kan-2*, which carried *BamHI* and *KpnI* restriction sites, respectively, and the fragment was cloned into the *SphI/BamHI*-digested plasmid pMXR1UP to give the pMXR1UP-Kan plasmid. This plasmid was digested with *KpnI/EcoRI* to generate a 2.2-kb fragment that was then inserted into

*KpnI/EcoRI*-digested pMXR1Down plasmid, yielding a *P. pastoris mxr1* deletion plasmid, pMD19-T-MXR1-del. The deletion cassette was released from pMD19-T-MXR1-del as a 2.7-kb *EcoRI/SphI*-digested fragment and transformed by electroporation into wild-type *P. pastoris* (strain X-33). G418-resistant transformants were isolated on YPD supplemented with 1 mg/mL G418. The correct integration of the deletion cassette into the genome and replacement of the *mxr1* open reading frame (ORF) in the transformants was confirmed by PCR analysis and Sanger sequencing.

### ***mxr1* and *tkl* overexpression strain construction**

The *mxr1* gene and *tkl* were amplified by PCR using genomic DNA as the template, and different primers. The fragment was ligated into the pMD™19-T plasmid (TAKARA) and sequenced. The recombinant plasmid and pGAPZB plasmid (Invitrogen) were then digested with *PmlI/XhoI*, and the *mxr1* fragment was inserted into pGAPZB to yield pGM. Finally, pGM was digested with *AvrII* and electro-transformed into the *P. pastoris X-33 Dmxr1* mutant and *P. pastoris X-33* wild-type, yielding Mxr1 overexpression strains *mxr1-Dmxr1* and *mxr1-wt*, respectively. *tkl1* were amplified by PCR using genomic DNA as the template. Purified PCR products were *PmlI/KpnI*-digested. This was followed by ligation into pGAPZB to create pGAPZB-*tkl1*. Then all the above plasmids were transformed into X-33 respectively by electro-transformation.

### **Real-time PCR**

Total RNA extraction was carried out according to the standard procedure described in the Quantscrip TR kit (TAKARA). The reaction consisted of 5' gDNA Eraser Buffer (2 µL), gDNA Eraser (1.0 µL), total RNA (4 µL, 500 ng) and RNase-Free ddH<sub>2</sub>O (3 µL). Samples were incubated at 42 °C for 2 min. cDNA served as the template for real-time PCR. The reverse transcription system contained 5 µL of the SYBR Premix Ex Taq II (2'), both forward and reverse primers (10 µM, 0.5 µL), cDNA (1 µL) and dH<sub>2</sub>O (3 mL). Each experiment was carried out in triplicate. The transcription level was normalized to an endogenous reference gene, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). The data processing method was 2<sup>-DDCT</sup> [31].

### **Western blotting detection**

Total protein samples were taken from cells cultured in YPD. Cultivating *E. coli BL21* cells were transferred from 1× YPD after 16 h to 2× YPD. Cells were harvested ~6 h later and the cell pellet washed twice with ice-cold 50 mM potassium phosphate buffer (pH 7.0). An electronic oscillator was used to disrupt cells to obtain soluble proteins. Before oscillation, 200 µL of ice-cold PEBF (0.7882% Tris-HCl, 0.0585% EDTA and 2 µL of 100 mM PMSF) and glass beads were also added to ensure efficient cell disruption. The lysed cells were centrifuged at 10,000 g for 1 min, and the supernatant was collected and used for SDS-PAGE analysis.

### **Xylulose detection**

Wild-type (*X-33*) cells and Tkl1p over-expression strain cells were harvested when the OD<sub>600</sub> reached ~6.0. The harvested cells were transferred from YPD to BMMY and incubated overnight at 30 °C and 230 rpm. Total protein extraction was the same as described above. The reaction system included formaldehyde (1–7%), xylulose (20 mM), and PEB and Mxr1 proteins (25 mL). The reaction was initiated by the addition of the crude cell extract (70 mL) and terminated with 20 mL H<sub>2</sub>SO<sub>4</sub>-Na<sub>2</sub>SO<sub>3</sub> (2 M, pH 2.0). High-performance liquid chromatography (HPLC) was performed to quantify the xylulose level. The HPLC parameters were as follows: an Aminexâ HPX-87H ion exclusion column, column temperature 60 °C, the flow rate of 0.6 mL/min and 5 mM H<sub>2</sub>SO<sub>4</sub> as eluent [32, 33].

### **Formaldehyde and Methanol detection**

Cells were incubated overnight in BMGY medium (0.5% methanol) and then transformed into fresh BMMY medium incubate for 12h, 30°C. Cells were centrifuged and supernatant was collected for methanol and formaldehyde detection. HPLC was used for detection followed by Zhan[11]. Formaldehyde concentration was determined via Nash [34].

## **Declarations**

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### **Author contributions**

Chunjun Zhan and Yankun Yang designed the research; Chunjun zhan and Yingyue Pan performed most of the experiments; Xiuxia Liu, Chunli Liu, Jinling Zhan, and Dinghua Xu analysed the data; Chunjun Zhan wrote the paper and Zhonghu Bai edited the manuscript.

### **Corresponding author**

Crorespondence to Yankun Yang

### **Ethical approval**

This article does not contain any studies with human participants performed by any of the authors.

### **Consent for publication**

Not applicable.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Tables

Table 1 Strains used in this study

Strain	Genotype <sup>R</sup>	Source of reference
<i>E.coli</i>		
<i>Trans5α</i>	F-φ80d lacZΔM15 Δ(lacZYA-argF) U169 end A1 recA1 hsdR17 (rk-,mk+) supE44λ- thi-1 gyrA96 relA1 phoA	TransGen Biotech
<i>Trans110</i>	<i>rpsL</i> (StrR) <i>thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44</i> Δ(lac- <i>proAB</i> ) /F' [ <i>traD36 proAB lacIq lacZΔM15</i> ]	TransGen Biotech
<i>Mxr1-BL21(DE3)</i>	Recombinant plasmid (Mxr-pSVT7) transformed into <i>X-33</i>	This study
<i>Pichia pastoris</i>		
<i>X-33</i>	Wide type Mut <sup>+</sup> ,His <sup>+</sup>	Invitrogen
<i>Dmxr1</i>	<i>X-33 mxr1:: Kan</i>	This study
<i>Mxr1-overexpression</i>	Recombinant plasmid (Mxr-pGAPZB) transformed into <i>X-33</i>	This study
strain		
<i>TKL1-X-33</i>	Recombinant plasmid (TKL1-pGAPZB) transformed into <i>X-33</i>	This study
<i>Tkl-mxr1p double over expression</i>		This study
<i>M2</i>	M2-pGAP transformed into <i>Dmxr1</i>	This study
<i>M3</i>	M3-pGAP transformed into <i>Dmxr1</i>	This study
<i>Saccharomyces cerevisias</i>		
<i>Y2HGold</i>	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ,  LYS2 : : GAL1 UAS -Gal1 TATA -His3, GAL2 UAS -Gal2 TATA -Ade2  URA3 : : MEL1 UAS -Mel1 TATA AUR1-C MEL1	TAKARA TAKARA
<i>Mxr1-1-Y2HGold</i>	Recombinant plasmid (Mxr1-1- pGBKT7) transformed into Y187	This study
<i>Mxr1-2-Y2HGold</i>	Recombinant plasmid (Mxr1-2 pGBKT7) transformed into Y187	This study
<i>Y187</i>	MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112,  gal4Δ, gal80Δ, met-,URA3 : : GAL1 UAS -Gal1 TATA -LacZ,MEL1	TAKARA
<i>TKL1-Y187</i>	Recombinant plasmid (DAS1- pGADT7)transformed into <i>Y187</i>	This study
<i>IMD-Y187</i>	Recombinant plasmid (IMD- pGADT7) transformed into <i>Y187</i>	This study
<i>UDP-187</i>	Recombinant plasmid (UDP- pGADT7) transformed into <i>Y187</i>	This study

Zeocin<sup>R</sup> Zeocin resistance

Table 2 Primers used in this study

Primer	Sequence
mxr150-F(M1-F)	CC CATATG ATGAGCAATCTACCCCAAC
mxr150-R(M1-R)	CC GGATCC TCA GTGGTGGTGGTGGTGGTG ATTTGAGTCCCGGCGGCC
mxr-400-F(M2-F)	GGGAATTC CATATG AGCAACTTACCGCTACCTTTG
mxr-400-R(M2-R)	CGC GGATCC TTACGGTGCGGTATCTTTTCGGC
mxr400B-F	CG GGATCC ATGAGCAACTTACCGCTACCTTTG
mxr400B-R	CCCTCGAGTTA ATGATGGTGATGATGGTGGCAGC
Mxr1-1-F	ATGAGCAATCTACCCCAAC
Mxr1-1-R	TCA GTGGTGGTGGTGGTGGTG ATTTGAGTCCCGGCGGCC
Mxr1-2-F	ATGAGCAACTTACCGCTACCTTTG
Mxr1-2-R	TTACGGTGCGGTATCTTTTCGGC
G1-F	TCAGAAAAGAACACCCTTCGTA CTG ATGAGCGCCTATCAATCTCCTA
G1-R	TCAGAAAAGAACACCCTTCGTA CTG CTAATGCTCCAAAATAGTAAGGTTACCTGT
G2-F	TCAGAAAAGAACACCCTTCGTA CTG ATGGCTAGAATTCCAAAAGCAGT
G2-R	TCAGAAAAGAACACCCTTCGTA CTG TTACA ACTTGTCATGCTTTGGTTTTTC
G3-F	TCAGAAAAGAACACCCTTCGTA CTG ATGACAGGAGAACATAAACGAAGTTTC
G3-R	TCAGAAAAGAACACCCTTCGTA CTG TCAGAAAAGAACACCCTTCGTA CTG

Table 3 Plasmids used in this study

Plasmid	Genotype <sup>a</sup>	Source or reference
pMD19-T Simple	Ampicillin <sup>R</sup> ; <i>E. coli</i> subcloning vector	This study
pGBKT7 mxr1-1-	Kanamycin <sup>R</sup> ; P <sub>TRP,1</sub> C-Myc epitope tag, GAL4(1-147) -based expression vector	TAKARA
pGBKT7 mxr1-2-	Kanamycin <sup>R</sup> ; P <sub>TRP,1</sub> C-Myc epitope tag, GAL4(1-147) -based expression vector P <sub>GAP</sub> -based expression vector containing <i>M1</i> gene	This study
pGBKT7 mxr1-3-	Kanamycin <sup>R</sup> ; P <sub>TRP,1</sub> C-Myc epitope tag, GAL4(1-147) -based expression vector P <sub>GAP</sub> -based expression vector containing <i>M2</i> gene	This study
pGBKT7 mxr1-3-	Kanamycin <sup>R</sup> ; P <sub>TRP,1</sub> C-Myc epitope tag, GAL4(1-147) -based expression vector P <sub>GAP</sub> -based expression vector containing <i>mxr150-400</i> gene	This study
pGADT7 DAS1-	Ampicillin <sup>R</sup> ; P <sub>TRP,1</sub> HA epitope tag, GAL4(768-881) -based expression vector	TAKARA
pGADT7 DAS1-	Ampicillin <sup>R</sup> ; P <sub>TRP,1</sub> HA epitope tag, GAL4(1-147) -based expression vector P <sub>GAP</sub> -based expression vector containing DAS1 gene	This study
pGADT7-M2	Ampicillin <sup>R</sup> ; P <sub>TRP,1</sub> HA epitope tag, GAL4(1-147) -based expression vector P <sub>GAP</sub> -based expression vector containing IMD gene	This study
pGADT7-M3	Ampicillin <sup>R</sup> ; P <sub>TRP,1</sub> HA epitope tag, GAL4(1-147) -based expression vector P <sub>GAP</sub> -based expression vector containing UDP gene	This study
M2-pGAPZB	Zeocin <sup>R</sup> ; P <sub>GAP</sub> -M2, -based expression vector P <sub>GAPZB</sub> -based expression vector	This study
M3-pGAPZB	Zeocin <sup>R</sup> ; P <sub>GAP</sub> -M3, -based expression vector P <sub>GAPZB</sub> -based expression vector	This study

## Figures

Genes	Induction	Regulation
AOX1	Methanol	Mxr1p
AOX2	Methanol	----
DAS1	Methanol	Mxr1p
DAS2	Methanol	Mxr1p
FLD	Methanol	----
FDH	Methanol	----
PEX8	Methanol	----
CTA1	----	----
DAK	Constitutive	----
FBA	Constitutive	----
FBP	Constitutive	----
TAL	Methanol	----
TKL	----	----
REP1	----	----
RKI1	----	----

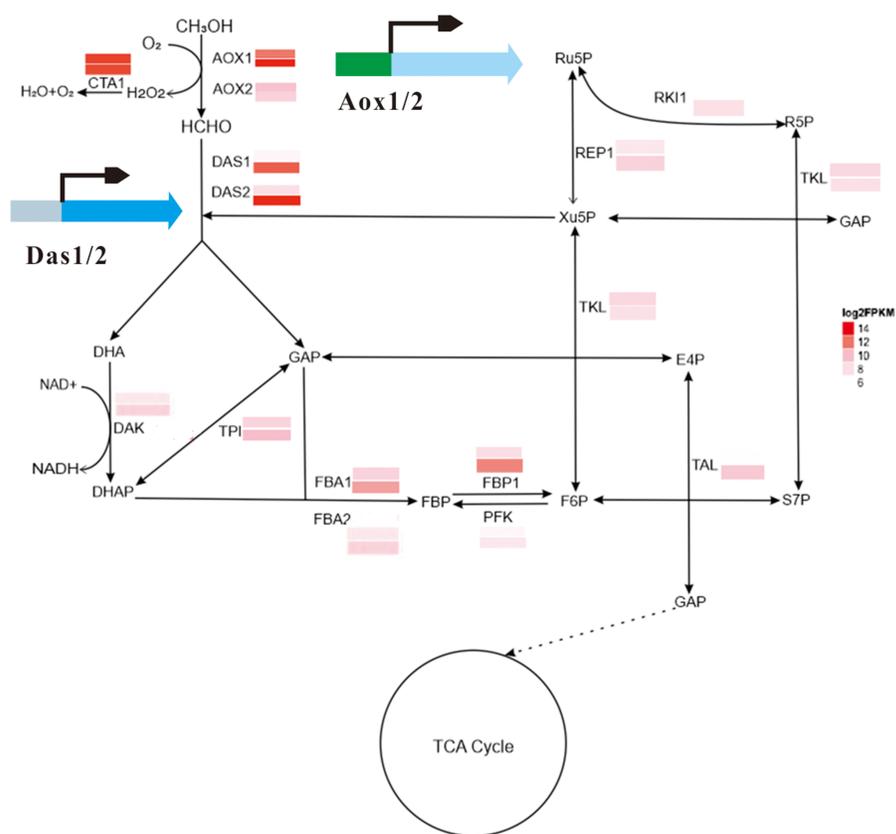
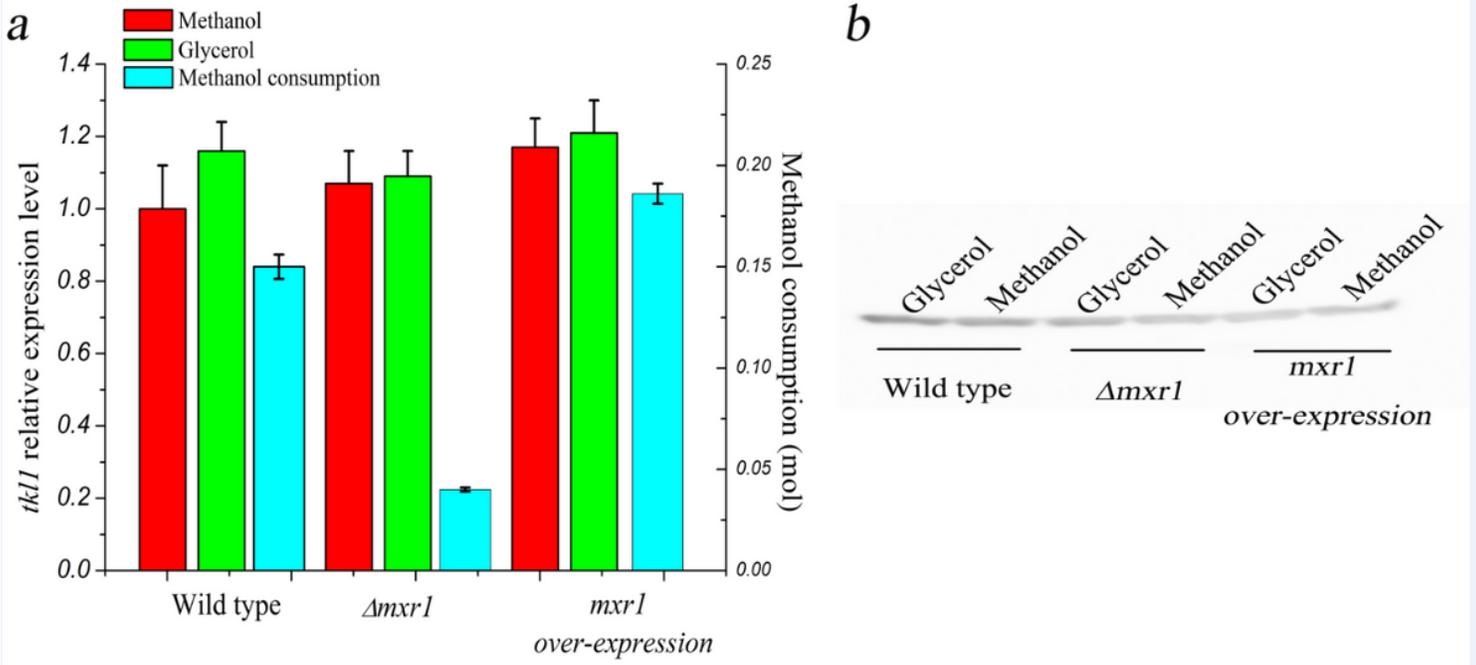


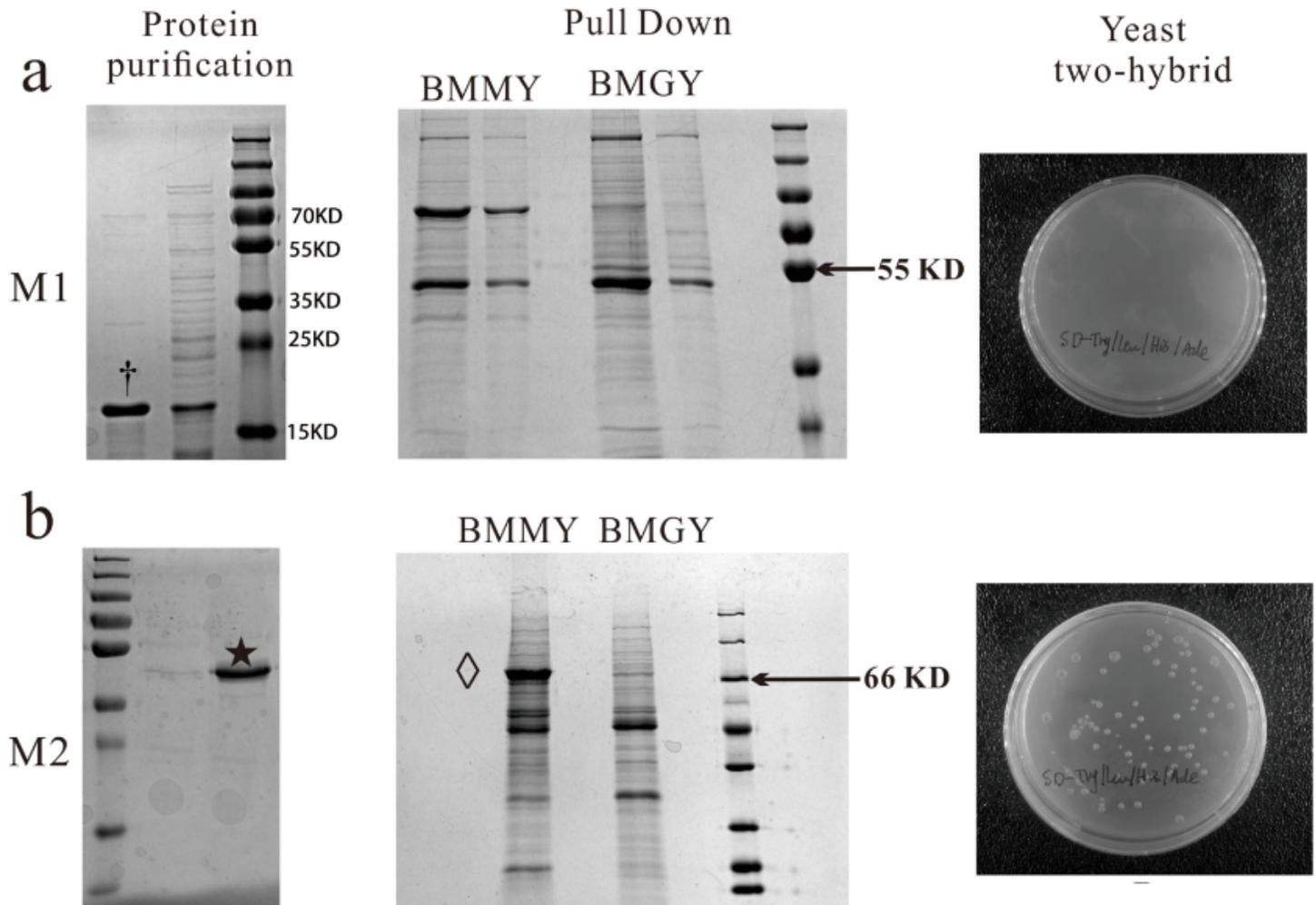
Figure 1

Schematic diagram of important genes related to methanol metabolic pathway (assimilation pathway). AOX1/2: alcohol oxidase1/2. DAS1/2: CTA1: catalase. DAK: dihydroxyacetone kinase. TPI: triosephosphate isomerase. FBA1/2: fructose-1,6-bisphosphate aldolase1/2. FBP: fructose-1,6-bisphosphate. TKL: transketolase. RPE1: ribulose-phosphate 3-epimerase. RPIA1: ribose 5-phosphate. TAL: transldolase. DHA: dihydroxyacetone. DHAP: dihydroxyacetone phosphate. GAP: glyceraldehyde-3-phosphate. FBP: fructose-1,6-bisphosphate. F6P: fructose-6-phosphate. E4P: S7P: sedoheptulose-7-phosphate. Xu5P: xylulose-5-phosphate. Ru5P: ribulose-5-phosphate. R5P: ribose-5-phosphate.



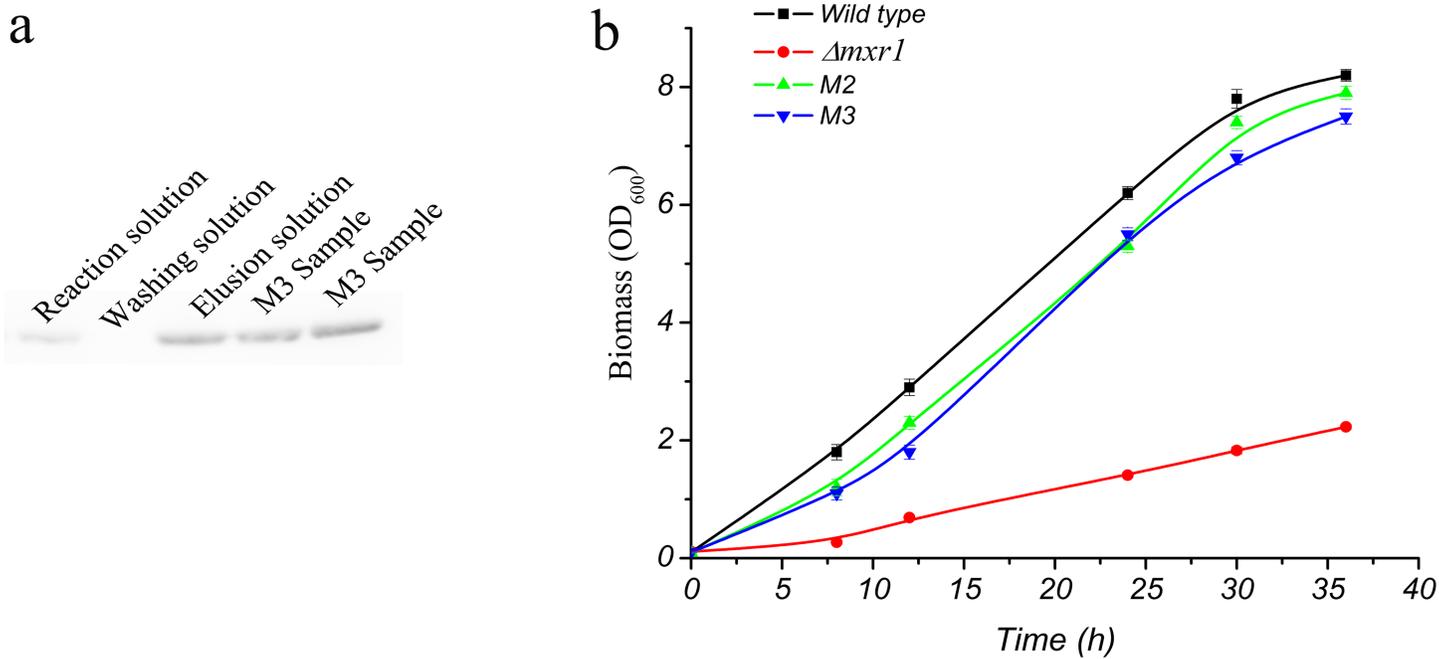
**Figure 2**

tk11 expression levels in different conditions. (a) tk11 transcriptional level and methanol consumption in different strains. (b) tk11 translational level in different cells.



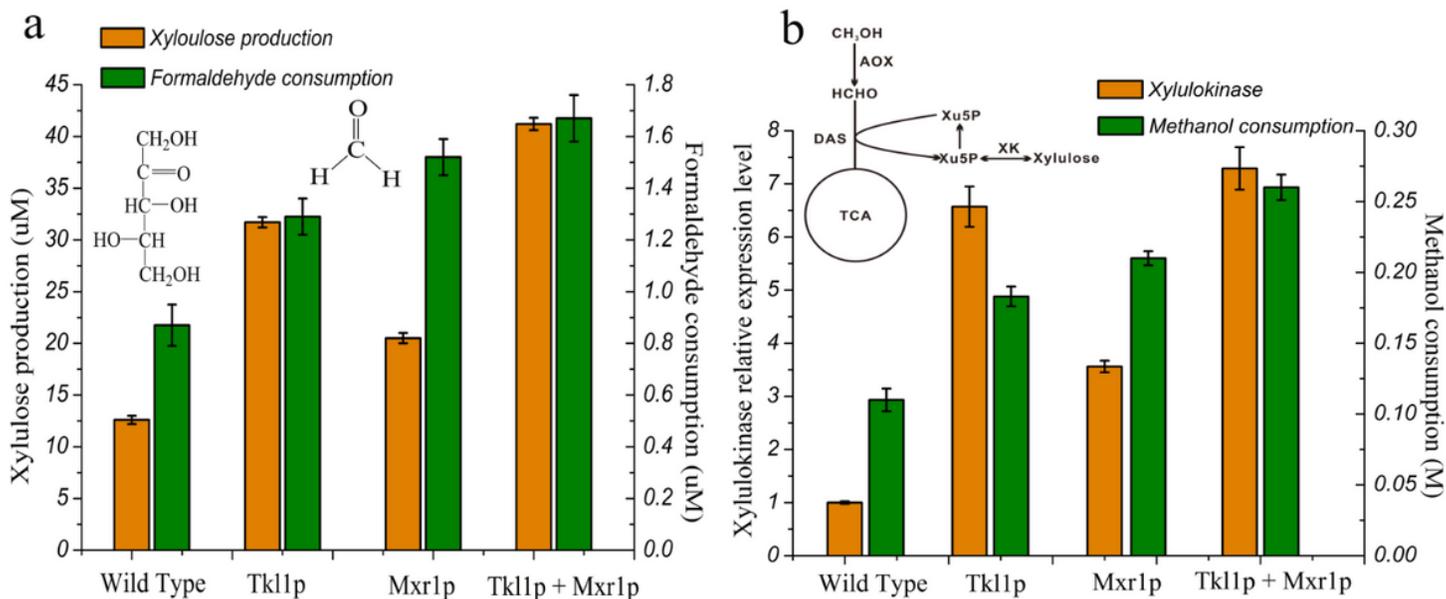
**Figure 3**

Mxr1p interacting protein diggnation. (a) Proteins interacted with M1 (Mxr1p-150AA). (b) Proteins interacted with M2 (Mxr1p-400AA). BMMY: Total proteins using for pull-down comes from BMMY medium. Total proteins using for pull-down comes from BMGY medium. †:M1. ★: M2. ◇: Targeted band.



**Figure 4**

Interaction site studies between Mxr1p mutant and Tkl1. (a) pull-down assay using Tkl1-His and M3-Flag purified protein. (b) Different strains growth curve in methanol.  $\square mxr1$ : *mxr1* deletion. M2: integrate M2 into  $\square mxr1$  mutant. M3: integrate M3 into  $\square mxr1$  mutant.



**Figure 5**

Evaluation Mxr1p-Tkl1p complex function. (a) Xylulose production and formaldehyde consumption in different reaction system. (b) Measurement of XK transcriptional level and methanol consumption in different reaction system

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