Supplementary Information

Methanol-Independent Protein Expression by *AOX1* Promoter with *trans*-Acting Elements Engineering and Glucose-Glycerol-Shift Induction in *Pichia pastoris*

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Construction of strains.

Standard procedures for the manipulation of plasmid DNA were described before[1]. ***(i) Mig1 and Mig2 subcellular localization strains.*** The sequence between the *Asu*II and *Not*I sites of pGAPZα A (Invitrogen) was replaced with a 743 bp *GFP* DNA (*Asu*II-*Not*I) amplified by PCR from pP-GFP (a vector capable of expressing a green fluorescent protein [GFP] under the control of P_{AOX1})[2] using primers GFP1 and GFP2, which included restriction sites for *Asu*II and *Not*I, respectively. Next, the resulting plasmid pGAP-GFP was further modified by replacing the sequence between the *Not*I and *Sal*I sites with a 1,355 bp *MIG1* DNA (*Not*I- *Sal*I) amplified by PCR from the genomic DNA of *P. pastoris*, thus generating a plasmid pGLM1. Finally, the plasmid pGLM1 was transformed by electroporation into wild-type *P. pastoris* strain GS115. Zeocin resistance transformants were isolated on YPD medium supplemented with 0.1 mg/ml zeocin. The method of construction of the GS115/pGLM2 strain was the same as that used for the GS115/pGLM1 strain. Both of the transformants were used to observe the subcellular localization of GFP-Migl and GFP-Mig2 under repressing (glucose or glycerol) or inducing (methanol) conditions, respectively. ***(ii) Δmig1, Δmig2 and Δnrg1 strains.*** To knock out the *MIG1* gene with the zeocin resistance gene *Sh ble* as a marker, we first constructed plasmid pUC18-MIG1-del, a *MIG1* deletion vector. This deletion vector was constructed as follows: a 728 bp fragment containing sequences of the upstream region of *MIG1* was amplified by PCR using genomic DNA of *P. pastoris* as a template with *Pyrobest* DNA polymerase (TaKaRa, Japan) and the primers for this PCR, MI1U1 and MI1U2, carried restriction sites for *Eco*RI and *Bam*HI, respectively. The upstream region fragment was inserted into *Eco*RI/*Bam*HI-digested plasmid pUC18 (Invitrogen), yielding pMIG1Up. Next, a 1011 bp fragment consisting of the downstream region of *MIG1* was amplified by PCR with the primers MI1D1 and MI1D2 carrying restriction sites for *Sal*I and *Sph*I, respectively. The downstream region fragment was inserted into *Sal*I/*Sph*I-digested plasmid pUC18 (Invitrogen), yielding pMIG1Down. Moreover, to obtain the zeocin resistance gene sequence with its own promoter and terminator, a 1,321 bp fragment was amplified by PCR using the plasmid pPICZ A (Invitrogen) as a template with primers Zeo1 and Zeo2, which included restriction sites for *Bam*HI and *Sal*I, respectively. Then the fragment was cloned into *Bam*HI/*Sal*I-digested vector pMIG1Up to create vector pMIG1Up-*ble*. A 2.05-kb DNA fragment by digesting this plasmid with *Eco*RI and *Sal*I was inserted into *Eco*RI-*Sal*I-digested pMIG1Down, thus generating a deletion vector for *MIG1* (pUC18-MIG1-del). The deletion cassette was released from pUC18-MIG1-del as a 3.06-kb *Eco*RI-*Sph*I-digested fragment and transformed by electroporation into WT *P. pastoris* strain GS115. Zeocin resistance transformants were

isolated on YPD (1% yeast extract, 2% peptone and 1% glucose) medium supplemented with 0.1 mg/ml zeocin. The correct integration of the deletion cassette into the genome and replacement of a 930 bp fragment near the upstream region of the full *MIG1* open reading frame (ORF) (1335bp) in the transformants were confirmed by PCR analysis and DNA sequencing. Knockout of the *MIG2* gene was the same as that used for the *MIG1* gene other than using the G418 resistance gene *KAN* as a marker, and the gene sequence with its own promoter and terminator (1,517 bp) was amplified by PCR using the plasmid pPIC3.5K (Invitrogen) as a template with primers Kan1 and Kan2. The full ORF of *MIG2* was totally replaced. Knockout of the *NRG1* gene was the same as that used for the *MIG1* gene other than using the hygromycin resistance gene *hph* as a marker, and the gene sequence with its own promoter and terminator (1,648 bp) was amplified by PCR using the plasmid pRDM054[3] (kindly provided by Prof. Suresh Subramani, University of California, San Diego) as a template with primers Hyg1 and Hyg2. The full ORF of *NRG1* was also totally replaced. ***(iii) Δmig1Δmig2 double-deletion strain.*** The Δ*mig1* and Δ*mig2* strains were crossed and diploid hybrids[4] were selected on YPD medium supplemented with zeocin (0.2 mg/ml) and G418 (1 mg/ml). After sporulation, the Δ*mig1*Δ*mig2* double-deletion mutant was selected among spore progeny on the same medium, and confirmed for the presence of *MIG1* and *MIG2* deletion cassettes as detailed above for single-deletion mutants. Mating and sporulation techniques were performed using established procedures[3]. ***(iv) Δmig1Δmig2Δnrg1 treble-deletion strain.*** We constructed the Δ*mig1*Δ*mig2*Δ*nrg1* strain by the deletion of the *NRG1* gene in the Δ*mig1*Δ*mig2* double-deletion strain. The treble-deletion strain was isolated by the gene replacement method in the same way as that used for the Δ*nrg1* single-deletion strain. ***(v) Strains overexpressing Mit1p and Prm1p.*** Constitutively expression Mit1p in yeast, the *MIT1* gene was cloned by PCR using GS115 genome as template with *Eco*RI-MIT1-5 and MIT1-3-XhoI primers. Then the resulting fragment was digested by *Eco*RI + *Xho*I and subsequently inserted into pGAPZA (Invitrogen) and resulted the plasmid pGGM1. Subsequently the pGAP-MIT1 fragment was realized from pGGM1 by *Bgl*II +*Xho*I, and subsequently inserted into *Bgl*II and *Xho*I of pPIC6A (Invitrogen), resulting plasmid pP6GM1. Then the pP6GM1 was linearized by *Bln*I and then transformed to WT, Δ*mig1*Δ*mig2* and Δ*mig1*Δ*mig2*Δ*nrg1* by electroporation, respectively. The transformants were screened by 3 mg/ml blasticidin, resulting strains designated WT-Mit1, Δ*mig1*Δ*mig2*-Mit1 and Δ*mig1*Δ*mig2*Δ*nrg1*-Mit1. The yeast strain constitutively expressing Prm1p was constructed as follows. The *PRM1* gene was amplified from GS115 genome with *Sfu*I-PRM1-5 + PRM1-3-*Xho*I primers. After digested by *Sfu*I and *Xho*I, the fragment was inserted into *Sfu*I and *Xho*I of

pGAPZA, resulting pGGP1. The pGGP1 was linearized by *Bln*I and then transformed to WT strain by electroporation. The transformants were screened by 0.1 mg/ml zeocin, resulting strains designated WT-Prm1. ***(vi) GFP and insulin precursor expression strains.*** To easily describe the following procedure, we then named the strain of Δ*mig1*Δ*mig2*Δ*nrg1*-Mit1 as MF1, based on which, the following strains were constructed. Firstly, pP-GFP plasmid[2] was linearized by *Sal*I and subsequently transformed into *P. pastoris* MF1. The transformants were screened on YND plates without histidine and then on YNM plates. The Mut^+ strains were selected and named as *P. pastoris* MF1-GFP. Similarly, a strain which expressing GFP in Δ*mig1*Δ*mig2* was constructed and named as Δ*mig1*Δ*mig2*-Mit1-GFP. Then the insulin precursor (IP) plasmid pPIC9K/IP, which was constructed previously[5], was linearized by *Pme*I and transformed into *P. pastoris* MF1. The Mut^+ transformants which could grow on the maximum concentration of 0.5, 1, 1.5, 2, 3, 4, 5 and 6 mg/mL G418 respectively were screened. Strains with the maximum production of IP in 500-mL baffled shake flask induced by glycerol were selected and designated as *P. pastoris* MF1-IP.

References

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Primer	Sequence[a]
MI1U1	5-CCGGAATTCGCCGACCTTATTCTTGGAAG-3
MI1U2	5-CGCGGATCCGATAAAAAAAATTTTATGGT-3
MI1D1	5-ACGCGTCGACCTACTCCTCCGACAATGTTG-3
MI1D2	5-ACATGCATGCTTCGGTCACTGATGGGTTGG-3
Zeo1	5-CGCGGATCCCCCACACACCATAGCT-3
Zeo2	5-ACGCGTCGACTAGTCCTGTCGGGTTT-3
MI2U1	5-CCGGAATTCAGCATGTCAAATGTGAACGT-3
MI2U2	5-CGCGGATCCTGCGAAGTATCTATTGGTGA-3
MI2D1	5-ACGCGTCGACTTGTGACATATAAGTTTGTT-3
MI2D2	5-ACATGCATGCGCAGTTGCCCGTAATCAGAC-3
Kan1	5-CGCGGATCCAAGGAGATGGCGCCCAAC-3
Kan2	5-ACGCGTCGACACTTGAAGTCGGACAGT-3
NRG15F	5-CGAGCTCCTGTGCCTATTACCCCCCTT-3
NRG15R	5-TCCCCCGGGAACAGATAACCAAAACGGACG-3
NRG13F	5-GCTCTAGAGTATTTATTTACGGATTGGA-3
NRG13R	5-ACATGCATGCTTCGGCTCCTCTTTCCCA-3
Hyg1	5-TCCCCCGGGAGCTTGCCTTGTCCCCGCCG-3
Hyg2	5-GCTCTAGATCGACACTGGATGGCGGCGT-3
MIG1F	5-AAGGCGGCCGCTACCACTGCCTATCC-3
MIG1R	5-ATGGTCGACTTATTTCTTTTCTTGATTTTC-3
MIG2F	5-AAAGCGGCCGCTCTACTACTGCTCCCC-3
MIG2R	5-GGGGTCGACTTAAGAGTCCGAGTTCATG-3
GFP1	5’-TATTTCGAACCATGGGATCTAAAGGTGAAGAATTATTC-3’
GFP2	5’-AGAGCGGCCGCCACCTTTGTACAATTCATC-3’
EcoRI-MIT1-5	5-CCGGAATTCACCATGGGTAGTACCGCAGCCCCAAT-3
MIT1-3-XhoI	5-CCGCTCGAGCTATTCTTCAACATTCCAGTA-3
SfuI-PRM1-5	5-CAACTATTTCGAAACCATGGGTCCTCCTAAACATCGGCTG-3
PRM1-3-XhoI	5-CCGCTCGAGTTAACTGTCAAAATTTATTGTATCT-3

Table S1. Primers used in this study. [a] Relevant restriction sites are underlined.

Plasmid	Characteristic(s)	Source or reference
pUC18	Ampicillin^R; *E. coli* subcloning vector	Invitrogen
pUC18-MIG1-del	pUC18 derivative carrying the Pp*MIG1* deletion cassette	This study
pUC18-MIG2-del	pUC18 derivative carrying the Pp*MIG2* deletion cassette	This study
pPIC3.5K	Ampicillin^R G418^R; P_{AOX1}-based expression vector	Invitrogen
pPICZ A	Zeocin^R; P_{AOX1}-based expression vector	Invitrogen
pRDM054	Ampicillin^R bleomycin^R hygromycin^R	2
pGAPZα A	Zeocin^R; P_{GAP}-based expression vector	Invitrogen
pGLM1	pGAPZα A derivative carrying the *GFP-MIG1* fusion gene	This study
pGLM2	pGAPZα A derivative carrying the *GFP-MIG2* fusion gene	This study
pGGM1	pGAPZA derivative carrying P_{GAP}-Mit1 expression cassette	This study
pPIC6A	Blasticidin^R; P_{AOX1}-based expression vector	Invitrogen
pP6GM1	pPIC6A derivative carrying P_{GAP}-Mit1 expression cassette	This study
pGGP1	pGAPZA derivative carrying P_{GAP}-Prm1 expression cassette	This study
pP-GFP	pPIC3.5K derivative carrying P_{AOX1}-GFP expression cassette	This study
pPIC9K/IP	pPIC9K derivative carrying P_{AOX1}-(insulin precursor) expression cassette	This study

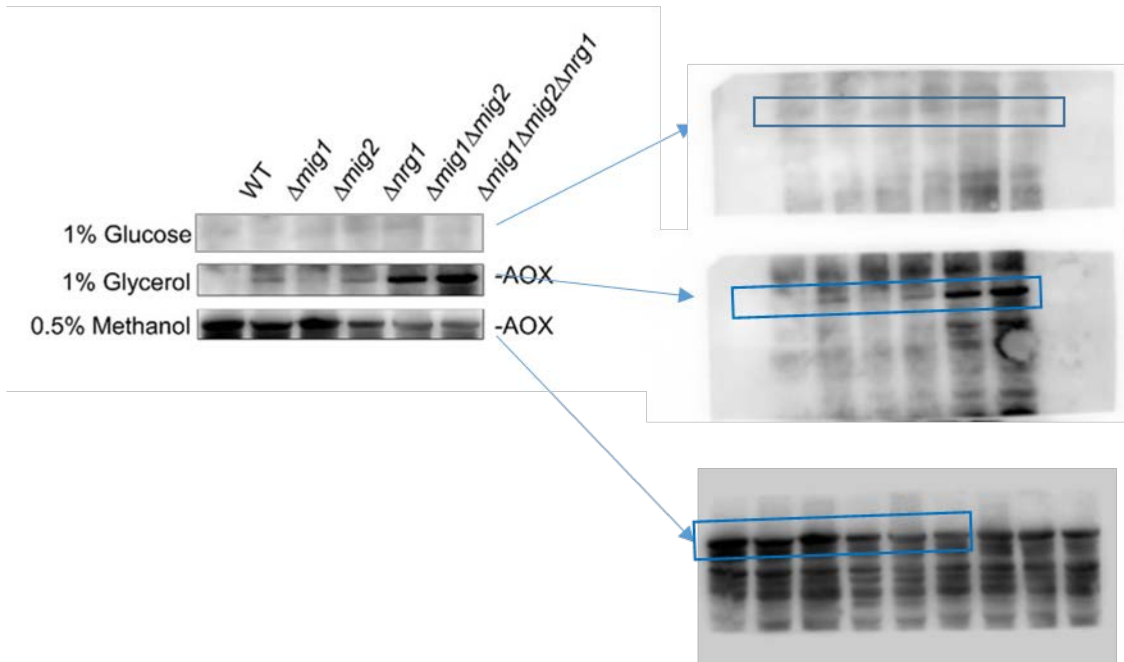
Table S2. Plasmids used in this study. ^{R}, resistance to indicated antimicrobial agent.

Primer	Sequence
RT-ACT1F	5-CTCCAATGAACCCAAAGTCCAAC-3
RT-ACT1R	5-GACAAAACGGCCTGAATAGAAAC-3
RT-MIT1F	5-GACTAATGACGATGAACTAAG-3
RT-MIT1R	5-TGCTGTTGTTGGTAGAAT-3
RT-PRM1F	5-CGAACTTGATGATGAGAACA-3
RT-PRM1R	5-CATTGGCTATTCCTGAACTG-3
RT-MXR1F	5-ATGCTGCTGATGCTATGA-3
RT-MXR1R	5-GCGGTCTGAATCGTTATTAC-3

Table S3. Sequence of gene-specific PCR primers used in the study.

Figure S1. Alignment of Mig proteins in *Saccharomyces cerevisiae* and *P. pastoris*.

Original gels/blots for Figure 1C



Original gels/blots for Figure 2C

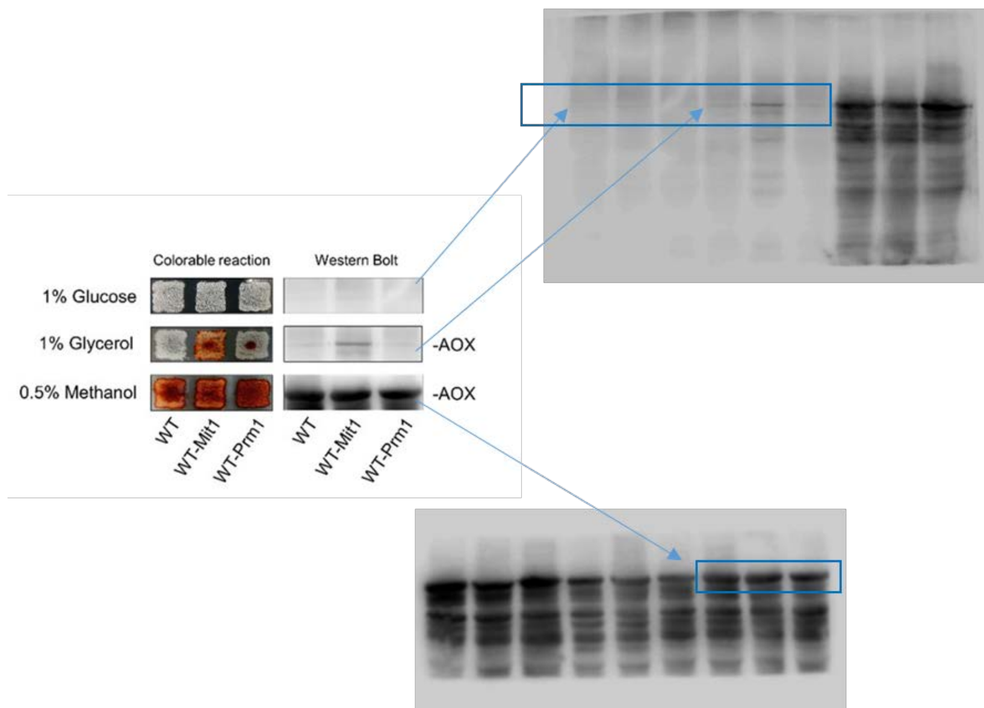


Figure S2. Original gels and blots for figure results (Figure 1C and Figure 2B) shown in the manuscript.

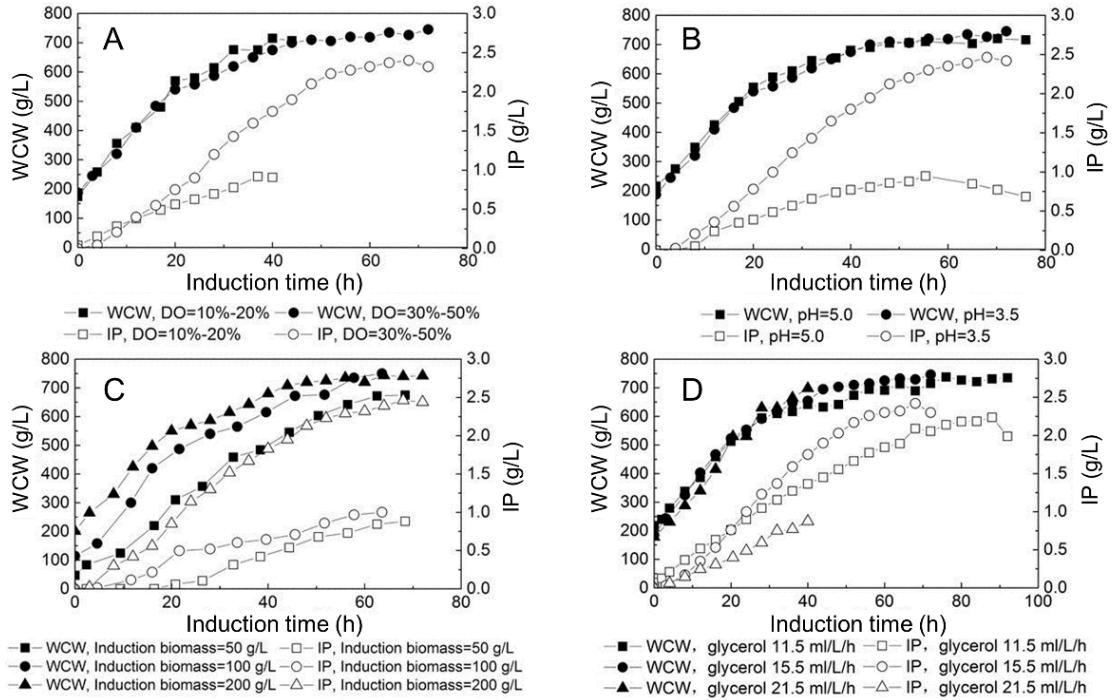


Figure S3. **Effects of DO, pH, induction biomass and induction feeding rate of glycerol on cell growth and insulin precursor expression of the MF1-IP strain.** (**A**) DO; (**B**) pH; (**C**) Induction biomass; (**D**) Glycerol feeding rate.