Supplementary information for

Engineering O-Glycosylation in modified N-linked oligosaccharides ($Man_{12}GlcNAc_2$~$Man_{16}GlcNAc_2$) *Pichia pastoris* strain

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Experimental section

Construction of *PMT1* insertional inactivation plasmid (*⊿pmt1*-pYES2)

(1). Construction of pYES2-URA3-AOX1TT plasmid.

The DNA sequence of complete AOX1TT gene was derived from the pPIC9 sequence and employed to design primers (Table SII) used for polymerase chain reaction (PCR) amplifications. The primers were synthesized by Sangonbiotech. PCR were performed with a Biometra PCR Amplifier using 2.5 unit of LA Taq DNA polymerase, 10 μg mL^{-1} of template DNA (pPIC9 plasmid), 0.4 mM of each deoxynucleotide triphosphate (dNTP), and 0.4 μM of the corresponding synthetic nucleotide primer (AOX1TT-5 and AOX1TT-3). The amplified DNA fragments were digested with Mlu I, inserted in pYES2-URA3 plasmid linearized by the same restriction enzymes to form pYES2-URA3-AOX1TT, and subsequently transformed into the *E. coli* DH5α competent cell. The transformants were selected on Luria-Bertani/Ampicillin (LB/Amp) plates (1% tryptone, 0.5% yeast extract, 1%sodium chloride, 1.5% agar and 0.01% Amp).

(2). Construction of *⊿pmt1*-pYES2 plasmid.

The DNA sequence of complete CYC1TT gene was derived from the pGE-GAP sequence and employed to design primers (Table S II) used for PCR amplifications. The primers were synthesized by Sangonbiotech. PCR were performed with a Biometra PCR Amplifier using 2.5 unit of LA Taq DNA polymerase, 10 μg mL^{-1} of template DNA (pGE-GAP plasmid), 0.4 mM of dNTP, and 0.4 μM of the corresponding synthetic nucleotide primer (CYC1TT-5 and CYC1TT-3).

Chromosomal DNA (1 mg mL^{-1}) of *P. pastoris* was extracted from yeast strain GS115 with a yeast genomic DNA kit and used for PCR. The DNA sequence of *PMT1* gene (63-900 bp) was derived from Gene bank (*PMT1*: CCA38772.1) and employed to design primers (Table S II) used for PCR amplifications. The primers were synthesized by Sangonbiotech. PCR were performed with a Biometra PCR Amplifier using 2.5 unit of LA Taq DNA polymerase, 10 μg mL^{-1} of template DNA, 0.4 mM of dNTP, and 0.4 μM of the corresponding synthetic nucleotide primer (*pmt1*-in-5 and *pmt1*-in-3).

Overlapped PCR was performed to fusion of *PMT1* gene (63-900 bp) and CYC1TT, 100 μL of reactions containing 2.5 unit of LA Taq DNA polymerase, 10 μg mL^{-1} of *PMT1*gene (63-900 bp) and CYC1TT gene, 0.4 mM of dNTP, and 0.4 μM of the corresponding synthetic nucleotide primer (*pmt1*-in-5 and CYC1TT-3). The amplified DNA fragments were digested with NsiI, inserted in pYES2-URA3-AOXTT plasmid linearized by the same restriction enzymes to form *⊿pmt1*-pYES2 recombinant plasmids, and subsequently transformed into the *E. coli* DH5α competent cell. The transformants were selected on LB/Amp plates.

Construction of *PMT5* gene deletion plasmid (*⊿ pmt5*-pYES2)

The DNA sequence of complete *PMT5* gene was derived from Gene bank (SCV11822.1) and employed to design primers (Table S II) used for PCR amplifications. The primers were synthesized by Sangonbiotech. PCR were performed with a Biometra PCR Amplifier using 2.5 unit of LA Taq DNA polymerase, 10 μg mL^{-1} of Chromosomal DNA, 0.4 mM of dNTP. For amplification of *PMT5* gene upstream and downstream, 0.4 μM primers of *pmt5*-5' arm-5 and *pmt5*-5' arm-3, and *pmt5*-3' arm-5 and *pmt5*-3' arm-3 were added into PCR reactions,

respectively.

Overlapped PCR was performed to fusion of *PMT5* gene upstream and downstream, 100 μL of reactions containing 2.5 unit of LA Taq DNA polymerase, 10 μg mL^{-1} of *PMT5* gene upstream and downstream, 0.4 mM of dNTP, and 0.4 μM of the corresponding synthetic nucleotide primer (*pmt5*-5' arm-5 and *pmt5*-3' arm-3). The amplified DNA fragments were digested with Mlu I, inserted in pYES2-URA3 plasmid linearized by the same restriction enzymes to form *⊿pmt5*-pYES2 recombinant plasmids, and subsequently transformed into the *E. coli* DH5α competent cell. The transformants were selected on LB/Amp plates.

Expression and purification of Anti Her-2 antibody

For construction of GJK15-HL (*Δoch1, Δpmt5*), pPIC9-HL recombinant plasmids linearized by SalI, and then electrotransformed into GJK15 (*Δoch1, Δpmt5*) competent cell, the transformants were selected on MD+UR plates (1.5% agar, 1%YNB, 1% glucose, 4×10^{-5} % biotin, 0.1% uracil and 0.01% arginine).

For expression of anti Her-2 antibody, GJK01-HL (*Δoch1*), GJK11-HL (*Δoch1, Δpmt1*), GJK15-HL (*Δoch1, Δpmt5*) was incubated in 100 mL of YPD+U at 25 °C for 36 h, respectively, at which point culture was added into BMGY+U (2% tryptone, 1% yeast extract, 1% YNB, 0.1% uracil, 4×10^{-5} % biotin, 1% glycerol and 100mM of PB, pH～6.0) at the final concentration of 5%. After incubation for 24 h, 0.5% methanol was added each 12 h, cell growth was continued for an additional 48 h (25 °C, 200 rpm), and then culture supernatant was collected by centrifugation (10 min, 8000 g/min).

Culture supernatant containing anti Her-2 antibodies from GJK01-HL (*Δoch1*), GJK11-HL (*Δoch1, Δpmt1*) and GJK15-HL (*Δoch1, Δpmt5*) were purified with a HiTrap rProtein A FF column, and eluted with citric acid buffer (500 mM, pH 3.0). Fractions containing anti Her-2 antibodies were adjusted pH to 9.0 by 1M of Tris-HCl. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (goat anti human IgG polyclonal antibody) were performed to confirm the purified proteins. Proteins concentrations were evaluated with a BCA protein quantitation kit, and the proteins were stored at -80 °C.

Comparison of growth rate of GJK01-HL (*Δoch1*), GJK11-HL (*Δoch1, Δpmt1*) and GJK15-HL (*Δoch1, Δpmt5*)

A desired strain was incubated 5 days in 2.5 mL of YPD+U medium. After growth 5 days (25 °C, 200 rpm), the culture was diluted to OD_{580} = 30, and then were 1:20 diluted with fresh BMGY+U medium typically to a total volume of 5 mL. The culture was collected and measured OD_{580} at point of 24, 60, 96, 120, 144, 168 and192 h, respectively.

Table SI. Strains and plasmids

Strain /plasmid	Relevant characteristics	Source/reference
Strains		
GS115	*Pichia pastoris* parent strain (*Δhis4*)	Invitrogen
GJK01	GS115 (*Δoch1, Δhis4, Δarg4, Δura3*)	[1]
GJK01-HL	GJK01strain with pPIC9-HL plasmid	[2]
GJK11-HL	GJK01-HL (*Δpmt1*)	This study
GJK15	GJK01 (*Δpmt5*)	This study
GJK15-HL	GJK15 strain with pPIC9-HL plasmid	This study
E.coli DH5α	Host for plasmid reproduction	Cwbiotech
Plasmids		
pYES2-URA3	Yeast overexpression vector containing *ura3* gene for selection	[3]
pYES2-URA3-AOX1TT	pYES2-URA3 plasmid with AOX1TT to terminate URA3 expression.	This study
Δpmt1-pYES2	pYES2-URA3-AOX1TT plasmid with *pmt1* (31-900 bp) and CYC1TT fusion sequence.	This study
Δpmt5-pYES2	pYES2-URA3 plasmid with *pmt5*-5' arm and *pmt5*-3' arm fusion sequence.	This study
pGE-GAP	Yeast overexpression vector containing CYC1TT used as template for PCR amplification of CYC1TT.	This study
pPIC9	Yeast overexpression vector containing his4 gene for selection, and AOX1 promotor and α-factor for production of secreted protein, and AOX1TT for PCR amplification of CYC1TT.	Invitrogen
pPIC9-HL	pPIC9 plasmid with antiHer-2 antibody genes.	[2]

Table SII. The primers used to clone and identify genes in this study.

Name	Direction	Primer sequence (5'→3')
AOX1TT-5	Forward	TCTACGCGTCCTTAGACATGACTGTTCCTCAGT
AOX1TT-3	Reverse	TCTACGCGTAAGCTTGCACAAACGAACTTC
CYC1TT--5	Forward	GCTTTCTTAGTCGTCCCCACTCTGATCTAATGATAGT TAATGACTAATAGATCATGTAATTAGTTATGTCA
CYC1TT-3	Reverse	GCAAATTAAAGCCTTCGAGCGTC
pmt1-in-5	Forward	TCTATGCATTAATGATAGTTAATGACTAATAGAGTA AAACAAGTCCTCAAGAGGT
pmt1-in-3	Reverse	TGACATAACTAATTACATGATCTATTAGTCATTAAC TATCATTAGATCAGAGTGGGGACGACTAAGAAAGC
pmt1-ORF-OUT-5	Forward	AAGACCCATGCCGAACACGAC
pmt1-ORF-OUT-3	Reverse	GCTCTGAGGCACCTTGGGTAA
pmt5-5' arm-5	Forward	TCTACGCGTGGGCAATGGAAACCCTGAAAC
pmt5-5' arm-3	Reverse	ATTCTAGCTCAAGCCCTCTTAGCGGCCGCCATTTCC ACCGCCGACTCATT
pmt5-3' arm-5	Forward	AATGAGTCGGCGGTGGAAATGGCGGCCGCTAAGAG GGCTTGAGCTAGAAT
pmt5-3' arm-3	Reverse	AGAACGCGTCTTTAGCGAAACCACTGACCT
pmt5-OUT-5	Forward	CCCGATTTGACACCAGACAGT
pmt5-OUT-3	Reverse	AAAGCCTGGTAATCACAAACC
pmt5-ORF-5	Forward	GAAGAAACCCGAAAGCCCTAA
pmt5-ORF-3	Reverse	ATACATGGCCGCCTTTGAGTA
pmt5-ORF-OUT-5	Forward	GCCAGCCATATCAGATAACAC
pmt5-ORF-OUT-3	Reverse	TCTGAGGGAAGGTTGCCGTGT

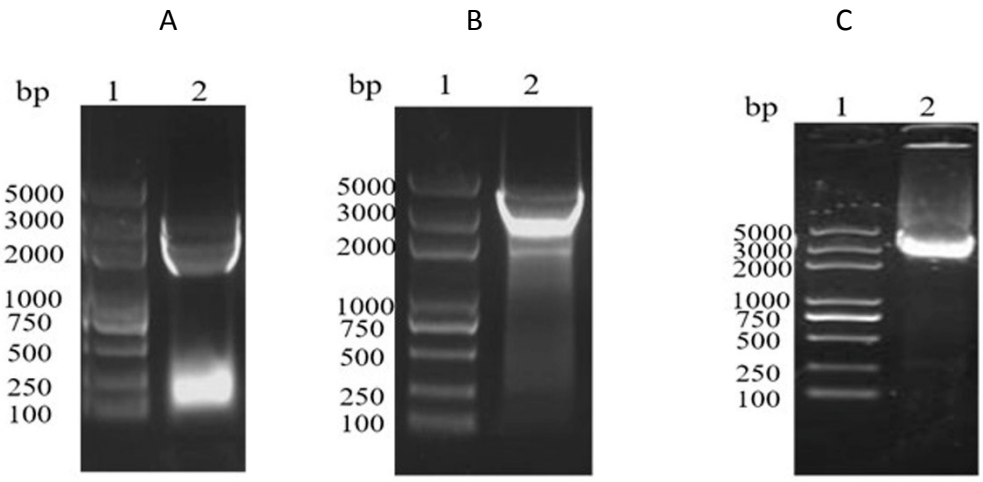

Figure. S1. PCR amplification of *pmt5* gene upstream (A, molecular marker, lane 1, *pmt5* gene upstream 1.7 kb, lane 2) and downstream flanking fragments (B, molecular marker, lane 1, *pmt5* gene downstream 2.1 kb, lane 2) and fusion of them (C, molecular marker, lane 1, fusion fragment3.8 kb, lane 2).

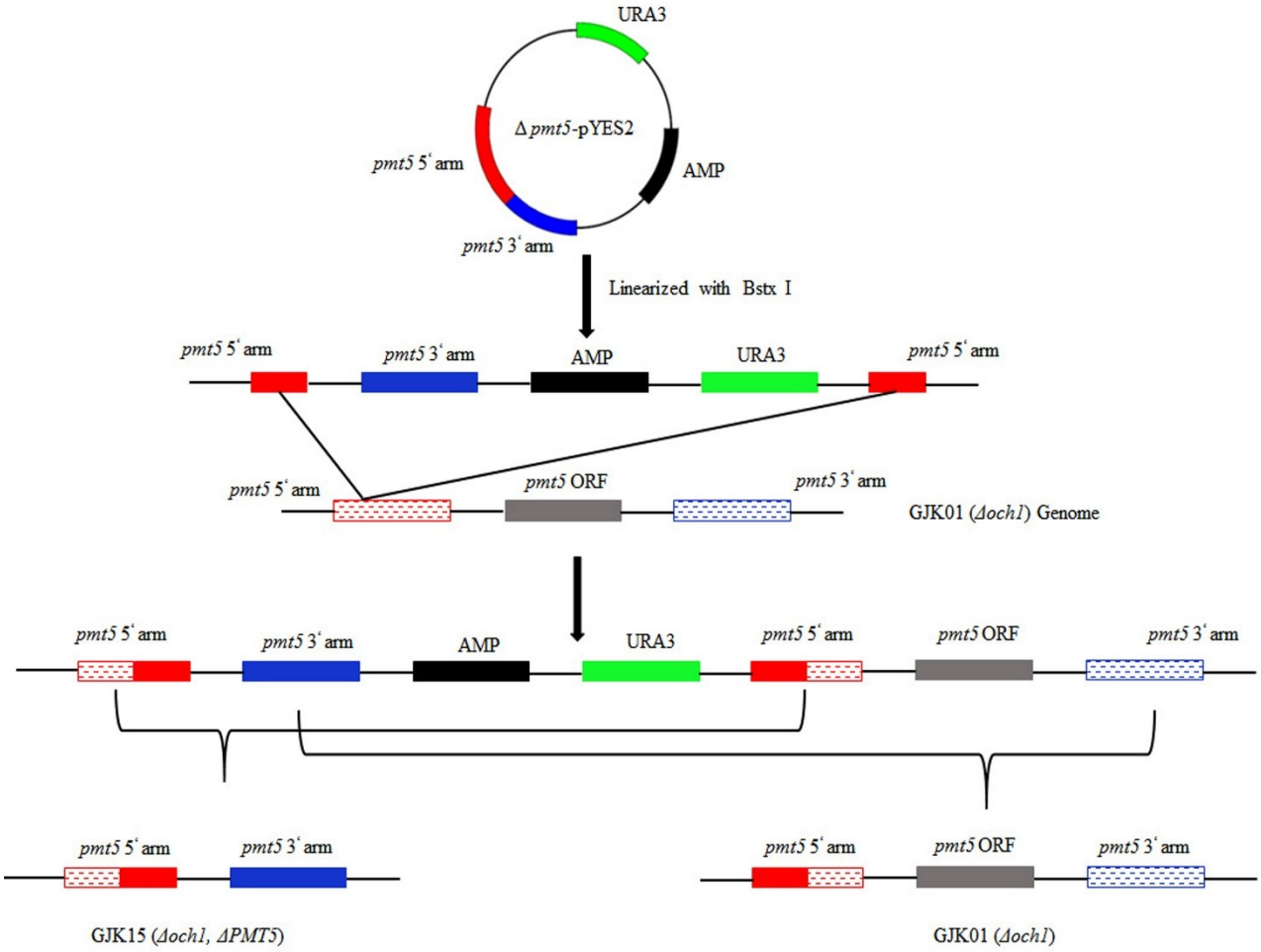

Figure. S2. *PMT5* gene knockout strategy-two-step homologous recombinant. Upon digestion *PMT5* 5 flanking region (5' arm) of *Δpmt5*-pYES2 with BstxI and transformation in GJK01(*Δoch1*), the construct integrates at the *PMT5* 5' arm. The first recombinant that was selected on MD+RH plates. The second recombinant was selected on MD+URH containing 5-Fluoroorotic Acid, two equivalent type strains were created, the *PMT5* deletion strains GJK15 (*Δoch1, Δpmt5*) or wild-type strains GJK01 (*Δoch1*).

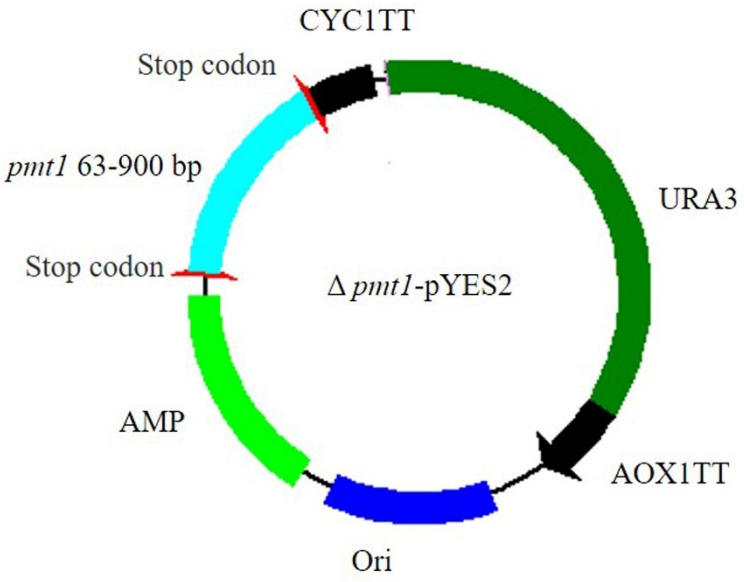

Figure. S3. Construction of *⊿pmt1*-pYES2.

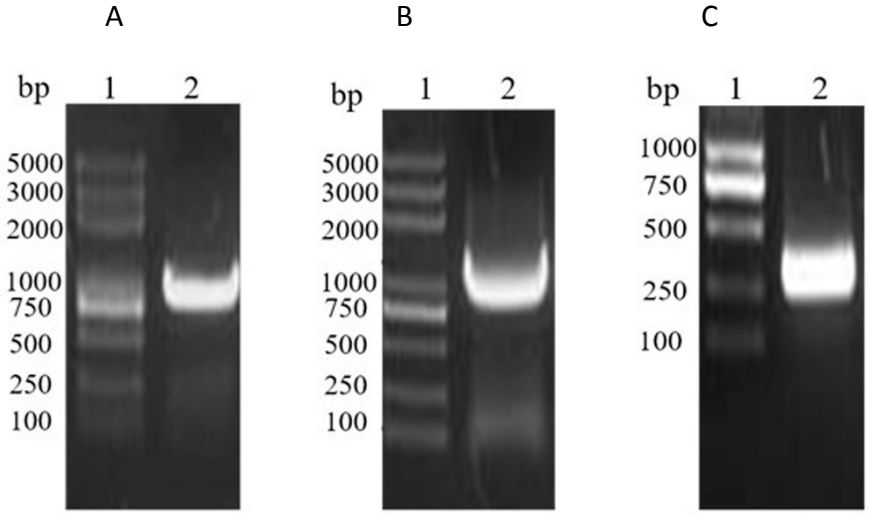

Figure. S4. PCR amplification of *pmt1* gene (A, molecular marker, lane 1, *pmt1* gene 837 bp, lane 2), CYC1TT (B, molecular marker, lane 1, CYC1TT 272 bp, lane 2) and fusion of them (C, molecular marker, lane 1, fusion of *pmt1* gene and CYC1TT 1.1 kb, lane 2).

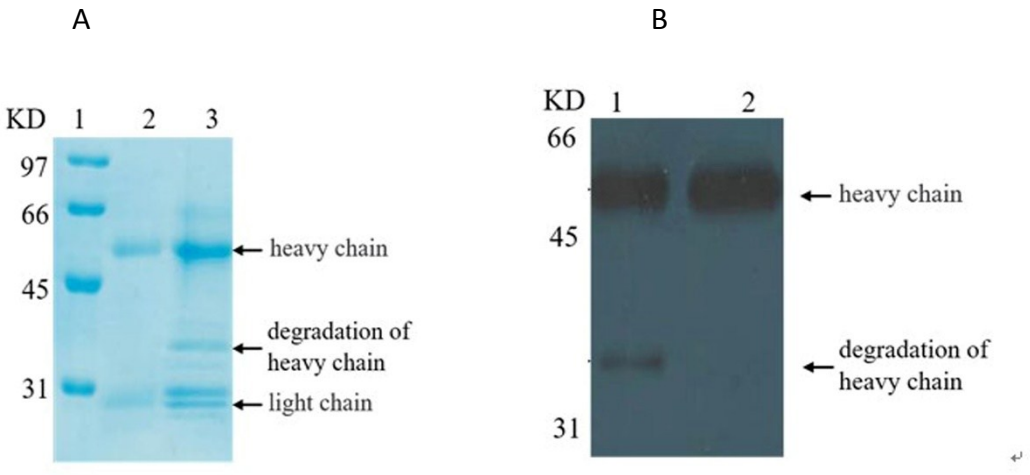

Figure. S5. (A) SDS-PAGE results of purified anti Her-2 antibody expressed by GJK15-HL (*Δoch1, Δpmt5*) (lane 3), human IgG standard (lane 2) and molecular marker (lane 1); and (B) western blot results of purified anti Her-2 antibody expressed by GJK15-HL (*Δoch1, Δpmt5*) (lane 1), human IgG standard (lane 2), stained with goat anti-human IgG Fc polyclonal antibody.

Table SIII. Comparison of anti-Her-2 antibody O-glycans from GJK01-HL (*Δoch1*) and GJK11-HL (*Δoch1, Δpmt1*)

Proteins	IOD	IOD/human IgG standard
RNB (10μg)	54607.67±12872.61	1.09±0.06
human IgG standard (10μg)	49777.67±8840.56	/
PNGase F+GJK01-HL (*Δoch1*) (10μg)	217706.9±22942.82	4.43±0.43
GJK01-HL (*Δoch1*) (10μg)	295924.2±41638.15	5.99±0.29
PNGase F +GJK11-HL (*Δoch1, Δpmt1*) (20μg)	154167.7±34839.09	3.09±0.34
GJK11-HL (*Δoch1, Δpmt1*) (20μg)	184287.1±34693.48	3.70±0.14

References:

1.Wang, Y., Gong, X., Chang, S., Liu, B., Song, M., Huang, H. and Wu, J., Chinese Journal of Biotechnology, 2007, 907-914.
2.Wang, L., Zhang, W. and Wu, J., Letters in biotechehnology, 2008, 843-847.
3.Wang, L., Liu, B., Gong, X., Chang, S., Wang, L., Song, M., Xu, W. and Wu, J., China Biotechnology, 2009, 44-49.