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Engineering the protein secretory pathway of *Saccharomyces cerevisiae* enables improved protein production

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Baker's yeast *Saccharomyces cerevisiae* is one of the most important and widely used cell factories for recombinant protein production. Many strategies have been applied to engineer this yeast for improving its protein production capacity, but productivity is still relatively low, and with increasing market demand, it is important to identify new gene targets, especially targets that have synergistic effects with previously identified targets. Despite improved protein production, previous studies rarely focused on processes associated with intracellular protein retention. Here we identified genetic modifications involved in the secretory and trafficking pathways, the histone deacetylase complex, and carbohydrate metabolic processes as targets for improving protein secretion in yeast. Especially modifications on the endosome-to-Golgi trafficking was found to effectively reduce protein retention besides increasing protein secretion. Through combinatorial genetic manipulations of several of the newly identified gene targets, we enhanced the protein production capacity of yeast by more than fivefold, and the best engineered strains could produce 2.5 g/L of a fungal α -amylase with less than 10% of the recombinant protein retained within the cells, using fed-batch cultivation.

cell engineering | protein secretion | yeast cell factories | intracellular protein retention | endosome-to-Golgi trafficking

Recombinant protein production by cell factories has been at the center of the biotech industry since it was established with the introduction of recombinant insulin and human growth hormone in the 1980s. Today, the market size of biopharmaceutical proteins and industrial enzymes reached hundreds of billions and tens of billions of US dollars per year worldwide, respectively (1, 2). The key for success in this industry is constant improvement of the protein production capacity of the applied cell factories. Although many organisms can be used as cell factories for protein production, some expression systems, such as *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, and Chinese hamster ovary cells, are preferred in consideration of quality, quantity, and cost of produced proteins (3, 4).

S. cerevisiae is widely used for production of biopharmaceutical proteins, but it is also used as a model organism for studying the protein secretory pathway (5–7). Continuous efforts to improve the yeast's protein production capacity has not only resulted in better cell factories for protein production, but also increased our understanding of the entire protein secretory pathway (8, 9). Given the model organism status of *S. cerevisiae*, knowledge and experience generated based on *S. cerevisiae* are also valuable to provide guidelines to engineer other cell factories for efficient protein production. However, full understanding of the underlying mechanisms of the molecular and genetic interactions underpinning protein secretion are still lacking. Therefore, identification of new gene targets, especially targets that have synergistic effects with previously identified targets, is desirable to further engineer this cell factory for efficient protein production (10, 11). Although

previous studies suggested various strategies for improving protein production at different levels including transcription, translation, posttranslational processes, etc. (12–17), they rarely focused on processes associated with intracellular retention of the protein in the secretory pathway.

In our earlier work (18), we isolated mutant yeast strains with increased protein secretion from yeast UV mutagenesis libraries through microfluidic droplet screening. Mutations in these mutant strains were identified through whole genome sequencing and classified according to the processes which they were involved in. As increased protein secretion of these mutant strains was caused by mutation in specific genes, these genes can potentially be used as metabolic engineering targets for the design of cell factories with improved protein secretory capacity. Single deletion of some of these targets was shown to have a positive effect on protein production (18), but not all targets identified were evaluated, and possible synergistic effects of these mutations were also not evaluated. Here we therefore used this study to identify several targets for genetic modifications, and we demonstrated that many of these targets have synergistic effects. Thus, through engineering yeast at these targets we could reduce protein retention and significantly increase protein secretion in yeast resulting in the production of 2.5 g/L of a fungal α -amylase in a fed-batch cultivation.

Significance

Recombinant proteins production by cell factories for medical and industrial use is one of the most prominent achievements in the modern biotech industry. It is constantly necessary to explore the underlying mechanisms of protein secretion to identify new targets for design and construction of improved cell factories. Here, we identified several gene targets, most of which are involved in the trafficking and secretory pathways, that could improve protein production by *Saccharomyces cerevisiae* to the gram per liter level. We also found that intracellular retention of recombinant proteins can be significantly reduced by engineering the endosome-to-Golgi trafficking. Genes and pathways identified here may provide general guidelines for engineering other cell factories for efficient protein secretion.

Author contributions: M.H. and J.N. designed research; M.H., G.W., and J.Q. performed research; M.H. and J.N. analyzed data; D.P. and J.N. supervised the study; and M.H., D.P., and J.N. wrote the paper.

Conflict of interest statement: M.H. and J.N. are partial applicants of a patent application entitled "Fungal cell with improved protein production capacity". The remaining authors declare no competing financial interests.

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Results and Discussion

Evaluation of Target Genes on Protein Secretion and Retention.

Based on a mutated gene list obtained from our previous study (18), genes involved in the secretory and trafficking pathways (such as *ECM3*, *EMC1*, *ERV29*, *GOS1*, *VPS5*, *TDA3*, *COG5*, and *SNC2*), genes with similar functions appearing in different strains (*HDA2* and *HDA3*) and genes with nonsense mutation from enriched GO terms (such as *TAN1* from tRNA processing, *PGM2* from the carbohydrate metabolic process, and *PXA1* from lipid transport) (*SI Appendix*, Fig. S1) were selected for evaluation of their association with protein secretion and retention using single gene deletions. To enable an initial screening of these different targets, we used the BY4742 strain background for which there is a single gene-deletion library available, but consistent with our earlier study we used amylase as a model protein. Amylase production varied in the BY4742 strains with single gene deletion with some having increased amylase secretion and others having reduced amylase secretion compared with the reference strain (*SI Appendix*, Fig. S2B). Besides changes in the amylase yield, intracellular amylase proportion was also found to be altered by gene deletion (*SI Appendix*, Fig. S2C). These results indicated that identified mutant genes involved in the secretory pathway and from enriched GO terms affected protein production and could be used as potential engineering targets for improving protein production.

Engineering of Endosome-to-Golgi Trafficking and the Histone Deacetylase Complex Increases Protein Secretion.

To explore these genes for cell factory construction, the four genes (*HDA2*, *VPS5*, *GOS1*, and *TDA3*) in which deletion led to the highest improvement in amylase production in the BY4742 strain background were chosen for further investigation in the CEN.PK strain background, which is the strain background used in our previous studies (8, 18). Single gene deletions of *HDA2*, *VPS5*, and *TDA3* also increased amylase secretion in the CEN.PK strain background (Fig. 1A). To test whether there was additivity in the enhancing effect on amylase secretion of the target genes, combinatorial gene deletions were performed. As *GOS1* deletion has a significant positive effect on amylase secretion in the BY4742 strain background, it was also included as a gene target in the combinatorial gene-deletion studies. Compared with the control strain K01, the amounts of secreted amylase after 96 h in tube fermentation increased fourfold and 4.5-fold in the triple gene-deletion strain K10 ($\Delta hda2$, $\Delta vps5$, and $\Delta tda3$) and the quadruple gene-deletion strain K11 ($\Delta hda2$, $\Delta vps5$, $\Delta tda3$ and $\Delta gos1$), respectively (Fig. 1A). The total amount of amylase production (secretion and intracellular retention) by strains with combinatorial gene deletion was presented in *SI Appendix*, Fig. S3. Furthermore, the final cell dry weight of the strains K10 and K11 were found to be slightly lower than that of the control strain K01 (Fig. 1C).

Hda2p is a subunit of the *HDA1* histone deacetylase complex, which is involved in the *TUP1*-specific repression and global deacetylation in yeast (19, 20). The likely mechanism for the beneficial effect of the *hda2* deletion on protein production was the release of *TUP1* repression, which has been shown to increase amylase production (8). *Gos1p* is involved in Golgi transport, and the deletion of *GOS1* reduces efficiency of retrograde Golgi traffic (21), which might have a beneficial effect on amylase secretion. Previously, balanced trafficking between the endoplasmic reticulum (ER) and the Golgi was shown to improve protein secretion (22), but clearly anterograde trafficking in the Golgi also represents a potential pathway to be engineered for achieving balanced trafficking within the Golgi.

The deletion of *VPS5* significantly reduced intracellular amylase retention, and only around 10% of totally produced amylase was retained intracellularly in the engineered strains with the

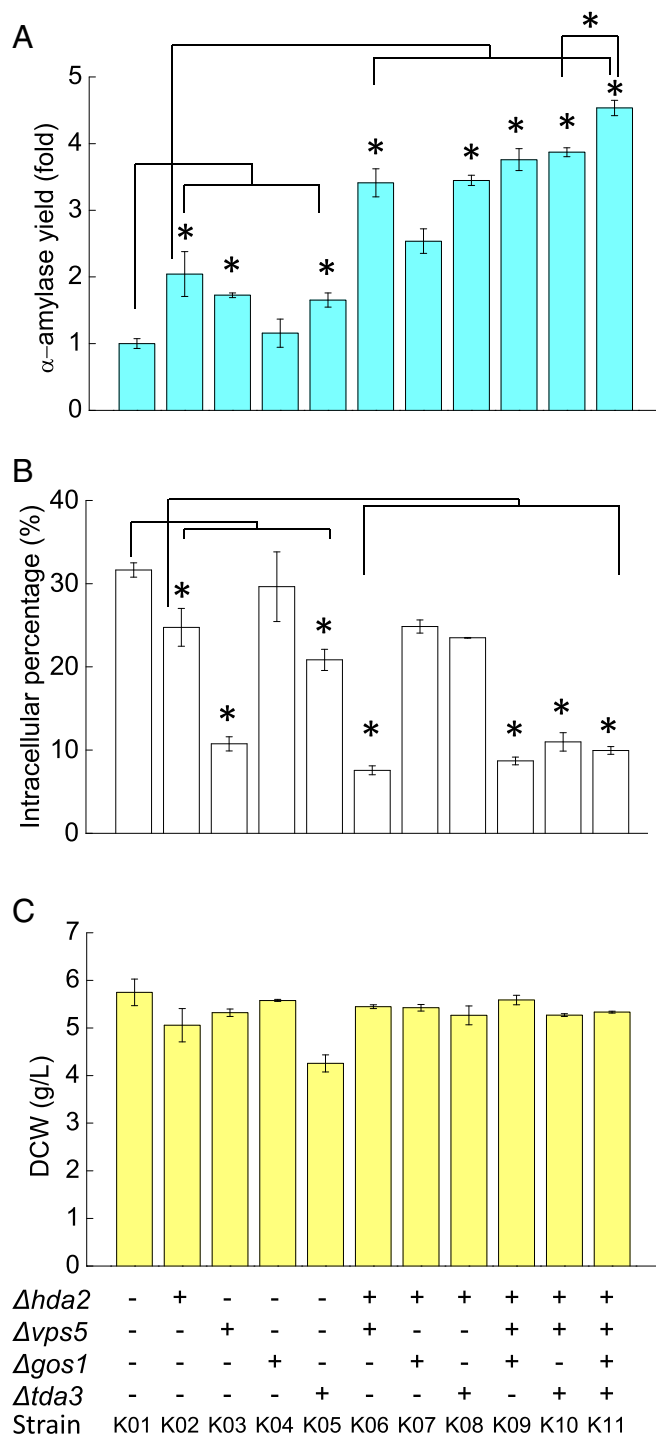


Fig. 1. Combinatorial gene deletions improve amylase secretion in a CEN.PK strain. (A) α -Amylase yield of engineered strains. (B) Intracellular α -amylase retention of engineered strains. (C) Biomass of engineered strains. Strains were cultivated in an SD-2xSCAA medium at 30 °C for 96 h for α -amylase production. Data shown are mean values \pm SDs of biological duplicates of single clones. The statistical significance was determined by a two-tailed homoscedastic (equal variance) *t* test and indicated with an asterisk if $P < 0.05$.

vps5 deletion (Fig. 1B). According to the literature, *Vps5p* forms a retromer subcomplex with *Vps17p* and interacts with another subcomplex, which consists of three proteins (*Vps26p*, *Vps29p*, and *Vps35p*), all involved in protein trafficking from the late

endosome to the late Golgi (23, 24) (Fig. 2A). Due to the interaction with a protein with a known effect on amylase secretion, the effects on amylase secretion by deletion of *VPS17* was also tested. A similar result to the effects of the *vps5* deletion was observed with both increased amylase yield and decreased intracellular amylase retention found in the *vps17* deletion strain after 96 h cultivation (Fig. 2B and C). The total amount of amylase produced by the *vps17* deletion strain was also similar to the *vps5* mutant strain (SI Appendix, Fig. S4). Increased amylase production as a consequence of both *vps5* and *vps17* deletions revealed the importance of trafficking between the Golgi apparatus

and the endosome in protein secretion. In a previous study, Vps10p was shown to have a shortened lifetime in *vps5* mutants (25). We therefore also constructed a *vps10* mutant strain and measured extracellular and intracellular amylase. The result showed that deletion of *vps10* reduced amylase retention (SI Appendix, Fig. S5), yet the protein secretion improvement was less than what was observed for the *vps5* mutant. This indicated that a disrupted retromer complex can improve protein secretion not only through *vps10* degradation, but also through other pathways. Another of our target genes, *TDA3*, is also involved in transport from the late endosome to the Golgi apparatus. Tda3p regulates endosomal protein trafficking of specific proteins (e.g., Kex2p and Yif1p) through negative regulation of Btn2p (SI Appendix, Fig. S6A), and deletion of *TDA3* has been shown to result in stronger Kex2p association with the Golgi apparatus (26). As the α factor leader sequence used for guiding amylase secretion contains a Kex2 cutting site (27), the amylase maturation in the Golgi requires Kex2p processing to remove the profragment (SI Appendix, Fig. S6B). Increased amylase secretion in the *TDA3* deletion strain was likely the result of more available Kex2p in the Golgi for amylase maturation. This was confirmed by overexpression of *BTN2*, which also increased amylase secretion (SI Appendix, Fig. S6C and D), whereas overexpression of *TDA3* did not significantly reduce amylase production (SI Appendix, Fig. S6C and D).

Strengthening of Anterograde Trafficking Increases Protein Secretion.

Two genes of the gene deletions found from our initial screening in the BY4742 strain background, namely, *ERV29* and *COG5*, reduced rather than increased amylase secretion (18). Erv29p is a transmembrane receptor for the incorporation of certain soluble cargo proteins into COPII-coated vesicles (28). The proregion of an α -factor signal peptide can mediate the cargo protein into COPII vesicles via Erv29p-dependent transport (29, 30). Cog5p is a subunit of the conserved oligomeric Golgi complex, which mediates retrograde vesicles trafficking to the Golgi and within the Golgi (31). This led us to speculate that overexpression rather than deletion of *ERV29* and *COG5* might increase amylase secretion, which we tested in the CEN.PK strain. As shown in the SI Appendix, Fig. S7, single gene overexpression of *COG5* and *ERV29* improved amylase secretion and/or decreased the intracellular amylase retention. As amylase was led by the α -factor signal peptide for secretion in this study, it is likely that the amylase changed in the *ERV29* overexpressed strain was related to enhancement of the Erv29p-dependent transport. This was in agreement with the decreased amylase production in the *erv29* deletion strain, which lost the Erv29p-dependent transport (SI Appendix, Fig. S2A). Overexpression of *COG5* might strengthen retrograde vesicles trafficking, which was beneficial for amylase production. However, combinatorial overexpression of the two genes (*ERV29* + *COG5*) only decreased intracellular amylase retention without a concomitant increase in amylase secretion. A possible explanation for the lack of increase in amylase secretion in the combined overexpressed strains is that the plasmid-based multiple gene overexpression imposed an extra burden on cells which led to the competition for resources with the amylase production. A similar phenomenon was observed in a previous study where single gene overexpression had a better performance than multiple gene overexpression for enhancing protein production (32).

Promotion of Protein Secretion by Multiple Genetic Manipulations.

To reduce the metabolic burden and increase cell stability for recombinant protein production, strong promoter replacement was applied for overexpression of the target genes instead of plasmid-based overexpression. As the looping out of the *amdS* marker gene from the quadruple gene-deletion strain K11 (deletions of *HDA2*, *VPS5*, *TDA3*, and *GOS1*) was not successful for unknown reasons, promoter replacement was performed on the

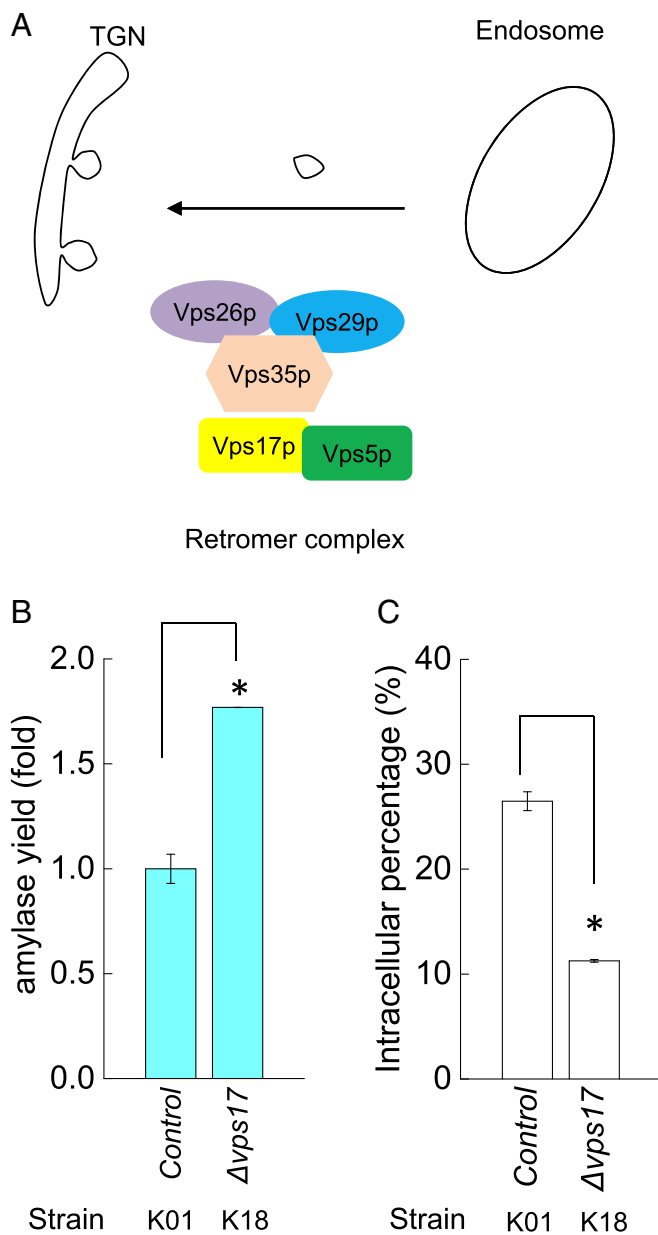


Fig. 2. The retromer complex affects amylase secretion. (A) Schematic of the retromer complex. (B) Amylase yield of the *vps17* deletion strain. Strain names are in the bottom line. (C) Intracellular amylase retention of the *vps17* deletion strain. Strains were cultivated in a SD-2xSCAA medium at 30 °C for 96 h for α -amylase production. Data shown are mean values \pm SDs of biological duplicates of single clones. The statistical significance was determined by a two-tailed homoscedastic (equal variance) *t* test and indicated with an asterisk if $P < 0.05$.

triple gene-deletion strain K30 (deletion of *HDA2*, *VPS5*, and *TDA3*) instead. Compared with strain K30, amylase secretion increased by 12% in the strain K13 where the native *COG5* promoter was replaced with a strong *PGK1p* promoter (Fig. 3A). However, replacement of the native *ERV29* promoter with the strong *TEF1p* promoter in strain K12 did not increase amylase production in the triple gene-deletion strain K30.

In our previous study (18), we found that chromosome III was duplicated in several mutant strains, including in the best protein secretion mutant strain B184. The *PDII* gene, which plays an important role in assisting the protein folding in the ER (33) and was shown to increase protein secretion when overexpressed (16, 34), is located on chromosome III. As amylase production was increased by *PDII* overexpression (*SI Appendix*, Fig. S8), elevation of *PDII* expression in the mutant strains was likely contributing to increased amylase secretion. We were interested in whether our gene modifications had additive effects on the overexpression of *PDII* and if the amylase production capacity of the engineered strains could be further increased. Therefore, both promoter replacement and gene integration were tested for the *PDII* gene. As deletion of *GOS1* had positive effects on the protein secretion (Fig. 1A), the position chosen for the integration of one copy of the *PDII* gene was the *GOS1* locus. The integration of *PDII* was thus achieved through replacement of *GOS1*. As shown in Fig. 3A, the overexpression of *PDII* had an additive effect on the enhancement of amylase secretion with the other gene modifications. An integration of *TEF1p-PDII* on the *GOS1* locus (strain K17) showed a 24% increase in amylase production, compared with strain K13. The levels of intracellular amylase retention remained low in all of the engineered strains with different combinatorial modifications (Fig. 3B). The biomass of the best engineered strain K17 was, however, lower than the reference strain (Fig. 3C). The total amount of amylase produced by strains with multiple genetic manipulations was shown in *SI Appendix*, Fig. S9.

Identified Genes Show General Positive Effects on Protein Secretion.

To test whether the selected genetic modifications have a general positive effect on protein secretion in addition to the positive effect on amylase secretion, another heterologous protein, glucan 1,4- α -glucosidase was expressed in the engineered strain CEN.PK 530-1CK303, which was generated by removing the amylase expression plasmid from strain K17 and contained optimized combinatorial gene modifications for amylase production (*SI Appendix*, Fig. S10). A higher glucan 1,4- α -glucosidase yield was achieved in the engineered strain compared with the reference strain. This result supported the hypothesis that the identified gene targets had a general positive effect on protein production by yeast.

Currently, many strains used for protein production in industry are already engineered or mutated. Therefore, it is interesting to evaluate newly identified gene targets not only using the reference strain, but also in engineered strains. We therefore evaluated if two of the identified gene targets, i.e., *VPS5* and *TDA3* involved in the endosome-to-Golgi trafficking pathway, could further improve protein secretion by strain B184, which we earlier generated by UV mutation and selection (18). As shown in *SI Appendix*, Fig. S11, the strain B184 with a *vps5* mutant or/and a *tda3* mutant had a higher amylase yield and less amylase intracellular retention. These results supported our findings that identified gene targets have a wide application on cell engineering for protein production.

Protein Production of the Best Engineered Strain K17 on the Bioreactor Scale.

To explore the industrial applicability of the engineered strain with selected gene modifications, the protein production capacity of the best engineered strain K17 was verified using batch fermentations in a bioreactor with the strain K01 used as a

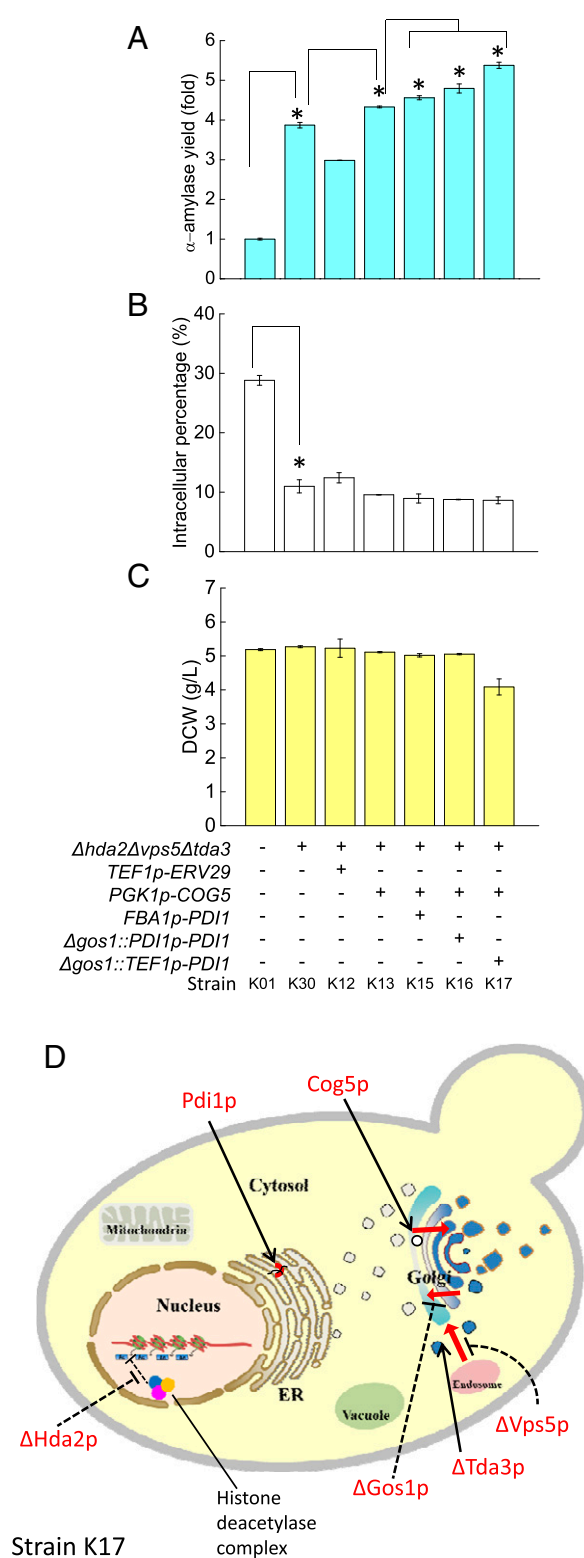


Fig. 3. Combinatorial engineering of strains with multiple genetic manipulations. (A) α -Amylase yield of engineered strains with gene deletions, promoter replacements, and gene integrations. (B) Intracellular α -amylase retention of engineered strains. (C) Biomass of engineered strains. (D) Schematic showing engineered target genes in the best strain K17. Strains were cultivated in a SD-2xSCAA medium at 30 °C for 96 h for α -amylase production. Data shown are mean values \pm SDs of biological duplicates of single clones. The statistical significance was determined by a two-tailed homoscedastic (equal variance) *t* test and indicated with an asterisk if $P < 0.05$.

reference. Strain K17 showed higher amylase yield throughout the whole fermentation process compared with the reference strain (Fig. 4A and B). Furthermore, the intracellular amylase

retention of strain K17 remained at a significantly lower level compared with the reference (Fig. 4C). Strain K17 had a reduced final biomass yield (Fig. 4D), which was in agreement

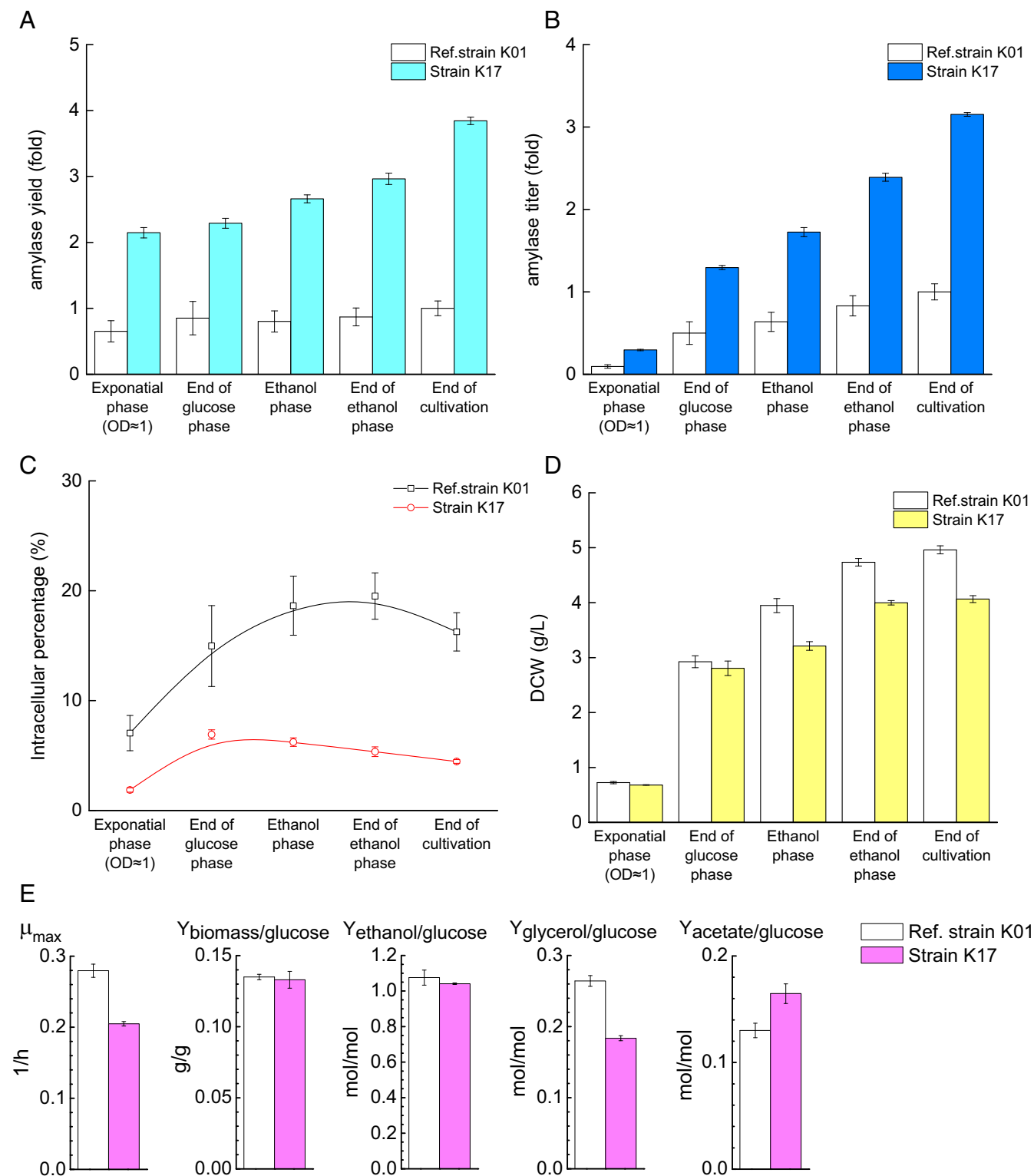


Fig. 4. Physiological characterization of the best amylase production strain K17 and the reference strain K01 in batch cultivation with a bioreactor system. The conditions in the bioreactor were controlled at 30 °C, 600 rpm agitation, 30 L/h airflow, and pH = 6 for cell growth. A SD-2×SCAA medium was used, and an initial OD₆₀₀ of cells was set at 0.01. α -Amylase yield (A), α -amylase titer (B), intracellular α -amylase retention (C), biomass (D), and physiological parameters (E). μ_{max} : maximum specific growth rate; $Y_{x/glucose}$: biomass or products yield from glucose. Data shown are mean values \pm SDs of biological triplicates of single clones.

with the previous finding that decreased biomass formation was favorable for protein production (8). A lower maximum specific growth rate and a lower glycerol production on glucose were found for strain K17 (Fig. 4E). Strain K17 also had a lower specific glucose uptake rate, a lower specific ethanol production rate, and a lower specific glycerol production rate (*SI Appendix, Fig. S12*). The lower specific glucose uptake rate of strain K17 resulted in a lower growth rate and decreasing overflow metabolism, which should lead to a higher biomass yield (35). In contrast, a reduced final biomass yield in strain K17 was observed, suggesting that resources were redirected from biomass formation to amylase production.

As the industrial application of *S. cerevisiae* for protein production is often performed using fed-batch processes (36), the protein production capacity of the best strain K17 was also explored in a fed-batch culture. Up to 98.5 g dry cell weight/L (g-DCW/L) of cell density was achieved at the end of a 156 h cultivation period with a total of 327 g/L glucose consumed (key process values shown in *SI Appendix, Fig. S13*). The final α -amylase titer reached 2.5 g/L (Fig. 5), which is a very high protein titer. Also, the intracellular α -amylase retention remained at a low level (with the peak value of 12% and below 10% for the majority of time) during the whole process. Medium feeding was controlled based on setting the growth rate and dissolved oxygen level to avoid an overflow metabolism, and virtually no ethanol accumulated during the feeding phase. Surprisingly, glycerol accumulated gradually with the

medium feeding and reached a peak value at around 108 h after which it was consumed. Strain K17 maintained a relatively stable specific amylase productivity (0.4–0.5 mg/g DCW·h) during the feeding phase but decreased during the last day (*SI Appendix, Fig. S14*). The biomass concentration kept increasing during the last day, and the cell density reached >90 g DCW/L. Decreased protein productivity in the last day suggested that the culture environment was unable to fully support cells releasing their potential for heterologous protein production as nutrient and oxygen demands for the cells basic metabolic consumption increased at a higher cell density.

Conclusion

To summarize, our work identified several targets involved in the secretory and trafficking pathways and histone deacetylase complex for the improvement of protein secretion. Our study demonstrates that deletions of *VPS5* and *VPS17* substantially enhance heterologous protein secretion and reduces intracellular heterologous protein retention. The involvement of both of these genes in the transport between Golgi and endosome suggests that this pathway is a promising avenue for further exploration in the design of efficient cell factories for protein production. Our results also indicate that, although gene overexpression may help to ease bottlenecks in protein production, it may also compete for resources with the production of the target protein. It was therefore important to balance benefits in the enhancement of

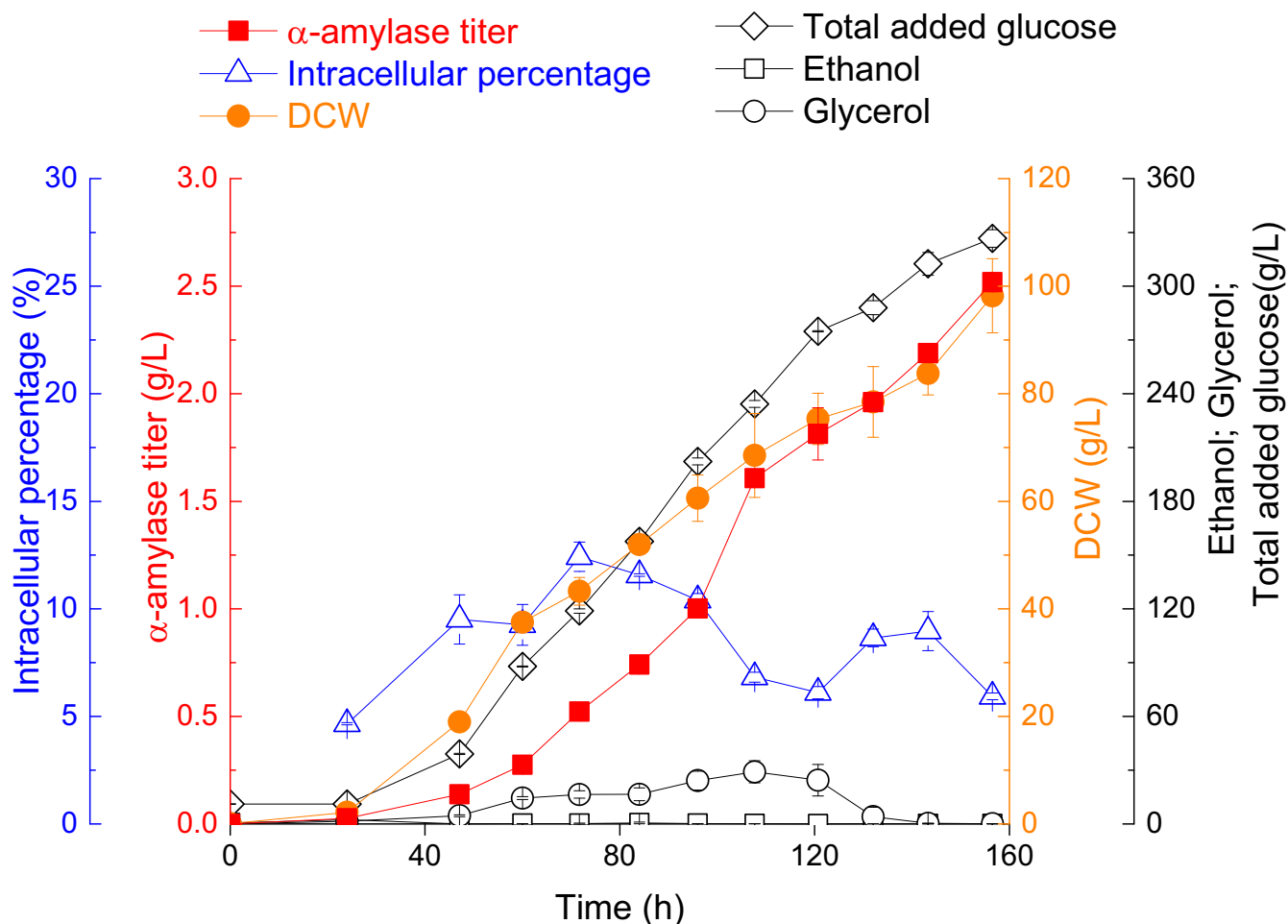


Fig. 5. Fed-batch cultivation of the best engineered strain K17. Cells were cultured in a SD-2×SCAA medium with condensed medium feeding. Detailed control parameters for the fed-batch cultivation are given in *Methods*. Data shown are mean values \pm SDs of biological duplicates of single clones.

protein production with this competition for resources, which can be achieved by controlling gene overexpression levels. Both batch and fed-batch cultivation results of strain K17 confirmed that the protein secretion was substantially improved in the yeast strain with combinatorial modifications. Strain K17 exhibited protein production capacity with titers at the gram-liter level (2.5 g/L amylase) in fed-batch cultures, a big leap from the milligram-liter level through strain engineering. The engineered strain was able to adapt to the high density cultivation and showed a potential for industrial application. Modifications of the gene targets presented in this study had generalized enhancement effects on protein production and were compatible with additional genetic modifications. As a consequence, they can be used for engineering both wild type strains and existing engineered strains to promote protein production. Here, by measuring the distribution of intracellular and extracellular amylase, we also showed the effects of gene modifications on protein secretion from another angle. Protein intracellular retention can be used as a useful parameter to evaluate gene targets and engineer strains in future studies. It should be mentioned that, although the intracellular amount of protein was determined by the secretion capacity of the cell, other mechanisms including intracellular degradation and/or increased protein synthesis might also affect the protein retention and secretion. Moreover, as *S. cerevisiae* is a model organism, targets and engineering pathways identified here are most likely to provide general guidelines for the design and construction of other cell factories for efficient protein production.

Methods

Strains, Plasmids, and Primers Used in This Study. All strains and plasmids used in this study are listed in *SI Appendix, Table S1*. All primers used in this study are listed in *SI Appendix, Table S2*. Gene deletions, promoter replacements, and gene replacements in the CEN.PK strain were performed through homologous recombination by using *amdS* as a selection marker (37). Gene-deletion cassettes were amplified from the plasmid pUG-*amdSYM* for the *amdS* marker flanking with homologous regions to the target genes. Gene replacement cassettes or promoter replacement cassettes were constructed by fusion of the integrated gene or promoter with the *amdS* marker, and the fusion fragment (integrated gene plus *amdS* marker) was also flanked by homologous regions of the target genes. Transformants were selected on the SM-Ac plates for homologous recombination, and the counterselection for looping out the *amdS* marker was performed on the SM-Fac plates by following the procedures described previously (37). Genes for overexpression were amplified from the yeast genome and then inserted into the plasmid pSP-GM1 (*SI Appendix, Fig. S4D*). Cassettes or plasmids were introduced into the yeast by the standard LiAc/SS DNA/PEG transformation method (38).

Media and Culture Conditions. The YPD medium contains 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. The YPE medium contains 10 g/L yeast extract, 20 g/L peptone, 10 g/L ethanol, and 0.5 g/L glucose. The SD-ura medium contains a 6.9 g/L yeast nitrogen base without amino acids, a 770 mg/L complete supplement mixture (CSM, without uracil), and 20 g/L glucose. The *amdS* marker is selected by using the SM-Ac medium (37), which contains 3 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6 g/L acetamide, 6.6 g/L K_2SO_4 , 1 mL/L of a trace element solution, and 1 mL/L of a vitamin solution. Counterselection of the *amdS* marker is carried out on SM-Fac medium, which contains 3 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/L $(\text{NH}_4)_2\text{SO}_4$, 2.3 g/L fluoroacetamide, 1 mL/L of a trace element solution, and 1 mL/L of a vitamin solution. Twenty gram-liter agar are added to the media when preparing agar plates. For protein

production in tubes or shake flasks, yeast strains were cultured at 30 °C and 200 rpm for 96 h in the SD-2xSCAA medium (39, 40) containing 20 g/L glucose, 6.9 g/L yeast nitrogen base without amino acids, 190 mg/L Arg, 400 mg/L Asp, 1260 mg/L Glu, 130 mg/L Gly, 140 mg/L His, 290 mg/L Ile, 400 mg/L Leu, 440 mg/L Lys, 108 mg/L Met, 200 mg/L Phe, 220 mg/L Thr, 40 mg/L Trp, 52 mg/L Tyr, 380 mg/L Val, 1 g/L bovine serum albumin (BSA), 5.4 g/L Na_2HPO_4 , and 8.56 g/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (pH = 6.0 by NaOH). For bioreactor cultivation, 5.4 g/L Na_2HPO_4 and 8.56 g/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ in the SD-2xSCAA medium were replaced by 2 g/L KH_2PO_4 (pH = 6.0 by NaOH).

Protein Quantification. The α -amylase assay kit (Megazyme K-CERA) was used for quantification of the α -amylase activity and a commercial α -amylase from *Aspergillus oryzae* (Sigma-Aldrich) was used as the standard. The weight of α -amylase can be converted with 69.6 U/mg as the α -amylase conversion coefficient (27). Five hundred microliter cell cultures were centrifuged at $12,000 \times g$ for 3 min with the liquid phase used for the measurement of α -amylase in the supernatant and the cell pellet used for intracellular α -amylase measurements. The cell pellet was washed with distilled water and resuspended in 0.5 mL phosphate-buffered saline containing a 5 μL halt protease inhibitor mixture (Thermo Fisher). The cell suspension was added to a lysing matrix tube, and cell lysis was processed in a FastPrep-24 tissue and cell homogenizer (MP Biomedicals) at the speed of 6.5 m/s for 2 min. Cell debris was removed by centrifugation, and the supernatant fraction was used for α -amylase quantification. The glucan 1,4- α -glucosidase was measured by using Amyloglucosidase Assay Reagent (Megazyme).

Batch Fermentation and Fed-Batch Fermentation. For the bioreactor batch cultivation, 5.4 g/L Na_2HPO_4 and 8.56 g/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ in the SD-2xSCAA medium were replaced by 2 g/L KH_2PO_4 (pH = 6.0 by NaOH). Strains were first grown in seed cultures to the late exponential phase and then were used to inoculate 500 mL SD-2xSCAA medium in 1 L bioreactor vessels (DasGip) with an initial OD_{600} of 0.01. The bioreactor system was run at 30 °C, 600 rpm agitation, 30 L/h airflow, and pH = 6 (controlled by KOH). Biological experiments were conducted in triplicate.

For the fed-batch cultivation, a 200 mL SD-2xSCAA medium was first inoculated with seed cultures of strain K17 (5.4 g/L Na_2HPO_4 and 8.56 g/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ were replaced by 2 g/L KH_2PO_4) with an initial OD_{600} of 0.1. The bioreactor system was run at 30 °C, 600 rpm [1 L bioreactor vessels (DasGip)] as the initial agitation speed and increased to maximally 1200 rpm, 18 L/h as the initial airflow and increased to maximally 48 L/h, pH = 6 (maintained by using 4 M KOH and 2 M HCl), and the dissolved oxygen level was maintained above 30% by controlling agitation speed, airflow, and medium feeding. The low glucose 10x feed medium contained: 200 g/L glucose, 69 g/L yeast nitrogen base without amino acids, 50 g/L casamino acids (Formedium), 1 g/L BSA, and 20 g/L KH_2PO_4 (pH = 5 by KOH). For the high glucose 10x feed medium, 200 g/L glucose in a low glucose 10x feed medium was replaced with 600 g/L glucose. After the glucose and ethanol were consumed in the batch culture (200 mL SD-2xSCAA medium), the exponential feed was started by using the low glucose 10x feed medium and controlled at a specific growth rate of 0.08 h^{-1} . When both the agitation speed and the airflow reached the maximum values (1200 rpm and 48 L/h, respectively) [1 L bioreactor vessels (DasGip)], medium feeding was triggered by a dissolved oxygen level >25%. After feeding about 330 mL of the low glucose 10x feed medium, a high glucose 10x feed medium was used. Fermentation was stopped when 330 mL of the high glucose 10x feed medium was fed in the bioreactor. In total, 660 mL of the feeding medium were added to the bioreactor. Biological experiments on the fed-batch cultivation were conducted in duplicate.

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