

# Scalable protein production by *Komagataella phaffii* enabled by ARS plasmids and carbon source-based selection

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27 **Table S1. List of primers used in this study.**

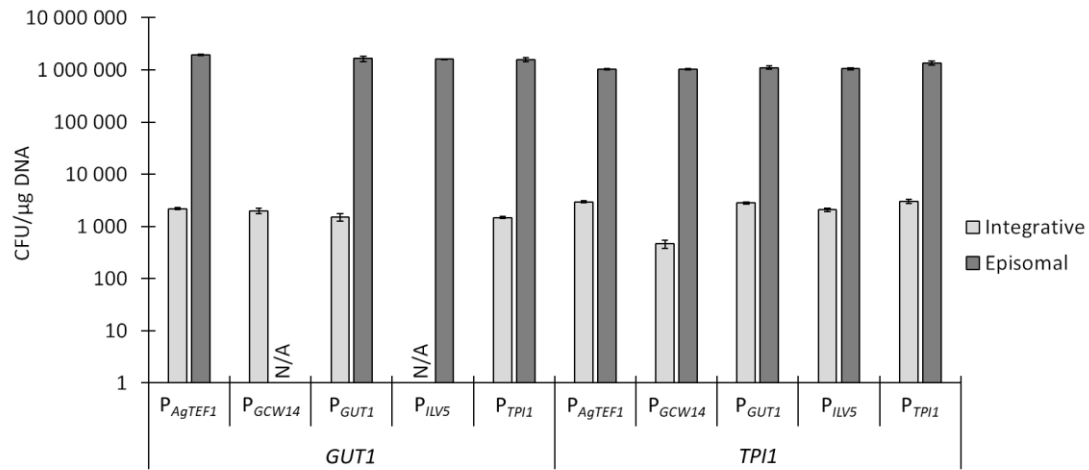
| Number    | Name                | Sequence 5' - 3'                                               |
|-----------|---------------------|----------------------------------------------------------------|
| Primer 1  | PARS1_PstI_AODTT    | GGATCTGATTACCTTAGCTGCAGTCGAGATAAGCTGGGGGAAC                    |
| Primer 2  | PARS1+KpnI_rnCTT    | CCTTAGCGAAAGCTAAGGATTTTTTTTAGGTACCTCGACAATTAATATTTACTTATTTTG   |
| Primer 3  | AOX1TT start rev    | GGCATTCTGACATCCTCTTG                                           |
| Primer 4  | CalB fwd            | TTGCCTTCAGGTTTCAGACCC                                          |
| Primer 5  | Dalpha_fwd_PDC      | ACTTGCTCTAGTCAAGACTTACAATTAAGAATTCATGAGATTCCCATCTATTTTCACCG    |
| Primer 6  | Dalpha_rev_CalB     | GAAGGCTGGGTCTGAACCTGAAGGCAAAGCTTCGGCCTCTCTCTC                  |
| Primer 7  | GUT1 fwd            | ATGGGAAAGGACTACACCCC                                           |
| Primer 8  | AODTT start rev     | CTCTCTAAATAATCGTAAGGTGTCAATTCC                                 |
| Primer 9  | TPI fwd             | ATGGTACGTGCATTGAGTCTCATAAG                                     |
| Primer 10 | TPI+NcoI_AODTT      | CTCTAAATAATCGTAAGGTGTCAATTCCATGGCTAGTTTCTGGAATTAATAATGTCCACG   |
| Primer 11 | PAgTEF1+SacI_AOX1TT | GATTAAGTGAGACCTTCGTTTGTGCGAGCTCAGCTTGCCTCGTCCCCGC              |
| Primer 12 | PAgTEF1+BamHI_GUT1  | CAAGAGGGGTGTAGTCCTTTCCCATGGATCCTTTTGTATTATGTTTCGGATGTGATGTGAG  |
| Primer 13 | PAgTEF1+BamHI_TPI1  | CTTATGAGACTCAATGCACGTACCATGGATCCTTTTGTATTATGTTTCGGATGTGATGTGAG |
| Primer 14 | PGCW14+SacI_AOX1TT  | GATTAAGTGAGACCTTCGTTTGTGCGAGCTCGGGTGAAAGCCAACCATCTTTG          |
| Primer 15 | PGCW14+BamHI_GUT1   | CAAGAGGGGTGTAGTCCTTTCCCATGGATCCTTTTGTGTTGAGTGAAGCGAGTG         |
| Primer 16 | PGCW14+BamHI_TPI1   | CACCTTATGAGACTCAATGCACGTACCATGGATCCTTTTGTGTTGAGTGAAGCGAGTG     |
| Primer 17 | PGUT1+SacI_AOX1TT   | GATTAAGTGAGACCTTCGTTTGTGCGAGCTCATACCGAAAGGTAAACAACCTTCGG       |
| Primer 18 | PGUT1+BamHI_GUT1    | CAAGAGGGGTGTAGTCCTTTCCCATGGATCCTATAGTAGATATATCTGTGGTATAGTGTG   |
| Primer 19 | PGUT1+BamHI_TPI1    | TTATGAGACTCAATGCACGTACCATGGATCCTATAGTAGATATATCTGTGGTATAGTGTG   |
| Primer 20 | PILV5+SacI_AOX1TT   | GATTAAGTGAGACCTTCGTTTGTGCGAGCTCTTCAGTAATGTCTTGTTCCTTTTGTTC     |
| Primer 21 | PILV5+BamHI_GUT1    | AAGAGGGGTGTAGTCCTTTCCCATGGATCCAAATTTTTTCGGATAATTTTTTAAAGCGAG   |
| Primer 22 | PILV5+BamHI_TPI1    | TATGAGACTCAATGCACGTACCATGGATCCAAATTTTTTCGGATAATTTTTTAAAGCGAG   |
| Primer 23 | PTPI1+SacI_AOX1TT   | GATTAAGTGAGACCTTCGTTTGTGCGAGCTCTCAACGAGACACTCTTCCGTC           |
| Primer 24 | PTPI1+BamHI_GUT1    | AAGAGGGGTGTAGTCCTTTCCCATGGATCCTGTGTTTGTGATAGATCTTGTATATCAATG   |
| Primer 25 | PTPI1+BamHI_TPI1    | TATGAGACTCAATGCACGTACCATGGATCCTGTGTTTGTGATAGATCTTGTATATCAATG   |
| Primer 26 | TPI check fwd       | CCCTCAAATGATGTCTCCTCTG                                         |
| Primer 27 | TPI check rev       | GAAGGAGATAATACGAGGGGTG                                         |
| Primer 28 | CalB sense          | CAGTTTTGGACGCAGGTTTGAC                                         |
| Primer 29 | CalB antisense      | GTTCCAGTACCTGGGACAAGC                                          |
| Primer 30 | ARG4 sense          | TCCTCCGGTGGCAGTTCTT                                            |
| Primer 31 | ARG4 antisense      | TCCATTGACTCCCGTTTTGAG                                          |
| Primer 32 | CalB antisense 2    | TGTGTCAATCCACCAGCGTTTC                                         |

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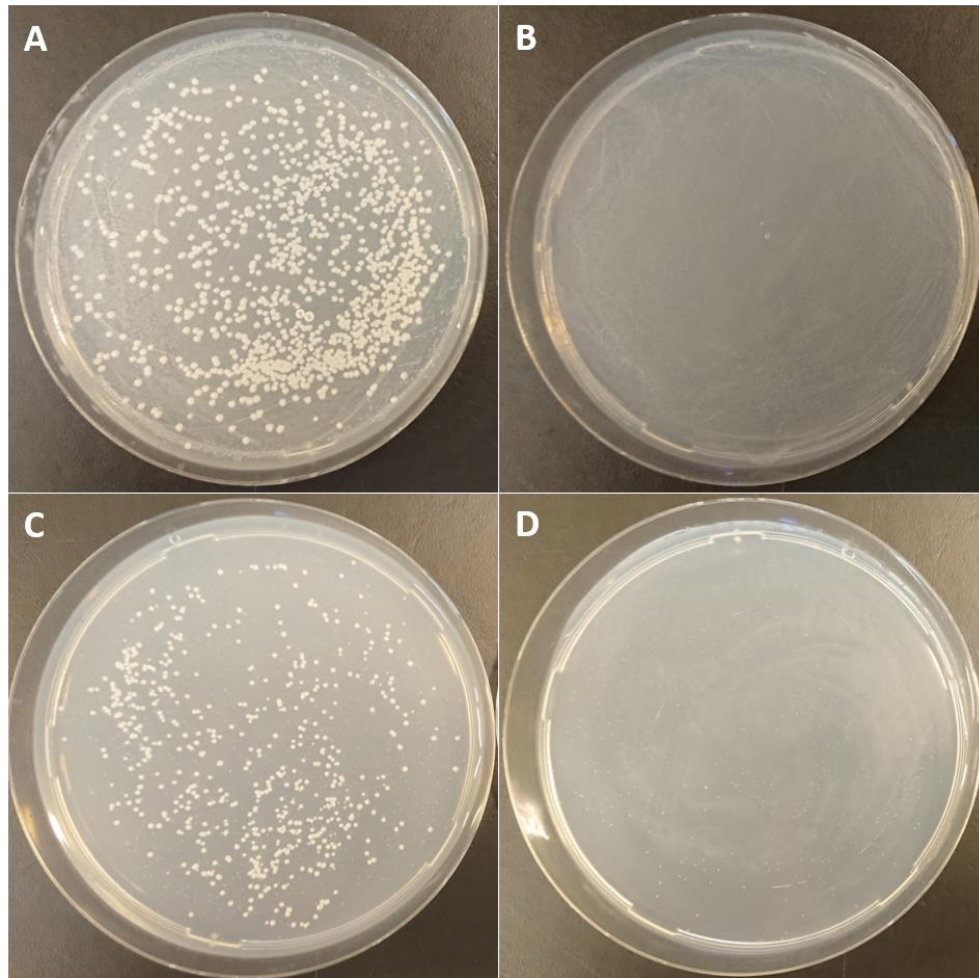
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30 **Table S2. Plating experiment for the determination of plasmid carrying cells.**

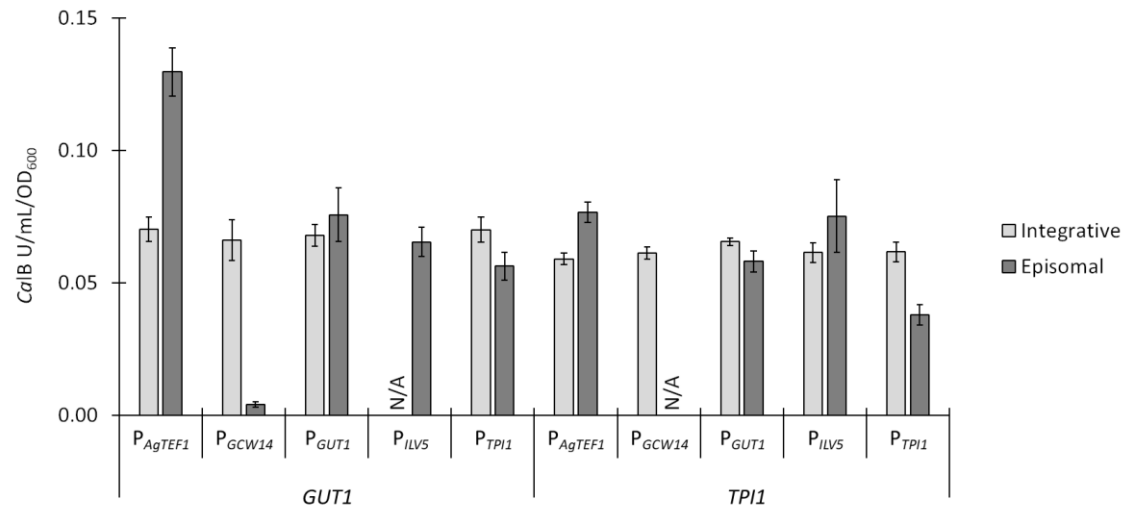
| Integrative |              |          |                    | Episomal |              |        |                    |
|-------------|--------------|----------|--------------------|----------|--------------|--------|--------------------|
| Sample      | Colony count |          | Cells with plasmid | Sample   | Colony count |        | Cells with plasmid |
|             | YPD          | BMG      |                    |          | YPD          | BMG    |                    |
| I0          | 207 ± 8      | 202 ± 3  | 97%                | E0       | 202 ± 14     | 45 ± 7 | 22%                |
| I1          | 47 ± 5       | 45 ± 7   | 97%                | E1       | 52 ± 4       | 6 ± 1  | 12%                |
| I4          | 219 ± 11     | 212 ± 6  | 97%                | E4       | 280 ± 30     | 20 ± 2 | 7%                 |
| I8          | 150 ± 13     | 147 ± 17 | 98%                | E8       | 85 ± 1       | 8 ± 3  | 9%                 |



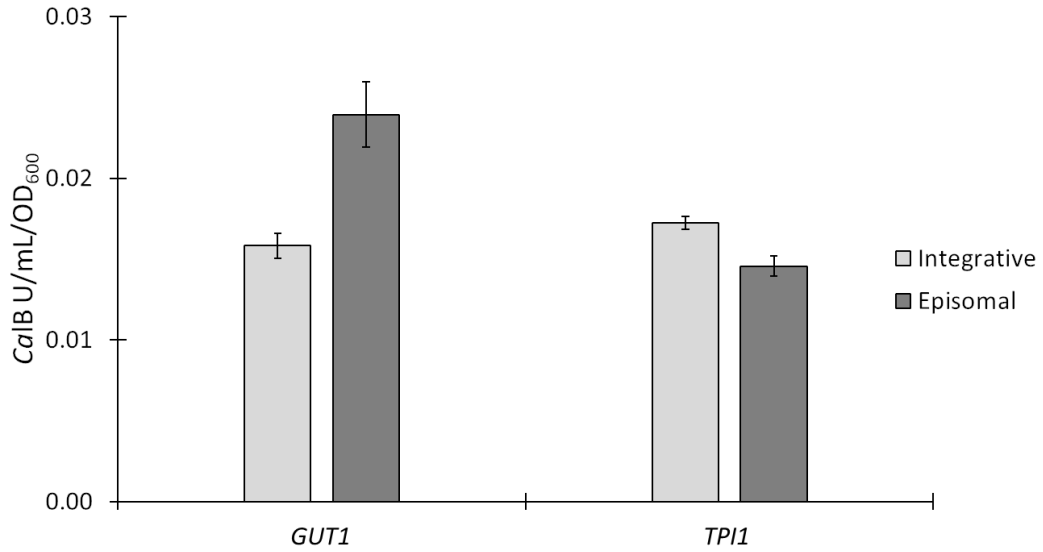
**Figure S1. Transformation efficiencies.** Integrative and episomal plasmids generated in this study were used to transform *K. phaffii* according to Lin-Cereghino *et al.* (2005). Transformations were plated on buffered minimal media plates containing 1 % glycerol as sole carbon source after 20 minutes of regeneration at 28 °C and 600 rpm agitation. Transformant selection was based on *TPI1* or *GUT1* marker systems. The obtained colonies per mL and μg of DNA were evaluated after 5 days of incubation at 28 °C.



**Figure S2: Pictures of transformation plates.** Pictures of BMG1 transformation plates were taken after 5d incubation at 28 °C. *K. phaffii* strain BSY11G1 was transformed with plasmid #6 (Table 2) (A) or plasmid #8 (B) and strain BSYBG11  $\Delta tpi1$  was transformed with plasmid 16 (C) and without DNA (D) according to Lin-Cereghino *et al.* 2005. The transformations were regenerated in BMG1 medium for 20 minutes and plated on BMG1 agar plates for selection of transformants. Image A and C represent selection plates with transformants that are clearly distinguishable from the background (*GUT1*- or *TPI1*-deficient strain without plasmid). Image B shows a transformation plate with non-visible or non-distinguishable transformant colonies. Image D represents a negative control of *TPI1*-plasmid transformants with reduced background compared to *GUT1* transformation plates (small white dots are tiny air bubbles that occur during incubation).

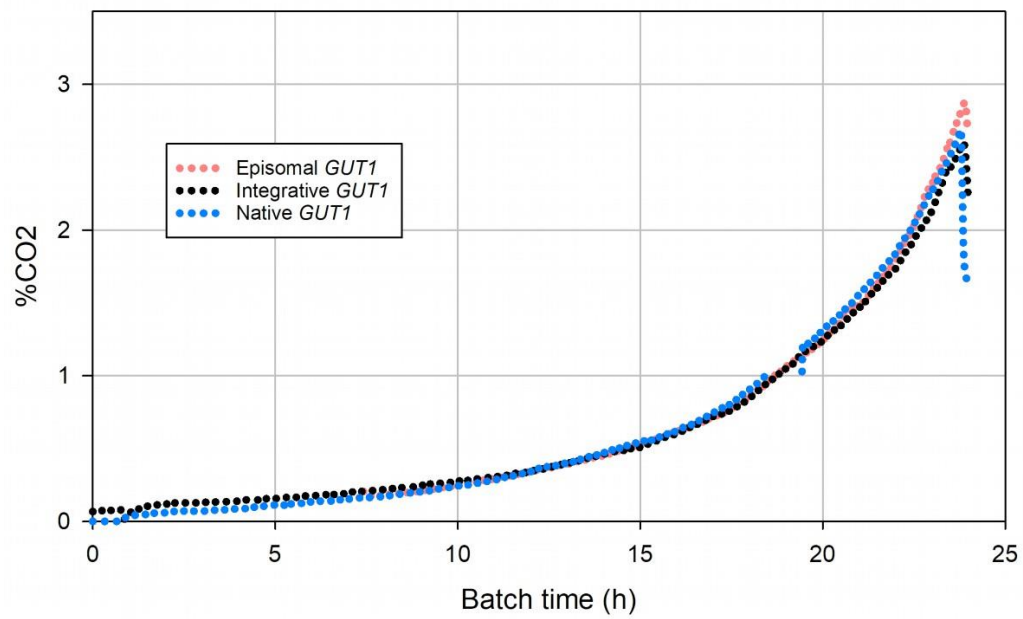


**Figure S3: Specific *CalB* activities obtained from deep-well plate cultivation of episomal plasmid transformants.** Seven transformants of various ARS plasmids with different combinations of selection marker and its promoter, together with 7 colonies of the respective integrative references, were cultivated in 96 deep-well plates containing 250  $\mu$ l BMG1. After 60 h of incubation at 28  $^{\circ}$ C and 320 rpm, 250  $\mu$ l of BMG0.5 were added, followed by 50  $\mu$ l BMG2.5 after 72 and 84 h to allow de-repression of P<sub>DC</sub> and consequent reporter gene expression. Cultivations were harvested after 108 h of cultivation by centrifugation and *CalB* activities in the supernatants were evaluated. The obtained *CalB* activities were normalized to the OD<sub>600</sub>, which was measured using 200  $\mu$ l of culture in microtiter plates using a Synergy MX plate reader (Biotek, Winooski, VT, USA).



**Figure S4: Specific CalB activities obtained from 250 mL shake flask cultivations.**

Five transformants of ARS plasmids with  $P_{AgTEF1}$  driving the *GUT1* or *TPI1* marker expression were used to directly inoculate baffled 250 mL shake flasks containing 50 mL of BMG1. The respective average, integrative controls were cultivated in triplicates. After 60 h, 72 h and 84 h *GUT1* plasmid cultivations and after 100 h, 112 h and 124 h *TPI1* plasmid cultivations were fed with 500  $\mu$ L of 50 % glycerol. After 108 h the *GUT1* plasmid cultivations and after 148 h the *TPI1* plasmid cultivations were harvested and CalB activities were measured in the supernatants. The obtained CalB activities were normalized to the OD<sub>600</sub>, which was measured in half-micro cuvettes with 12.5 mm distance using an Eppendorf BioPhotometer plus.

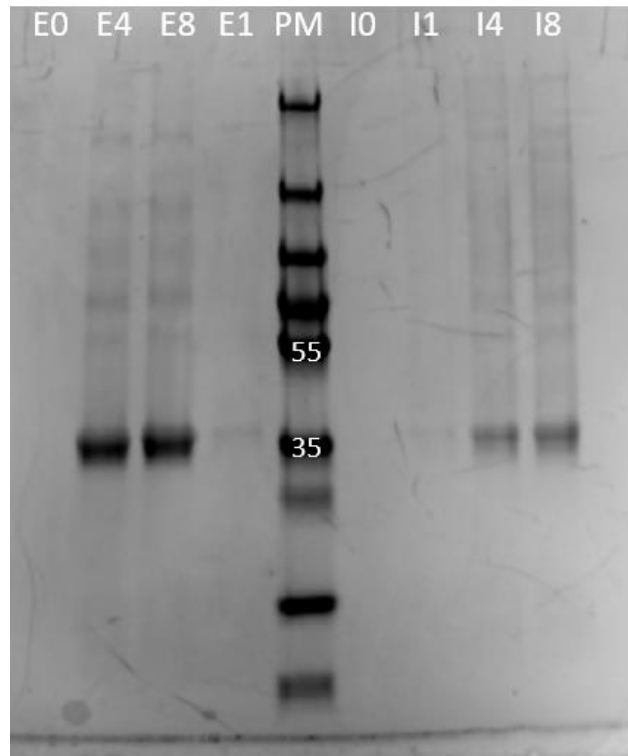


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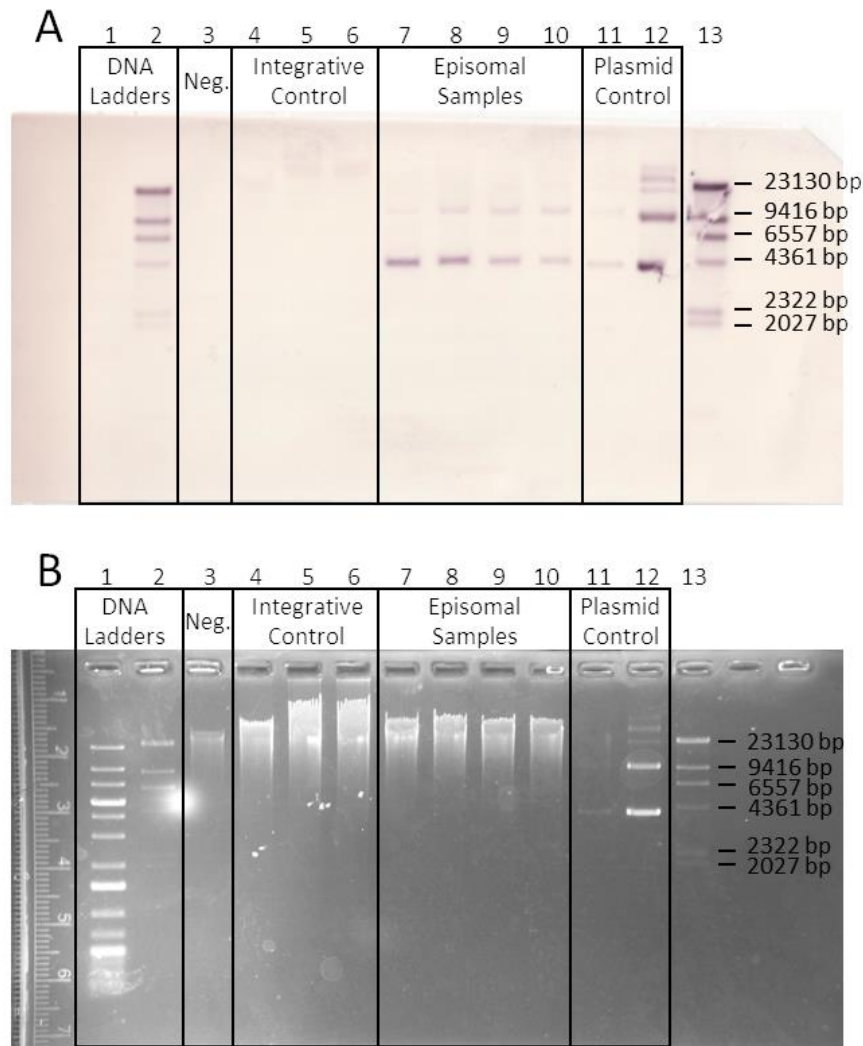
74 **Figure S5: Maximum specific growth rates during the batch phase of a bioreactor**

75 **cultivation based on off-gas analysis.** Strains harbouring the native *GUT1* locus and

76 episomal or integrative *GUT1* plasmids were analyzed.

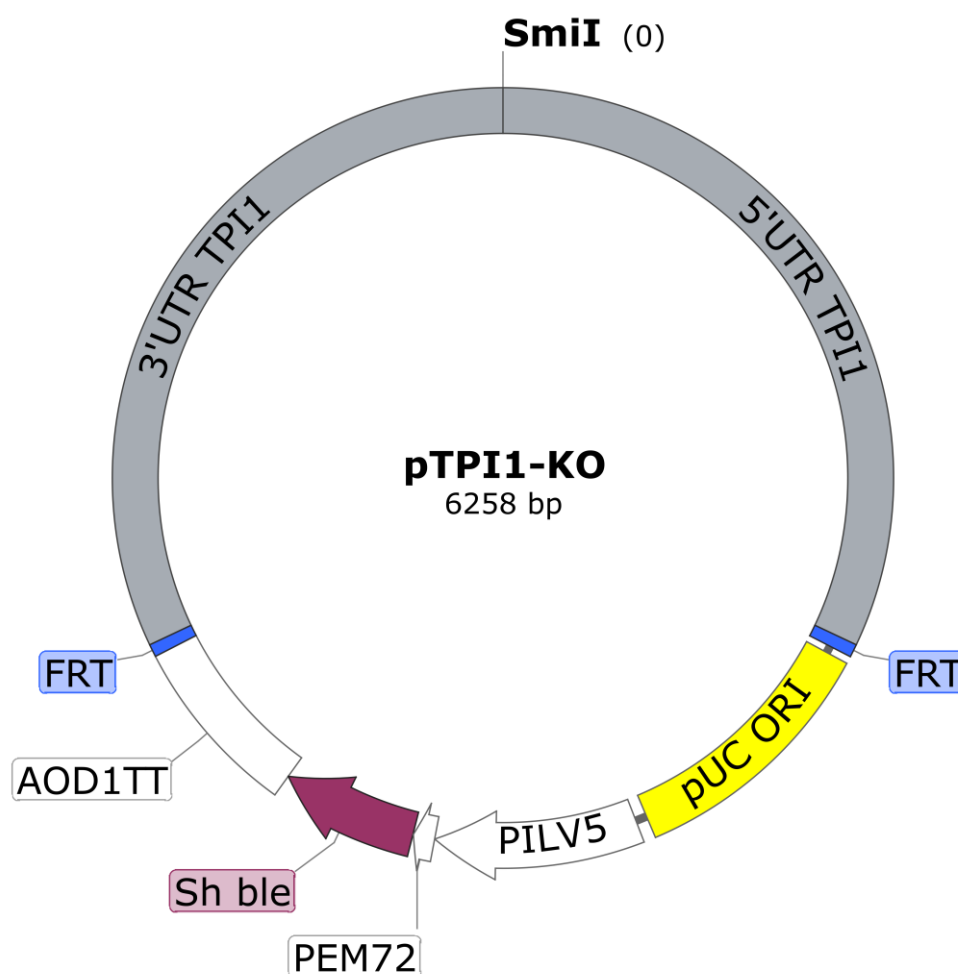


**Figure S6. SDS page of bioreactor samples.** 15  $\mu$ l of bioreactor samples E0, E1, E4, E8, I0, I1, I4 and I8 were added to 5  $\mu$ l of Laemmli Sample Buffer (Bio-Rad Laboratories, USA) and heated to 100  $^{\circ}$ C for 10 minutes. 10  $\mu$ l of the sample preparations were loaded on a 5-15 % Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> Precast Gel with 15 combs (Bio-Rad Laboratories, USA) together with the peqGOLD Protein Marker V (VWR International, USA) marked as PM in the image. The gel was stained with InstantBlue protein stain. The relevant 35 KDa and 55 KDa bands of the protein marker are indicated in the image to allow identification of the 33 KDa-sized *CalB* bands.



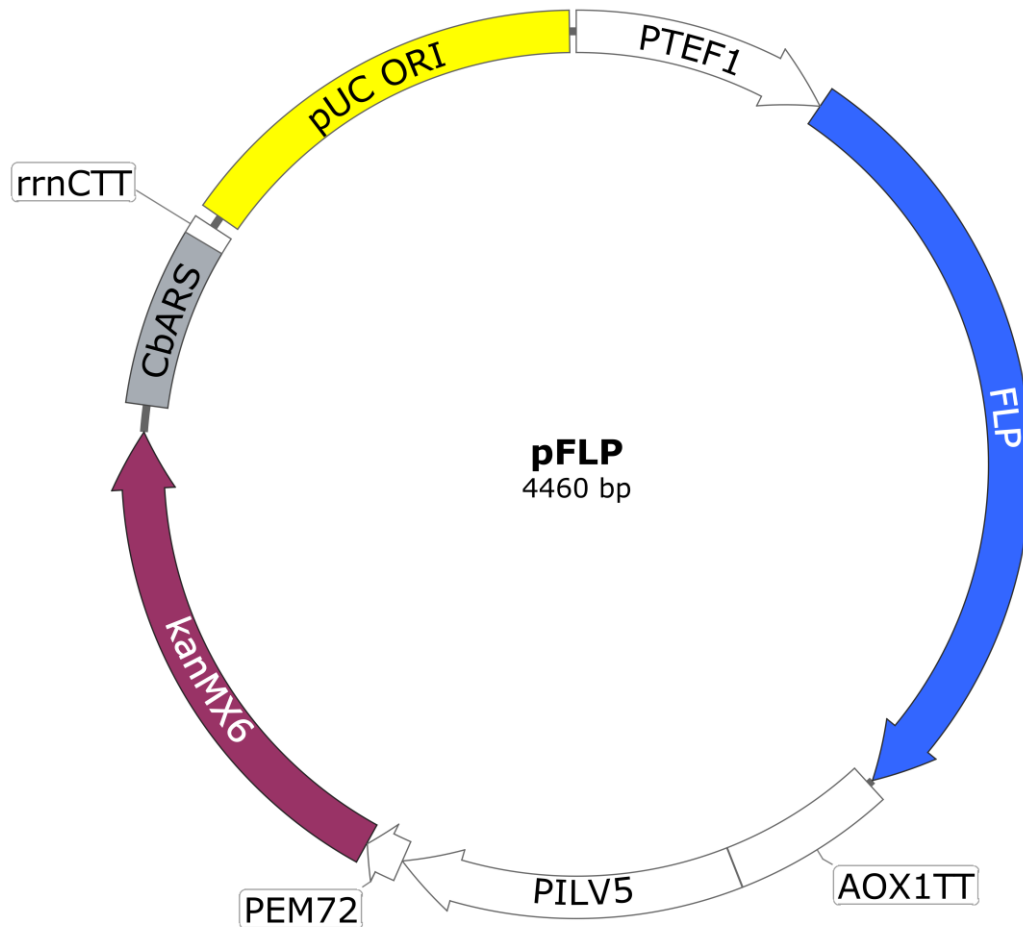
**Figure S7. Southern Blot of *GUT1*-based ARS plasmid bioreactor cultivation.**

Genomic DNA was isolated from samples taken from the bioreactor cultivation of the episomal expression system as well as the integrative reference strain. Gel-electrophoresis (B), Southern blot and subsequent detection of the *CalB* gene with a DIG-labeled probe (A) was performed. 1: Gene Ruler 1 kb plus not dig-labeled 0,5 µg; 2: DNA Molecular Weight Marker II, DIG-labeled (20 ng); 3: BSY11G1 empty strain; 4: BSY11G1\_FW001 A; 5: BSY11G1\_FW001 B; 6: BSY11G1\_FW001 C; 7: Bioreactor sample E0; 8: Bioreactor sample E1; 9: Bioreactor sample E4; 10: Bioreactor sample E8; 11: FW002 10 ng; 12: FW002 200 ng; 13: DNA Molecular Weight Marker II, DIG-labeled (20 ng).

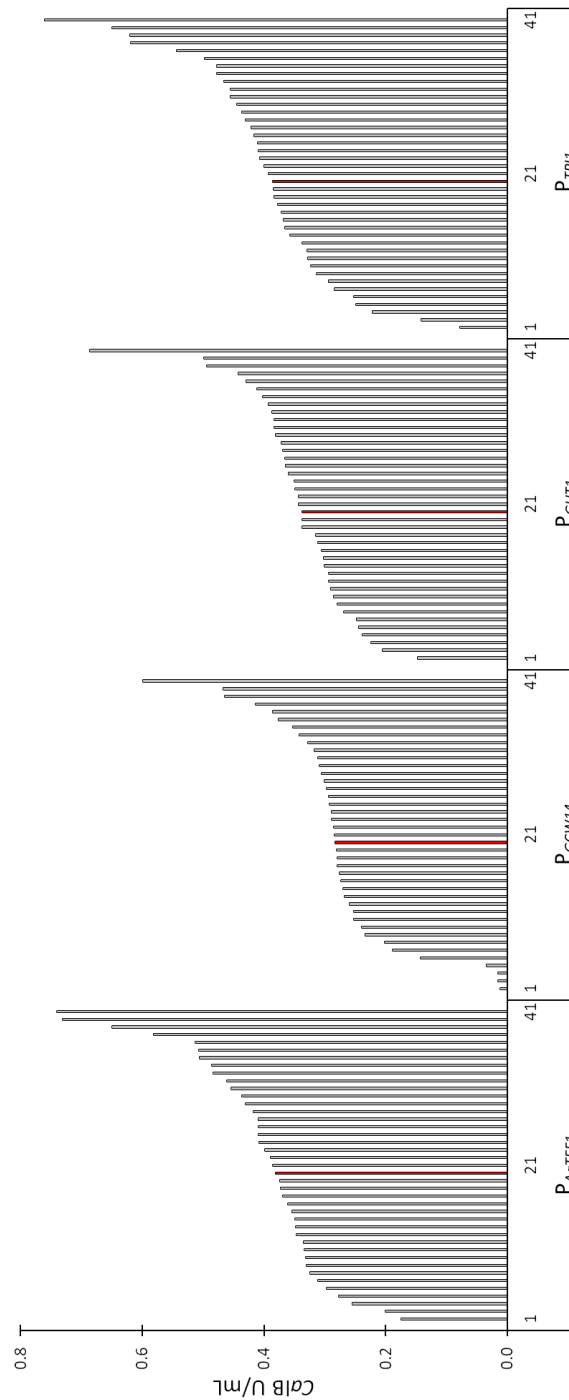


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98 **Figure S8. Vector map of the plasmid used for the deletion of *TPI1*.** The shuttle vector  
 99 shown in this figure harbors a Zeocin resistance cassette for both *E. coli* and *K. phaffii*,  
 100 an origin of replication for *E. coli* and sequences homologous to the genomic sequences  
 101 adjacent to the *TPI1* coding sequence in *K. phaffii*, in order to facilitate homologous  
 102 recombination-based gene replacement and knock-out of the *TPI1* gene. The *SmiI*  
 103 linearized plasmid was used to transform *K. phaffii* according to Lin-Cereghino *et al.*  
 104 (2005). The transformation was plated on agar plates containing 2 % (w/v) peptone, 1 %  
 105 (w/v) yeast extract, 0.1 % (w/v) glucose, 2 % (v/v) ethanol, 1.5 (w/v) % agar and 100  
 106 mg/L Zeocin and was incubated at 28 °C for 7-10 days.

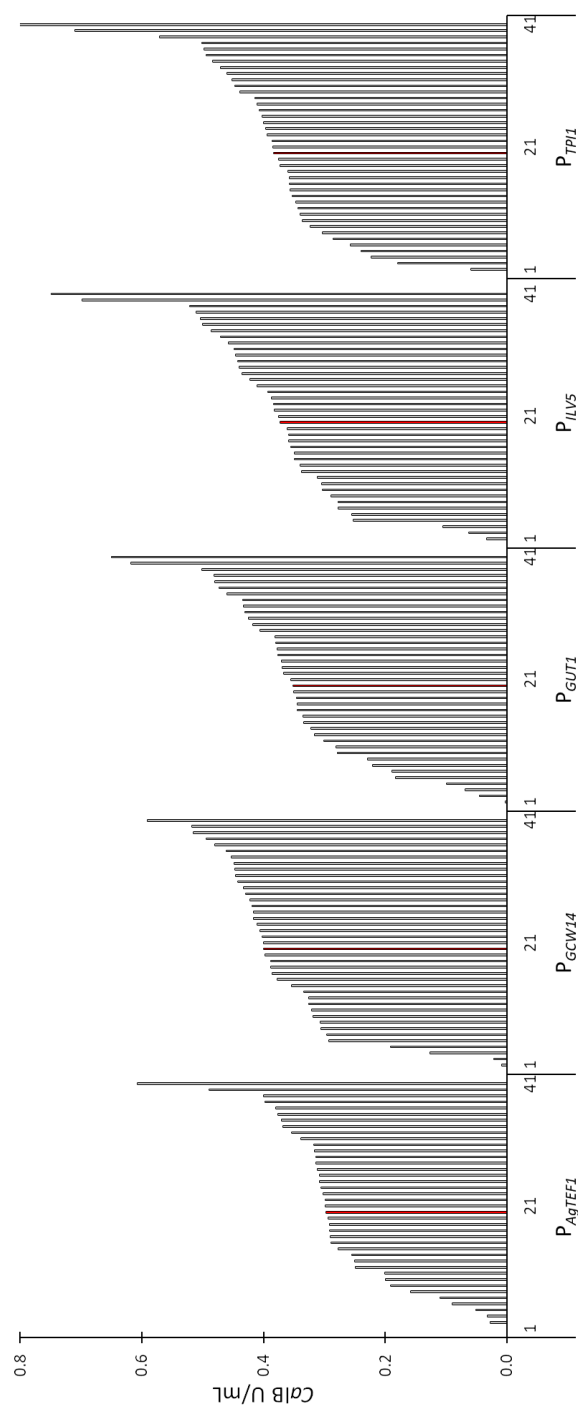


**Figure S9. Vector map of the plasmid used for marker recycling in the *TPI1* knock-out strain.** The shuttle vector shown in this figure harbors a Kanamycin/Geneticin resistance cassette, an origin of replication and the *CbARS* sequence for the replication in both *E. coli* and *K. Phaffii*, respectively. Additionally, a flippase expression cassette with the constitutive *TEF1* promoter is included, too. This plasmid was used to transform *TPI1* knock-out strains in order to recycle the Zeocin resistance marker with the Flp/FRT system.



**Figure S10. Median Screening of integrative *GUT1* plasmid transformants. *K. phaffii***

BSY11G1 was transformed with *SmiI* linearized plasmids 5, 7, 9, 11 and 13 according to Lin-Cereghino *et al.* (2005). 41 transformants of each transformation (no transformants obtained for plasmid 11) were cultivated in 96 deep-well plates and the *CalB* activity was determined. The median clone of each plasmid was used as reference strain for the respective episomal plasmid in following experiments.



**Figure S11. Median Screening of integrative *TPI1* plasmid transformants.** *K. phaffii* BSYBG11  $\Delta tpi1$  was transformed with *SmiI* linearized plasmids 15, 17, 19, 21 and 23 according to Lin-Cereghino *et al.* (2005). 41 transformants of each transformation were cultivated in 96 deep-well plates and the *CalB* activity was determined. The median clone of each plasmid was used as reference strain for the respective episomal plasmid in following experiments.