

Supplemental Material

A novel regulated hybrid promoter that permits auto-induction of heterologous protein expression in *Kluyveromyces lactis*

Hassan Sakhtah^{1*}, Juliane Behler^{1,2*}, Alana Ali-Reynolds¹, Thomas B. Causey¹, Saulius Vainauskas^{1‡} and Christopher H. Taron^{1‡§}

*Authors contributed equally to this work

‡Authors share senior authorship

¹ New England Biolabs, 240 County Road, Ipswich, MA 01938, USA

²Genetics & Experimental Bioinformatics, University of Freiburg, Institute of Biology III, Schänzlestraße 1, D-79104 Freiburg, Germany

§Corresponding author. Email: taron@neb.com

This document provides information (tables and figures) supplemental to the main text

Table S1. DNA primers used in this study.

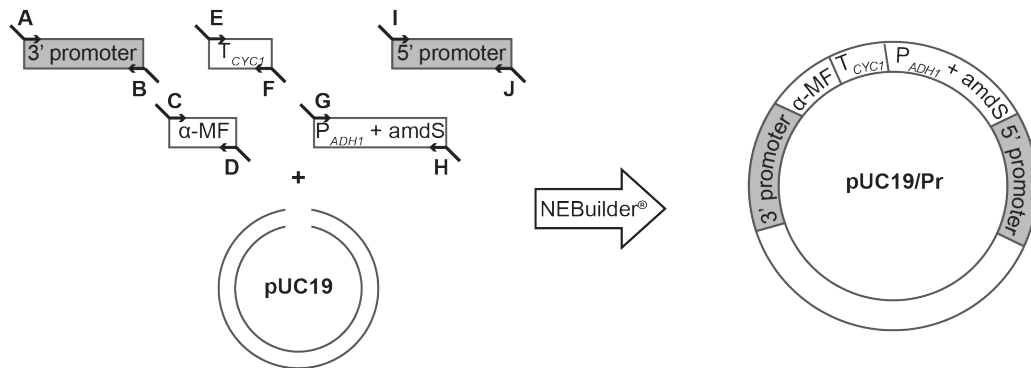
Primer Description	¹ Name	² Sequence 5' - 3'
<i>KIADH3</i> expression cassette assembly	ADH3_S1A ADH3_S1B ADH3_S1C ADH3_S1H ADH3_S1I ADH3_S1J	cgttgtaaaacgacggccagtgaaattCAAACCCGTAGTGATGCAC gagaatttcatCTTAATATTTCTTGTTTTAAATTTGATGGG gaaatattaagATGAAATTCTCTACTATATTAGCCG ccatccgaaatCTATGGAGTCACCACATTTT tgactccatagATTTTCGGATGGGTGCTTG ggatccccgggtaccgagctcgaattcATGTAGAACTCCGATTTTACATC
<i>KIGAP1</i> expression cassette assembly	GAP1_S1A GAP1_S1B GAP1_S1C GAP1_S1H GAP1_S1I GAP1_S1J	cgttgtaaaacgacggccagtgaaattcACCTCGGAAGATTTCCCAATTAC gagaatttcatTGTGTAATATTTCTTTTTTTTACTTGAAACTG aatattacacaATGAAATTCTCTACTATATTAGCCG tcggtactgacCTATGGAGTCACCACATTTT tgactccatagGTCAGTACCGACTGGGATC ggatccccgggtaccgagctcgaattcAGTATGACATGTCCGGTG
<i>KIGUT2</i> expression cassette assembly	GUT2_S1A GUT2_S1B GUT2_S1C GUT2_S1H GUT2_S1I GUT2_S1J	cgttgtaaaacgacggccagtgaaattCCATCAACCCAGACAAAAAC gagaatttcatCGTTTGTAACTGTGAGCG gtttacaaacgATGAAATTCTCTACTATATTAGCCG tcggtcagcagCTATGGAGTCACCACATTTT tgactccatagCTGCTGAACGAAAAAATTAAGG ggatccccgggtaccgagctcgaattcGTTTTGTTTTGCGTGGTTTTG
<i>KIICL1</i> expression cassette assembly	ICL1_S1A ICL1_S1B ICL1_S1C ICL1_S1H ICL1_S1I ICL1_S1J	cgttgtaaaacgacggccagtgaaattcATCTAGATAATCGGGTATGATTAAC gagaatttcatTATGTTGGGTTTGTATGTTTTG aaccacaacataATGAAATTCTCTACTATATTAGCCG aaaggtaaaagCTATGGAGTCACCACATTTT tgactccatagCTTTTACCTTTGTTGTCTTATGTG ggatccccgggtaccgagctcgaattcGCAGATTAGGTGAGCTTAC
<i>KISTR3</i> expression cassette assembly	STR3_S1A STR3_S1B STR3_S1C STR3_S1H STR3_S1I STR3_S1J	cgttgtaaaacgacggccagtgaaattcTAACTAAATCAAAGTTGACTTAATC gagaatttcatGTTGGAAGTTTATTGTTGG aaactccaacATGAAATTCTCTACTATATTAGCCG ttatgaagcctCTATGGAGTCACCACATTTT tgactccatagAGCCTTCATAATATTACTGGAC ggatccccgggtaccgagctcgaattCTACCTTACTGGTGAACCTTG
<i>KILAC4</i> expression cassette assembly	LAC4_S1A LAC4_S1B LAC4_S1C LAC4_S1H LAC4_S1I LAC4_S1J	cgttgtaaaacgacggccagtgaaattcGATCGACTCATAAAATAGTAACC gagaatttcatTTTTTCAAGCTTCTCGATG gctgaaaaaaaATGAAATTCTCTACTATATTAGCCG atctgtccttCTATGGAGTCACCACATTTT tgactccatagAAGGAACAGATAGATAAAATTCGG ggatccccgggtaccgagctcgaattCCGCGGAAATTTAGGAATTTAAAC
Expression cassette assembly	S1D S1E S1F S1G	aaggggctgtTCTTTTCTCGAGATCATCC tcgagaaaaagaACAGGCCCTTTTCTTTGTGCG caccggaaacAGCTTGCAAATTAAGCCTTC atgtcaagctGTTTCCGGGTGTACAATATG
Promoter specific backbone amplification	S2K S2L	ACAGGCCCTTTTCTTTGTGCG TCTTTTCTCGAGATCATCCTTGTGTCAG
Gaussia luciferase	Gluc_S2M Gluc_S2N	gacaaggatgatctcgagaaaagaAAGCCCACCGAGAACAACGAAG atcgacaaaggaaggggctgtTAGTCACCACCGGCCCTTG
Fucosidase	Fuc_S2M Fuc_S2N	gacaaggatgatctcgagaaaagaTCATCATCATCATCATTACC atcgacaaaggaaggggctgtTCAGATAGCAGATATTTGTGAATG
Enterokinase	EK_S2M EK_S2N	gacaaggatgatctcgagaaaagaATTGTTGGTGGTTCTGATTCT atcgacaaaggaaggggctgtCTAATGTAGAAAATTTGTATC
Expression cassette amplification primers	S3O S3P	TATTACGCCAGCTGGCGAAAG GTGAGCGGATAACAATTTACACACAGG
pUC19/ICL1 partial backbone for fusion	PICL1(-680)_rev PICL1_ amdS_fwd	GGCTGAGGAACCAAAATAGAGTC TGTTTTCCGGGTGACAATATGG
P1000 promoter	PICL1_PGAP1_fwd PICL1_PGAP1_rev	atgtgtcctcagccACCTCGGAAGATTTCCCAATTAC tgtacaccggaaacaAGCTTGCAAATTAAGCCTTCG
P500 promoter	PGAP1-500fusion_fwd PGAP1-500fusion_rev	gactctatttggtcctcagccTGGACAGGAAGAGAAAATC ctaataatagtagaatttcatTGTGTAATATTCTTTTTTTTACTTGAAC
P450 promoter	PGAP1-450_fusion_fwd	gactctatttggtcctcagccACTTTCACCAGATCCCAATG
P400 promoter	PGAP1-400_fusion_fwd	gactctatttggtcctcagccTACCATAACTTACCATTTTCATCAC
P350 promoter	PGAP1-350_fusion_fwd	gactctatttggtcctcagccTCGACTGCTTTGCTTCATC
P220 promoter	PGAP1-220_fusion_fwd	gactctatttggtcctcagccTAATTTTGTATATAAAGGGTGGATC
P125 promoter	PGAP1-125_fusion_fwd	gactctatttggtcctcagccTTCTTATTAACCTTTTTTAAAGTCAAAC
P74 promoter	PGAP1-74_fusion_fwd	gactctatttggtcctcagccCAAAGGTATTTCAAGTTATCATAC

¹Primers correlate to specific expression vector cloning steps outlined in Figure S1. Primers with S1, S2 or S3 in their name pertain to cloning steps 1, 2 or 3, respectively. The letter immediately following denotes the exact location of the primer within that schematic.

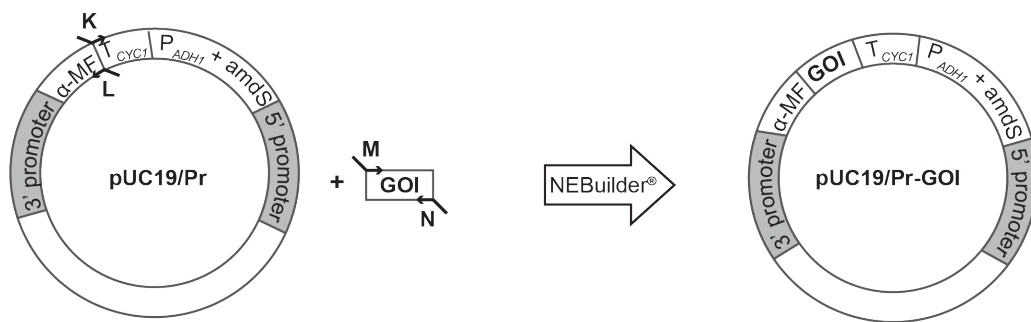
For example, EK-S2M and EK-S2N that are used in Step 2 of the cloning strategy, anneal to locations M and N, and are used to amplify DNA encoding enterokinase. P### primers were used to prepare truncated PICL1-PGAP fusion promoters.

²Uppercase letters represent insert-specific DNA sequences; lowercase letters are sequences that overlap with the vector backbone or an adjacent DNA region.

Step 1: first round of PCR and *in-vitro* assembly



Step 2: second round of PCR and *in-vitro* assembly



Step 3: final round of PCR to generate linear expression cassette

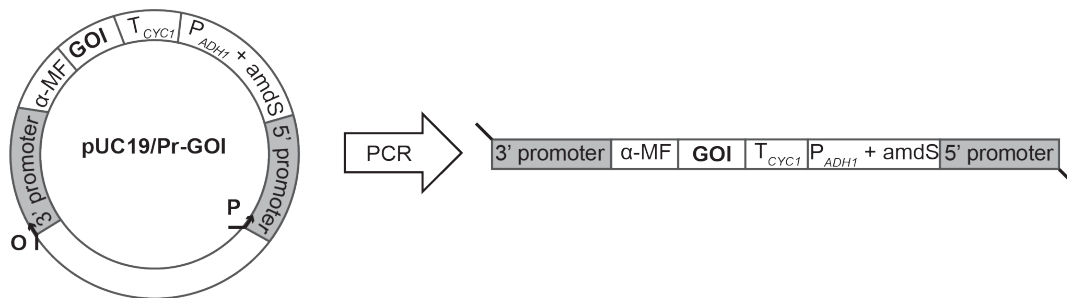


Figure S1. Assembly of *K. lactis* expression cassettes *in vitro*. (Step 1) Different promoters used in this study (3' Promoter) and their flanking upstream regions needed for homologous recombination (5' Promoter) were amplified from *K. lactis* GG779 genomic DNA. *Saccharomyces cerevisiae* YJM193 genomic DNA was used for the amplification of the *CYC1* transcription terminator sequence (T_{CYC1}). The pKLAC2 vector (NEB, Ipswich, MA) was used as template for amplifying the *Aspergillus nidulans* acetamidase expression cassette (P_{ADH1} + *amdS*) and *K. lactis* α -mating factor secretion leader sequence (α -MF). Primers were designed to create homologous overlaps between the different fragments (black arrows) and the letters (A to P) denoting each primer correspond to primer sequences presented in Table S1. The *EcoRI* digested vector pUC19 was used to assemble individual expression constructs. The fragments were assembled *in vitro* into *EcoRI*-linearized pUC19 plasmid DNA using NEBuilder® HiFi DNA Assembly Master Mix, resulting in a plasmid pUC19/Pr. (Step 2) DNA fragments from a reporter gene of interest (GOI) and from pUC19/Pr were amplified by PCR (both using primers with homologous overlaps) and assembled to create a new plasmid containing a complete integrative expression cassette. (Step 3) A linear integrative expression cassette was prepared by PCR using specific primers and the assembled plasmid from Step 2 as a DNA template. This linear cassette was used for integrative transformation of *K. lactis* cells.

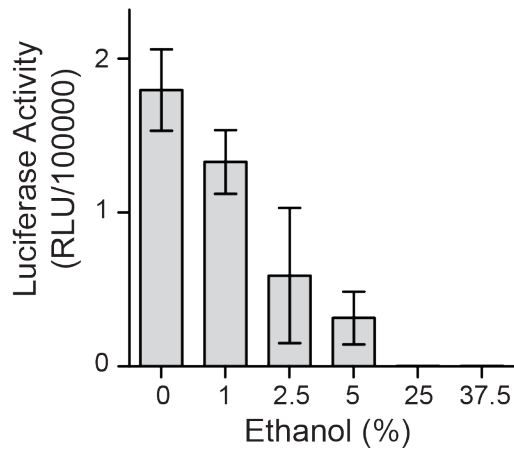


Figure S2. Sensitivity of *Gaussia princeps* luciferase to ethanol. *K. lactis* P₃₅₀-Gluc was grown in a fermenter in YDFM containing 2% glucose. After 41.5 h, 1 mL of culture was removed and centrifuged at 13000 x g for 1 min to pellet the cells. The cleared spent medium was diluted 1:20 in phosphate buffered saline (PBS). An aliquot of the dilution (2 μ L) was added to PBS containing different quantities of ethanol, and Gluc activity was measured. Error bars represent standard deviation from the mean of three technical replicates.

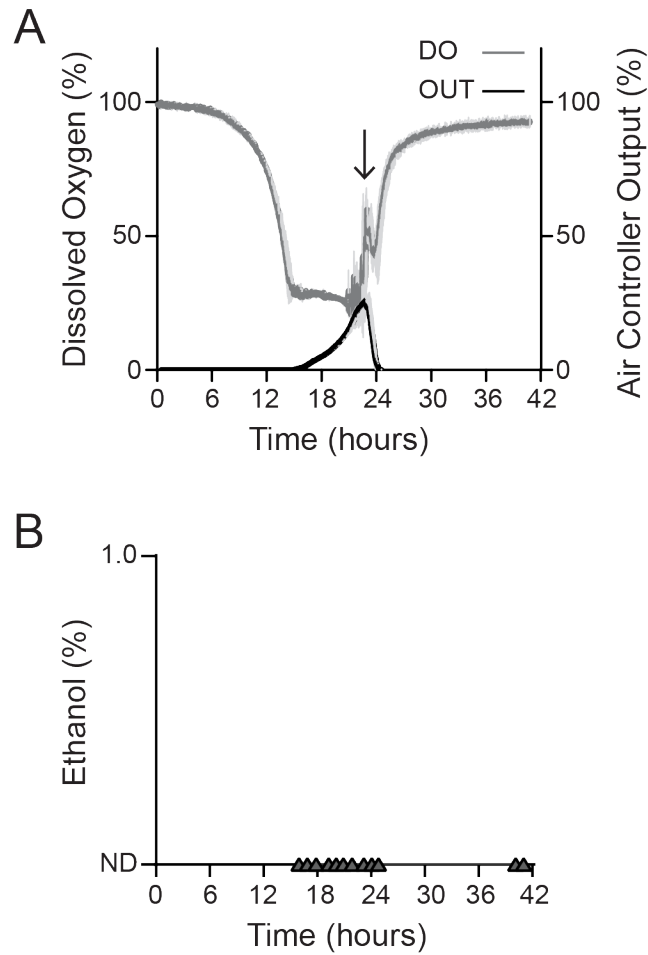


Figure S3. Additional bioprocessing measurements for *K. lactis* fermentations presented in Figure 5A-B of the main text. (A) Shown is average oxygen consumption (dark gray line), dissolved oxygen controller output (black line) relative to the time of culturing. The arrow indicates time at which glucose became growth-limiting to the cells. Light grey shading represents standard deviation from the mean of three replicate fermentations. (B) Shown is ethanol production relative to the time of culturing. ND represents the ethanol (<0.05%) detection limit of our instrumentation.

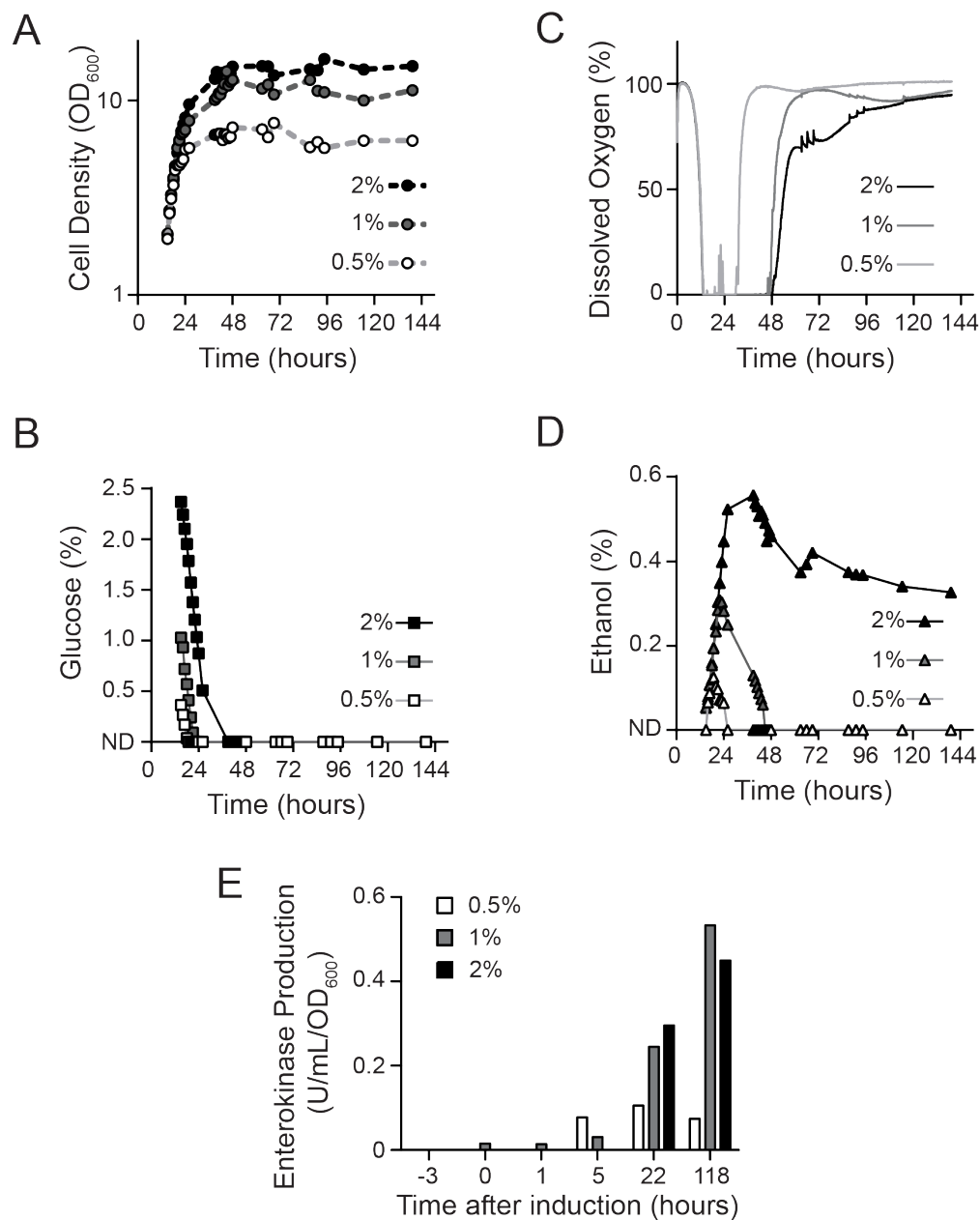


Figure S4. Shake flask production of enterokinase in growth medium having different glucose concentrations. Shown are (A) cell growth, (B) glucose consumption, (C) oxygen consumption, and (D) ethanol production, each relative to the time of culturing. (E) Shown is EK_L production relative to the time at which glucose became depleted and P_{350} - EK_L was induced (induction time) in each culture. Enterokinase production was calculated by normalizing EK_L activity (U/mL) to the cell density (OD_{600}) of each culture at each timepoint post-induction. In panels B and D, ND represents the glucose ($<0.002\%$) and ethanol ($<0.05\%$) detection limits of our instrumentation. In all panels, light gray lines, grey lines, and black lines represent cultures grown in 0.5%, 1% and 2% glucose, respectively.

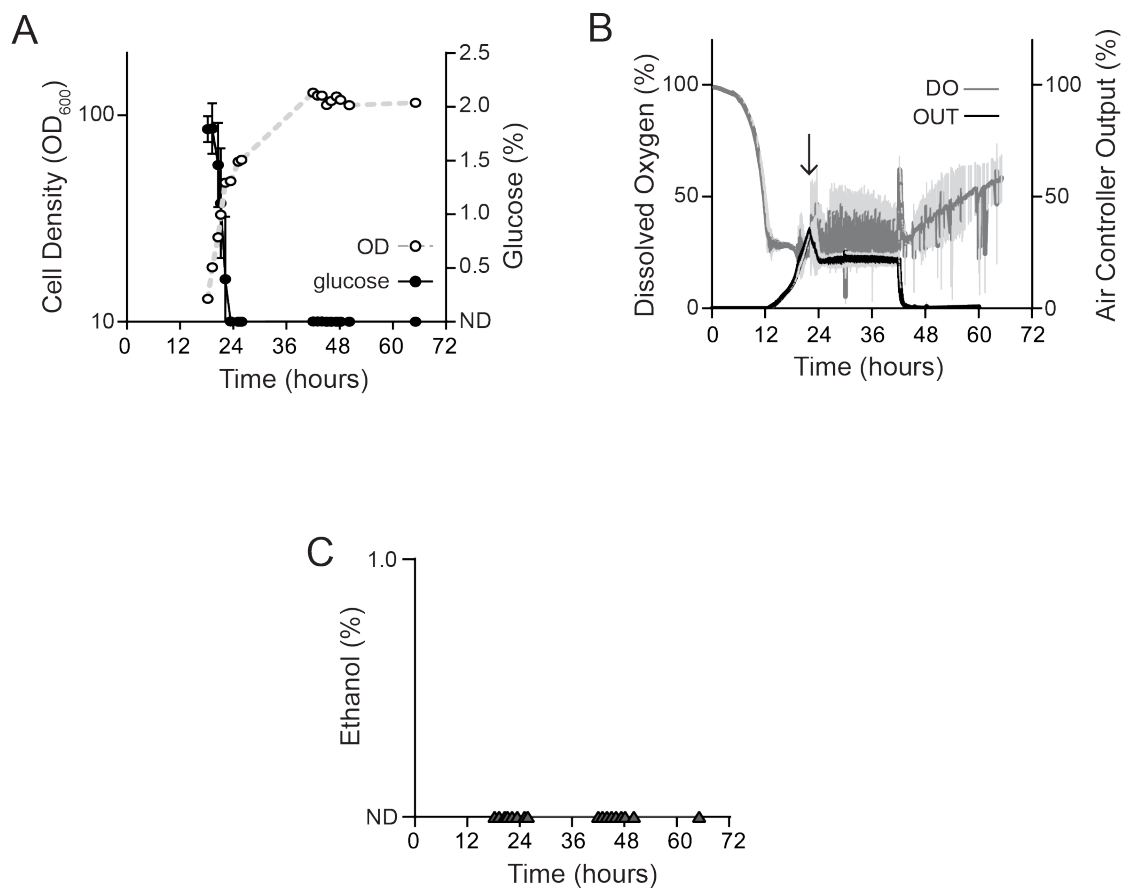


Figure S5: Additional bioprocessing measurements for *K. lactis* high cell density fermentations presented in Figure 5C of the main text. Shown are (A) glucose consumption, (B), oxygen consumption, and (C) ethanol production, each relative to the time of culturing. In panel B, dark gray lines represent average dissolved oxygen (DO) and the black line represents average oxygen controller output (OUT) of three replicate cultures. The arrow indicates time at which glucose became growth-limiting to the cells. In panels A and C, ND represents the glucose (<0.002%) and ethanol (<0.05%) detection limits of our instrumentation. In all panels, error bars or light gray shading represents standard deviation from the mean of three replicate fermentations.

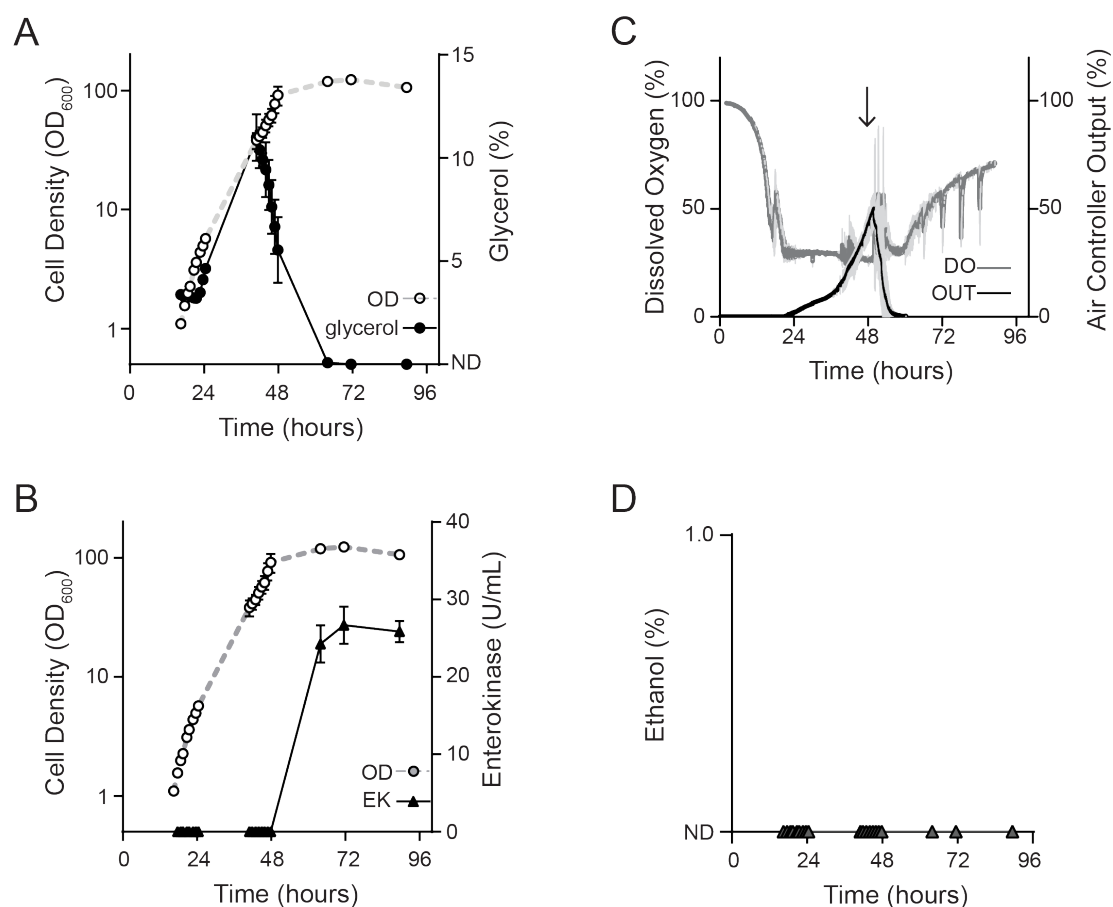


Figure S6. P_{350} -dependent production of enterokinase by *K. lactis* YCT1267 grown in high cell density fermentations using glycerol as a carbon source. Triplicate fermentations of *K. lactis* YCT1267 were performed in bioreactors. A 60% glycerol feed solution containing ampicillin and vitamins was added at a constant rate of 5 mL/h from 22.5 h ($OD_{600} = \sim 4.3$) to 41 h ($OD_{600} = \sim 38.2$) to achieve high cell density. Shown are (A) glycerol consumption, (B) EK_L activity, (C) oxygen consumption, and (D) ethanol production, each relative to the time of culturing. In panel B, dark gray lines represent average dissolved oxygen (DO) and the black line represents average oxygen controller output (OUT) of the three replicate cultures. The arrow indicates time at which glucose became growth-limiting to the cells. In panels A and D, ND represents the glycerol (<0.0009%) and ethanol (<0.05%) detection limits of our instrumentation. In all panels, error bars or light gray shading represents standard deviation from the mean of three replicate fermentations.