

Combined engineering of disaccharide transport and phosphorolysis for enhanced ATP yield from sucrose fermentation in *Saccharomyces cerevisiae*

Marques, Wesley Leoricy; Mans, Robert; Henderson, Ryan K.; Marella, Eko Roy; Horst, Jolanda ter; Hulster, Erik de; Poolman, Bert; Daran, Jean Marc; Pronk, Jack T.; Gombert, Andreas K.

DOI

[10.1016/j.ymben.2017.11.012](https://doi.org/10.1016/j.ymben.2017.11.012)

Publication date

2018

Document Version

Accepted author manuscript

Published in

Metabolic Engineering

Citation (APA)

Marques, W. L., Mans, R., Henderson, R. K., Marella, E. R., Horst, J. T., Hulster, E. D., Poolman, B., Daran, J. M., Pronk, J. T., Gombert, A. K., & van Maris, A. J. A. (2018). Combined engineering of disaccharide transport and phosphorolysis for enhanced ATP yield from sucrose fermentation in *Saccharomyces cerevisiae*. *Metabolic Engineering*, 45, 121-133. <https://doi.org/10.1016/j.ymben.2017.11.012>

Important note

To cite this publication, please use the final published version (if applicable).
Please check the document version above.

Copyright

Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy

Please contact us and provide details if you believe this document breaches copyrights.
We will remove access to the work immediately and investigate your claim.

1 **Combined engineering of disaccharide transport and phosphorolysis for**
2 **enhanced ATP yield from sucrose fermentation in *Saccharomyces***
3 ***cerevisiae***

4 Wesley Leoricy Marques^{a,b,c,†}, Robert Mans^{a,†,*}, Ryan K. Henderson^d, Eko Roy Marella^a,
5 Jolanda ter Horst^a, Erik de Hulster^a, Bert Poolman^d, Jean-Marc Darana^a, Jack T. Pronk^a,
6 Andreas K. Gombert^b, Antonius J.A. van Maris^{a,1}

7
8 ^aDepartment of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629
9 HZ Delft, The Netherlands

10 ^bSchool of Food Engineering, University of Campinas, Rua Monteiro Lobato 80, Campinas-
11 SP 13083-862, Brazil

12 ^cDepartment of Chemical Engineering, University of São Paulo, Avenida Professor Lineu
13 Prestes, 580 - Bloco 20, São Paulo-SP, 05424-970, Brazil

14 ^dDepartment of Biochemistry, Groningen Biomolecular Sciences and Biotechnology
15 Institute & Zernike Institute for Advanced Materials, University of Groningen, Nijenborgh 4,
16 9747 AG Groningen, The Netherlands

17 ¹ Current address: Division of Industrial Biotechnology, School of Biotechnology, KTH Royal
18 Institute of Technology, AlbaNova University Center, SE 106 91, Stockholm, Sweden.

19

20 **Manuscript for publication in: Metabolic engineering**

21 **Running title:** Increased ATP yield of sucrose fermentation by yeast

22 * To whom correspondence should be addressed. Email R.mans@tudelft.nl

23 † These authors contributed equally to this publication.

1 **ABBREVIATIONS**

- 2 SPase, sucrose phosphorylase;
- 3 *LmSPase, Leuconostoc mesenteroides* sucrose phosphorylase;
- 4 *LmSPase, Leuconostoc mesenteroides* sucrose phosphorylase coding gene;
- 5 Mal11, alpha-glucoside proton symporter 11;
- 6 *MAL11, alpha-glucoside proton symporter 11* coding gene;
- 7 Mal12, maltose hydrolase 12;
- 8 *MAL12, maltose hydrolase 12* coding gene;
- 9 PvSUF1, *Phaseolus vulgaris* sucrose facilitator 1;
- 10 *PvSUF1, Phaseolus vulgaris* sucrose facilitator 1 coding gene;
- 11 *PsSUF1, Pisum sativum* sucrose facilitator 1 coding gene;
- 12 *PsSUF4, Pisum sativum* sucrose facilitator 4 coding gene;
- 13 *AtSWEET12, Arabidopsis thaliana* SWEET12 coding gene;
- 14 *OsSWEET11, Oryza sativa* SWEET11 coding gene;
- 15 SM, synthetic medium;
- 16 ORF, open reading frame;
- 17 G418, Geneticin (aminoglycoside antibiotic);
- 18 bp, basepair;
- 19 SHR-sequence, synthetic homologous recombination sequence;
- 20 v/v, volume per volume (%);
- 21 w/v, weight per volume;
- 22 LB, lysogeny broth;
- 23 U (units), the amount of enzyme catalysing the conversion of 1 μ mol of substrate per minute;
- 24 μ , biomass specific growth rate;
- 25 $q_{\text{metabolite}}$, biomass specific consumption or production rate

1 **HIGHLIGHTS**

- 2 • Functional replacement of native *S. cerevisiae* sucrose hydrolysis and monosaccharide
3 transport by a *Phaseolus vulgaris* putative sucrose facilitator (PvSUF1) and *Leuconostoc*
4 *mesenteroides* sucrose phosphorylase (*LmSPase*).
- 5 • Replacement of sucrose hydrolysis by intracellular phosphorolysis increased anaerobic
6 biomass yield on sucrose by 31%.
- 7 • Additional replacement of proton-coupled sucrose uptake through Mal11 by transport via
8 PvSUF1 further increased the yield by 8%.
- 9 • Overexpression of endogenous phosphoglucomutase *PGM2* increased the anaerobic growth
10 rate on sucrose of *LmSPase* expressing strains.

1 **ABSTRACT** (247 words)

2 Anaerobic industrial fermentation processes do not require aeration and intensive mixing
3 and the accompanying cost savings are beneficial for production of chemicals and fuels.
4 However, the free-energy conservation of fermentative pathways is often insufficient for
5 the production and export of the desired compounds and/or for cellular growth and
6 maintenance. To increase free-energy conservation during fermentation of the industrially
7 relevant disaccharide sucrose by *Saccharomyces cerevisiae*, we first replaced the native
8 yeast α -glucosidases by an intracellular sucrose phosphorylase from *Leuconostoc*
9 *mesenteroides* (*LmSPase*). Subsequently, we replaced the native proton-coupled sucrose
10 uptake system by a putative sucrose facilitator from *Phaseolus vulgaris* (*PvSUF1*). The
11 resulting strains grew anaerobically on sucrose at specific growth rates of $0.09 \pm 0.02 \text{ h}^{-1}$
12 (*LmSPase*) and $0.06 \pm 0.01 \text{ h}^{-1}$ (*PvSUF1*, *LmSPase*). Overexpression of the yeast *PGM2* gene,
13 which encodes phosphoglucomutase, increased anaerobic growth rates on sucrose of these
14 strains to $0.23 \pm 0.01 \text{ h}^{-1}$ and $0.08 \pm 0.00 \text{ h}^{-1}$, respectively. Determination of the biomass
15 yield in anaerobic sucrose-limited chemostat cultures was used to assess the free-energy
16 conservation of the engineered strains. Replacement of intracellular hydrolase with a
17 phosphorylase increased the biomass yield on sucrose by 31%. Additional replacement of
18 the native proton-coupled sucrose uptake system by *PvSUF1* increased the anaerobic
19 biomass yield by a further 8%, resulting in an overall increase of 41%. By experimentally
20 demonstrating an energetic benefit of the combined engineering of disaccharide uptake
21 and cleavage, this study represents a first step towards anaerobic production of
22 compounds whose metabolic pathways currently do not conserve sufficient free-energy.

- 1 **Keywords:** Free-energy conservation, ATP, Facilitated diffusion, Phosphoglucomutase,
- 2 Chemostat, Yeast physiology
- 3

1 **1. INTRODUCTION**

2 Microbial conversion of sugars from renewable feedstocks into chemicals and fuels offers a
3 sustainable alternative to conventional petroleum-based production processes (Nielsen et
4 al., 2013). In microbial processes for production of commodity chemicals, the cost of the
5 sugar substrate can be up to 70% of the variable cost price. This impact of substrate costs
6 on process economics necessitates high yield of product on substrate (Borodina and
7 Nielsen, 2014; De Kok et al., 2012). The efficiency of free-energy conservation in central
8 metabolism, expressed as conversion of ADP and phosphate to ATP, has a big impact on the
9 product yield. For products whose synthesis from sugar requires a net input of ATP and
10 therefore are produced in aerobic bioreactors, an increased efficiency of energy
11 conservation would imply that less substrate has to be respired to provide the ATP
12 required for product formation. As a result, more substrate carbon can be channelled
13 towards the desired product. Additionally, the product yield on oxygen increases, which
14 improves volumetric productivity (often limited by oxygen transfer (Meadows et al.,
15 2016)) and/or decreases the cost of aeration and cooling (Luong and Volesky, 1980).
16 Where possible, anaerobic conversion of sugars into fuels and chemicals would be even
17 more beneficial (Cueto-Rojas et al., 2015; De Kok et al., 2012; Weusthuis et al., 2011).

18 Although many conversions of sugars into industrially relevant products are feasible
19 based on thermodynamics and mass conservation, ATP formation by substrate-level
20 phosphorylation in central metabolism can be insufficient to provide the energy required
21 for product-formation pathways, product export, cellular growth and/or maintenance
22 (Cueto-Rojas et al., 2015; De Kok et al., 2012). For example, in the conversion of glucose
23 into lactic acid by *Saccharomyces cerevisiae*, all ATP formed by substrate-level

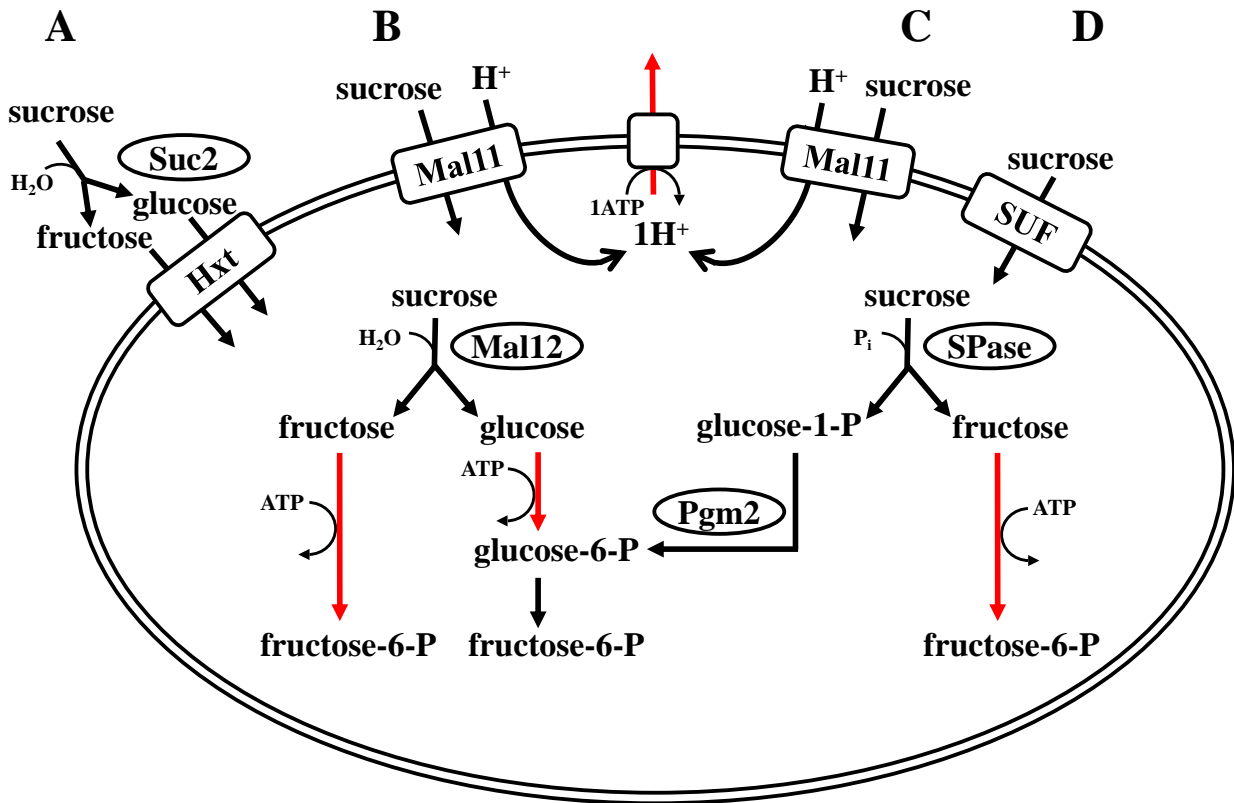
1 phosphorylation in glycolysis is required for export of product (Derek A Abbott et al., 2009;
2 Van Maris et al., 2004). In this specific example, increased free-energy (ATP) conservation
3 could enable homofermentative, anaerobic lactate production.

4 A negative Gibbs free-energy change for the conversion of substrate into product
5 can either be conserved in the form of ATP, or used to drive the reaction. Therefore, a
6 trade-off often exists between high energetic efficiency and high reaction rates (Pfeiffer et
7 al., 2001). In nature, competition for resources is often more important than optimal free-
8 energy conservation. Consequently, microbial evolution has in many cases yielded
9 pathways with high turnover rates that facilitate fast substrate utilization at the expense of
10 energetic efficiency (Pfeiffer et al., 2001). This evolutionary trade-off between yield and
11 rate creates metabolic engineering opportunities for increasing free-energy conservation in
12 industrial microorganisms.

13 The conversion of cheap and abundant substrates such as the disaccharide sucrose,
14 which is mainly derived from sugar cane and sugar beet, is especially interesting for
15 industrial applications (Marques et al., 2015). The yeast *Saccharomyces cerevisiae* is very
16 well suited for large-scale industrial fermentation processes due to its robustness and
17 tolerance towards industrial conditions (Derek A. Abbott et al., 2009; Hong and Nielsen,
18 2012). *S. cerevisiae* can metabolize sucrose in two ways: extracellular hydrolysis followed
19 by facilitated diffusion of the monosaccharides glucose and fructose (**Figure 1A**) or uptake
20 of sucrose by a proton-symport mechanism followed by intracellular hydrolysis (**Figure**
21 **1B**) (Batista et al., 2005; Santos et al., 1982; Stambuk et al., 2000).

22

23



1
 2 **Figure 1.** Schematic representation of different strategies for the uptake and cleavage of
 3 sucrose. **A)** Extracellular hydrolysis of sucrose, facilitated by the external invertase Suc2,
 4 followed by facilitated diffusion of the monosaccharides. **B)** Uptake of sucrose via the
 5 proton-symporter Mal11, followed by ATP-driven export of the proton and intracellular
 6 hydrolysis of sucrose catalysed by Mal12. **C)** Uptake of sucrose via the proton-symporter
 7 Mal11, followed by ATP-driven export of the proton and intracellular phosphorylation of
 8 sucrose catalysed by sucrose phosphorylase (SPase). **D)** Uptake of sucrose via facilitated
 9 diffusion using a sucrose facilitator (SUF), followed by intracellular phosphorylation of
 10 sucrose catalysed by SPase. In red: metabolic steps that require hydrolysis of ATP.

11
 12 *S. cerevisiae* does not conserve the free energy of sucrose hydrolysis ($\Delta G_0' = -29$
 13 kJ/mol). In some anaerobic microorganisms sucrose is cleaved by phosphorylation instead

1 of hydrolysis. In the latter cleavage process, sucrose phosphorylase (SPase) converts
2 inorganic phosphate and sucrose into glucose-1-phosphate and fructose. Glucose-1-
3 phosphate can subsequently be converted into glucose-6-phosphate by
4 phosphoglucomutase. As this phosphorolytic cleavage circumvents the ATP-requiring
5 hexokinase reaction, it enables higher overall free-energy conservation than sucrose
6 hydrolysis (**Figure 1C**). Genes encoding SPase are known from various bacterial species
7 (Kawasaki et al., 1996). Other disaccharide phosphorylases, such as maltose- and cellobiose
8 phosphorylase, have previously been functionally expressed in *S. cerevisiae* (De Kok et al.,
9 2011; Sadie et al., 2011).

10 While intracellular phosphorolysis theoretically enables a higher free-energy
11 conservation (gain of 1 ATP per sucrose molecule), it requires transport of extracellular
12 sucrose to the cytosol. However, in wild-type *S. cerevisiae*, uptake of sucrose via a proton-
13 symporter (e.g. Mal11 (Stambuk et al., 1999)) and subsequent export of the proton via the
14 H⁺-ATPase results in a net expense of 1 ATP (Weusthuis et al., 1993). Therefore, an
15 improved free-energy conservation can be achieved when the proton-symport system is
16 replaced by transport via facilitated diffusion (SUF, **Figure 1D**). Sucrose transporters from
17 *Phaseolus vulgaris* and *Pisum sativum* have been functionally expressed in *S. cerevisiae* and
18 were described as probable sucrose facilitators (SUFs) (Zhou et al. (2007)). Additionally,
19 sucrose transporters from the SWEET family, e.g. from *Arabidopsis thaliana* and *Oryza*
20 *sativa*, have also been proposed to catalyse facilitated diffusion (Chen et al., 2012, 2010; Lin
21 et al., 2014).

22 The goal of this study was to explore whether free-energy conservation from
23 sucrose fermentation by *S. cerevisiae* can be improved by replacing the first two steps of

1 the native sucrose metabolism by facilitated uptake of the disaccharide and subsequent
2 phosphorolytic cleavage. A previously constructed *S. cerevisiae* strain lacking all native
3 sucrose proton-symporters and hydrolases, which remained sucrose-negative upon strong
4 selective pressures (Marques et al., 2017), was used as a platform to avoid interference by
5 native sucrose metabolising enzymes. For the phosphorolytic cleavage reaction, SPase from
6 *Leuconostoc mesenteroides* ATTC 12291 was chosen in view of the compatibility of its
7 temperature and pH optima with expression in yeast (Aerts et al., 2011; Goedl et al., 2010,
8 2007; Kawasaki et al., 1996; Lee et al., 2008). Several proposed sucrose facilitators from
9 plant origins were screened for their ability to support growth of the platform strain on
10 sucrose: *Phaseolus vulgaris* *SUF1* (*PvSUF1*), *Pisum sativum* *SUF1* and *SUF4* (*PsSUF1* and
11 *PsSUF4*), *Arabidopsis thaliana* *SWEET12* (*AtSWEET12*) and *Oryza sativa* *SWEET11*
12 (*OsSWEET11*). The impact of these modifications on free-energy conservation was studied
13 by analysis of biomass yields of engineered *S. cerevisiae* strains in anaerobic, sucrose-
14 limited chemostat cultures.

15 **2. MATERIALS AND METHODS**

16 **2.1 Strain storage and maintenance**

17 The *S. cerevisiae* strains used in this study (**Table 1**) share the CEN.PK genetic
18 background (Entian and Kötter, 2007; Nijkamp et al., 2012) with the exception of BY4742
19 which is derived from S288C (Brachmann et al., 1998). Cultures were grown at 30°C in 500
20 mL shake flasks containing 100 mL synthetic medium (SM) (Verduyn et al., 1992) with 20
21 g/L glucose as a carbon source in an Innova incubator shaker (Eppendorf, Hamburg,
22 Germany) set at 200 rpm. Frozen stocks were prepared by addition of glycerol (30% v/v)

- 1 to exponentially growing shake-flask cultures of *S. cerevisiae* and stored aseptically in 1 mL
- 2 aliquots at -80°C.

3 **Table 1**

4 The *Saccharomyces cerevisiae* strains that were used in this study.

Name	Relevant genotype	Origin
CEN.PK113-7D	MAT α <i>URA3 LEU2 MAL2-8^c SUC2</i>	Entian and Kötter, 2007
BY4742	MAT α <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Brachmann et al., 1998
IMK291	MAT α <i>ura3-52 leu2-112 MAL2-8^c mal11-mal12::loxP mal21-mal22::loxP mal31-mal32::loxP mph2/3::loxP mph2/3::loxP-hphNT1-loxP suc2::loxP-kanMX-loxP</i>	Marques et al., 2017
IMZ570	MAT α <i>ura3-52 leu2-112 MAL2-8^c malΔ mphΔ suc2Δ pUDC156 (URA3 cas9)</i>	This study
IMK698	MAT α <i>ura3-52 leu2-112 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ</i>	This study
IMX935	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ</i>	This study
IMZ616	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ pUDC156 (URA3 cas9)</i>	This study
IMZ627	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::MAL12</i>	This study
IMZ664	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::MAL12 pUDE432 (URA3 MAL11)</i>	This study
IMZ633	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::MAL12 pUDE413 (URA3 PvSUF1)</i>	This study
IMZ630	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase</i>	This study
IMZ665	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE432 (URA3 MAL11)</i>	This study
IMZ666	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE439 (URA3 OsSWEET11)</i>	This study
IMZ667	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE438 (URA3 AtSWEET12)</i>	This study
IMZ671	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE442 (URA3 PsSUF4)</i>	This study
IMZ672	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE441 (URA3 PsSUF1)</i>	This study
IMZ636	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE413 (URA3 PvSUF1)</i>	This study
IMZ692	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE260 (URA3)</i>	This study
IMX1272	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE453 (URA3 MAL11-YPet)</i>	This study
IMX1273	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE471 (URA3 PvSUF1-YPet)</i>	This study
IMZ696	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE486 (URA3 PvSUF1 PGM2)</i>	This study
IMZ709	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE496 (URA3 MAL11 PGM2)</i>	This study
IMX1274	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ pUDE260 (URA3)</i>	This study
IMX1275	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ pUDE413 (URA3 PvSUF1)</i>	This study
IMX1276	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ pUDE432 (URA3 MAL11)</i>	This study
IMX1277	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ pUDE453 (URA3 MAL11-YPet)</i>	This study
IMX1278	MAT α <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ pUDE471 (URA3 PvSUF1-YPet)</i>	This study

1 2.2 Plasmid construction

2 All plasmids used in this study are listed in **Table 2**. Plasmid pUDC156 was
3 assembled by *in vivo* homologous recombination (Kuijpers et al., 2013; Marques et al.,
4 2017), in strain IMK291 resulting in strain IMZ570 (**Table 1**). pUDC156 was then isolated
5 from strain IMZ570 and transformed into *E. coli* for storage and plasmid propagation.
6 Plasmid pUDR128 was constructed in the same way as pUDR127 (Marques et al., 2017)
7 with the exception that in this study pROS15 (Mans et al., 2015) was used as template for
8 backbone amplification. A gene encoding *Leuconostoc mesenteroides* ATCC 12291 sucrose
9 phosphorylase (*LmSPase*) (NCBI accession number D90314.1) was codon optimized
10 (**Supplementary material**) by JCat (Grote et al., 2005), ordered from Baseclear B.V.
11 (Leiden, The Netherlands) and delivered within pUD155. For construction of pUDE262, the
12 open reading frame (ORF) of *LmSPase* was excised from pUD155 with BamHI and Sall
13 restriction enzymes and cloned into the vector backbone of pUDE063 (De Kok et al., 2011),
14 which had been digested with the same enzymes, thereby removing the *pgmβ* gene.
15 Plasmid pUDE260 (empty vector) was made by digestion of pUDE063 with PvuII to excise
16 the *pgmβ* ORF followed by recircularization of the vector. Plasmid p426TEF-*amdSYM* was
17 constructed by replacing the marker of p426TEF (Mumberg et al., 1995) by the *amdSYM*
18 marker. The marker cassette was amplified from pUG-*amdSYM* (Solis-Escalante et al.,
19 2013) using primers 3093 & 3094 (**Table S1**). The p426TEF backbone was amplified with
20 primers 6845 & 6846 and ligation was done via Gibson assembly.

21 Plasmid pUDE379 was constructed via Gibson assembly of the *MAL11* ORF amplified
22 from pUDI035 (De Kok et al., 2011) using primers 8379 & 8380 and a vector backbone
23 amplified from plasmid p426TEF-*amdSYM* using primers 7998 & 7999. Plasmid pUDE432

1 was constructed via Gibson assembly of a *MAL11* expression cassette, amplified from
2 pUDE379 using primers 9043 & 9044 and a vector backbone linearized from p426GPD
3 (Mumberg et al., 1995) using KpnI and SacI restriction sites.

4 Plasmid pUDE485 was constructed via Gibson assembly of the *S. cerevisiae* *PGM2*
5 ORF amplified from genomic DNA of strain CEN.PK113-7D (**Table 1**) using primers 10303
6 & 10304 and a vector backbone amplified from pUDE206 (González-Ramos et al., 2016)
7 with primers 6486 & 9719. Plasmid pUDE496 was constructed via *in vivo* assembly of a
8 *PGM2* expression cassette, amplified from pUDE485 using primers 10305 & 10306 and
9 vector backbone amplified from pUDE432 with primers 10307 & 10308. Both amplicons
10 were transformed into strain IMZ630, resulting in strain IMZ709 (**Table 1**). Plasmid
11 pUDE486 was constructed via Gibson assembly of a *PGM2* expression cassette amplified
12 from pUDE485 using primers 10305 & 10306 and vector backbone amplified from
13 pUDE413 using primers 10307 & 10308.

14 Sequences coding for *AtSWEET12* (NCBI gene ID: 832431) and *OsSWEET11* (NCBI
15 gene ID: 4346153) were purchased from GeneArt (Regensburg, Germany) and were
16 delivered in vectors pMA-T (*AtSWEET12*, plasmid named pUD400) and pMK-RQ
17 (*OsSWEET11*, plasmid named pUD401). From pDR196-*PsSUF1* and pDR196-*PsSUF4* (Zhou
18 et al., 2007), *PsSUF1* (NCBI accession number DQ221698.1) and *PsSUF4* (NCBI accession
19 Number DQ221697.2) gene cassettes were obtained via digestion with XbaI and Sall. These
20 cassettes were ligated into the backbone of pUDI035, which was obtained via digestion
21 with the same enzymes, resulting in plasmids pUDI085 and pUDI086, respectively. *PvSUF1*
22 (NCBI accession number DQ221700.1) was codon optimized for expression in *S. cerevisiae*
23 with OptimumGene™ (GenScript, **Supplementary material**), purchased from GenScript

1 USA Inc. (Piscataway, NJ) and delivered within pUC57-*PvSUF1*. From pUC57-*PvSUF1*, the
2 *PvSUF1* ORF was obtained and subsequently cloned into pUDI035 via the *SpeI* and *Sall*
3 restriction sites, replacing the *MAL11* ORF, resulting in pUDI087 (**Table 1**). Plasmids
4 pUDE367, pUDE368, pUDE369, pUDE370 and pUDE374 were constructed by assembly of
5 cassettes containing the ORF of the transporter gene and a plasmid backbone. The
6 transporter expression cassettes containing the *PsSUF1*, *PsSUF4* and *PvSUF1* ORFs were
7 amplified from pUDI085, pUDI086 and pUDI087 using primers 8018 & 8019, 8020 & 8021
8 and 8022 & 8023, respectively and *AtSWEET12* and *OsSWEET11* ORFs were amplified from
9 pUD400 and pUD401 using primers 8000 & 8001, respectively. The vector backbone was
10 amplified from plasmid p426TEF-*amdSYM* using primers 7998 & 7999 and the transporter
11 genes were inserted in the vector backbone via Gibson assembly. Plasmids pUDE413,
12 pUDE438, pUDE439, pUDE441 and pUDE442 were constructed via Gibson assembly of
13 transporter expression cassettes amplified from pUDE369, pUDE370, pUDE374, pUDE367
14 and pUDE368 respectively with primers 9043 & 9044 and the p426GPD vector backbone
15 digested with *KpnI* and *SacI*.

16 For fluorescent tagging of Mal11 and *PvSUF1* with the YPet fluorescent protein
17 (Nguyen and Daugherty, 2005), first plasmid pRHA00 was made by amplifying the
18 backbone of pFB001 (Bianchi et al., 2016), using primers 5273 & 5274 and the *MAL11* gene
19 from the genomic DNA of *S. cerevisiae* strain BY4742 (EUROSCARF, Accession No. Y10000
20 (Brachmann et al., 1998)) with primers 5271 & 5272 followed by *in vivo* assembly. *MAL11*
21 from BY4742 is identical to *MAL11* from CEN.PK-derived strains. Then, the *URA3* marker
22 was omitted from pRHA00 via PCR with primers 5437 & 5438 and replaced with the *LEU2*
23 gene, amplified from pRS315 (Sikorski and Hieter, 1989) with primers 5435 & 5436 via *in*

1 *vivo* assembly. pR151 was then made by Gibson assembly of three fragments; the plasmid
2 backbone and *MAL11* gene amplified from pRHA00L with primers 5959 & 6324 and 5961
3 & 5272 respectively and the *TEF1* promoter amplified from pUDE379 with primers 4995 &
4 5960. Plasmid pUDE453 was made via Gibson assembly of a *MAL11*-YPet expression
5 cassette, amplified from pR151 using primers 6717 & 580 and vector backbone amplified
6 from pUDE413 with primers 5921 & 7812. For construction of plasmid pUDE471 via
7 Gibson assembly, the *MAL11* gene from pUDE453 was replaced by *PvSUF1* via amplification
8 of the plasmid backbone, including the YPet-tag, from pUDE453 with primers 5921 & 9772
9 and amplification of the *PvSUF1* ORF from pUDE413 with primers 6717 & 9763 before
10 assembly of both fragments.

11
12 **Table 2**
13 Plasmids used in this study

Name	Relevant characteristics	Origin
pUDC156	<i>ARS4-CEN6 URA3 pTEF1-cas9-tCYC1</i>	Marques et al., 2017
pUDR128	<i>2μ natNT2 gRNA-IMA5 gRNA-IMA1,2,3,4</i>	This study
pROS15	<i>2μ natNT2 gRNA-CAN1.Y gRNA-ADE2.Y</i>	Mans et al., 2015
pUD155	<i>attB1-LmSPase-attB2</i>	Baseclear B.V., Leiden, NL
pUDE262	<i>2μ URA3 pTDH3-LmSPase-tADH1</i>	This study
pUDE063	<i>2μ URA3 pTDH3-pgmB-tADH1</i>	de Kok et al., 2011
pUDE260	<i>2μ URA3 pTDH3-tADH1</i>	This study
p426TEF- <i>amdSYM</i>	<i>2μ amdSYM pTEF1-tCYC1</i>	This study
p426TEF	<i>2μ URA3 pTEF1-tCYC1</i>	Mumberg et al., 1995
pUG- <i>amdSYM</i>	<i>amdSYM</i>	Solis-Escalante et al., 2013
pUDE379	<i>2μ amdSYM pTEF1-MAL11-tCYC1</i>	This study
pUDI035	<i>Integrative plasmid, LEU2 pTDH3-MAL11-tCYC1</i>	de Kok et al., 2011
pUDE432	<i>2μ URA3 pTEF1-MAL11-tCYC1</i>	This study
p426GPD	<i>2μ URA3 pTDH3-tCYC1</i>	Mumberg et al., 1995
pUDE485	<i>2μ natNT1 pTPI1-PGM2-tTEF1</i>	This study
pUDE206	<i>2μ natNT1 pTPI1-I-Scel-tTEF1</i>	González-Ramos et al., 2016
pUDE496	<i>2μ URA3 pTEF1-MAL11-tCYC1 pTPI1-PGM2-tTEF1</i>	This study
pUD400	<i>pMA-T AtSWEET12</i>	GeneArt, Regensburg, Germany
pUD401	<i>pMK-RQ OsSWEET11</i>	GeneArt, Regensburg, Germany
pDR196- <i>PsSUF1</i>	<i>2μ URA3 pPMA1-PsSUF1-tADH1</i>	Zhou et al., 2007
pDR196- <i>PsSUF4</i>	<i>2μ URA3 pPMA1-PsSUF4-tADH1</i>	Zhou et al., 2007
pUDI085	<i>Integrative plasmid, LEU2 pTDH3-PsSUF1-tCYC1</i>	This study
pUDI086	<i>Integrative plasmid, LEU2 pTDH3-PsSUF4-tCYC1</i>	This study
pUC57- <i>PvSUF1</i>	<i>PvSUF1-codon optimized</i>	GenScript, Piscataway, NJ
pUDI087	<i>Integrative plasmid, LEU2 pTDH3-PvSUF1-tCYC1</i>	This study
pUDE367	<i>2μ amdSYM pTEF1-PsSUF1-tCYC1</i>	This study
pUDE368	<i>2μ amdSYM pTEF1-PsSUF4-tCYC1</i>	This study

pUDE369	<i>2μ amdSYM pTEF1-PvSUF1-tCYC1</i>	This study
pUDE370	<i>2μ amdSYM pTEF1-AtSWEET12-tCYC1</i>	This study
pUDE374	<i>2μ amdSYM pTEF1-OsSWEET11-tCYC1</i>	This study
pUDE413	<i>2μ URA3 pTEF1-PvSUF1-tCYC1</i>	This study
pUDE438	<i>2μ URA3 pTEF1-AtSWEET12-tCYC1</i>	This study
pUDE439	<i>2μ URA3 pTEF1-OsSWEET11-tCYC1</i>	This study
pUDE441	<i>2μ URA3 pTEF1-PsSUF1-tCYC1</i>	This study
pUDE442	<i>2μ URA3 pTEF1-PsSUF4-tCYC1</i>	This study
pFB001	<i>2μ URA3 YPet-tCYC1</i>	Bianchi et al., 2016
pRHA00	<i>2μ URA3 MAL11-YPet-tCYC1</i>	This study
pRS315	<i>ARS4-CEN6 LEU2</i>	Sikorski and Hieter, 1989
pRHA00L	<i>2μ LEU2 MAL11-YPet-tCYC1</i>	This study
pR151	<i>2μ LEU2 pTEF1-MAL11-YPet-tCYC1</i>	This study
pUDE453	<i>2μ URA3 pTEF1-MAL11-YPet-tCYC1</i>	This study
pUDE471	<i>2μ URA3 pTEF1-PvSUF1-YPet-tCYC1</i>	This study
pUDE486	<i>2μ URA3 pTEF1-PvSUF1-tCYC1 pTPI1-PGM2-tTEF1</i>	This study
pUDR119	<i>2μ amdSYM gRNA-SGA1</i>	van Rossum et al., 2016
pUDE044	<i>2μ URA3 pTDH3-MAL12-tADH1</i>	de Kok et al., 2011

1

2 2.3 Strain construction

3 *S. cerevisiae* transformations were carried out according to Gietz and Woods (2002)

4 using 1 μg of DNA per transformation, if not stated otherwise. Transformants were selected

5 on 2% (w/v) agar plates containing synthetic medium (SM) (Verduyn et al., 1992) with 20

6 g/L glucose plus the following components when necessary: G418 (200 mg/L); uracil (0.15

7 g/L); L-leucine (0.5 g/L) (Pronk, 2002). Cells expressing the *amdSYM* marker were selected

8 on plates according to Solis-Escalante et al. (2013). Cells expressing the *natNT2* marker

9 were selected on plates containing nourseothricin (100 mg/L) (Jena Bioscience, Jena,

10 Germany) in SM with 1 g/L glutamic acid as sole nitrogen source. IMZ570 was made via *in*

11 *vivo* assembly of plasmid pUDC156 in IMK291 (**Table 1**). IMZ570 was transformed with 1

12 μg of plasmid pUDR128, 4 μg dsDNA repair fragment for *IMA1-4* and 4 μg dsDNA repair

13 fragment for *IMA5* (Mans et al., 2015; Marques et al., 2017). Subsequently, pUDR128 and

14 pUDC156 were cured from IMZ570 by cultivation on YPD plates with 20 g/L glucose and 1

15 g/L 5'-fluoroorotic acid (Boeke et al., 1984), resulting in IMK698. The *LEU2* marker

16 fragment was amplified from pUDI035 with primers 1742 & 1743 and integrated in

17 IMK698 resulting in strain IMX935. IMZ616 was made via transformation of IMX935 with

1 pUDC156. For the construction of IMZ627 and IMZ630, cassettes with homology to the
2 *SGA1* locus were amplified from pUDE044 (De Kok et al., 2011) and pUDE262 with primers
3 9355 & 9356 resulting in expression cassettes containing *MAL12* and *LmSPase* respectively.
4 IMZ616 was then transformed with 1 µg of pUDR119 (gRNA-*SGA1*) (Van Rossum et al.,
5 2016) together with either 1 µg *MAL12* expression cassette or 1 µg *LmSPase* expression
6 cassette and subsequent removal of pUDR119 and pUDC156 resulted in strains IMZ627
7 (*MAL12* expression) and IMZ630 (*LmSPase* expression), respectively. IMZ636, IMZ666,
8 IMZ667, IMZ671, IMZ672 and IMZ692 were made by transformation of plasmids pUDE413,
9 pUDE439, pUDE438, pUDE442, pUDE441 and pUDE260 into IMZ630, respectively. IMZ633
10 was made via transformation of plasmid pUDE413 into IMZ627. pUDE432 was transformed
11 into IMZ627 and IMZ630, resulting in IMZ664 and IMZ665, respectively. IMZ696, IMX1272
12 and IMX1273 were constructed via transformation of pUDE486, pUDE453 and pUDE471
13 into IMZ630, respectively. IMZ709 was made via *in vivo* assembly of pUDE469 in IMZ630.
14 IMX935 was transformed with pUDE260, pUDE413, pUDE432, pUDE453 and pUDE471,
15 resulting in IMX1274-1278 respectively.

16 **2.4 Molecular biology techniques**

17 PCR amplification for strain construction was performed with Phusion Hot Start II
18 High Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA) according to the
19 manufacturer's instructions using PAGE-purified oligonucleotide primers (Sigma-Aldrich,
20 St. Louis, MO). Diagnostic PCR was done via colony PCR on randomly picked yeast colonies,
21 using DreamTaq (Thermo Fisher Scientific) and desalted primers (Sigma-Aldrich). The
22 primers used in this study are listed in **Table S1**. Yeast genomic DNA was isolated using the
23 YeaStar Genomic DNA kit (D2002, Zymo Research, Irvine, CA). DNA fragments obtained by

1 PCR were separated by gel electrophoresis on 1% (w/v) agarose gels (Thermo Fisher
2 Scientific,) in Tris-acetate-EDTA buffer (Thermo Fisher Scientific) at 100 V for 30 min. DNA
3 fragments were excised from gel and purified by gel purification (D2004, Zymo Research).
4 Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich)
5 according to the supplier's manual and from yeast with the Zymoprep Yeast Plasmid
6 Miniprep II kit (D2004, Zymo Research). Restriction analysis with enzymes was performed
7 using FastDigest enzymes (Thermo Fisher Scientific) according to the manufacturer's
8 manual. Plasmid assembly was performed using *in vivo* recombination in yeast (Kuijpers et
9 al., 2013) , T4 DNA ligase (Thermo Fisher Scientific), Gibson Assembly Cloning Kit (New
10 England Biolabs, Ipswich, MA) or NEBuilder HiFi DNA Assembly Cloning Kit (New England
11 Biolabs). Assembly of plasmids was done according to the suppliers' protocols, but
12 downscaled to a total volume of 5 μ L for the Gibson and NEBuilder HiFi DNA Assembly
13 Cloning kit. Ligation of plasmids was performed using T4 DNA ligase according to
14 manufacturer's instructions (New England Biolabs). *E. coli* DH5 α (18258-012, Thermo
15 Fisher Scientific) or XL1-Blue (GE Healthcare Life Sciences, Uppsala, Sweden) were used for
16 chemical transformation (T3001, Zymo Research) or for electroporation. Chemical
17 transformation of *E. coli* was done according to the supplier's instructions. Electroporation
18 was done in a 2 mm cuvette (165-2086, BioRad, Hercules, CA) using a Gene PulserXcell
19 Electroporation System (BioRad), following the manufacturer's protocol. Electrocompetent
20 *E. coli* cells were prepared according to the BioRad protocol, except for the use of lysogeny
21 broth (LB) (Bertani, 1951) without NaCl when pre-growing the cells.

22 **2.5 Media and cultivation**

1 Synthetic medium (SM) was prepared according to Verduyn et al., (1992) and
2 autoclaved at 120°C for 20 min. Glucose, sucrose and vitamins (Verduyn et al., 1992) were
3 prepared separately and filter sterilized (sucrose and vitamins) or heat sterilized at 110°C
4 for 20 min (glucose). For anaerobic cultures, the growth factors ergosterol (10 mg/L) and
5 Tween80 (420 mg/L) were dissolved in ethanol and added to the media. Aerobic shake-
6 flask cultures were grown in an Innova incubator shaker (New Brunswick Scientific,
7 Edison, NJ) and anaerobic shake-flask cultures were grown in a Bactron Anaerobic
8 Chamber (Sheldon Manufacturing, Cornelius, OR) with an atmosphere consisting of 5% H₂
9 6% CO₂ and 89% N₂. Trace amounts of oxygen entering the chamber (e.g. when sampling)
10 were removed by reacting with the hydrogen using a palladium catalyst. Cultures were
11 shaken at 200 rpm at 30°C.

12 Precultures were prepared by inoculation of shake flasks containing SM with 20 g/L
13 glucose as the carbon source with a -80°C frozen stock and subsequent overnight
14 incubation under aerobic conditions. 1 mL of the grown culture was transferred to fresh
15 SM with 20 g/L sucrose and incubated under aerobic conditions. Exponentially growing
16 cultures were washed and used as precultures for aerobic experiments. For anaerobic
17 precultures, 1 mL of the growing aerobic sucrose culture was transferred to fresh SM with
18 20 g/L sucrose and incubated anaerobically. Exponentially growing cultures were washed
19 and used as inoculum for anaerobic experiments.

20 Strain characterization in shake flasks was carried out in SM containing 20 g/L
21 sucrose. For aerobic cultures, 100 mL SM in a 500 mL shake flask was used and for
22 anaerobic cultures 50 mL SM in a 100 mL shake flask.

1 Strain characterization in bioreactors was carried out at 30°C in aerobic and
2 anaerobic 2-L laboratory bioreactors (Applikon, Delft, The Netherlands) with a working
3 volume of 1 L. After heat sterilization (120°C for 20 min), the SM was supplemented with
4 heat sterilized (120°C for 20 min) Antifoam Emulsion C (Sigma-Aldrich) to a final
5 concentration of 0.15 g/L, sucrose to a final concentration of 25 g/L, anaerobic growth
6 factors and vitamins (Verduyn et al., 1992). The culture pH was maintained at 5.0 by
7 automated addition of 2 M KOH and was stirred at 800 rpm. To maintain anaerobic
8 conditions, the bioreactors were sparged with 500 mL N₂/min (<5 ppm O₂) and equipped
9 with Norprene tubing to minimize oxygen diffusion or 500 mL/min compressed air for
10 aerobic experiments. For anaerobic experiments, the medium vessels were also sparged
11 with N₂. For the batch phase, the reactors were inoculated with *S. cerevisiae* strains to an
12 initial optical density of 0.2 - 0.5, and culture growth was monitored via determination of
13 the CO₂-concentration in the off-gas. After the batch phase, medium pumps were switched
14 on, resulting in the continuous addition of synthetic medium (25 g/L sucrose for anaerobic
15 and 7.5 g/L sucrose for aerobic conditions) to the cultures. During the chemostat phase of
16 the anaerobic cultures, a continuously stirred Antifoam Emulsion C (100 g/L) was added
17 separately at a rate of 2-5 drops per hour and for the aerobic cultures, 0.15 g/L Antifoam
18 Emulsion C was added to the medium. To minimize differences between the aerobic and
19 anaerobic cultures, both cultures were supplemented with anaerobic growth factors. The
20 working volume was kept constant at 1.0 L using an effluent pump controlled by an electric
21 level sensor, resulting in a constant dilution rate. The exact working volume and medium
22 flow rate were measured at the end of each experiment. Chemostat cultures were assumed
23 to be in steady state when, after five volume changes, the culture dry weight, extracellular

1 metabolite concentrations of ethanol and glycerol and the CO₂ production rate varied by
2 less than 2% over at least a further 2 volume changes.

3 **2.6 Analytical methods**

4 Optical density was monitored using a Libra S11 spectrophotometer (Biochrom,
5 Cambridge, United Kingdom) at 660 nm. Culture dry weights were determined via filtration
6 of well-mixed samples over dry nitrocellulose filters with a pore size of 0.45 µm (Gelman
7 laboratory, Ann Arbor, USA). Prior to filtration, the filters were dried and weighed. After
8 filtration of the sample, the filters were washed using demineralized water and dried in a
9 microwave oven for 20 min at 360 W and weighed again. Supernatant was obtained via
10 centrifugation of the culture broth and samples for residual sugars were obtained via rapid
11 quenching using cold stainless-steel beads (Mashego et al., 2003). Residual sucrose
12 concentration was analysed enzymatically (10716260035, R-Biopharm AG, Darmstadt,
13 Germany) according to manufacturer's instructions and via HPLC. HPLC analysis of the
14 supernatant, residual sugar samples and ingoing medium was performed as described
15 previously (De Kok et al., 2011; Marques et al., 2017) and ethanol concentrations were
16 corrected for ethanol evaporation (Guadalupe-Medina et al., 2010). Cellular protein content
17 was determined as described previously (Verduyn et al., 1990), with the exception that 1 M
18 NaOH was used instead of 1 M KOH and the absorbance was measured at 510 nm instead of
19 550 nm. Off-gas was cooled in a condenser and dried with a Perma Pure Dryer (Perma
20 Pure, Lakewood, NJ) before CO₂ concentrations were measured with a NGA 2000
21 Rosemount gas analyser (Emerson, St. Louis, MO).

22 **2.7 Determination of sucrose hydrolase, sucrose phosphorylase and** 23 **phosphoglucomutase activities**

1 For enzyme-activity assays, culture samples corresponding to 62.5 mg dry weight were
2 harvested during the steady state of the chemostat cultures. Strain IMZ616, was grown in
3 shake-flasks with SM containing 2% (v/v) ethanol and harvested during exponential
4 growth for cell extract preparation. 4 hours before harvesting, 20 g/L sucrose was added to
5 the culture of IMZ616 in order to induce any sucrose responsive genes. Cell extracts were
6 prepared by sonication and centrifugation as described previously (Postma et al., 1989).
7 Protein concentrations in cell extracts were determined with the Lowry method (Lowry et
8 al., 1951). Sucrose hydrolytic activity was measured as described previously for maltase
9 activity (De Kok et al., 2011), with the exception that 250 mM sucrose was used to start the
10 reaction. Sucrose-phosphorylase activity was measured at 30°C by monitoring the
11 reduction of NADP⁺ at 340 nm in a 1 ml reaction mixture containing 200 mM potassium
12 phosphate buffer (pH 7.0), 10 mM EDTA, 10 mM MgCl₂, 10 μM α-D-glucose 1,6-
13 bisphosphate (activator of Pgm2 (Tedokon et al., 1992)), 2 mM NADP⁺, 2.65 U
14 phosphoglucomutase, 5.25 U glucose 6-phosphate dehydrogenase and 1–100 μl cell extract
15 (adapted from Goedl et al., 2007). The reaction was started by the addition of sucrose to a
16 final concentration of 250 mM. Phosphoglucomutase activity was determined according to
17 van den Brink et al., 2009. An extinction coefficient of 6.3 mM⁻¹ was assumed for NADPH.

18 **2.8 Microscopy of YPet tagged Mal11 and PvSUF1**

19 For fluorescence microscopy, samples were taken from aerobic, steady-state chemostat
20 cultures with *S. cerevisiae* strains IMX1272 (*Mal11-YPet, LmSPase*) and IMX1273 (*PvSUF1-*
21 *YPet, LmSPase*). Cells were then imaged by phase-contrast microscopy using a Zeiss D1
22 Imager with a 100x objective (EC Plan-Neofluar 100x/1.30 Oil Ph 3 M27), equipped with an
23 AxioCamMR camera (Zeiss, Jena, Germany). For fluorescence microscopy a HAL100

1 fluorescent lamp and Filter set 10 (Ex 450-490 nm/Em 515-565 nm) (Zeiss, Jena, Germany)
2 were used.

3 **2.9 Proton-solute symport assays**

4 Cells for proton-solute symport assays were harvested from aerobic, sucrose-limited
5 chemostat cultures at a dilution rate of 0.030 h⁻¹. The reactors were prepared as described
6 above, with the exceptions of the use of 7.5 g/L sucrose in the medium and aeration with
7 500 mL compressed air/min. Proton-solute symport measurements were done according
8 to Van Urk et al., 1989 with the following modifications: The culture of IMZ696 culture was
9 centrifuged at room temperature, washed once with distilled water and resuspended in
10 1.25 mM potassium phthalate buffer (pH 5) to a final concentration of 12 g dry weight/L. In
11 view of the higher proton-solute symport activity of Mal11 expressing strains, IMZ709 was
12 resuspended to 6 g dry weight/L. The assay was performed in a magnetically stirred vessel
13 containing 5 mL of cell suspension, kept at 30°C. A pH-probe connected to a S220
14 SevenCompact™ pH/Ion (Mettler Toledo, Greifensee, Switzerland) was used to record
15 buffer alkalization upon addition of sucrose, maltose, glucose or fructose to a final
16 concentration of 20 mM. Data was recorded using LabX™ (Mettler Toledo, Greifensee,
17 Switzerland). Pulses of 100 nanomoles NaOH were used to estimate the correlation
18 between the voltage change measured by the pH-probe and the change in H⁺ concentration
19 for each strain tested.

20 **2.10 Transport of radiolabelled sucrose**

21 Yeast strains were grown aerobically on synthetic medium with 2% ethanol as carbon
22 source. Cells from exponential cultures were harvested by centrifugation and then washed
23 twice and resuspended in potassium citrate-phosphate (KCP) buffer at pH 5, containing

1 0.2% (v/v) ethanol. Cell suspensions in buffer were kept on ice for no longer than 4 hours
2 before use. Transport assays were performed at 30°C using cell suspensions with an optical
3 density (OD₆₀₀) of 8. Cells were incubated at 30°C for 5 min and then [U-¹⁴C] sucrose (600
4 mCi/mmol; American Radiolabeled Chemicals, Inc.) was added to approximately 48100
5 Bq/mL (final sucrose concentration of 1 mM) to start the uptake reaction. After 20 min of
6 uptake, 10 μM of the protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
7 (FCCP) was added to the uptake reactions. At given time intervals, 50 μL samples of the
8 uptake reaction mixture were mixed with 2 mL ice-cold KCP and then rapidly filtered using
9 cellulose-nitrate filters with 0.45 μm pores (GE-Healthcare, Little Chalfont, UK). Prior to
10 filtration of the cell suspension, the filters were pre-soaked in KCP with 1 mM of sucrose to
11 block non-specific adsorption of ¹⁴C-sucrose. Filters were washed once with 2 mL KCP and
12 dissolved in 2 mL scintillation solution (Emulsifier^{plus}, PerkinElmer, Waltham, MA, USA).
13 The radioactivity on each filter paper was measured using a liquid scintillation counter
14 (Tri-Carb 2800TR liquid scintillation analyzer, PerkinElmer). The amount of sucrose in
15 each sample was normalized to 10⁶ cells by counting cells using a Accuri C6 flow cytometer
16 (BD AccuriTM, Durham, USA) and an estimate of 60 fL internal volume per cell was used to
17 calculate the concentration of intracellular sucrose.

18 **2.11 Flow cytometry**

19 Yeast strains were grown aerobically on synthetic medium with 2% ethanol as carbon
20 source. Cells from exponential cultures were harvested and diluted to an optical density
21 (OD₆₀₀) between 0.25-0.4, and then 20 μL samples were analysed using an Accuri C6 flow
22 cytometer (BD Biosciences, Durham, USA). YPet fluorescence was detected using a 488 nm
23 laser and an "FL1" emission detector (533/30 nm).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

3. RESULTS

3.1 Replacement of invertase by *Leuconostoc mesenteroides* sucrose phosphorylase increases the ATP yield from sucrose fermentation in *Saccharomyces cerevisiae*.

The first step towards improving the free-energy conservation of sucrose fermentation was to functionally replace the native yeast invertase with a sucrose phosphorylase (SPase). Deletion of the native sucrose hydrolysing enzymes and sucrose-proton symporters was achieved by targeted deletion of *SUC2*, the *MAL* loci, the α -glucoside permease genes *MPH2* and *MPH3* and the isomaltase genes *IMA1-5* (De Kok et al., 2011; Marques et al., 2017). The resulting strain IMZ616 (**Table 1**) was unable to grow on sucrose over a period of up to 2 months and no sucrose hydrolysis activity could be detected in cell extracts (**Table 3**). Therefore, IMZ616 was used as a platform strain to express SPase. Chromosomal integration of an expression cassette carrying the *Leuconostoc mesenteroides* sucrose-phosphorylase gene (*LmSPase*), in combination with expression of the native sucrose-proton symporter *MAL11* from a multi-copy plasmid resulted in strain IMZ665. Functional expression of *LmSPase* was confirmed via measurement of *in vitro* sucrose phosphorylase activity of 0.90 ($\mu\text{mol}/\text{min}$)/mg protein (**Table 3**). Under anaerobic conditions, strain IMZ665 was able to grow in synthetic medium with sucrose as the sole carbon source, at a specific growth rate of $0.09 \pm 0.02 \text{ h}^{-1}$ (**Table 3**). Even after prolonged incubation, strains with a similar genetic background that expressed only *MAL11* were unable to grow in medium with sucrose as the sole carbon source (Marques et al., 2017). Additionally, a reference strain expressing *MAL11* combined with a native glucosidase (*MAL12*) was constructed (IMZ664, **Table 1**). This strain grew

1 anaerobically on sucrose, at a specific growth rate of $0.19 \pm 0.01 \text{ h}^{-1}$ (**Table 3**). The higher
 2 specific growth rate of this isogenic strain indicated that the sucrose transporter *MAL11*
 3 was not rate limiting for growth of IMZ665 (*MAL11*, *LmSPase*).

4
 5 **Table 3**

6 Specific growth rates on sucrose and enzyme activities of sucrose hydrolase and sucrose
 7 phosphorylase of *S. cerevisiae* strains expressing either the sucrose transporter Mal11 or
 8 PvSUF1 in combination with either the sucrose hydrolase Mal12 or sucrose phosphorylase
 9 *LmSPase* in anaerobic bioreactors. Average growth rates were determined from triplicate
 10 experiments and based on CO₂ production in anaerobic batch fermentations at pH 5.0 and
 11 25 g/L sucrose. Enzyme activities represent the average of measurements on duplicate
 12 steady-state chemostat cultures at pH 5.0, 25 g/L sucrose and a dilution rate of 0.030 h^{-1}
 13 (IMZ664, IMZ665 and IMZ636) or 0.07 h^{-1} (IMZ709 and IMZ696). IMZ616 was incubated in
 14 aerobic shake flasks with 20 g/L sucrose for growth assessment and 20 g/L ethanol and 20
 15 g/L sucrose for enzyme activity assays.

Strain	Relevant Genotype	Growth rate (h^{-1})	Enzyme activity (($\mu\text{mol}/\text{min}$)/mg protein)		
			Sucrose hydrolase	Sucrose phosphorylase	Phosphogluco mutase
IMZ616	<i>malΔ mphΔ suc2Δ imaΔ</i>	No growth ^{a,b}	B.D.	B.D.	N.D.
IMZ664	<i>malΔ mphΔ suc2Δ imaΔ MAL11 MAL12</i>	0.19 ± 0.01	4.23 ± 0.21	B.D.	0.43 ± 0.06
IMZ665	<i>malΔ mphΔ suc2Δ imaΔ MAL11 LmSPase</i>	0.09 ± 0.02	N.D.	0.90 ± 0.20	1.04 ± 0.20
IMZ636	<i>malΔ mphΔ suc2Δ imaΔ PvSUF1 LmSPase</i>	0.06 ± 0.01^a	N.D.	2.96 ± 0.36	1.60 ± 0.13
IMZ709	<i>malΔ mphΔ suc2Δ imaΔ MAL11 LmSPase PGM2</i>	0.23 ± 0.01	0.07 ± 0.01	1.74 ± 0.14	20.06 ± 3.91
IMZ696	<i>malΔ mphΔ suc2Δ imaΔ PvSUF1 LmSPase PGM2</i>	0.08 ± 0.00	0.06 ± 0.01	1.67 ± 0.14	34.15 ± 1.33

16 N.D. = not determined

17 B.D. = below detection ($< 0.04 \text{ (}\mu\text{mol}/\text{min})/\text{mg protein}$)

18 ^a Measured in duplicate

19 ^b No growth observed after 2 months

20

1 To investigate the impact of *LmSPase* expression on the ATP yield from sucrose
2 fermentation, biomass yields on sucrose were measured. Alcoholic fermentation of one
3 sucrose molecule via the proton-coupled symporter *MAL11* and sucrose hydrolase *MAL12*
4 generates 3 ATP (Figure 1B). Replacement of sucrose hydrolysis by sucrose phosphorylase
5 increases the theoretical yield to 4 ATP per sucrose (Figure 1C). The anaerobic biomass
6 yield on sucrose can be used as an *in vivo* read-out of the energetic difference between
7 strains (De Kok et al., 2011; Weusthuis et al., 1993), where a strain yielding 4 ATP per
8 sucrose is predicted to have a 33% higher biomass yield than a 3 ATP strain (Basso et al.,
9 2011). To minimize effects of specific growth rate on the biomass yield, IMZ664 (*MAL11*,
10 *MAL12*) and IMZ665 (*MAL11*, *LmSPase*) were investigated under identical conditions and at
11 identical specific growth rates in anaerobic steady-state chemostat cultures. In view of the
12 maximum anaerobic specific growth rate of IMZ665 on sucrose ($\mu = 0.09 \pm 0.02 \text{ h}^{-1}$, **Table**
13 **3**), a dilution rate of 0.030 h^{-1} was chosen.

14 IMZ665 (*MAL11*, *LmSPase*) showed an increased biomass yield compared to the
15 *MAL11*, *MAL12* expressing strain IMZ664 (0.069 ± 0.000 vs 0.053 ± 0.001 g/g glucose
16 equivalent (**Table 4**). This observed difference of 31% is close to the theoretical value of
17 33%. Increased free-energy conservation in the catabolic pathway dictates that less
18 sucrose needs to be fermented to ethanol and CO_2 to provide the same amount of ATP
19 required for biomass formation. Accordingly, the biomass specific uptake rate of sucrose
20 was 32% lower (1.21 ± 0.00 vs 1.59 ± 0.05 mmol/g biomass/h) and rates of ethanol and
21 CO_2 production were 39% (4.17 ± 0.01 vs 5.81 ± 0.15 mmol/g biomass/h) and 34% ($4.17 \pm$
22 0.01 vs 5.81 ± 0.15 mmol/g biomass/h) lower in strain IMZ655 (*MAL11*, *LmSPase*) as
23 compared to those in IMZ664 (*MAL11*, *MAL12*). Replacement of sucrose hydrolysis by

1 phosphorolysis also resulted in a decrease in the residual sucrose concentration from 0.12
 2 ± 0.02 g/L to 0.07 ± 0.01 g/L (**Table 4**).

3 **Table 4**

4 Growth characteristics of IMZ664 (*MAL11*, *MAL12*), IMZ665 (*MAL11*, *LmSPase*), IMZ636
 5 (*PvSUF1*, *LmSPase*), IMZ709 (*MAL11*, *LmSPase*, *PGM2*) and IMZ696 (*PvSUF1*, *LmSPase*,
 6 *PGM2*) in sucrose-limited anaerobic chemostat cultures. The cultures of IMZ664, IMZ665
 7 and IMZ636 were grown at a dilution rate of 0.030 h^{-1} and the cultures of IMZ709 and
 8 IMZ696 at a dilution rate of 0.07 h^{-1} . Biomass specific production- or consumption rates are
 9 shown with the denotation $q_{\text{metabolite}}$. Averages, mean deviations and standard deviations
 10 were, respectively, obtained from duplicate (IMZ664, IMZ665 and IMZ636) or triplicate
 11 (IMZ709 and IMZ696) experiments.

Strain	IMZ664	IMZ665	IMZ636	IMZ709	IMZ696
Relevant genotype	<i>MAL11 MAL12</i>	<i>MAL11 LmSPase</i>	<i>PvSUF1 LmSPase</i>	<i>MAL11 LmSPase PGM2</i>	<i>PvSUF1 LmSPase PGM2</i>
Biomass yield (g/g glucose equivalent)	0.053 ± 0.001	0.069 ± 0.000	0.075 ± 0.000	0.080 ± 0.001	0.087 ± 0.002
q_{sucrose} (mmol/g biomass/h)	-1.59 ± 0.05	-1.21 ± 0.00	-1.11 ± 0.00	-2.25 ± 0.08	-2.27 ± 0.06
q_{ethanol} (mmol/g biomass/h)	5.81 ± 0.15	4.17 ± 0.01	3.69 ± 0.06	7.17 ± 0.31	7.05 ± 0.29
q_{CO_2} (mmol/g biomass/h)	5.73 ± 0.17	4.29 ± 0.13	3.79 ± 0.05	8.34 ± 0.20	8.35 ± 0.10
q_{glycerol} (mmol/g biomass/h)	0.31 ± 0.00	0.29 ± 0.01	0.26 ± 0.01	0.57 ± 0.02	0.57 ± 0.02
q_{lactate} (mmol/g biomass/h)	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.06 ± 0.00	0.02 ± 0.00
q_{pyruvate} (mmol/g biomass/h)	B.D. ^b	B.D.	B.D.	0.01 ± 0.00	B.D.
q_{acetate} (mmol/g biomass/h)	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Protein content (g/g biomass)	0.46 ± 0.01	0.44 ± 0.02	0.46 ± 0.02	N.D.	N.D.
Residual sucrose (g/L)	0.12 ± 0.02	0.07 ± 0.01	0.24 ± 0.01	0.14 ± 0.00	0.71 ± 0.17
Carbon recovery (%)	103 ± 1	103 ± 1	100 ± 1	101 ± 1	101 ± 1
Actual dilution rate (/h)	0.030 ± 0.002	0.030 ± 0.000	0.030 ± 0.000	0.065 ± 0.002	0.070 ± 0.001

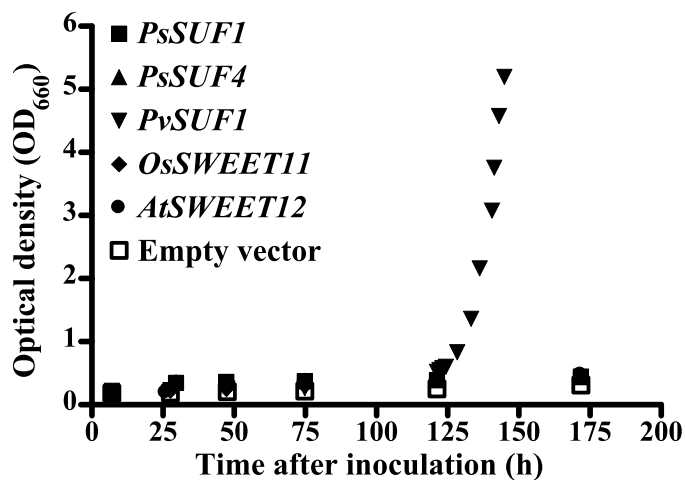
12 N.D. = not determined

13 B.D. = below detection (< 0.01 mmol/g biomass/h)

14

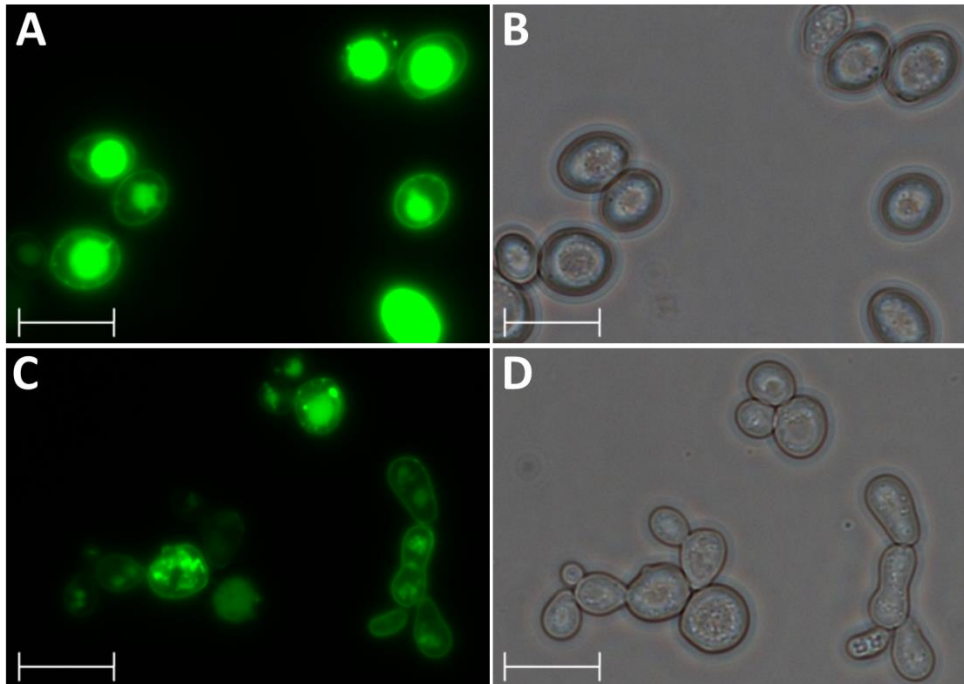
15 **3.2 Functional expression of *Phaseolus vulgaris* sucrose facilitator 1 in *S. cerevisiae*.**

1 The next objective in increasing the ATP yield from sucrose fermentation was to replace
2 the native proton-coupled uptake of sucrose in *S. cerevisiae* by a sucrose facilitator. To this
3 end, the genes *PsSUF1*, *PsSUF4*, *PvSUF1*, *OsSWEET11* and *AtSWEET12*, all encoding for
4 transporters that were described in literature as sucrose facilitators (Chen et al., 2012,
5 2010; Lin et al., 2014; Zhou et al., 2007), were individually expressed from multi-copy
6 plasmids in a strain carrying an integrated copy of *LmSPase*. To test for functional
7 expression of the sucrose transporters, the resulting strains (IMZ672, IMZ671, IMZ636,
8 IMZ666 and IMZ667, respectively) were pre-grown on glucose-based medium and then
9 incubated aerobically in medium containing sucrose as the sole carbon source. After 5 d,
10 growth was observed for strain IMZ636 (*PvSUF1*, *LmSPase*) at a specific growth rate of 0.12
11 $\pm 0.02 \text{ h}^{-1}$ (**Figure 2**). Neither the control strain IMZ692 (expressing only *LmSPase*) nor any
12 of the other strains expressing plant transporters exhibited growth after 7 d of incubation.
13 In anaerobic bioreactors, strain IMZ636 grew on sucrose at a specific growth rate of $0.06 \pm$
14 0.01 h^{-1} (**Table 3**). A strain expressing *PvSUF1* from a multi-copy plasmid with an
15 integrated copy of *MAL12* (IMZ633) was unable to grow on sucrose and therefore the
16 *PvSUF1*- and *LmSPase*-expressing strain IMZ636 was subjected to a further
17 characterization.
18



1
2 **Figure 2.** Growth curves of *S. cerevisiae* strains expressing *LmSPase* in combination with
3 either *PsSUF1* (IMZ683 (■)), *PsSUF4* (IMZ682 (▲)), *PvSUF1* (IMZ636 (▼)), *OsSWEET11*
4 (IMZ677 (◆)), *AtSWEET12* (IMZ678 (●)) or an empty vector (IMZ692 (□)) in aerobic
5 shake flasks containing synthetic medium with sucrose as the sole carbon source. One
6 representative culture of duplicate cultivations is shown in the figure. All the symbols
7 overlap, except for ▼ from 125 h onwards.

8
9 Cellular localisation of both endogenous YPet-tagged Mal11 (IMX1272) and YPet-
10 tagged PvSUF1 (IMX1273) was investigated microscopically (**Figure 3**). Both strains
11 showed a similar fluorescence at the periphery of cytosol, confirming that both Mal11 and
12 PvSUF1 were indeed targeted to the plasma membrane. However, distribution of the
13 remainder of the fluorescence differed significantly between the two strains. Whereas
14 intracellular fluorescence in strain IMX1272 (Mal11) was predominantly associated with
15 vacuoles (**Figure 3A**), fluorescence of IMX1273 (PvSUF1) appeared to be distributed over
16 multiple smaller intracellular compartments (**Figure 3C**). Lastly, cells from IMX1273
17 (PvSUF1) appeared to form cell clusters and were slightly elongated.



1
2 **Figure 3.** Fluorescent and phase-contrast pictures of *S. cerevisiae* strains IMX1272 (*MAL11-*
3 *YPet, LmSPase, A & B*) and IMX1273 (*PvSUF1-YPet, LmSPase, C & D*). Cells were collected
4 from aerobic, steady-state, sucrose-limited chemostat cultures grown at a dilution rate of
5 0.030 h^{-1} . The scale bar represents $10 \mu\text{m}$.

6
7 In theory, facilitated uptake of sucrose via a uniporter, combined with
8 phosphorolytic cleavage of sucrose via SPase and anaerobic alcoholic fermentation, should
9 lead to the formation of 5 ATP per sucrose (**Figure 1D**). This increased ATP yield is
10 predicted to result in a 25% increase in the anaerobic biomass yield when compared to a
11 strain yielding 4 ATP per sucrose (Basso et al., 2011; De Kok et al., 2011; Weusthuis et al.,
12 1993). To quantitatively investigate the impact of combined expression of *LmSPase* and
13 *PvSUF1*, strain IMZ636 (*PvSUF1, LmSPase*) was grown in anaerobic sucrose-limited
14 chemostat cultures at a dilution rate of 0.030 h^{-1} . The observed anaerobic biomass yield on
15 sucrose was increased by 8% from $0.069 \pm 0.000 \text{ g/g}$ glucose equivalent for strain IMZ655

1 (*MAL11*, *LmSPase*) to 0.075 ± 0.000 g/g glucose equivalent for IMZ636 (*PvSUF1*, *LmSPase*)
2 (**Table 4**). In line with an increased ATP yield, the biomass specific sucrose uptake rate
3 (1.11 ± 0.00 vs 1.21 ± 0.00 mmol/g biomass/h), specific ethanol production rate ($3.69 \pm$
4 0.06 vs 4.17 ± 0.01 mmol/g biomass/h) and CO₂ production rate (3.79 ± 0.05 vs 4.29 ± 0.13
5 mmol/g biomass/h) decreased for IMZ636 (*PvSUF1*, *LmSPase*) compared to IMZ665
6 (*MAL11*, *SPase*). The observed 8% increase in the anaerobic biomass yield was lower than
7 the predicted 25%. Mislocalization and/or increased protein turnover of *PvSUF1* could
8 have increased the maintenance energy requirement and thereby resulted in a lower-than-
9 expected increase in the biomass yield. The impact of the cellular maintenance energy
10 requirements on the biomass yield decreases with an increase in the specific growth rate
11 (Leuenberger, 1971; Pirt, 1965). Therefore testing of *PvSUF1*- and *MAL11*-expressing
12 strains at higher dilution rates could provide insight into maintenance energy related
13 effects on the biomass yield.

14

15 **3.3 Overexpression of phosphoglucomutase in *LmSPase*-dependent strains enables** 16 **faster anaerobic growth on sucrose.**

17 Increasing the maximum specific growth rate of the engineered strains on sucrose
18 would benefit both the intended industrial applications of this strategy as well as further
19 investigations into their physiology. To investigate whether the conversion of glucose-1-
20 phosphate, the product of the *SPase* reaction, to glucose-6-phosphate by
21 phosphoglucomutase was limiting growth, *PGM2* was overexpressed from a multi-copy
22 plasmid. Introduction of this vector resulted in an approximately 20-fold increase of
23 phosphoglucomutase activity in cell extracts (**Table 3**). Combined overexpression of *PGM2*

1 and *LmSPase* with *MAL11* (IMZ709) or *PvSUF1* (IMZ696) increased the maximum specific
2 growth rate in anaerobic, sucrose-grown cultures from 0.09 h⁻¹ to 0.23 h⁻¹ and from 0.06 h⁻¹
3 to 0.08 h⁻¹ respectively (**Table 3**). The higher maximum specific growth rates of *PGM2*-
4 expressing strains allowed for a chemostat-based, quantitative evaluation of the impact of
5 the combined expression of *PvSUF1* and *LmSPase* at higher dilution rates and an additional
6 set of steady-state chemostat cultures was performed at a dilution rate of 0.07 h⁻¹.

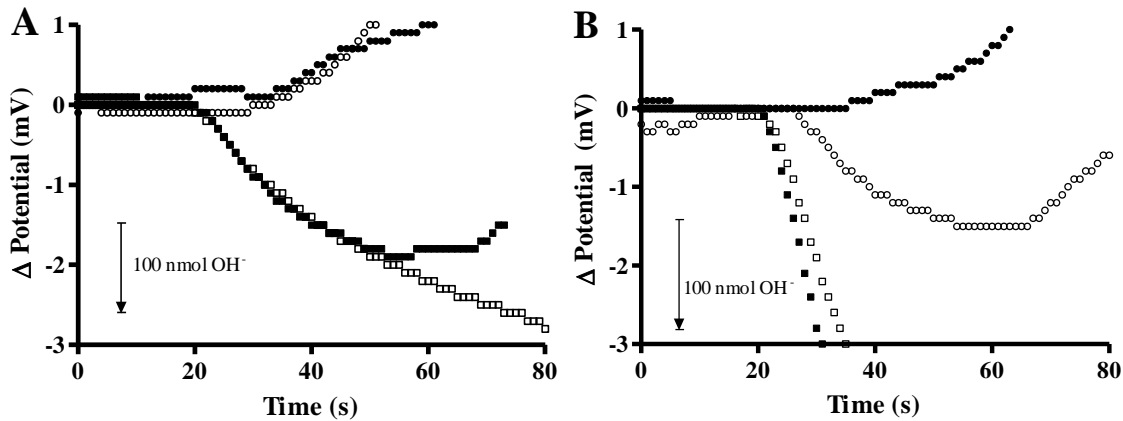
7 As expected for a microorganism with a growth-rate independent maintenance
8 requirement (Boender et al., 2009; Pirt, 1965), with the increase in dilution rate from 0.030
9 h⁻¹ to 0.07 h⁻¹, the anaerobic biomass yield of IMZ696 (*PvSUF1*, *LmSPase*, *PGM2*) increased,
10 from 0.075 ± 0.000 to 0.087 ± 0.002 g/g glucose equivalent and from 0.069 ± 0.000 to 0.080
11 ± 0.001 g/g glucose equivalent for the isogenic *MAL11*-expressing strain IMZ709 (**Table 4**).
12 However, the difference in biomass yield between the strains was identical (8%) to the
13 difference observed at 0.030 h⁻¹.

14

15 **3.4 Sucrose uptake by *PvSUF1* expressing strains leads to alkalinisation of the** 16 **extracellular environment.**

17 While *PvSUF1* has been described as a sucrose facilitator or uniporter (Zhou et al.,
18 2007), involvement of protons in sucrose uptake (partly coupled transport (Lolkema and
19 Poolman, 1995; Poolman et al., 1995), mediated by *PvSUF1*, could explain the lower-than-
20 expected anaerobic biomass yield of *PvSUF1*-expressing strains on sucrose. To investigate
21 this possibility, IMZ696 (*PvSUF1*, *LmSPase*, *PGM2*) and the isogenic *MAL11*-expressing
22 strain IMZ709 were subjected to a sucrose-proton symport assay (**Figure 4**). To minimize
23 residual sucrose concentrations, cells for this assay were collected from aerobic, sucrose-

1 limited bioreactors at a dilution rate of 0.030 h⁻¹. In the essay, an alkalinisation upon
2 sucrose addition to the cell suspension indicates the presence of a proton-coupled sucrose-
3 uptake mechanism (Stambuk et al., 2000). Indeed, such an alkalinisation of the
4 extracellular medium was observed upon addition of either sucrose or maltose to cell
5 suspensions of the *MAL11*-expressing strain IMZ709 (**Figure 4B**). Surprisingly,
6 alkalinisation of the extracellular medium was also observed upon disaccharide addition to
7 cell suspensions of the *PvSUF1*-expressing strain IMZ696 (**Figure 4A**), although at a lower
8 initial rate (8.2 ± 2.2 and 7.9 ± 1.0 $\mu\text{mol H}^+/\text{g biomass}/\text{min}$ for sucrose and maltose
9 respectively) than observed for IMZ709 (51.4 ± 8.6 and 42.6 ± 6.2 $\mu\text{mol H}^+/\text{g biomass}/\text{min}$
10 for sucrose and maltose respectively). As a control, fructose and glucose were added to cell
11 suspensions of both strains. In accordance with hexose uptake via facilitated diffusion
12 mediated by the hexose transporters, no pH change upon addition of glucose or fructose to
13 cell suspensions of IMZ696 (*PvSUF1*, *LmSPase*, *PGM2*) was observed. Addition of fructose to
14 cell suspensions of IMZ709 (*MAL11*, *LmSPase*, *PGM2*) did not result in a pH change. In line
15 with previous observations from Wieczorke et al., 1999, some alkalinisation of the
16 extracellular medium was observed upon glucose addition to this strain. Resequencing of
17 the *PvSUF1* genes at the end of both aerobic chemostat cultures revealed 2 point mutations
18 (**Supplementary material**), leading to different amino acid substitutions in both cultures
19 (H67G and T302I respectively). These mutations did not involve acidic residues making it
20 unlikely that they affected proton coupling of sucrose transport (Lemoine, 2000).

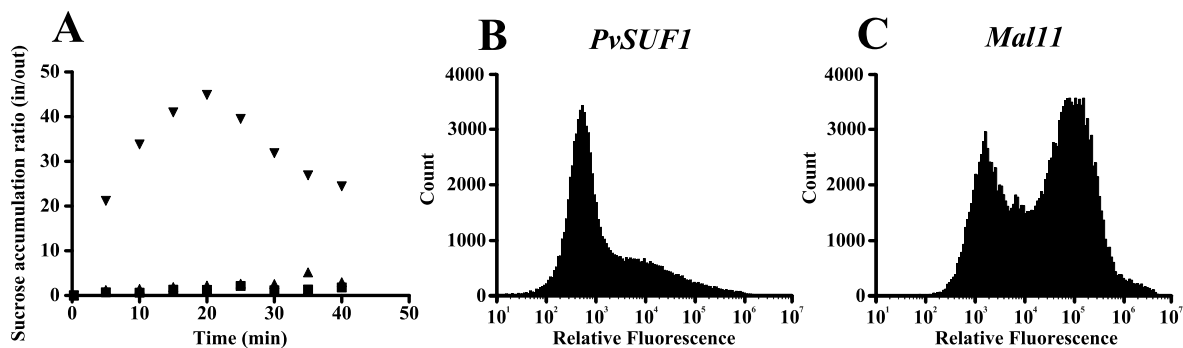


1
2 **Figure 4.** Proton uptake induced by addition of sugars to cell suspensions of two yeast
3 strains. A) Strain IMZ696 expressing *PvSUF1*, *LmSPase* and *PGM2*. B) Strain IMZ709
4 expressing *MAL11*, *LmSPase* and *PGM2*. Cells were pre-grown in aerobic, sucrose-limited
5 chemostat cultures, harvested from steady-state cultures and incubated in K-phthalate
6 buffer (1.25 mM pH 5.0) in a 30°C thermostated vessel with magnetic stirring. The graphs
7 show the response of a sensitive pH probe (mV) upon addition of 20 mM of either sucrose
8 (■), maltose (□), glucose (○) or fructose (●). The response of the electrode was calibrated
9 by pulse-wise addition of 100 nmol NaOH to the assays. Assays were performed with cells
10 from independent duplicate chemostat cultures; the graphs show data from a
11 representative single experiment for each strain. Biomass concentrations in the assay were
12 12 g dry weight/L for IMZ696 and 6 g dry weight/L for IMZ709.

13
14 If *PvSUF1* indeed exhibits (partial) proton-coupled sucrose transport, the additional
15 driving force for sucrose uptake provided by the proton motive force can lead to
16 intracellular sucrose accumulation in the absence of a catabolic pathway (Zhou et al.,
17 2007). To investigate whether *PvSUF1* expression resulted in intracellular sucrose

1 accumulation, strains were constructed that expressed either PvSUF1 or Mal11 without a
2 sucrose cleavage enzyme and used for radioactively labelled sucrose uptake measurements
3 (**Figure 5**). As expected, sucrose accumulation was observed in the *MAL11*-expressing
4 strain IMX1276 to an accumulation ratio of 45 (intracellular/extracellular sucrose
5 concentration) before the protonophore FCCP was added (**Figure 5A**). Upon addition of
6 FCCP, the proton motive force was dissipated, which resulted in the efflux of sucrose
7 following the sucrose concentration gradient. Uptake of sucrose by the *PvSUF1*-expressing
8 strain IMX1275 was very close to that of the empty vector reference strain IMX1274 and no
9 intracellular accumulation was observed, which is consistent with a uniporter mechanism
10 or poorly coupled transporter. In line with the observed lower average fluorescence of the
11 *PvSUF1*-expressing strain (**Figure 3**), also flow cytometry data from ethanol-grown
12 isogenic strains expressing YPet-tagged transport proteins showed relatively low
13 expression levels of PvSUF1 compared to Mal11 (**Figure 5B&C**).

14



15

1 **Figure 5.** A) Transport of 1 mM ^{14}C -sucrose by *S. cerevisiae* IMX1274 (empty vector, ■),
2 IMX1275 (*PvSUF1*, ▲), and IMX1276 (*MAL11*, ▼). Cells were grown and prepared as
3 described above. After 20 minutes of incubation, 10 μM FCCP was added to the reactions.
4 B) Flow cytometry analysis of *S. cerevisiae* IMX1278 (*PvSUF1-YPet*) and C) IMX1277
5 (*MAL11-YPet*), showing the relative fluorescence of each strain. Cells were harvested from
6 aerobic cultures grown on synthetic medium with 2% (v/v) ethanol as a carbon source and
7 diluted in K-citrate-phosphate buffer at pH 5 for measurement.

9 **4. DISCUSSION**

10 The native sucrose hydrolases of *S. cerevisiae* were functionally replaced by a
11 prokaryotic phosphorylase. The resulting increase in the anaerobic biomass yield on
12 sucrose (31%) is very close to the theoretically expected difference (33%, i.e. from 3 to 4
13 moles of ATP per mole of sucrose consumed), proving the energetic benefit of replacing
14 sucrose hydrolysis by phosphorolysis. Besides the energetic benefit for the cleavage of
15 intracellular sucrose via SPase, also the kinetics of *L. mesenteroides* SPase, $K_m = 5.7$ mM and
16 $k_{cat} = 165$ s $^{-1}$ (Goedl et al., 2007), are better than those of Mal12, $K_m = 12$ mM and $k_{cat} = 0.45$
17 s $^{-1}$ (Voordeckers et al., 2012), which could explain why strains expressing *PvSUF1* were
18 only able to grow on sucrose when co-expressing *LmSPase* (as opposed to *MAL11*) and
19 could also explain the lower residual sucrose concentrations observed under nutrient
20 limitation in *LmSPase* expressing strains (**Table 4**). This provides an additional advantage
21 for fed-batch or continuous industrial applications by lowering the fraction of unused
22 substrate. Other potential benefits for fed-batch or continuous industrial applications
23 ensue from changing extracellular hydrolysis to intracellular metabolism. In wild type *S.*

1 *cerevisiae*, extracellular hydrolysis and subsequent alcoholic fermentation of the
2 monosaccharides result in 4 ATP per sucrose (**Figure 1A**). An engineered strain using
3 proton-coupled uptake (e.g. Mal11) and intracellular phosphorolysis has an identical ATP
4 yield (**Figure 1C**), but has the potential to overcome two sucrose-associated challenges
5 encountered in industry: *i*) accumulation of residual fructose at the end of cultivation
6 processes due to preferred uptake of the released glucose over fructose by yeast (Beato et
7 al., 2016; Berthels et al., 2004; Prijambada et al., 2013; Wu et al., 2010) and *ii*) competition
8 for the extracellularly released monosaccharides by bacterial contaminants or wild yeasts
9 (Celiker and Gore, 2012; Gore et al., 2009; Greig and Travisano, 2004; Reis et al., 2014).
10 Although some of these benefits might also translate to industrial batch fermentations,
11 further study would be required to investigate the impact of the composition of molasses
12 together with glucose repression of, for instance, *MAL11*.

13 Although *S. cerevisiae* constitutively expresses *PGM2* at a basal level (Oh and
14 Hopper, 1990), overexpression of *PGM2* improved the maximum specific growth rate on
15 sucrose of cells expressing sucrose phosphorylase. The increased growth rate indicates
16 that the conversion of glucose-1-P to glucose-6-P was rate limiting in sucrose-grown
17 *LmSPase*-expressing *S. cerevisiae* strains and co-expression of a phosphoglucomutase is
18 essential for faster sucrose conversion.

19 Replacement of a hydrolase by a phosphorylase has previously been demonstrated
20 for maltose and cellobiose (De Kok et al., 2011; Sadie et al., 2011). However, only by
21 combining the energetic benefits of disaccharide uniport and phosphorolysis can the
22 energetic efficiency of wild type strains be exceeded. In this study, combined expression of
23 *LmSPase* and *PvSUF1* resulted in an 8% increase in the biomass yield on sucrose compared

1 to the Mal11/*LmSPase*-based reference strain and 41% compared to the Mal11/Mal12-
2 based reference strain (**Figure 1, Table 4**). Although promising, the observed 8% increase
3 in biomass yield was below the theoretical prediction of 25%. Four parameters that could
4 have contributed to this lower than anticipated increase of the biomass yield were
5 evaluated in this study: increased cellular maintenance energy requirements in *PvSUF1*-
6 expressing strains, a change in biomass composition, sucrose hydrolysis activity and/or
7 (partially) proton-coupled sucrose uptake catalysed by *PvSUF1*.

8 Cellular maintenance energy requirements have been shown to be growth rate
9 independent in anaerobic, sugar-limited *S. cerevisiae* cultures (Boender et al., 2009).
10 Especially at low specific growth rates, changes in genotype or experimental conditions
11 that lead to changes in maintenance-energy requirements can strongly affect biomass yield
12 (Leuenberger, 1971). We hypothesized that the abundance of fluorescence originating from
13 YPet-tagged *PvSUF1* observed in various intracellular compartments (**Figure 3C**), as well
14 as the observed changes in morphology, could indicate increased protein turnover and/or
15 maintenance in *PvSUF1*-expressing strains. However, the observation that the difference in
16 the biomass yield between strains expressing *LmSPase* with either Mal11 or *PvSUF1* was
17 independent of the dilution rate (**Table 4**) indicated that a difference in maintenance
18 energy requirements was probably not responsible for the lower-than-predicted increase
19 in biomass yield. Secondly, a change in biomass composition could result in a lower
20 biomass yield on sucrose in *PvSUF1* expressing strains. Since protein synthesis is the main
21 contributor to the energetic cost of biomass formation (Stouthamer, 1973), the protein
22 content of IMZ664 (*MAL11, MAL12*), IMZ665 (*MAL11, LmSPase*) and IMZ636 (*PvSUF1,*
23 *LmSPase*) was determined in steady-state chemostat cultures at a dilution rate of 0.030 h⁻¹.

1 No significant differences in cellular protein content were observed between the steady
2 state chemostat cultures of the relevant strains grown at 0.030 h⁻¹ (**Table 4**). Alternatively,
3 sucrose hydrolytic activity catalysed by *LmSPase*, which has previously been described
4 (Goedl et al., 2010), has the potential to lower the biomass yield on sucrose due to
5 competition with phosphorolytic cleavage. A low but significant (0.06 (μmol/min)/mg
6 protein, **Table 3**) sucrose hydrolase activity was measured in cell extracts of IMZ696
7 (*PvSUF1*, *LmSPase*), grown in steady-state chemostat cultures at a dilution rate of 0.07 h⁻¹.
8 Since sucrose hydrolytic activity was also observed in IMZ709 (*MAL11*, *LmSPase*, 0.07
9 (μmol/min)/mg protein, **Table 3**), and no activity could be detected in a strain void of
10 either SPase or Mal12 (IMZ616, **Table 3**), this hydrolytic activity might originate from
11 *LmSPase*. Hydrolytic activity by *LmSPase* is likely absent *in vivo* when expressed in *S.*
12 *cerevisiae*, as it has been described to be ≥50 times slower than phosphorolysis, repressed
13 in the presence of sucrose and glycerol (Goedl et al., 2010) and abolished in the presence of
14 phosphate (Silverstein et al., 1967). Additionally, the fact that the benefit of solely replacing
15 intracellular sucrose hydrolysis by phosphorolysis was very close to the theoretical
16 prediction (31% vs 33%), makes it unlikely that this *in vitro* hydrolytic activity is
17 responsible for the lower than predicted increase in the biomass yield in the combined
18 strategy. Lastly, proton-coupled sucrose uptake catalysed by *PvSUF1*, resulting in
19 subsequent ATP-dependent proton extrusion, could lower the biomass yield compared to a
20 sucrose uniporter. In contrast to a previous characterisation of *PvSUF1* in literature (Zhou
21 et al., 2007), the presented study revealed proton-dependent sucrose uptake in cell
22 suspensions of the *PvSUF1*-expressing strain IMZ696 (*PvSUF1*, *LmSPase*, *PGM2*) (**Figure**
23 **4A**). The stoichiometry between sucrose and proton uptake could not be determined due to

1 the low activity of sucrose uptake (**Figure 5A**). Nonetheless, it seems likely that proton-
2 coupled sucrose uptake is at least partly responsible for the lower-than-expected biomass
3 yield in *PvSUF1*-expressing strains. In this study, PvSUF1 was the only one of five
4 investigated heterologous transporters that supported growth on sucrose (**Figure 2**).
5 Subsequent analysis of PvSUF1, showed low protein levels (**Figure 5B**), partial localization
6 to the plasma membrane (**Figure 3**) and a potential benefit from additional mutations for
7 growth on sucrose. These observations illustrate the challenge of expressing heterologous
8 transporters as part of metabolic engineering strategies.

9 The strategy of replacing extracellular hydrolysis with a combination of uptake
10 through facilitated diffusion and intracellular phosphorylase can also be applied to
11 increase free-energy conservation for other oligosaccharides. Phosphorylases have been
12 described for maltose, cellobiose, trehalose, lactose and cellodextrin (Alexander, 1968;
13 Belocopitow and Maréchal, 1970; De Groeve et al., 2009; De Kok et al., 2011; Kishore and
14 Alexander, 1967; Sadie et al., 2011) and functional expression of maltose, cellobiose and
15 cellodextrin phosphorylase has already been demonstrated in *S. cerevisiae* (De Kok et al.,
16 2011; Ha et al., 2012; Sadie et al., 2011). In addition to sucrose facilitators, a putative
17 maltose facilitator from *Arabidopsis thaliana* (Niittylä et al., 2004; Reidel et al., 2008; Rost
18 et al., 1996) has been described and a cellodextrin facilitator from *Neurospora crassa* has
19 previously been expressed and evolved for efficient transport in *S. cerevisiae* (Lian et al.,
20 2014).

21 Combined facilitated diffusion and intracellular phosphorylase of oligosaccharides
22 results in a lower requirement of carbon to provide ATP, thereby increasing product yields
23 and improving the volumetric productivity for anabolic products. Furthermore, this

1 concept may enable homofermentative production of fuels and chemicals whose formation
2 currently has a very low, zero or negative ATP yield, improving process economics (Cueto-
3 Rojas et al., 2015; De Kok et al., 2012; Van Maris et al., 2004). An additional advantage of
4 engineering such a homofermentative pathway is that it directly couples cell growth to
5 product formation. Evolutionary engineering of such a strain can be applied to select for
6 energy-efficient mutants with a higher sucrose conversion rate and the resulting strains
7 can then be used as a platform for the production of other industrially relevant products.

8

9 **5. ACKNOWLEDGEMENTS**

10 We thank Professor Dr. John W. Patrick from the School of Environmental & Life
11 Sciences (The University of Newcastle, Australia) for sending the plasmids containing
12 *PsSUF1* and *PsSUF4* expression cassettes. We thank our colleagues Marijke Luttik, Mark
13 Bisschops and Xavier Hakkaart for assistance and advice regarding the fluorescence
14 microscopy pictures. We additionally thank Marijke Luttik for assistance with proton-
15 uptake assays. We thank Mandy Hulst, Yannick Buys, Hein van der Wall, Jordi Geelhoed and
16 Annabel Giezekamp, who contributed to this work as part of their BSc/MSc studies, for
17 their involvement in screening for functional sucrose facilitators. This work was supported
18 by the BE-Basic R&D Program (FS6.003) (<http://www.be-basic.org/>), which was granted a
19 FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation
20 (EL&I) and by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São
21 Paulo, Brazil), grant numbers 2012/05548-1 and 2012/16630-0. The work of Wesley
22 Marques was carried out as part of a Dual Degree PhD project under the agreement
23 between UNICAMP and Delft University of Technology.

6. REFERENCES

- 1
2 Abbott, D.A., Van den Brink, J., Minneboo, I.M.K., Pronk, J.T., Van Maris, A.J.A., 2009.
3 Anaerobic homolactate fermentation with *Saccharomyces cerevisiae* results in
4 depletion of ATP and impaired metabolic activity. FEMS Yeast Res. 9, 349–57.
5 doi:10.1111/j.1567-1364.2009.00506.x
- 6 Abbott, D.A., Zelle, R.M., Pronk, J.T., van Maris, A.J.A., 2009. Metabolic engineering of
7 *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and
8 challenges. FEMS Yeast Res. 9, 1123–1136. doi:10.1111/j.1567-1364.2009.00537.x
- 9 Aerts, D., Verhaeghe, T.F., Roman, B.I., Stevens, C. V., Desmet, T., Soetaert, W., 2011.
10 Transglucosylation potential of six sucrose phosphorylases toward different classes of
11 acceptors. Carbohydr. Res. 346, 1860–1867. doi:10.1016/j.carres.2011.06.024
- 12 Alexander, J.K., 1968. Purification and specificity of cellobiose phosphorylase from
13 *Clostridium thermocellum*. J. Biol. Chem. 243, 2899–2904.
- 14 Basso, T.O., De Kok, S., Dario, M., do Espirito-Santo, J.C. a, Müller, G., Schlögl, P.S., Silva, C.P.,
15 Tonso, A., Daran, J.-M., Gombert, A.K., Van Maris, A.J. a, Pronk, J.T., Stambuk, B.U., 2011.
16 Engineering topology and kinetics of sucrose metabolism in *Saccharomyces cerevisiae*
17 for improved ethanol yield. Metab. Eng. 13, 694–703.
18 doi:10.1016/j.ymben.2011.09.005
- 19 Batista, A.S., Miletti, L.C., Stambuk, B.U., 2005. Sucrose fermentation by *Saccharomyces*
20 *cerevisiae* lacking hexose transport. J. Mol. Microbiol. Biotechnol. 8, 26–33.
21 doi:10.1159/000082078
- 22 Belocopitow, E., Maréchal, L.R., 1970. Trehalose phosphorylase from *Euglena gracilis*.
23 Biochim. Biophys. Acta - Enzymol. 198, 151–154. doi:10.1016/0005-2744(70)90045-8

1 Bertani, G., 1951. Studies on lysogenesis I.: The mode of phage liberation by lysogenic
2 Escherichia coli. J. Bacteriol. 62, 293–300. doi:citeulike-article-id:149214

3 Bianchi, F., Van 't Klooster, J.S., Ruiz, S.J., Luck, K., Pols, T., Urbatsch, I.L., Poolman, B., 2016.
4 Asymmetry in inward- and outward-affinity constant of transport explain
5 unidirectional lysine flux in *Saccharomyces cerevisiae*. Sci. Rep. 6, 31443.
6 doi:10.1038/srep31443

7 Boeke, J.D., La Croute, F., Fink, G.R., 1984. A positive selection for mutants lacking orotidine-
8 5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen.
9 Genet. MGG 197, 345–346. doi:10.1007/BF00330984

10 Boender, L.G.M., De Hulster, E.A.F., Van Maris, A.J.A., Daran-Lapujade, P.A.S., Pronk, J.T.,
11 2009. Quantitative physiology of *Saccharomyces cerevisiae* at near-zero specific growth
12 rates. Appl. Environ. Microbiol. 75, 5607–5614. doi:10.1128/AEM.00429-09

13 Borodina, I., Nielsen, J., 2014. Advances in metabolic engineering of yeast *Saccharomyces*
14 *cerevisiae* for production of chemicals. Biotechnol. J. 9, 609–620.
15 doi:10.1002/biot.201300445

16 Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., Boeke, J.D., 1998. Designer
17 deletion strains derived from *Saccharomyces cerevisiae* S288C: A useful set of strains
18 and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115–
19 132. doi:10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2

20 Celiker, H., Gore, J., 2012. Competition between species can stabilize public-goods
21 cooperation within a species. Mol. Syst. Biol. 8, 621. doi:10.1038/msb.2012.54

22 Chen, L.-Q., Hou, B.-H., Lalonde, S., Takanaga, H., Hartung, M.L., Qu, X.-Q., Guo, W.-J., Kim, J.-
23 G., Underwood, W., Chaudhuri, B., Chermak, D., Antony, G., White, F.F., Somerville, S.C.,

1 Mudgett, M.B., Frommer, W.B., 2010. Sugar transporters for intercellular exchange and
2 nutrition of pathogens. *Nature* 468, 527–32. doi:10.1038/nature09606

3 Chen, L.-Q., Qu, X.-Q., Hou, B.-H., Sosso, D., Osorio, S., Fernie, A.R., Frommer, W.B., 2012.
4 Sucrose efflux mediated by SWEET proteins as a key step for phloem transport.
5 *Science* 335, 207–11. doi:10.1126/science.1213351

6 Cueto-Rojas, H.F., Van Maris, A.J.A., Wahl, S.A., Heijnen, J.J., 2015. Thermodynamics-based
7 design of microbial cell factories for anaerobic product formation. *Trends Biotechnol.*
8 33, 534–546. doi:10.1016/j.tibtech.2015.06.010

9 De Groeve, M.R.M., De Baere, M., Hoflack, L., Desmet, T., Vandamme, E.J., Soetaert, W., 2009.
10 Creating lactose phosphorylase enzymes by directed evolution of cellobiose
11 phosphorylase. *Protein Eng. Des. Sel.* 22, 393–399. doi:10.1093/protein/gzp017

12 De Kok, S., Kozak, B.U., Pronk, J.T., Van Maris, A.J.A., 2012. Energy coupling in
13 *Saccharomyces cerevisiae*: Selected opportunities for metabolic engineering. *FEMS*
14 *Yeast Res.* 12, 387–397. doi:10.1111/j.1567-1364.2012.00799.x

15 De Kok, S., Yilmaz, D., Suir, E., Pronk, J.T., Daran, J.-M., Van Maris, A.J.A., 2011. Increasing
16 free-energy (ATP) conservation in maltose-grown *Saccharomyces cerevisiae* by
17 expression of a heterologous maltose phosphorylase. *Metab. Eng.* 13, 518–26.
18 doi:10.1016/j.ymben.2011.06.001

19 Entian, K.-D., Kötter, P., 2007. Yeast genetic strain and plasmid collections, in: *Methods in*
20 *Microbiology.* pp. 629–666. doi:10.1016/S0580-9517(06)36025-4

21 Gietz, D.R., Woods, R.A., 2002. Transformation of yeast by lithium acetate/single-stranded
22 carrier DNA/polyethylene glycol method. *Methods Enzymol., Methods in Enzymology*
23 350, 87–96. doi:10.1016/S0076-6879(02)50957-5

1 Goedel, C., Sawangwan, T., Wildberger, P., Nidetzky, B., 2010. Sucrose phosphorylase: a
2 powerful transglucosylation catalyst for synthesis of α -D-glucosides as industrial fine
3 chemicals. *Biocatal. Biotransformation* 28, 10–21. doi:10.3109/10242420903411595
4 Goedel, C., Schwarz, A., Minani, A., Nidetzky, B., 2007. Recombinant sucrose phosphorylase
5 from *Leuconostoc mesenteroides*: Characterization, kinetic studies of
6 transglucosylation, and application of immobilised enzyme for production of α -D-
7 glucose 1-phosphate. *J. Biotechnol.* 129, 77–86. doi:10.1016/j.jbiotec.2006.11.019
8 González-Ramos, D., Gorter de Vries, A.R., Grijseels, S.S., van Berkum, M.C., Swinnen, S., van
9 den Broek, M., Nevoigt, E., Daran, J.-M.G., Pronk, J.T., van Maris, A.J.A., 2016. A new
10 laboratory evolution approach to select for constitutive acetic acid tolerance in
11 *Saccharomyces cerevisiae* and identification of causal mutations. *Biotechnol. Biofuels* 9,
12 173. doi:10.1186/s13068-016-0583-1
13 Gore, J., Youk, H., Van Oudenaarden, A., 2009. Snowdrift game dynamics and facultative
14 cheating in yeast. *Nature* 459, 253–256. doi:10.1038/nature07921
15 Greig, D., Travisano, M., 2004. The Prisoner's Dilemma and polymorphism in yeast *SUC*
16 genes. *Proc. R. Soc. B Biol. Sci.* 271, S25–S26. doi:10.1098/rsbl.2003.0083
17 Grote, A., Hiller, K., Scheer, M., Münch, R., Nörtemann, B., Hempel, D.C., Jahn, D., 2005. JCat: A
18 novel tool to adapt codon usage of a target gene to its potential expression host.
19 *Nucleic Acids Res.* 33, 526–531. doi:10.1093/nar/gki376
20 Guadalupe-Medina, V., Almering, M.J.H., Van Maris, A.J.A., Pronk, J.T., 2010. Elimination of
21 glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain
22 engineered to use acetic acid as an electron acceptor. *Appl. Environ. Microbiol.* 76,
23 190–195. doi:10.1128/AEM.01772-09

- 1 Ha, S.-J., Galazka, J.M., Joong Oh, E., Kordic, V., Kim, H., Jin, Y.-S., Cate, J.H.D., 2012. Energetic
2 benefits and rapid cellobiose fermentation by *Saccharomyces cerevisiae* expressing
3 cellobiose phosphorylase and mutant cellodextrin transporters. *Metab. Eng.*
4 doi:10.1016/j.ymben.2012.11.005
- 5 Hong, K.K., Nielsen, J., 2012. Metabolic engineering of *Saccharomyces cerevisiae*: A key cell
6 factory platform for future biorefineries. *Cell. Mol. Life Sci.* 69, 2671–2690.
7 doi:10.1007/s00018-012-0945-1
- 8 Kawasaki, H., Nakamura, N., Ohmori, M., Amari, K., Sakai, T., 1996. Screening for bacteria
9 producing sucrose phosphorylase and characterization of the enzymes. *Biosci.*
10 *Biotechnol. Biochem.* 60, 319–321. doi:10.1271/bbb.60.319
- 11 Kishore, S., Alexander, J.K., 1967. Cellodextrin phosphorylase from *Clostridium*
12 *thermocellum*. *Biochim. Biophys. Acta - Gen. Subj.* 148, 808–810. doi:10.1016/0304-
13 4165(67)90057-8
- 14 Kuijpers, N.G.A., Solis-Escalante, D., Bosman, L., van den Broek, M., Pronk, J.T., Daran, J.-M.,
15 Daran-Lapujade, P., 2013. A versatile, efficient strategy for assembly of multi-fragment
16 expression vectors in *Saccharomyces cerevisiae* using 60 bp synthetic recombination
17 sequences. *Microb. Cell Fact.* 12, 47. doi:10.1186/1475-2859-12-47
- 18 Lee, J.-H., Moon, Y.-H., Kim, N., Kim, Y.-M., Kang, H.-K., Jung, J.-Y., Abada, E., Kang, S.-S., Kim,
19 D., 2008. Cloning and expression of the sucrose phosphorylase gene from *Leuconostoc*
20 *mesenteroides* in *Escherichia coli*. *Biotechnol. Lett.* 30, 749–754. doi:10.1007/s10529-
21 007-9608-y
- 22 Lemoine, R., 2000. Sucrose transporters in plants: update on function and structure.
23 *Biochim. Biophys. Acta - Biomembr.* 1465, 246–262. doi:10.1016/S0005-

1 2736(00)00142-5

2 Leuenberger, H.G.W., 1971. Cultivation of *Saccharomyces cerevisiae* in continuous culture -
3 I. Growth kinetics of a respiratory deficient yeast strain grown in continuous culture.
4 Arch. Mikrobiol. 79, 176–186. doi:10.1007/BF00424924

5 Lian, J., Li, Y., Hamedirad, M., Zhao, H., 2014. Directed evolution of a cellodextrin
6 transporter for improved biofuel production under anaerobic conditions in
7 *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 111, 1521–1531. doi:10.1002/bit.25214

8 Lin, I.W., Sosso, D., Chen, L.-Q., Gase, K., Kim, S.-G., Kessler, D., Klinkenberg, P.M., Gorder,
9 M.K., Hou, B.-H., Qu, X.-Q., Carter, C.J., Baldwin, I.T., Frommer, W.B., 2014. Nectar
10 secretion requires sucrose phosphate synthases and the sugar transporter SWEET9.
11 Nature 508, 546–9. doi:10.1038/nature13082

12 Lolkema, J.S., Poolman, B., 1995. Uncoupling in secondary transport proteins. J. Biol. Chem.
13 270, 12670–12676. doi:10.1074/jbc.270.21.12670

14 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the
15 Folin phenol reagent. J. Biol. Chem. 193, 265–275. doi:10.1016/0304-3894(92)87011-
16 4

17 Luong, J.H.T., Volesky, B., 1980. Determination of the heat of some aerobic fermentations.
18 Can. J. Chem. Eng. 58, 497–504. doi:10.1002/cjce.5450580412

19 Mans, R., Van Rossum, H.M., Wijsman, M., Backx, A., Kuijpers, N.G.A., Van den Broek, M.,
20 Daran-Lapujade, P., Pronk, J.T., Van Maris, A.J.A., Daran, J.-M.G., 2015. CRISPR/Cas9: a
21 molecular Swiss army knife for simultaneous introduction of multiple genetic
22 modifications in *Saccharomyces cerevisiae*. FEMS Yeast Res. 15.
23 doi:10.1093/femsyr/fov004

1 Marques, W.L., Mans, R., Marella, E.R., Cordeiro, R.L., van den Broek, M., Daran, J.-M.G.,
2 Pronk, J.T., Gombert, A.K., van Maris, A.J.A., 2017. Elimination of sucrose transport and
3 hydrolysis in *Saccharomyces cerevisiae*: a platform strain for engineering sucrose
4 metabolism. FEMS Yeast Res. fox006. doi:10.1093/femsyr/fox006

5 Marques, W.L., Raghavendran, V., Stambuk, B.U., Gombert, A.K., 2015. Sucrose and
6 *Saccharomyces cerevisiae*: a relationship most sweet. FEMS Yeast Res. 1–16.
7 doi:10.1093/femsyr/fov107

8 Mashego, M.R., Van Gulik, W.M., Vinke, J.L., Heijnen, J.J., 2003. Critical evaluation of sampling
9 techniques for residual glucose determination in carbon-limited chemostat culture of
10 *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 83, 395–399. doi:10.1002/bit.10683

11 Meadows, A.L., Hawkins, K.M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., Dahl, R.H., Tai, A.,
12 Mahatdejkul-Meadows, T., Xu, L., Zhao, L., Dasika, M.S., Murarka, A., Lenihan, J., Eng, D.,
13 Leng, J.S., Liu, C.-L., Wenger, J.W., Jiang, H., Chao, L., Westfall, P., Lai, J., Ganesan, S.,
14 Jackson, P., Mans, R., Platt, D., Reeves, C.D., Saija, P.R., Wichmann, G., Holmes, V.F.,
15 Benjamin, K., Hill, P.W., Gardner, T.S., Tsong, A.E., 2016. Rewriting yeast central carbon
16 metabolism for industrial isoprenoid production. Nature 537, 694–697.
17 doi:10.1038/nature19769

18 Mumberg, D., Müller, R., Funk, M., 1995. Yeast vectors for the controlled expression of
19 heterologous proteins in different genetic backgrounds. Gene 156, 119–122.
20 doi:10.1016/0378-1119(95)00037-7

21 Nguyen, A.W., Daugherty, P.S., 2005. Evolutionary optimization of fluorescent proteins for
22 intracellular FRET. Nat. Biotechnol. 23, 355–360. doi:10.1038/nbt1066

23 Nielsen, J., Larsson, C., Van Maris, A., Pronk, J., 2013. Metabolic engineering of yeast for

1 production of fuels and chemicals. *Curr. Opin. Biotechnol.* 24, 398–404.
2 doi:10.1016/j.copbio.2013.03.023

3 Niittylä, T., Messerli, G., Trevisan, M., Chen, J., Smith, A.M., Zeeman, S.C., 2004. A previously
4 unknown maltose transporter essential for starch degradation in leaves. *Science* 303,
5 87–89. doi:10.1126/science.1091811

6 Nijkamp, J.F., Van den Broek, M., Datema, E., de Kok, S., Bosman, L., Luttik, M.A., Daran-
7 Lapujade, P., Vongsangnak, W., Nielsen, J., Heijne, W.H.M., Klaassen, P., Paddon, C.J.,
8 Platt, D., Kötter, P., van Ham, R.C., Reinders, M.J.T., Pronk, J.T., de Ridder, D., Daran, J.-
9 M., 2012. *De novo* sequencing, assembly and analysis of the genome of the laboratory
10 strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial
11 biotechnology. *Microb. Cell Fact.* 11, 36. doi:10.1186/1475-2859-11-36

12 Oh, D., Hopper, J.E., 1990. Transcription of a yeast phosphoglucomutase isozyme gene is
13 galactose inducible and glucose repressible. *Mol. Cell. Biol.* 10, 1415–22.
14 doi:10.1128/MCB.10.4.1415

15 Pfeiffer, T., Schuster, S., Bonhoeffer, S., 2001. Cooperation and competition in the evolution
16 of ATP-producing pathways. *Science* 292, 504–507. doi:10.1126/science.1058079

17 Pirt, S.J., 1965. The maintenance energy of bacteria in growing cultures. *Proc. R. Soc. B Biol.*
18 *Sci.* 163, 224–231. doi:10.1098/rspb.1965.0069

19 Poolman, B., Knol, J., Lolkema, J.S., 1995. Kinetic analysis of lactose and proton coupling in
20 Glu379 mutants of the lactose transport protein of *Streptococcus thermophilus*. *J. Biol.*
21 *Chem.*

22 Postma, E., Verduyn, C., Scheffers, W.A., van Dijken, J.P., 1989. Enzymic analysis of the
23 crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*.

1 Appl. Environ. Microbiol. 55, 468–477.

2 Pronk, J.T., 2002. Auxotrophic yeast strains in fundamental and applied research. Appl.
3 Environ. Microbiol. 68, 2095–2010. doi:10.1128/AEM.68.5.2095

4 Reidel, E.J., Turgeon, R., Cheng, L., 2008. A maltose transporter from apple is expressed in
5 source and sink tissues and complements the *Arabidopsis* maltose export-defective
6 mutant. Plant Cell Physiol. 49, 1607–1613. doi:10.1093/pcp/pcn134

7 Reis, A.L.S., De Fátima Rodrigues de Souza, R., Baptista Torres, R.R.N., Leite, F.C.B., Paiva,
8 P.M.G., Vidal, E.E., De Morais, M.A., 2014. Oxygen-limited cellobiose fermentation and
9 the characterization of the cellobiase of an industrial *Dekkera/Brettanomyces*
10 *bruxellensis* strain. Springerplus 3, 38. doi:10.1186/2193-1801-3-38

11 Rost, S., Frank, C., Beck, E., 1996. The chloroplast envelope is permeable for maltose but not
12 for maltodextrins. Biochim. Biophys. Acta - Gen. Subj. 1291, 221–227.
13 doi:10.1016/S0304-4165(96)00068-2

14 Sadie, C.J., Rose, S.H., Den Haan, R., van Zyl, W.H., 2011. Co-expression of a cellobiose
15 phosphorylase and lactose permease enables intracellular cellobiose utilisation by
16 *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 90, 1373–1380.
17 doi:10.1007/s00253-011-3164-z

18 Santos, E., Rodriguez, L., Elorza, M.V., Sentandreu, R., 1982. Uptake of sucrose by
19 *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. 216, 652–660. doi:10.1016/0003-
20 9861(82)90255-7

21 Sikorski, R.S., Hieter, P., 1989. A system of shuttle vectors and yeast host strains designed
22 for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122, 19–27.
23 doi:0378111995000377

1 Silverstein, R., Voet, J., Reed, D., Abeles, R.H., 1967. Purification and mechanism of action of
2 sucrose phosphorylase. *J. Biol. Chem.* 242, 1338–1346.

3 Solis-Escalante, D., Kuijpers, N.G.A., Bongaerts, N., Bolat, I., Bosman, L., Pronk, J.T., Daran, J.-
4 M., Daran-Lapujade, P., 2013. *amdSYM*, a new dominant recyclable marker cassette for
5 *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 13, 126–39. doi:10.1111/1567-1364.12024

6 Stambuk, B.U., Batista, A.S., De Araujo, P.S., 2000. Kinetics of active sucrose transport in
7 *Saccharomyces cerevisiae*. *J. Biosci. Bioeng.* 89, 212–214. doi:10.1016/S1389-
8 1723(00)88742-3

9 Stambuk, B.U., Silva, M.A., Panek, A.D., Araujo, P.S. De, 1999. Active α -glucoside transport in
10 *Saccharomyces cerevisiae* 170, 73–78.

11 Stouthamer, A.H., 1973. A theoretical study on the amount of ATP required for synthesis of
12 microbial cell material. *Antonie Van Leeuwenhoek* 39, 545–565.
13 doi:10.1007/BF02578899

14 Tedokon, M., Suzuki, K., Kayamori, Y., Fujita, S., Katayama, Y., 1992. Enzymatic assay of
15 inorganic phosphate with use of sucrose phosphorylase and phosphoglucomutase.
16 *Clin. Chem.* 38, 512–515.

17 Van den Brink, J., Akeroyd, M., Van der Hoeven, R., Ponk, J.T., De Winde, J.H., Daran-
18 Lapujade, P.A.S., 2009. Energetic limits to metabolic flexibility: Responses of
19 *Saccharomyces cerevisiae* to glucose-galactose transitions. *Microbiology* 155, 1340–
20 1350. doi:10.1099/mic.0.025775-0

21 Van Maris, A.J.A., Winkler, A.A., Porro, D., Van Dijken, J.P., Pronk, J.T., 2004.
22 Homofermentative lactate production cannot sustain anaerobic growth of engineered
23 *Saccharomyces cerevisiae*: Possible consequence of energy-dependent lactate export.

1 Appl. Environ. Microbiol. 70, 2898–2905. doi:10.1128/AEM.70.5.2898-2905.2004

2 Van Rossum, H.M., Kozak, B.U., Niemeijer, M.S., Duine, H.J., Luttik, M.A.H., Boer, V.M., Kötter,
3 P., Daran, J.M.G., Van Maris, A.J.A., Pronk, J.T., 2016. Alternative reactions at the
4 interface of glycolysis and citric acid cycle in *Saccharomyces cerevisiae*. FEMS Yeast
5 Res. 16, 1–13. doi:10.1093/femsyr/fow017

6 Van Urk, H., Postma, E., Scheffers, W.A., Van Dijken, J.P., 1989. Glucose transport in crabtree-
7 positive and crabtree-negative yeasts. Microbiology 135, 2399–2406.
8 doi:10.1099/00221287-135-9-2399

9 Verduyn, C., Postma, E., Scheffers, W.A., van Dijken, J.P., 1992. Effect of benzoic acid on
10 metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration
11 and alcoholic fermentation. Yeast 8, 501–17. doi:10.1002/yea.320080703

12 Verduyn, C., Postma, E., Scheffers, W.A., van Dijken, J.P., 1990. Physiology of *Saccharomyces*
13 *cerevisiae* in anaerobic glucose-limited chemostat cultures. J. Gen. Microbiol. 136, 395–
14 403. doi:10.1099/00221287-136-3-395

15 Voordeckers, K., Brown, C.A., Vanneste, K., van der Zande, E., Voet, A., Maere, S., Verstrepen,
16 K.J., 2012. Reconstruction of ancestral metabolic enzymes reveals molecular
17 mechanisms underlying evolutionary innovation through gene duplication. PLoS Biol.
18 10. doi:10.1371/journal.pbio.1001446

19 Weusthuis, R.A., Adams, H., Scheffers, W.A., van Dijken, J.P., 1993. Energetics and kinetics of
20 maltose transport in *Saccharomyces cerevisiae*: a continuous culture study. Appl.
21 Environ. Microbiol. 59, 3102–3109.

22 Weusthuis, R.A., Lamot, I., Van der Oost, J., Sanders, J.P.M., 2011. Microbial production of
23 bulk chemicals: Development of anaerobic processes. Trends Biotechnol. 29, 153–158.

1 doi:10.1016/j.tibtech.2010.12.007

2 Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C.P., Boles, E., 1999.

3 Concurrent knock-out of at least 20 transporter genes is required to block uptake of
4 hexoses in *Saccharomyces cerevisiae*. FEBS Lett. 464, 123–128. doi:10.1016/S0014-
5 5793(99)01698-1

6 Zhou, Y., Qu, H., Dibley, K.E., Offler, C.E., Patrick, J.W., 2007. A suite of sucrose transporters
7 expressed in coats of developing legume seeds includes novel pH-independent
8 facilitators. Plant J. 49, 750–764. doi:10.1111/j.1365-313X.2006.03000.x

9

10

1 **SUPPLEMENTARY MATERIAL**

2 **Table S1**

3 Primers used in this study.

Primer	Sequence (5' → 3')	Purpose
3093	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACAT TCCGCCAGATCATCAATAGGCACCTTCGTACGCTGCA GGTCGAC	<i>amdSYM</i> amplification
3094	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCC ATAGCCATGCCTTCACATATAGTGACGGATCGCTTGC CTGTAAC	<i>amdSYM</i> amplification
6845	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCC ATAGCCATGCCTTCACATATAGTACAGGCAACACGCA GATATAGG	p426TEF backbone amplification
6846	CACCTTTCGAGAGGACGATGCCCCGTGTCTAAATGATT CGACCAGCCTAAGAATGTTCAACGGCCCACTACGTGA ACCATC	p426TEF backbone amplification
8379	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGA TCCATGAAAAATATCATTTCATTGGTAAGCAAGAAG	<i>MAL11</i> ORF amplification
8380	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAG CTTCCTAACATTTATCAGCTGCATTTAATTC	<i>MAL11</i> ORF amplification
7998	AAGCTTATCGATACCGTCGACC	p426TEF <i>amdSYM</i> backbone amplification
7999	GGATCCACTAGTTCTAGAAAACTTAGATTAG	p426TEF <i>amdSYM</i> backbone amplification
9043	TCACAGAGGGATCCCGTTACCCATCTATGCTGAAGAT TTATCATACTATTCTCCGCTCGGCGATCGCGTGTGG AAGAAC	<i>MAL11</i> expressing cassette amplification
9044	CGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAG CTCGCCGCAAATTAAGCCTTCG	<i>MAL11</i> expressing cassette amplification
10303	TTAAATCTATAACTACAAAAACACATACATAAACT AAAAATGTCATTTCAAATTGAAACG	<i>PGM2</i> ORF amplification
10304	ACTACAATATAAAAAACTATACAAAATGACAAGTTC TTGATTAAGTACGAACCGTTGGTTCCTCAG	<i>PGM2</i> ORF amplification
6486	TTTTAGTTTATGTATGTGTTTTTTGTAGTTATAGATT TAAGCAAG	pUDE206 backbone amplification
9719	TCAAGAACTTGTCAATTTGTATAG	pUDE206 backbone amplification
10305	GGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCT GTTTAGTGTGAGCGGGATTTAAACTGTGAGG	<i>PGM2</i> expressing cassette amplification
10306	GTTGTGTGGAATTGTGAGCGGATAACAATTCACACA GGACAGTATAGCGACCAGCATTTC	<i>PGM2</i> expressing cassette amplification
10307	AACAGCTATGACCATGATTA	pUDE432 or pUDE413 backbone amplification
10308	TCCTGTGTGAAATTGTTATC	pUDE432 or pUDE413 backbone amplification
8018	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGA TCCCTAGAATGGATAATCCTTCCACCAATG	Transporter expression cassette amplification
8019	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAG CTTCTAATGAAATCCACCCGCAATTGG	Transporter expression cassette amplification
8020	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGA TCCAATGCCGAATCCCGACTCTTC	Transporter expression cassette amplification
8021	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAG CTTGTCGACTCATACTGGGTTTCTAGG	Transporter expression cassette amplification
8022	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGA TCCATGGAAGCCCCATCCCCAAC	Transporter expression cassette amplification

8023	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAG CTTACTTAATGAAAACCACCTGCTACCATAC	Transporter expression cassette amplification
8000	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTG	Transporter expression cassette amplification
8001	ACTAATTACATGACTCGAGGTCGACG	Transporter expression cassette amplification
1742	GGTCGCCTGACGCATATACC	<i>LEU2</i> integration (<i>LEU2</i> homology)
1743	TAAGGCCGTTTCTGACAGAG	<i>LEU2</i> integration (<i>LEU2</i> homology)
5271	CAAGGAGAAAAAACCCCGGATTCTAGAACTAGTGGA TCCCCCATGAAAAATATCATTTCATTGGTAAG	<i>MAL11</i> amplification from BY4742
5272	GAATAATTCCTTACCTTTAGAACCTTGAAAATATAAA TTTTCCCTCCACATTTATCAGCTGCATTTAATTC	<i>MAL11</i> amplification from BY4742 and pRHA00L
5273	GGAGGGGAAAAATTTATATTTTCAAGGTTTC	Backbone amplification from pFB001
5274	GGGGGATCCACTAGTTCTAGAATC	Backbone amplification from pFB001
5435	CGCATCTGTGCGGTATTTTC	<i>LEU2</i> amplification from pRS315
5436	GGCTTAACTATGCGGCATC	<i>LEU2</i> amplification from pRS315
5437	TGCACTCTCAGTACAATCTGCTC	Backbone amplification from pRHA00
5438	TGCGGTGTGAAATACCGC	Backbone amplification from pRHA00
5959	AGGGGAAAAATTTATATTTTCAAGG	Backbone amplification from pRHA00L
6324	TTGTTCCCTTTAGTGAGGG	Backbone amplification from pRHA00L
5961	TCTAGAACTAGTGGATCCAAAATGAAAAATATCAT TTCATTGG	<i>MAL11</i> amplification from pRHA00L
4995	AATTAACCTCACTAAAGGG	<i>TEF1</i> -promoter amplification from pUDE379
5960	CATTTTGGGATCCACTAGTTCTAG	<i>TEF1</i> -promoter amplification from pUDE379
6717	CTCATTAGAAAAGAAAGCATAGCAATC	Cassette amplification from pR151 or pUDE413
580	GAATGTAAGCGTGACATAAC	Cassette amplification from pR151
5921	AAAACTTAGATTAGATTGCTATGCTTTCTTTCTAATG AGC	pUDE413 backbone amplification or YPet-tag amplification from pUDE453
7812	TCATGTAATTAGTTATGTCACGCTTACATTC	pUDE413 backbone amplification
9772	CGAAGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTT CATGGAGGGGAAAAATTTATATTTTCAAGGTTCTAAA G	YPet-tag amplification from pUDE453
9763	ATGAAAACCACCTGCTACCATAC	Cassette amplification from pUDE413
9355	TGTAAATATCTAGGAAATACACTTGTGTATACTTCTC GCTTTTCTTTTATTTTTTTTTTTGTAGTTTATCATTATC AATACTCGCCATTTTC	<i>LmSPase</i> or <i>MAL12</i> integration (<i>SGA1</i> homology)
9356	TTTACAATATAGTGATAATCGTGGACTAGAGCAAGA TTTCAAATAAGTAACAGCAGCAAAGTGTGGAAGAAC GATTACAACAG	<i>MAL12</i> or <i>LmSPase</i> integration (<i>SGA1</i> homology)

1 ***LmSPase* codon optimized:**

2 ATGGAATCCAAAACAAGGCTATGTTGATCACTTACGCTGACTCTTTGGGTAAGAACTTGAAGG
3 ACGTTCACCAAGTTTTGAAGGAAGACATCGGTGACGCTATCGGTGGTGTTCACTTGTTGCCATTC
4 TTCCCATCTACTGGTGACAGAGGTTTCGCTCCAGCTGACTACACTAGAGTTGACGCTGCTTTCGG
5 TGA CTGGGCTGACGTTGAAGCTTTGGGTGAAGAATACTACTTGATGTTCGACTTCATGATCAACC
6 ACATCTCTAGAGAATCTGTTATGTACCAAGACTTCAAGAAGAACCACGACGACTCTAAGTACAA
7 GGACTTCTTCATCAGATGGGAAAAGTTCTGGGCTAAGGCTGGTGAAAACAGACCAACTCAAGCT
8 GACGTTGACTTGATCTACAAGAGAAAGGACAAGGCTCCA ACTCAAGAAATCACTTTCGACGACG
9 GTACTACTGAAA ACTTGTGGAACACTTTCGGTGAAGAACA AATCGACATCGACGTTAACTCTGCT
10 ATCGCTAAGGAATTCATCAAGACTACTTTGGAAGACATGGTTAAGCACGGTGCTAACTTGATCA
11 GATTGGACGCTTTCGCTTACGCTGTTAAGAAGGTTGACACTAACGACTTCTTCGTTGAACCAGAA
12 ATCTGGGACACTTTGAACGAAGTTAGAGAAATCTTGACTCCATTGAAGGCTGAAATCTTGCCAG
13 AAATCCACGAACACTACTCTATCCCAAAGAAGATCAACGACCACGGTTACTTCACTTACGACTTC
14 GCTTTGCCAATGACTACTTTGTACACTTTGTACTCTGGTAAGACTAACCAATTGGCTAAGTGGTT
15 GAAGATGTCTCCAATGAAGCAATTCACTACTTTGGACACTCACGACGGTATCGGTGTTGTTGACG
16 CTAGAGACATCTTGACTGACGACGAAATCGACTACGCTTCTGAACAATTGTACAAGGTTGGTGCT
17 AACGTTAAGAAGACTTACTCTTCTGCTTCTTACAACA ACTTGGACATCTACCAAATCAACTCTAC
18 TTACTACTCTGCTTTGGGTAACGACGACGCTGCTTACTTGTGTCTAGAGTTTTCCAAGTTTTCG
19 CTCCAGGTATCCCAAATCTACTACGTTGGTTTGTGGCTGGTGAAAACGACATCGCTTGTGTTG
20 GAATCTACTAAGGAAGGTAGAAACATCAACAGACACTACTACACTAGAGAAGAAGTTAAGTCTG
21 AAGTTAAGAGACCAGTTGTTGCTAACTTGTGAAGTTGTTGTCTTGGAGAAACGAATCTCCAGC
22 TTTCGACTTGGCTGGTTCTATCACTGTTGACACTCCA ACTGACACTACTATCGTTGTTACTAGAC

1 AAGACGAAAACGGTCAAACAAGGCTGTTTTGACTGCTGACGCTGCTAACAAGACTTTCGAAAT

2 CGTTGAAAACGGTCAAACGTTATGTCTTCTGACAACTTGACTCAAACCTAA

3

1 ***PvSUF1* codon optimized:**

2 ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCACATTATCCGT
3 AGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTGCGCCGTTGCTTCAATAGCTGCA
4 GGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGTTAACCCCATATGTCCAATTGTTAGGTGT
5 ACCTCATGCCGCTGCATCCTTTATATGGTTATGTGGTCCAATCAGTGGTTTGGTTGTCCAACCTA
6 TCGTTGGTTACTACTCTGATAGATCTACTTCTAGATACGGTAGAAGAAGACCTTTTATTTTAGGT
7 GGTGCAGTCGCCGTAGCTATCGCAGTTTTCTTGATTGGTTATGCCGCTGATATTGGTTACTCAGC
8 TGGTGACGACATAACCAAAAAGACTAGACCAAGAGCCGTTGCTGTCTTCGTAATTGGTTTCTGG
9 ATCTTGGACGTTGCAAACAACATGTTGCAAGGTCCTTGCAGAGCCTTTTTGGCTGATTTGGCAGC
10 CGGAGATCAAAGAAAGACTAGAATCGCAAACGGTTTCTTTTCTTTCTTTATGGCCGTTGGTAACG
11 TTTTAGGTTATGCTGCAGGTTCTTTTTCAGGTTTGCACAAAATTTCCCTTTTACTCAAACAAAG
12 GCATGTGATGTTTTCTGCGCCAATTTGAAGTCTTGTTTTCTTTTTCTCTATCTTGTTGTTGTTATT
13 TTTGTCCACAGTTGCTTTGATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGCCGTTCAAGAAG
14 ACGCTCAACCTTCTTGCTTCTTTCAATTGTTCCGGTGCTTTGAAGGAATTGAAGAGACCAATGTGG
15 ATGTTGATGTTAGTCACCGCAGTAAATTGGATTGGTTGGTTTCCTTATTTCTTGTTTTGATACTGA
16 CTGGATGGGTAGAGAAGTTTATGGTGGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAGA
17 GTCGGTTCCTTGGGTTTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAACC
18 ATTGGGTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTTAACTTCATCTTGGCTATC
19 GGTTTCGGTATGACAGTCGTAATAACCAAAAATGGCAGAACATCAAAGACACTTAAACCCAGCAG
20 CCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGGTATTCTTTGCTGTATTGGGTGTT
21 CCATTAGCAATCACATTCTCTGTTCTTTTTGCTTTGGCATCCATCTATTCTTCAGCAAGTGGTGC
22 CGGTCAAGGTTTGTCAATTAGGTGTTTTGAATTTGGCTATTGTTGTCCACAAATGGTAGTTTCCG
23 CCTTAAGTGGTTCCTTGGGACGCTTTATTCGGTGGTGGTAACTTGCCAGCTTTTATGGTTGGTGTCT

1 GCAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTGTTACCTACCCCTAAGCCAGCCGACGA

2 AGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTTCATTAA

3

1 ***PvSUF1* codon optimized T201G (proton uptake study replicate #1):**

2 ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCACATTATCCGT
3 AGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTGCGCGTTGCTTCAATAGCTGCA
4 GGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGTTAACCCCATATGTCCAATTGTTAGGTGT
5 ACCTCAGGCCGCTGCATCCTTTATATGGTTATGTGGTCCAATCAGTGGTTTGGTTGTCCAACCTA
6 TCGTTGGTTACTACTCTGATAGATCTACTTCTAGATACGGTAGAAGAAGACCTTTTATTTTAGGT
7 GGTGCAGTCGCCGTAGCTATCGCAGTTTTCTTGATTGGTTATGCCGCTGATATTGGTTACTCAGC
8 TGGTGACGACATAACCAAAAAGACTAGACCAAGAGCCGTTGCTGTCTTCGTAATTGGTTTCTGG
9 ATCTTGGACGTTGCAAACAACATGTTGCAAGGTCCTTGCAGAGCCTTTTTGGCTGATTTGGCAGC
10 CGGAGATCAAAGAAAGACTAGAATCGCAAACGGTTTCTTTCTTTCTTTATGGCCGTTGGTAACG
11 TTTTAGGTTATGCTGCAGGTTCTTTTTCAGGTTTGCACAAAATTTCCCTTTTACTCAAACAAAG
12 GCATGTGATGTTTTCTGCGCCAATTTGAAGTCTTGTTTTCTTTTCTCTATCTTGTTGTTGTTATT
13 TTTGTCCACAGTTGCTTTGATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGCCGTTCAAGAAG
14 ACGCTCAACCTTCTTGCTTCTTTCAATTGTTCCGGTGCTTTGAAGGAATTGAAGAGACCAATGTGG
15 ATGTTGATGTTAGTCACCGCAGTAAATTGGATTGGTTGGTTTCCTTATTTCTTGTTTTGATACTGA
16 CTGGATGGGTAGAGAAGTTTATGGTGGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAGA
17 GTCGGTTCCTTGGGTTTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAACC
18 ATTGGGTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTTAACTTCATCTTGGCTATC
19 GGTTTCGGTATGACAGTCGTAATAACCAAAAATGGCAGAACATCAAAGACACTTAAACCCAGCAG
20 CCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGGTATTCTTTGCTGTATTGGGTGTT
21 CCATTAGCAATCACATTCTCTGTTCTTTTGGCTTTGGCATCCATCTATTCTTCAGCAAGTGGTGC
22 CGGTCAAGGTTTGTCAATTAGGTGTTTTGAATTTGGCTATTGTTGTCCCACAAATGGTAGTTTCCG
23 CCTTAAGTGGTTCCTTGGGACGCTTTATTCGGTGGTGGTAACTTGCCAGCTTTTATGGTTGGTGTCT

1 GCAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTGTTACCTACCCCTAAGCCAGCCGACGA

2 AGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTTCATTAA

3

1 ***PvSUF1* codon optimized C905T (proton uptake study replicate #2):**

2 ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCACATTATCCGT
3 AGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTGCGCGTTGCTTCAATAGCTGCA
4 GGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGTTAACCCCATATGTCCAATTGTTAGGTGT
5 ACCTCATGCCGCTGCATCCTTTATATGGTTATGTGGTCCAATCAGTGGTTTGGTTGTCCAACCTA
6 TCGTTGGTTACTACTCTGATAGATCTACTTCTAGATACGGTAGAAGAAGACCTTTTATTTTAGGT
7 GGTGCAGTCGCCGTAGCTATCGCAGTTTTCTTGATTGGTTATGCCGCTGATATTGGTTACTCAGC
8 TGGTGACGACATAACCAAAAAGACTAGACCAAGAGCCGTTGCTGTCTTCGTAATTGGTTTCTGG
9 ATCTTGGACGTTGCAAACAACATGTTGCAAGGTCCTTGCAGAGCCTTTTTGGCTGATTTGGCAGC
10 CGGAGATCAAAGAAAGACTAGAATCGCAAACGGTTTCTTTTCTTTCTTTATGGCCGTTGGTAACG
11 TTTTAGGTTATGCTGCAGGTTCTTTTTCAGGTTTGCACAAAATTTCCCTTTTACTCAAACAAAG
12 GCATGTGATGTTTTCTGCGCCAATTTGAAGTCTTGTTTTCTTTTTCTCTATCTTGTTGTTGTTATT
13 TTTGTCCACAGTTGCTTTGATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGCCGTTCAAGAAG
14 ACGCTCAACCTTCTTGCTTCTTTCAATTGTTCCGGTGCTTTGAAGGAATTGAAGAGACCAATGTGG
15 ATGTTGATGTTAGTCACCGCAGTAAATTGGATTGGTTGGTTTCCTTATTTCTTGTTTTGATA**T**GTG
16 ACTGGATGGGTAGAGAAGTTTATGGTGGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAG
17 AGTCGGTTCCTTGGGTTTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAA
18 CCATTGGGTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTAACTTCATCTTGGCTA
19 TCGGTTTCGGTATGACAGTCGTAATAACCAAAATGGCAGAACATCAAAGACACTTAAACCCAGC
20 AGCCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGGTATTCTTTGCTGTATTGGGGTG
21 TTCCATTAGCAATCACATTCTCTGTTCCTTTTGCTTTGGCATCCATCTATTCTTCAGCAAGTGGT
22 GCCGGTCAAGGTTTGTCAATTAGGTGTTTTGAATTTGGCTATTGTTGTCCCACAAATGGTAGTTTC
23 CGCCTTAAGTGGTCCTTGGGACGCTTTATTCGGTGGTGGTAACTTGCCAGCTTTTATGGTTGGTG

1 CTGCAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTGTTACCTACCCCTAAGCCAGCCGAC

2 GAAGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTTCATTAA

3