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

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ABBREVIATIONS

SPase, sucrose phosphorylase;

*Lm*SPase, *Leuconostoc mesenteroides* sucrose phosphorylase;

LmSPase, *Leuconostoc mesenteroides* sucrose phosphorylase coding gene;

Mal11, alpha-glucoside proton symporter 11;

MAL11, alpha-glucoside proton symporter 11 coding gene;

Mal12, maltose hydrolase 12;

MAL12, maltose hydrolase 12 coding gene;

PvSUF1, *Phaseolus vulgaris* sucrose facilitator 1;

PvSUF1, *Phaseolus vulgaris* sucrose facilitator 1 coding gene;

PsSUF1, *Pisum sativum* sucrose facilitator 1 coding gene;

PsSUF4, *Pisum sativum* sucrose facilitator 4 coding gene;

AtSWEET12, *Arabidopsis thaliana* SWEET12 coding gene;

OsSWEET11, *Oryza sativa* SWEET11 coding gene;

SM, synthetic medium;

ORF, open reading frame;

G418, Geneticin (aminoglycoside antibiotic);

bp, basepair;

SHR-sequence, synthetic homologous recombination sequence;

v/v, volume per volume (%);

w/v, weight per volume;

LB, lysogeny broth;

U (units), the amount of enzyme catalysing the conversion of 1 μmol of substrate per minute;

μ, biomass specific growth rate;

$q_{metabolite}$, biomass specific consumption or production rate

HIGHLIGHTS

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

ABSTRACT (247 words)

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

Keywords: Free-energy conservation, ATP, Facilitated diffusion, Phosphoglucomutase, Chemostat, Yeast physiology

1. INTRODUCTION

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

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

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

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

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

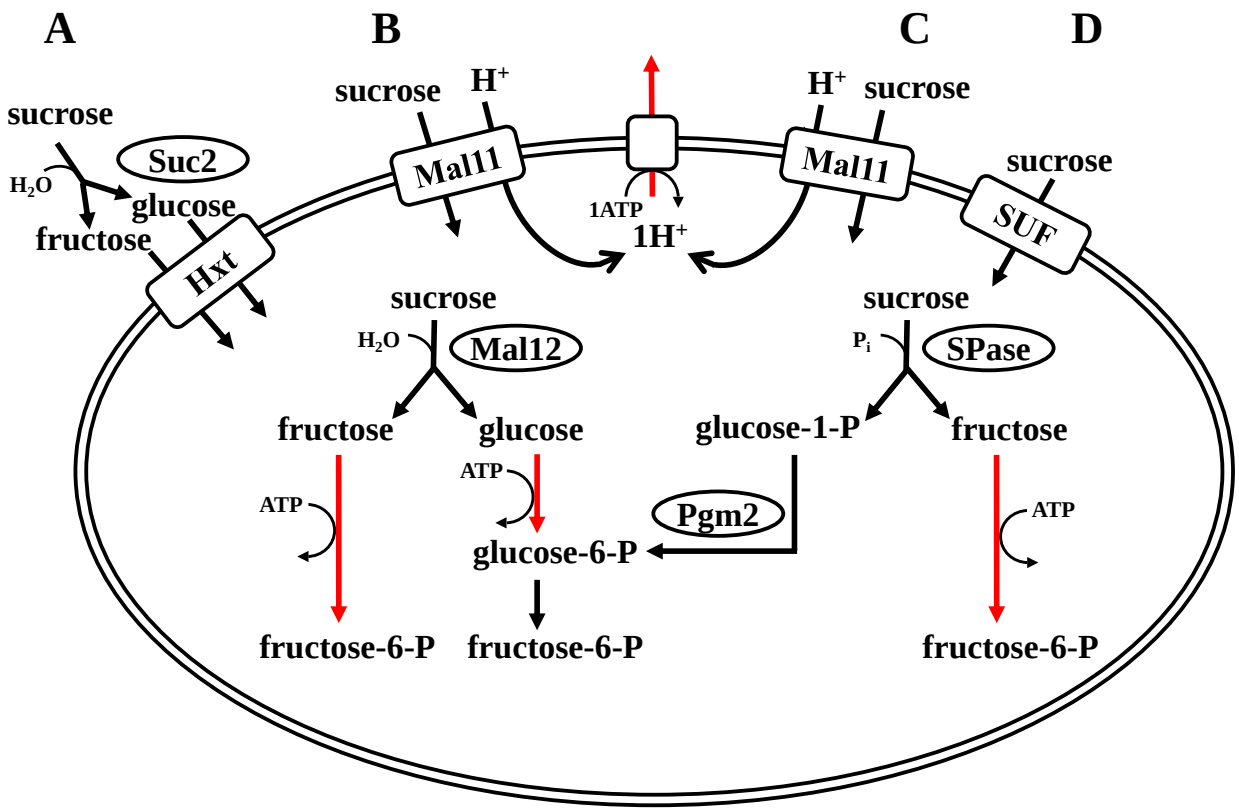

Figure 1. Schematic representation of different strategies for the uptake and cleavage of sucrose. **A**) Extracellular hydrolysis of sucrose, facilitated by the external invertase Suc2, followed by facilitated diffusion of the monosaccharides. **B**) Uptake of sucrose via the proton-symporter Mal11,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

S. cerevisiae does not conserve the free energy of sucrose hydrolysis (ΔG_0' = -29 kJ/mol). In some anaerobic microorganisms sucrose is cleaved by phosphorolysis instead

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

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

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

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

2. MATERIALS AND METHODS

2.1 Strain storage and maintenance

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

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

Table 1

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

Name	Relevant genotype	Origin
CEN.PK113-7D	MATa *URA3 LEU2 MAL2-8^{C} SUC2*	Entian and Kötter, 2007
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	Brachmann et al., 1998
IMK291	MATa *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*	Marques et al., 2017
IMZ570	MATa *ura3-52 leu2-112 MAL2-8^{C} malΔ mphΔ suc2Δ* pUDC156 (*URA3 cas9*)	This study
IMK698	MATa *ura3-52 leu2-112 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ*	This study
IMX935	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ*	This study
IMZ616	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDC156 (*URA3 cas9*)	This study
IMZ627	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::MAL12*	This study
IMZ664	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::MAL12* pUDE432 (*URA3 MAL11*)	This study
IMZ633	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::MAL12* pUDE413 (*URA3 PvSUF1*)	This study
IMZ630	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: LmSPase*	This study
IMZ665	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: LmSPase* pUDE432 (*URA3 MAL11*)	This study
IMZ666	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE439 (*URA3 OsSWEET11*)	This study
IMZ667	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE438 (*URA3 AtSWEET12*)	This study
IMZ671	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE442 (*URA3 PsSUF4*)	This study
IMZ672	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE441 (*URA3 PsSUF1*)	This study
IMZ636	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE413 (*URA3 PvSUF1*)	This study
IMZ692	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE260 (*URA3*)	This study
IMX1272	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE453 (*URA3 MAL11-YPet*)	This study
IMX1273	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase* pUDE471 (*URA3 PvSUF1-YPet*)	This study
IMZ696	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: LmSPase* pUDE486 (*URA3 PvSUF1 PGM2*)	This study
IMZ709	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ:: LmSPase* pUDE496 (*URA3 MAL11 PGM2*)	This study
IMX1274	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDE260 (*URA3*)	This study
IMX1275	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDE413 (*URA3 PvSUF1*)	This study
IMX1276	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDE432 (*URA3 MAL11*)	This study
IMX1277	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDE453 (*URA3 MAL11-YPet*)	This study
IMX1278	MATa *ura3-52 LEU2 MAL2-8^{C} malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ* pUDE471 (*URA3 PvSUF1-YPet*)	This study

2.2 Plasmid construction

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

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

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

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

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

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

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

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

Table 2

Plasmids used in this study

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

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

2.3 Strain construction

S. cerevisiae transformations were carried out according to Gietz and Woods (2002) using 1 μg of DNA per transformation, if not stated otherwise. Transformants were selected on 2% (w/v) agar plates containing synthetic medium (SM) (Verduyn et al., 1992) with 20 g/L glucose plus the following components when necessary: G418 (200 mg/L); uracil (0.15 g/L); L-leucine (0.5 g/L) (Pronk, 2002). Cells expressing the *amdSYM* marker were selected on plates according to Solis-Escalante et al. (2013). Cells expressing the *natNT2* marker were selected on plates containing nourseothricin (100 mg/L) (Jena Bioscience, Jena, Germany) in SM with 1 g/L glutamic acid as sole nitrogen source. IMZ570 was made via *in vivo* assembly of plasmid pUDC156 in IMK291 (**Table 1**). IMZ570 was transformed with 1 μg of plasmid pUDR128, 4 μg dsDNA repair fragment for *IMA1-4* and 4 μg dsDNA repair fragment for *IMA5* (Mans et al., 2015; Marques et al., 2017). Subsequently, pUDR128 and pUDC156 were cured from IMZ570 by cultivation on YPD plates with 20 g/L glucose and 1 g/L 5'-fluoroorotic acid (Boeke et al., 1984), resulting in IMK698. The *LEU2* marker fragment was amplified from pUDI035 with primers 1742 & 1743 and integrated in IMK698 resulting in strain IMX935. IMZ616 was made via transformation of IMX935 with

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

2.4 Molecular biology techniques

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

PCR were separated by gel electrophoresis on 1% (w/v) agarose gels (Thermo Fisher Scientific,) in Tris-acetate-EDTA buffer (Thermo Fisher Scientific) at 100 V for 30 min. DNA fragments were excised from gel and purified by gel purification (D2004, Zymo Research). Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich) according to the supplier's manual and from yeast with the Zymoprep Yeast Plasmid Miniprep II kit (D2004, Zymo Research). Restriction analysis with enzymes was performed using FastDigest enzymes (Thermo Fisher Scientific) according to the manufacturer's manual. Plasmid assembly was performed using *in vivo* recombination in yeast (Kuijpers et al., 2013) , T4 DNA ligase (Thermo Fisher Scientific), Gibson Assembly Cloning Kit (New England Biolabs, Ipswich, MA) or NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). Assembly of plasmids was done according to the suppliers' protocols, but downscaled to a total volume of 5 μL for the Gibson and NEBuilder HiFi DNA Assembly Cloning kit. Ligation of plasmids was performed using T4 DNA ligase according to manufacturer's instructions (New England Biolabs). *E. coli* DH5α (18258-012, Thermo Fisher Scientific) or XL1-Blue (GE Healthcare Life Sciences, Uppsala, Sweden) were used for chemical transformation (T3001, Zymo Research) or for electroporation. Chemical transformation of *E. coli* was done according to the supplier's instructions. Electroporation was done in a 2 mm cuvette (165-2086, BioRad, Hercules, CA) using a Gene PulserXcell Electroporation System (BioRad), following the manufacturer's protocol. Electrocompetent *E. coli* cells were prepared according to the BioRad protocol, except for the use of lysogeny broth (LB) (Bertani, 1951) without NaCl when pre-growing the cells.

2.5 Media and cultivation

Synthetic medium (SM) was prepared according to Verduyn et al., (1992) and autoclaved at 120°C for 20 min. Glucose, sucrose and vitamins (Verduyn et al., 1992) were prepared separately and filter sterilized (sucrose and vitamins) or heat sterilized at 110°C for 20 min (glucose). For anaerobic cultures, the growth factors ergosterol (10 mg/L) and Tween80 (420 mg/L) were dissolved in ethanol and added to the media. Aerobic shake-flask cultures were grown in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ) and anaerobic shake-flask cultures were grown in a Bactron Anaerobic Chamber (Sheldon Manufacturing, Cornelius, OR) with an atmosphere consisting of 5% H_2 6% CO_2 and 89% N_2. Trace amounts of oxygen entering the chamber (e.g. when sampling) were removed by reacting with the hydrogen using a palladium catalyst. Cultures were shaken at 200 rpm at 30°C.

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

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

Strain characterization in bioreactors was carried out at 30°C in aerobic and anaerobic 2-L laboratory bioreactors (Applikon, Delft, The Netherlands) with a working volume of 1 L. After heat sterilization (120°C for 20 min), the SM was supplemented with heat sterilized (120°C for 20 min) Antifoam Emulsion C (Sigma-Aldrich) to a final concentration of 0.15 g/L, sucrose to a final concentration of 25 g/L, anaerobic growth factors and vitamins (Verduyn et al., 1992). The culture pH was maintained at 5.0 by automated addition of 2 M KOH and was stirred at 800 rpm. To maintain anaerobic conditions, the bioreactors were sparged with 500 mL N_2/min (<5 ppm O_2) and equipped with Norprene tubing to minimize oxygen diffusion or 500 mL/min compressed air for aerobic experiments. For anaerobic experiments, the medium vessels were also sparged with N_2. For the batch phase, the reactors were inoculated with *S. cerevisiae* strains to an initial optical density of 0.2 - 0.5, and culture growth was monitored via determination of the CO_2-concentration in the off-gas. After the batch phase, medium pumps were switched on, resulting in the continuous addition of synthetic medium (25 g/L sucrose for anaerobic and 7.5 g/L sucrose for aerobic conditions) to the cultures. During the chemostat phase of the anaerobic cultures, a continuously stirred Antifoam Emulsion C (100 g/L) was added separately at a rate of 2-5 drops per hour and for the aerobic cultures, 0.15 g/L Antifoam Emulsion C was added to the medium. To minimize differences between the aerobic and anaerobic cultures, both cultures were supplemented with anaerobic growth factors. The working volume was kept constant at 1.0 L using an effluent pump controlled by an electric level sensor, resulting in a constant dilution rate. The exact working volume and medium flow rate were measured at the end of each experiment. Chemostat cultures were assumed to be in steady state when, after five volume changes, the culture dry weight, extracellular

metabolite concentrations of ethanol and glycerol and the CO_2 production rate varied by less than 2% over at least a further 2 volume changes.

2.6 Analytical methods

Optical density was monitored using a Libra S11 spectrophotometer (Biochrom, Cambridge, United Kingdom) at 660 nm. Culture dry weights were determined via filtration of well-mixed samples over dry nitrocellulose filters with a pore size of 0.45 µm (Gelman laboratory, Ann Arbor, USA). Prior to filtration, the filters were dried and weighed. After filtration of the sample, the filters were washed using demineralized water and dried in a microwave oven for 20 min at 360 W and weighed again. Supernatant was obtained via centrifugation of the culture broth and samples for residual sugars were obtained via rapid quenching using cold stainless-steel beads (Mashego et al., 2003). Residual sucrose concentration was analysed enzymatically (10716260035, R-Biopharm AG, Darmstadt, Germany) according to manufacturer's instructions and via HPLC. HPLC analysis of the supernatant, residual sugar samples and ingoing medium was performed as described previously (De Kok et al., 2011; Marques et al., 2017) and ethanol concentrations were corrected for ethanol evaporation (Guadalupe-Medina et al., 2010). Cellular protein content was determined as described previously (Verduyn et al., 1990), with the exception that 1 M NaOH was used instead of 1 M KOH and the absorbance was measured at 510 nm instead of 550 nm. Off-gas was cooled in a condenser and dried with a Perma Pure Dryer (Perma Pure, Lakewood, NJ) before CO_2 concentrations were measured with a NGA 2000 Rosemount gas analyser (Emerson, St. Louis, MO).

2.7 Determination of sucrose hydrolase, sucrose phosphorylase and phosphoglucomutase activities

For enzyme-activity assays, culture samples corresponding to 62.5 mg dry weight were harvested during the steady state of the chemostat cultures. Strain IMZ616, was grown in shake-flasks with SM containing 2% (v/v) ethanol and harvested during exponential growth for cell extract preparation. 4 hours before harvesting, 20 g/L sucrose was added to the culture of IMZ616 in order to induce any sucrose responsive genes. Cell extracts were prepared by sonication and centrifugation as described previously (Postma et al., 1989). Protein concentrations in cell extracts were determined with the Lowry method (Lowry et al., 1951). Sucrose hydrolytic activity was measured as described previously for maltase activity (De Kok et al., 2011), with the exception that 250 mM sucrose was used to start the reaction. Sucrose-phosphorylase activity was measured at 30°C by monitoring the reduction of $NADP^+$ at 340 nm in a 1 ml reaction mixture containing 200 mM potassium phosphate buffer (pH 7.0), 10 mM EDTA, 10 mM $MgCl_2$, 10 µM α-D-glucose 1,6-bisphosphate (activator of Pgm2 (Tedokon et al., 1992)), 2 mM $NADP^+$, 2.65 U phosphoglucomutase, 5.25 U glucose 6-phosphate dehydrogenase and 1–100 µl cell extract (adapted from Goedl et al., 2007). The reaction was started by the addition of sucrose to a final concentration of 250 mM. Phosphoglucomutase activity was determined according to van den Brink et al., 2009. An extinction coefficient of 6.3 mM^{-1} was assumed for NADPH.

2.8 Microscopy of YPet tagged Mal11 and PvSUF1

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

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

2.9 Proton-solute symport assays

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

2.10 Transport of radiolabelled sucrose

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

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

2.11 Flow cytometry

Yeast strains were grown aerobically on synthetic medium with 2% ethanol as carbon source. Cells from exponential cultures were harvested and diluted to an optical density (OD_{600}) between 0.25-0.4, and then 20 µL samples were analysed using an Accuri C6 flow cytometer (BD Biosciences, Durham, USA). YPet fluorescence was detected using a 488 nm laser and an "FL1" emission detector (533/30 nm).

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 *Lm*SPase was confirmed via measurement of *in vitro* sucrose phosphorylase activity of 0.90 (μmol/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 ± 0.02 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

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

Table 3

Specific growth rates on sucrose and enzyme activities of sucrose hydrolase and sucrose phosphorylase of *S. cerevisiae* strains expressing either the sucrose transporter Mal11 or PvSUF1 in combination with either the sucrose hydrolase Mal12 or sucrose phosphorylase *Lm*SPase in anaerobic bioreactors. Average growth rates were determined from triplicate experiments and based on CO_2 production in anaerobic batch fermentations at pH 5.0 and 25 g/L sucrose. Enzyme activities represent the average of measurements on duplicate steady-state chemostat cultures at pH 5.0, 25 g/L sucrose and a dilution rate of 0.030 h^{-1} (IMZ664, IMZ665 and IMZ636) or 0.07 h^{-1} (IMZ709 and IMZ696). IMZ616 was incubated in aerobic shake flasks with 20 g/L sucrose for growth assessment and 20 g/L ethanol and 20 g/L sucrose for enzyme activity assays.

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

N.D. = not determined

B.D. = below detection (< 0.04 (µmol/min)/mg protein)

[a] Measured in duplicate

[b] No growth observed after 2 months

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

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

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

Table 4

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

Strain	**IMZ664**	**IMZ665**	**IMZ636**	**IMZ709**	**IMZ696**
Relevant genotype	*MAL11 MAL12*	*MAL11 LmSPase*	*PvSUF1 LmSPase*	*MAL11 LmSPase PGM2*	*PvSUF1 LmSPase PGM2*
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

N.D. = not determined

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

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

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

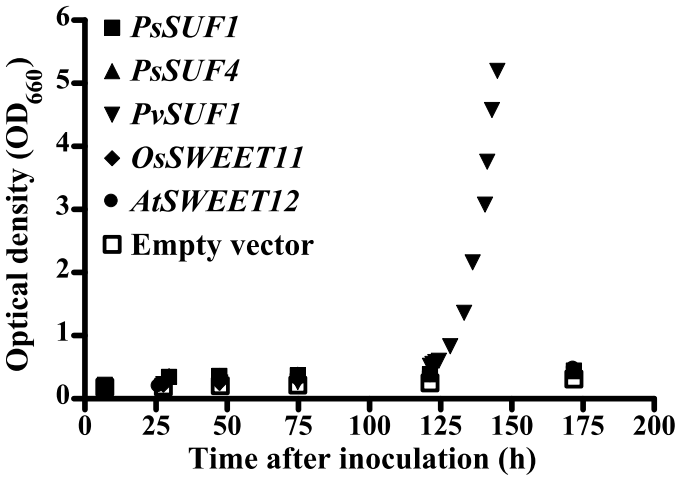

Figure 2. Growth curves of *S. cerevisiae* strains expressing *LmSPase* in combination with either *PsSUF1* (IMZ683 (■)), *PsSUF4* (IMZ682 (▲)), *PvSUF1* (IMZ636 (▼)), *OsSWEET11* (IMZ677 (◆)), *AtSWEET12* (IMZ678 (●)) or an empty vector (IMZ692 (□)) in aerobic shake flasks containing synthetic medium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

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

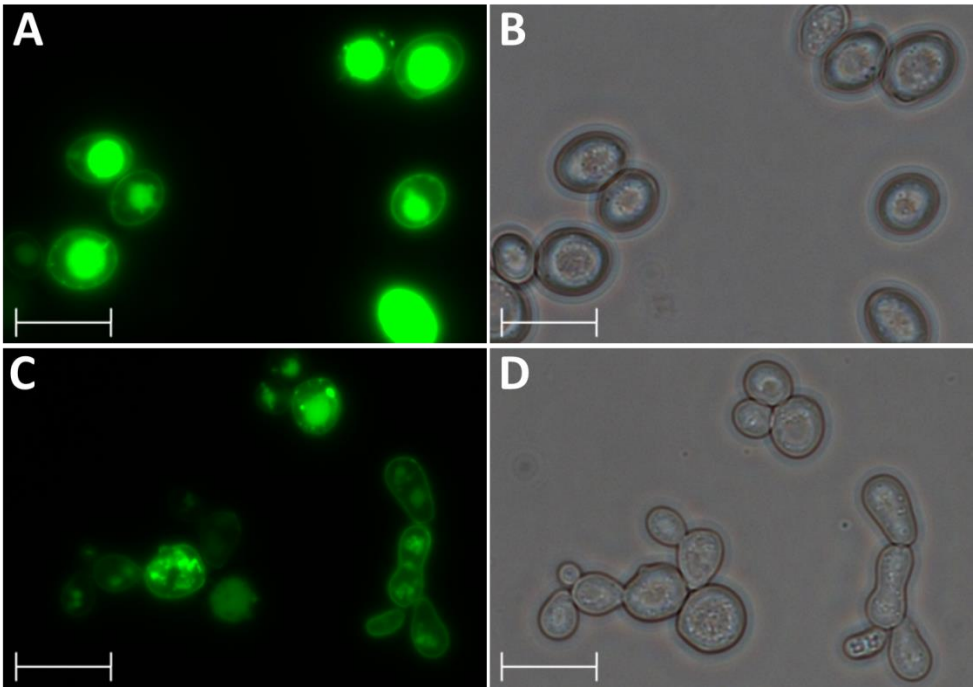

Figure 3. Fluorescent and phase-contrast pictures of *S. cerevisiae* strains IMX1272 (*MAL11-YPet, LmSPase*, **A & B**) and IMX1273 (*PvSUF1-YPet, LmSPase*, **C & D**). Cells were collected from aerobic, steady-state, sucrose-limited chemostat cultures grown at a dilution rate of 0.030 h^{-1}. The scale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

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

(*MAL11*, *LmSPase*) to 0.075 ± 0.000 g/g glucose equivalent for IMZ636 (*PvSUF1*, *LmSPase*) (**Table 4**). In line with an increased ATP yield, the biomass specific sucrose uptake rate (1.11 ± 0.00 vs 1.21 ± 0.00 mmol/g biomass/h), specific ethanol production rate (3.69 ± 0.06 vs 4.17 ± 0.01 mmol/g biomass/h) and CO_2 production rate (3.79 ± 0.05 vs 4.29 ± 0.13 mmol/g biomass/h) decreased for IMZ636 (*PvSUF1*, *LmSPase*) compared to IMZ665 (*MAL11*, *SPase*). The observed 8% increase in the anaerobic biomass yield was lower than the predicted 25%. Mislocalization and/or increased protein turnover of PvSUF1 could have increased the maintenance energy requirement and thereby resulted in a lower-than-expected increase in the biomass yield. The impact of the cellular maintenance energy requirements on the biomass yield decreases with an increase in the specific growth rate (Leuenberger, 1971; Pirt, 1965). Therefore testing of *PvSUF1*- and *MAL11*-expressing strains at higher dilution rates could provide insight into maintenance energy related effects on the biomass yield.

3.3 Overexpression of phosphoglucomutase in *Lm*SPase-dependent strains enables faster anaerobic growth on sucrose.

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

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

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

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

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

limited bioreactors at a dilution rate of 0.030 h^{-1}. In the essay, an alkalinisation upon sucrose addition to the cell suspension indicates the presence of a proton-coupled sucrose-uptake mechanism (Stambuk et al., 2000). Indeed, such an alkalinisation of the extracellular medium was observed upon addition of either sucrose or maltose to cell suspensions of the *MAL11*-expressing strain IMZ709 (**Figure 4B**). Surprisingly, alkalinisation of the extracellular medium was also observed upon disaccharide addition to cell suspensions of the *PvSUF1*-expressing strain IMZ696 (**Figure 4A**), although at a lower initial rate (8.2 ± 2.2 and 7.9 ± 1.0 µmol H^+/g biomass/min for sucrose and maltose respectively) than observed for IMZ709 (51.4 ± 8.6 and 42.6 ± 6.2 µmol H^+/g biomass/min for sucrose and maltose respectively). As a control, fructose and glucose were added to cell suspensions of both strains. In accordance with hexose uptake via facilitated diffusion mediated by the hexose transporters, no pH change upon addition of glucose or fructose to cell suspensions of IMZ696 (*PvSUF1*, *LmSPase*, *PGM2*) was observed. Addition of fructose to cell suspensions of IMZ709 (*MAL11*, *LmSPase*, *PGM2*) did not result in a pH change. In line with previous observations from Wieczorke et al., 1999, some alkalinisation of the extracellular medium was observed upon glucose addition to this strain. Resequencing of the *Pv*SUF1 genes at the end of both aerobic chemostat cultures revealed 2 point mutations (**Supplementary material**), leading to different amino acid substitutions in both cultures (H67G and T302I respectively). These mutations did not involve acidic residues making it unlikely that they affected proton coupling of sucrose transport (Lemoine, 2000).

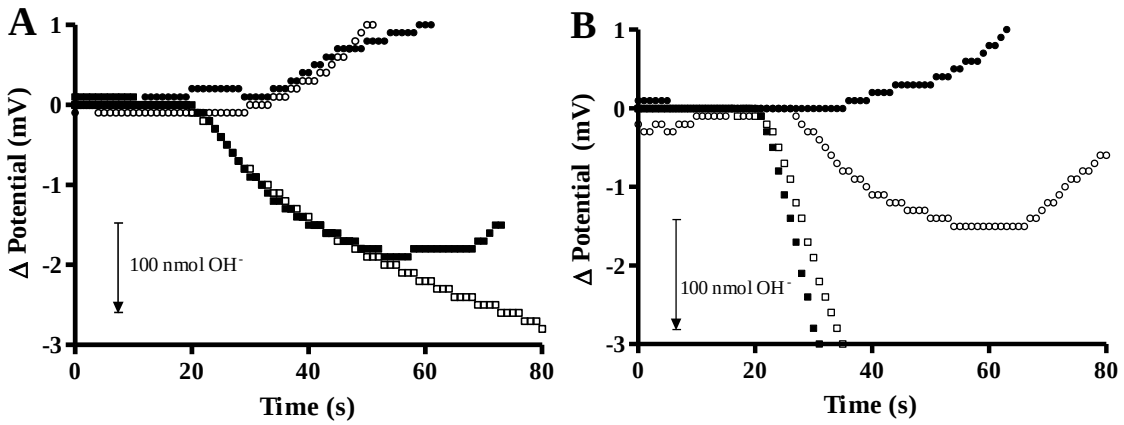

Figure 4. Proton uptake induced by addition of sugars to cell suspensions of two yeast strains. A) Strain IMZ696 expressing *PvSUF1*, *LmSPase* and *PGM2*. B) Strain IMZ709 expressing *MAL11*, *LmSPase* and *PGM2*. Cells were pre-grown in aerobic, sucrose-limited chemostat cultures, harvested from steady-state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

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

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

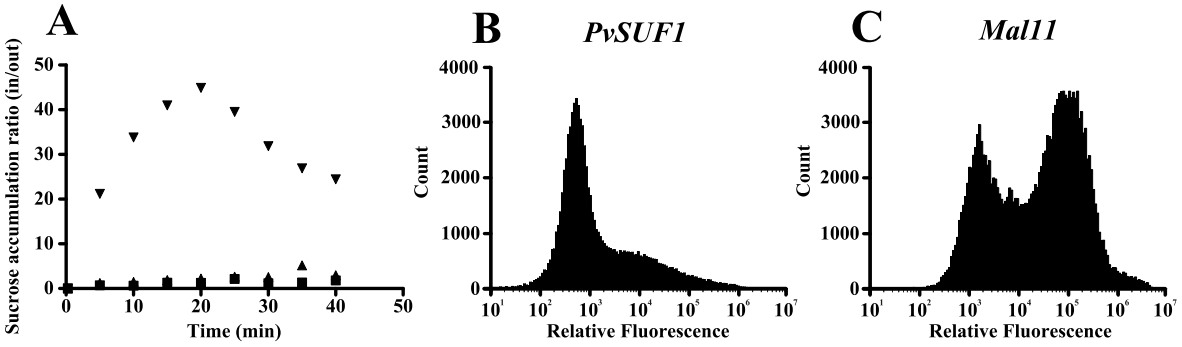

Figure 5. A) Transport of 1 mM ^{14}C-sucrose by *S. cerevisiae* IMX1274 (empty vector, ■), IMX1275 (*PvSUF1*, ▲), and IMX1276 (*MAL11*,▼). Cells were grown and prepared as described above. After 20 minutes of incubation, 10 µM FCCP was added to the reactions. B) Flow cytometry analysis of *S. cerevisiae*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

4. DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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SUPPLEMENTARY MATERIAL

Table S1

Primers used in this study.

Primer	Sequence (5′ → 3′)	Purpose
3093	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACAT TCCGCCAGATCATCAATAGGCACCTTCGTACGCTGCA GGTCGAC	*amdSYM* amplification
3094	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCC ATAGCCATGCCTTCACATATAGTGACGGATCGCTTGC CTGTAAC	*amdSYM* amplification
6845	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCC ATAGCCATGCCTTCACATATAGTACAGGCAACACGCA GATATAGG	p426TEF backbone amplification
6846	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATT CGACCAGCCTAAGAATGTTCAACGGCCCACTACGTGA ACCATC	p426TEF backbone amplification
8379	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGA TCCATGAAAAATATCATTTCATTGGTAAGCAAGAAG	*MAL11* ORF amplification
8380	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAG CTTCCTTAACATTTATCAGCTGCATTTAATTC	*MAL11* ORF amplification
7998	AAGCTTATCGATACCGTCGACC	p426TEF *amdSYM* backbone amplification
7999	GGATCCACTAGTTCTAGAAAACTTAGATTAG	p426TEF *amdSYM* backbone amplification
9043	TCACAGAGGGATCCCGTTACCCATCTATGCTGAAGAT TTATCATACTATTCCTCCGCTCGGCGATCGCGTGTGG AAGAAC	*MAL11* expressing cassette amplification
9044	CGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAG CTCGCCGCAAATTAAAGCCTTCG	*MAL11* expressing cassette amplification
10303	TTAAATCTATAACTACAAAAAACACATACATAAACT AAAAATGTCATTTCAAATTGAAACG	*PGM2* ORF amplification
10304	ACTACAATATAAAAAAACTATACAAATGACAAGTTC TTGATTAAGTACGAACCGTTGGTTCTTCAG	*PGM2* ORF amplification
6486	TTTTAGTTTATGTATGTGTTTTTTGTAGTTATAGATT TAAGCAAG	pUDE206 backbone amplification
9719	TCAAGAACTTGTCATTTGTATAG	pUDE206 backbone amplification
10305	GGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCT GTTTAGTGTGAGCGGGATTTAAACTGTGAGG	*PGM2* expressing cassette amplification
10306	GTTGTGTGGAATTGTGAGCGGATAACAATTTCACACA GGACAGTATAGCGACCAGCATTC	*PGM2* 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	*LEU2* integration (*LEU2* homology)
1743	TAAGGCCGTTTCTGACAGAG	*LEU2* integration (*LEU2* homology)
5271	CAAGGAGAAAAAACCCCGGATTCTAGAACTAGTGGA TCCCCCATGAAAAATATCATTTCATTGGTAAG	*MAL11* amplification from BY4742
5272	GAATAATTCTTCACCTTTAGAACCTTGAAAATATAAA TTTTCCCCTCCACATTTATCAGCTGCATTTAATTC	*MAL11* amplification from BY4742 and pRHA00L
5273	GGAGGGGAAAATTTATATTTTCAAGGTTC	Backbone amplification from pFB001
5274	GGGGGATCCACTAGTTCTAGAATC	Backbone amplification from pFB001
5435	CGCATCTGTGCGGTATTTC	*LEU2* amplification from pRS315
5436	GGCTTAACTATGCGGCATC	*LEU2* amplification from pRS315
5437	TGCACTCTCAGTACAATCTGCTC	Backbone amplification from pRHA00
5438	TGCGGTGTGAAATACCGC	Backbone amplification from pRHA00
5959	AGGGGAAAATTTATATTTTCAAGG	Backbone amplification from pRHA00L
6324	TTGTTCCCTTTAGTGAGGG	Backbone amplification from pRHA00L
5961	TCTAGAACTAGTGGATCCCAAAATGAAAAATATCAT TTCATTGG	*MAL11* amplification from pRHA00L
4995	AATTAACCCTCACTAAAGGG	*TEF1*-promoter amplification from pUDE379
5960	CATTTTGGGATCCACTAGTTCTAG	*TEF1*-promoter amplification from pUDE379
6717	CTCATTAGAAAGAAAGCATAGCAATC	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 CATGGAGGGGAAAATTTATATTTTCAAGGTTCTAAA G	YPet-tag amplification from pUDE453
9763	ATGAAAACCACCTGCTACCATAC	Cassette amplification from pUDE413
9355	TGTAAATATCTAGGAAATACACTTGTGTATACTTCTC GCTTTTCTTTTATTTTTTTTTGTAGTTTATCATTATC AATACTCGCCATTTC	*LmSPase* or *MAL12* integration (*SGA1* homology)
9356	TTTACAATATAGTGATAATCGTGGACTAGAGCAAGA TTTCAAATAAGTAACAGCAGCAAAGTGTGGAAGAAC GATTACAACAG	*MAL12* or *LmSPase* integration (*SGA1* homology)

1

***LmSPase* codon optimized:**

ATGGAAATCCAAAACAAGGCTATGTTGATCACTTACGCTGACTCTTTGGGTAAGAACTTGAAGG
ACGTTCACCAAGTTTTGAAGGAAGACATCGGTGACGCTATCGGTGGTGTTCACTTGTTGCCATTC
TTCCCATCTACTGGTGACAGAGGTTTCGCTCCAGCTGACTACACTAGAGTTGACGCTGCTTTCGG
TGACTGGGCTGACGTTGAAGCTTTGGGTGAAGAATACTACTTGATGTTCGACTTCATGATCAACC
ACATCTCTAGAGAATCTGTTATGTACCAAGACTTCAAGAAGAACCACGACGACTCTAAGTACAA
GGACTTCTTCATCAGATGGGAAAAGTTCTGGGCTAAGGCTGGTGAAAACAGACCAACTCAAGCT
GACGTTGACTTGATCTACAAGAGAAAGGACAAGGCTCCAACTCAAGAAATCACTTTCGACGACG
GTACTACTGAAAACTTGTGGAACACTTTCGGTGAAGAACAAATCGACATCGACGTTAACTCTGCT
ATCGCTAAGGAATTCATCAAGACTACTTTGGAAGACATGGTTAAGCACGGTGCTAACTTGATCA
GATTGGACGCTTTCGCTTACGCTGTTAAGAAGGTTGACACTAACGACTTCTTCGTTGAACCAGAA
ATCTGGGACACTTTGAACGAAGTTAGAGAAATCTTGACTCCATTGAAGGCTGAAATCTTGCCAG
AAATCCACGAACACTACTCTATCCCAAAGAAGATCAACGACCACGGTTACTTCACTTACGACTTC
GCTTTGCCAATGACTACTTTGTACACTTTGTACTCTGGTAAGACTAACCAATTGGCTAAGTGGTT
GAAGATGTCTCCAATGAAGCAATTCACTACTTTGGACACTCACGACGGTATCGGTGTTGTTGACG
CTAGAGACATCTTGACTGACGACGAAATCGACTACGCTTCTGAACAATTGTACAAGGTTGGTGCT
AACGTTAAGAAGACTTACTCTTCTGCTTCTTACAACAACTTGGACATCTACCAAATCAACTCTAC
TTACTACTCTGCTTTGGGTAACGACGACGCTGCTTACTTGTTGTCTAGAGTTTTCCAAGTTTTCG
CTCCAGGTATCCCACAAATCTACTACGTTGGTTTGTTGGCTGGTGAAAACGACATCGCTTTGTTG
GAATCTACTAAGGAAGGTAGAAACATCAACAGACACTACTACACTAGAGAAGAAGTTAAGTCTG
AAGTTAAGAGACCAGTTGTTGCTAACTTGTTGAAGTTGTTGTCTTGGAGAAACGAATCTCCAGC
TTTCGACTTGGCTGGTTCTATCACTGTTGACACTCCAACTGACACTACTATCGTTGTTACTAGAC

AAGACGAAAACGGTCAAAACAAGGCTGTTTTGACTGCTGACGCTGCTAACAAGACTTTCGAAAT

CGTTGAAAACGGTCAAACTGTTATGTCTTCTGACAACTTGACTCAAAACTAA

***PvSUF1* codon optimized:**

ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCACATTATCCGT
AGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTCGCCGTTGCTTCAATAGCTGCA
GGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGTTAACCCCATATGTCCAATTGTTAGGTGT
ACCTCATGCCGCTGCATCCTTTATATGGTTATGTGGTCCAATCAGTGGTTTGGTTGTCCAACCTA
TCGTTGGTTACTACTCTGATAGATCTACTTCTAGATACGGTAGAAGAAGACCTTTTATTTTAGGT
GGTGCAGTCGCCGTAGCTATCGCAGTTTTCTTGATTGGTTATGCCGCTGATATTGGTTACTCAGC
TGGTGACGACATAACCAAAAAGACTAGACCAAGAGCCGTTGCTGTCTTCGTAATTGGTTTCTGG
ATCTTGGACGTTGCAAACAACATGTTGCAAGGTCCTTGCAGAGCCTTTTTGGCTGATTTGGCAGC
CGGAGATCAAAGAAAGACTAGAATCGCAAACGGTTTCTTTTCTTTCTTTATGGCCGTTGGTAACG
TTTTAGGTTATGCTGCAGGTTCTTTTTCAGGTTTGCACAAAATTTTCCCTTTTACTCAAACAAAG
GCATGTGATGTTTTCTGCGCCAATTTGAAGTCTTGTTTCTTTTTCTCTATCTTGTTGTTGTTATT
TTTGTCCACAGTTGCTTTGATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGCCGTTCAAGAAG
ACGCTCAACCTTCTTGCTTCTTTCAATTGTTCGGTGCTTTGAAGGAATTGAAGAGACCAATGTGG
ATGTTGATGTTAGTCACCGCAGTAAATTGGATTGGTTGGTTTCCTTATTTCTTGTTTGATACTGA
CTGGATGGGTAGAGAAGTTTATGGTGGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAGA
GTCGGTTCCTTGGGTTTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAACC
ATTGGGTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTTAACTTCATCTTGGCTATC
GGTTTCGGTATGACAGTCGTAATAACCAAAATGGCAGAACATCAAAGACACTTAAACCCAGCAG
CCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGGTATTCTTTGCTGTATTGGGTGTT
CCATTAGCAATCACATTCTCTGTTCCTTTTGCTTTGGCATCCATCTATTCTTCAGCAAGTGGTGC
CGGTCAAGGTTTGTCATTAGGTGTTTTGAATTTGGCTATTGTTGTCCCACAAATGGTAGTTTCCG
CCTTAAGTGGTCCTTGGGACGCTTTATTCGGTGGTGGTAACTTGCCAGCTTTTATGGTTGGTGCT

GCAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTGTTACCTACCCCTAAGCCAGCCGACGA

AGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTCATTAA

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

ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCACATTATCCGT

AGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTCGCCGTTGCTTCAATAGCTGCA

GGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGTTAACCCCATATGTCCAATTGTTAGGTGT

ACCTCAGGCCGCTGCATCCTTTATATGGTTATGTGGTCCAATCAGTGGTTTGGTTGTCCAACCTA

TCGTTGGTTACTACTCTGATAGATCTACTTCTAGATACGGTAGAAGAAGACCTTTTATTTTAGGT

GGTGCAGTCGCCGTAGCTATCGCAGTTTTCTTGATTGGTTATGCCGCTGATATTGGTTACTCAGC

TGGTGACGACATAACCAAAAAGACTAGACCAAGAGCCGTTGCTGTCTTCGTAATTGGTTTCTGG

ATCTTGGACGTTGCAAACAACATGTTGCAAGGTCCTTGCAGAGCCTTTTTGGCTGATTTGGCAGC

CGGAGATCAAAGAAAGACTAGAATCGCAAACGGTTTCTTTTCTTTCTTTATGGCCGTTGGTAACG

TTTTAGGTTATGCTGCAGGTTCTTTTTCAGGTTTGCACAAAATTTTCCCTTTTACTCAAACAAAG

GCATGTGATGTTTTCTGCGCCAATTTGAAGTCTTGTTTCTTTTTCTCTATCTTGTTGTTGTTATT

TTTGTCCACAGTTGCTTTGATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGCCGTTCAAGAAG

ACGCTCAACCTTCTTGCTTCTTTCAATTGTTCGGTGCTTTGAAGGAATTGAAGAGACCAATGTGG

ATGTTGATGTTAGTCACCGCAGTAAATTGGATTGGTTGGTTTCCTTATTTCTTGTTTGATACTGA

CTGGATGGGTAGAGAAGTTTATGGTGGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAGA

GTCGGTTCCTTGGGTTTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAACC

ATTGGGTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTTAACTTCATCTTGGCTATC

GGTTTCGGTATGACAGTCGTAATAACCAAAATGGCAGAACATCAAAGACACTTAAACCCAGCAG

CCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGGTATTCTTTGCTGTATTGGGTGTT

CCATTAGCAATCACATTCTCTGTTCCTTTTGCTTTGGCATCCATCTATTCTTCAGCAAGTGGTGC

CGGTCAAGGTTTGTCATTAGGTGTTTTGAATTTGGCTATTGTTGTCCCACAAATGGTAGTTTCCG

CCTTAAGTGGTCCTTGGGACGCTTTATTCGGTGGTGGTAACTTGCCAGCTTTTATGGTTGGTGCT

GCAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTGTTACCTACCCCTAAGCCAGCCGACGA

AGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTCATTAA

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

ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCACATTATCCGT

AGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTCGCCGTTGCTTCAATAGCTGCA

GGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGTTAACCCCATATGTCCAATTGTTAGGTGT

ACCTCATGCCGCTGCATCCTTTATATGGTTATGTGGTCCAATCAGTGGTTTGGTTGTCCAACCTA

TCGTTGGTTACTACTCTGATAGATCTACTTCTAGATACGGTAGAAGAAGACCTTTTATTTTAGGT

GGTGCAGTCGCCGTAGCTATCGCAGTTTTCTTGATTGGTTATGCCGCTGATATTGGTTACTCAGC

TGGTGACGACATAACCAAAAAGACTAGACCAAGAGCCGTTGCTGTCTTCGTAATTGGTTTCTGG

ATCTTGGACGTTGCAAACAACATGTTGCAAGGTCCTTGCAGAGCCTTTTTGGCTGATTTGGCAGC

CGGAGATCAAAGAAAGACTAGAATCGCAAACGGTTTCTTTTCTTTCTTTATGGCCGTTGGTAACG

TTTTAGGTTATGCTGCAGGTTCTTTTTCAGGTTTGCACAAAATTTTCCCTTTTACTCAAACAAAG

GCATGTGATGTTTTCTGCGCCAATTTGAAGTCTTGTTTCTTTTTCTCTATCTTGTTGTTGTTATT

TTTGTCCACAGTTGCTTTGATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGCCGTTCAAGAAG

ACGCTCAACCTTCTTGCTTCTTTCAATTGTTCGGTGCTTTGAAGGAATTGAAGAGACCAATGTGG

ATGTTGATGTTAGTCACCGCAGTAAATTGGATTGGTTGGTTTCCTTATTTCTTGTTTGATATTG

ACTGGATGGGTAGAGAAGTTTATGGTGGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAG

AGTCGGTTCCTTGGGTTTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAA

CCATTGGGTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTTAACTTCATCTTGGCTA

TCGGTTTCGGTATGACAGTCGTAATAACCAAAATGGCAGAACATCAAAGACACTTAAACCCAGC

AGCCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGGTATTCTTTGCTGTATTGGGTG

TTCCATTAGCAATCACATTCTCTGTTCCTTTTGCTTTGGCATCCATCTATTCTTCAGCAAGTGGT

GCCGGTCAAGGTTTGTCATTAGGTGTTTTGAATTTGGCTATTGTTGTCCCACAAATGGTAGTTTC

CGCCTTAAGTGGTCCTTGGGACGCTTTATTCGGTGGTGGTAACTTGCCAGCTTTTATGGTTGGTG

CTGCAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTGTTACCTACCCCTAAGCCAGCCGAC

GAAGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTCATTAA