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1 **Laboratory evolution and physiological analysis of**
2 ***Saccharomyces cerevisiae* strains dependent on sucrose**
3 **uptake via the *Phaseolus vulgaris* Suf1 transporter**

4

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20 **ABSTRACT**

21 Knowledge on the genetic factors important for efficient expression of plant transporters in
22 yeast is still very limited. Here, we investigated if laboratory evolution could improve the
23 expression a sucrose transporter in yeast and which mutations were crucial for the evolved
24 phenotype. *PvSuf1* (*Phaseolus vulgaris* sucrose facilitator 1) was key in a previously published
25 strategy aimed at increasing ATP yield in *S. cerevisiae* since it has been described as an
26 uniporter. However, attempts to construct yeast strains in which sucrose metabolism was
27 dependent on *PvSUF1* led to slow sucrose uptake. Here, *PvSUF1*-dependent *S. cerevisiae* strains
28 were evolved for faster growth. Of five independently evolved strains, two showed an
29 approximately two-fold higher anaerobic growth rate on sucrose than the parental strain ($\mu =$
30 0.19 h^{-1} and $\mu = 0.08 \text{ h}^{-1}$, respectively). All five mutants displayed sucrose-induced proton uptake
31 ($13\text{-}50 \mu\text{mol H}^+ (\text{g biomass})^{-1} \text{ min}^{-1}$). Their ATP yield from sucrose dissimilation, as estimated
32 from biomass yields in anaerobic chemostat cultures, was the same as that of a congeneric strain
33 expressing the native sucrose symporter Mal11p. Four out of six observed amino acid
34 substitutions encoded by evolved *PvSUF1* alleles removed or introduced a cysteine residue and
35 may be involved in transporter folding and/or oligomerization. Expression of one of the evolved
36 *PvSUF1* alleles (*PvSUF1*^{I209F C265F G326C}) in an unevolved strain enabled it to grow on sucrose at the
37 same rate (0.19 h^{-1}) as the corresponding evolved strain. This study shows how laboratory
38 evolution can be used to improve sucrose uptake via heterologous plant transporters; sheds
39 light into the importance of cysteine residues for efficient plant transporter expression in yeast
40 and warrant reinvestigation of *PvSuf1*'s mechanism in a plant context.

41

42 **KEY WORDS:** plant transporter expression, laboratory evolution, yeast physiology, plant
43 sucrose facilitator, sucrose uptake.

44 INTRODUCTION

45 Sucrose (α -D-glucopyranosyl-(1 \leftrightarrow 2)- β -D-fructofuranoside) is an intensively used carbon source
46 in microbial biotechnology (Maiorella, Blanch, & Wilke, 1984; Marques, Raghavendran, Stambuk,
47 & Gombert, 2016; Peters, Rose, & Moser, 2010) and plays a vital role as a carbon and energy
48 carrier in plants (Salerno & Curatti, 2003). In plants, sucrose exits source cells via
49 plasmodesmata as well as via membrane transport mediated by sucrose uniporters (Doidy et al.,
50 2012).

51 In *Saccharomyces cerevisiae*, a yeast employed in many different industrial bioprocesses, the
52 predominant pathway for sucrose catabolism starts with its extracellular hydrolysis, catalysed
53 by invertase. The free energy available from sucrose hydrolysis ($\Delta G_{o'} = -29$ kJ/mol)(Goldberg,
54 Tewari, & Ahluwalia, 1989) is not conserved by the yeast cells. The resulting monomers glucose
55 and fructose are subsequently transported into the cells by facilitated diffusion. Alternatively,
56 after its uptake via proton symporters, sucrose can also be hydrolysed in the cytosol (Marques et
57 al., 2017; Stambuk, Silva, Panek, & Araujo, 1999).

58 Inspired by previous studies on free-energy conservation by *S. cerevisiae* during maltose
59 consumption (de Kok et al., 2011), Marques et al. (2018) replaced yeast invertase by an
60 intracellular sucrose phosphorylase from the bacterium *Leuconostoc mesenteroides*. Sucrose
61 phosphorylase converts sucrose and inorganic phosphate into fructose and glucose-1-phosphate
62 (glucose-1P) (Weimberg & Doudoroff, 1954), which can subsequently be isomerised to glucose-
63 6P via the *S. cerevisiae* phosphoglucomutase (Pgm2). By circumventing the ATP-requiring
64 hexokinase reaction for one of the monomers, this phosphorolysis pathway saves one mole of
65 ATP per mole of sucrose consumed. However, since phosphorolysis takes place in the cytosol
66 and uptake of sucrose in yeast involves proton symport via α -glucoside transporters (e.g. Mal11,
67 Mal21, Mal31, Mph2, Mph3) (Badotti et al., 2008; Stambuk et al., 1999), protons have to be
68 exported back to the extracellular space via plasma membrane H⁺-ATPase (Pma1), which has a
69 H⁺/ATP stoichiometry of 1:1 (Van Leeuwen, Weusthuis, Postma, Van den Broek, & Van Dijken,
70 1992). For this reason, the overall free-energy conservation in the engineered 'phosphorolytic'

71 strain did not change relative to a wild-type strain: in both cases, anaerobic fermentation of
72 sucrose yielded 4 mol ATP per mol of sucrose. To gain one additional mol of ATP per mol of
73 sucrose consumed, the native sucrose transporters should be replaced by a transporter that
74 mediates facilitated diffusion (de Kok, Kozak, Pronk, & van Maris, 2012). If the ATP yield of
75 sucrose fermentation by *S. cerevisiae* could be increased to 5 mol of ATP per mole of sucrose, this
76 could theoretically expand the range of products that can be made in anaerobic yeast-based
77 processes. Such anaerobic production processes have considerable cost advantages relative to
78 aerated processes since less power has to be devoted to air compression, reactor agitation and
79 cooling (de Kok, 2012; Weusthuis, Lamot, van der Oost, & Sanders, 2011; Mans, 2017).

80 Many studies have demonstrated functional expression of heterologous transporters in *S.*
81 *cerevisiae* (He, Wang, & Yan, 2014; Kim, Lee, Galazka, Cate, & Jin, 2014; Lin et al., 2014; Ton &
82 Rao, 2004). However, efficient transporter sorting, folding and stability in yeast cells can be a
83 major challenge (Froissard et al., 2006; Hernández, 2005). Therefore, functional characteristics
84 of plant transporters expressed in *S. cerevisiae* cannot always be derived from *in planta* results
85 (Bassham & Raikhel, 2000). Few previous studies have investigated the expression of plant
86 sucrose uniporters in yeast (Chen, 2014; Lin et al., 2014; Zhou, Grof, & Patrick, 2014; Zhou, Qu,
87 Dibley, Offler, & Patrick, 2007). Marques et al. (2018) expressed 5 candidate genes for sucrose
88 uniporters in *S. cerevisiae*, of which only one, encoding the *Phaseolus vulgaris* Sucrose Facilitator
89 1 (*PvSUF1*), supported growth of a strain (after one week time) in which extracellular invertase
90 and sucrose symport have been eliminated. However, the anaerobic specific growth rate on
91 sucrose of this strain was only 0.05 h⁻¹, which was six-fold lower than that of the reference strain
92 CEN.PK113-7D. Indeed, rates of uptake of radiolabelled sucrose by the *PvSUF1*-expressing strain
93 were close to the detection limit. While some YPet-tagged *PvSuf1* was found at the yeast plasma
94 membrane, it also accumulated in intracellular compartments, suggesting poor intracellular
95 targeting and/or high turnover of heterologously expressed *PvSuf1* (Marques et al., 2018).

96 In view of the potential relevance of expressing *PvSUF1* and other plant sugar transporter genes
97 in the metabolic engineering of *Saccharomyces cerevisiae*, this study aimed at investigating

98 genetic factors involved in optimal functional expression of *PvSUF1* in this yeast. To this end, we
99 used laboratory evolution to select for *PvSuf1*-dependent *S. cerevisiae* strains with improved
100 sucrose-uptake kinetics and analysed causal mutations for improved sucrose consumption by
101 evolved strains. To study the energy coupling of sucrose transport by evolved and unevolved
102 *PvSuf1* variants, we analysed sucrose-induced proton-uptake by reference and evolved strains
103 and measured biomass yields of yeast strains expressing different *PvSuf1* variants in anaerobic,
104 sucrose-limited chemostat cultures.

105

106 **MATERIALS AND METHODS**

107

108 **Microbial strains and cultivation medium**

109 The *S. cerevisiae* strains used in this study (**Table 1, Figure 1**) share the CEN.PK genetic
110 background (Entian & Kötter, 2007; Nijkamp et al., 2012). Cultures were grown in an Innova
111 incubator shaker (Eppendorf, Hamburg, Germany) at 200 rpm, 30 °C, in 500 mL shake flasks
112 containing 100 mL of either yeast-peptone-dextrose medium (YPD) or synthetic medium
113 (Verduyn, Postma, Scheffers, & van Dijken, 1992) with 20 g/L glucose as the carbon source
114 (SMD). Frozen stock cultures were prepared by adding glycerol (30 % v/v final concentration)
115 to exponentially growing cells, followed by aseptic freezing and storage of 1 mL aliquots at -80
116 °C.

117

118 **Molecular biology techniques**

119 PCR amplifications for strain construction were performed with Phusion Hot Start II High
120 Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's
121 instructions. Diagnostic PCR was carried out using DreamTaq (Thermo Fisher Scientific). The
122 primers used in this study (**Table S1**) were purchased from Sigma-Aldrich. Yeast genomic DNA
123 was isolated using the YeaStar Genomic DNA kit (D2002, Zymo Research, Irvine, CA). DNA
124 fragments obtained by PCR were separated by gel electrophoresis using 1% (w/v) agarose gels

125 (Thermo Fisher Scientific) in Tris-acetate-EDTA buffer (Thermo Fisher Scientific). DNA
126 fragments were excised from the gels and purified by gel purification kit (D2004, Zymo
127 Research). Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich)
128 according to the supplier's manual and from *S. cerevisiae* using Zymoprep Yeast Plasmid
129 Miniprep II kit (Zymo Research). Restriction analysis was performed using FastDigest enzymes
130 (Thermo Fisher Scientific) according to the manufacturer's manual. *E. coli* DH5 α cells (18258-
131 012, Thermo Fisher Scientific) were transformed via electroporation using a Gene Pulser Xcell
132 Electroporation System (Bio-Rad), following the manufacturers protocol.

133

134 **Sanger and whole-genome sequencing**

135 Genome-integrated and episomal expression cassettes present in the evolved strains (IMS644,
136 IMS646, IMS647, IMS648 and IMS649) were Sanger sequenced at BaseClear BV (Leiden, The
137 Netherlands). Primers 6018&7822 (**Table S1**) were used to amplify the *SPase*-expression
138 cassette for sequencing. Similarly, primers 5606&7827 were used to amplify the *PvSUF1* allele of
139 strain IMS648, before Sanger sequencing. Plasmids expressing the *PGM2* and *PvSUF1* genes were
140 extracted from yeast using Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research) and
141 transformed into *E. coli* (DH5 α cells, 18258-012, Thermo Fisher Scientific) via electroporation
142 using a Gene Pulser Xcell Electroporation System (Bio-Rad) for propagation. After extraction
143 from *E. coli* using the Sigma GenElute Plasmid kit (Sigma-Aldrich), plasmids were used as a
144 template to sequence the *PGM2* and *PvSUF1* cassettes. Genes were sent for Sanger sequencing
145 using the primers listed in **Table S1** resulting in a two times coverage of each base pair. The
146 promoter and terminator regions sequenced were: 420 bp upstream and 280 bp downstream of
147 the *PvSUF1* ORF, 500 bp upstream and 170 bp downstream of the *PGM2* ORF and 670 bp
148 upstream and 370 bp downstream of the *LmSPase* ORF. Genomic DNA for whole-genome
149 sequencing was extracted using the Qiagen 100/G kit following the manufacturer's protocol
150 (Qiagen, Hilden, Germany), from shake-flask cultures grown on SMD. Whole-genome sequencing
151 was performed by Novogene (HK) Company Ltd (Hong Kong, China). A PCR-free insert library of

152 350-bp genomic fragments was created and sequenced paired end (150-bp reads). A minimum
153 data quantity of 2600 MB was generated per strain, representing a minimum 216-fold coverage.
154 Data analysis was performed by mapping the sequence reads to the CEN.PK113-7D reference
155 (Salazar et al., 2017) using the Burrows-Wheeler alignment (BWA) tool (Li & Durbin, 2009) and
156 processed with Pilon (Walker et al., 2014). The sequencing data of the parental strain IMZ630
157 and of the evolved isolates (IMS644, IMS646, IMS647, IMS648 and IMS649) were deposited at
158 NCBI under the BioProject ID: PRJNA471800.

159

160 **Plasmid construction**

161 Plasmid assembly was performed by *in vivo* homologous recombination in *S. cerevisiae*
162 transformed according to Gietz and Woods (2002) using 0.5 - 1.0 µg of DNA. Construction of the
163 "empty" vector plasmid pUDE690 (**Table 2**) was done by amplifying pUDE486 in two parts,
164 leaving out the *PvSUF1* ORF. Both parts were amplified using primers 11846 & 5975 and 5974 &
165 11847 (**Table S1**). The ends of the amplified fragments shared 60 bp homology regions and
166 were joined *in vivo* (Kuijpers et al., 2013) by transforming both parts in strain IMZ630 (**Table 1**).
167 Plasmids pUDE544, pUDE546, pUDE547, pUDE559 and pUDE560 were extracted from sucrose-
168 evolved strains IMS644, IMS646, IMS647, IMS648 and IMS649, respectively, and transformed
169 into *E. coli* DH5α cells for plasmid multiplication and storage (**Table 1, Table 2**). *PvSUF1*-
170 expression cassettes from pUDE413 and pUDE486 were replaced by *PvSUF1*-expression
171 cassettes containing mutations in the *PvSUF1* ORF. For this purpose, pUDE413 or pUDE486
172 backbone was amplified using primers 9041&5975 and assembled *in vivo* with *PvSUF1*-
173 expression cassette amplified from pUDE545 or pUDE546 using primers 2889&10307 in strain
174 IMZ630, resulting in plasmids pUDE565 and pUDE566, respectively, when pUDE413-backbone
175 was used, and plasmids pUDE567 and pUDE568, respectively, when backbone from pUDE486
176 was used (**Table 2**). pUDE691 was constructed in the same way as pUDE567 and pUDE568 with
177 the exception that, for construction of this plasmid, the *PvSUF1*-expression cassette was
178 amplified from pUDE560 (**Table 2**).

179

180 Strain construction

181 Strain IMZ730 was constructed by transforming pUDE690 into IMZ630 (**Figure 1**). Plasmids
182 present in the evolved strains, IMS644, IMS646, IMS647, IMS648 and IMS649, were removed by
183 overnight cultivation on YPD medium followed by selection on SMD agar plates supplemented
184 with 0.15 g/L uracil and 1 g/L 5'-fluoroorotic acid (Boeke, La Croute, & Fink, 1984), resulting in
185 strains IMS652, IMS653, IMS654, IMS655 and IMS656, respectively (**Figure 1**). Expression
186 cassettes (*TEF1p-PvSUF1-CYC1t*) were extracted from the evolved strains, cloned into a 2- μ m
187 plasmid (with and without *PGM2*) resulting in plasmids pUDE565, pUDE566, pUDE567,
188 pUDE568 and pUDE691, which were subsequently transformed into an unevolved strain
189 background (IMZ630), resulting in strains IMZ712, IMZ713, IMZ714, IMZ715 and IMZ729,
190 respectively. Similarly, the *PvSUF1*-containing plasmids extracted from the evolved strains
191 (pUDE545, pUDE546 and pUDE560) were transformed into an evolved background, IMS656
192 (obtained after plasmid removal from strain IMS649), resulting in strains IMZ724, IMZ725 and
193 IMZ727, respectively (**Figure 1**).

194

195 Cultivation conditions

196 Shake flask cultivations were carried out in 500 mL flasks containing 100 mL synthetic medium
197 with 20 g/L initial sucrose (SMS) (if not stated, sugar concentration in SMD or SMS was 20 g/L),
198 in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ) set at 200 rpm and 30 °C.
199 For growth rate determinations, frozen stock cultures were first inoculated in a shake flask
200 containing SMD. After reaching stationary phase, cultures were transferred to SMS (initial
201 $OD_{660nm} \leq 0.2$) and incubated under an anaerobic atmosphere (5% H₂ 6% CO₂ and 89% N₂) in a
202 Bactron X anaerobic chamber (Shell Lab, Cornelius, OR) until exponential growth was observed.
203 Inside the anaerobic chamber, exponentially growing cultures were then transferred to fresh
204 SMS (initial $OD_{660nm} = 0.2$) and samples were taken hourly until stationary phase was reached.
205 Specific growth rates were calculated from at least five OD measurements evenly distributed

206 over the exponential growth phase. For anaerobic cultivations, synthetic medium was
207 supplemented with 10 mg/L ergosterol and 420 mg/L Tween 80. Since stock solutions of these
208 anaerobic growth factors were prepared with ethanol, the initial ethanol concentration in media
209 for anaerobic growth was 0.67 g/L. Chemostat cultivations were performed in 1.5 L bioreactors
210 (800 rpm, 30°C) (Applikon, Delft, The Netherlands) with 1 L SMS supplemented with 0.15 g/L
211 Antifoam C (Sigma-Aldrich), which was autoclaved separately (120 °C for 20 min) (Verduyn et
212 al., 1992). The culture pH was maintained at 5.0 by automated addition of 2 M KOH. For aerobic
213 cultivation, 500 mL min⁻¹ compressed air was sparged in the reactor. To maintain anaerobic
214 conditions, the bioreactors were sparged with 500 mL N₂ min⁻¹ (<5 ppm O₂) (also the medium
215 vessels were sparged with N₂) and equipped with Norprene tubing to minimize oxygen diffusion.
216 After the batch phase, medium pumps were switched on, resulting in the continuous addition of
217 SMS containing 25 g/L sucrose to the cultures. The working volume was kept constant at 1.0 L
218 using an effluent pump controlled by an electric level sensor, resulting in a constant dilution
219 rate. The exact working volume and medium flow rate were measured at the end of each
220 experiment. Chemostat cultures were assumed to be in steady state when, after five volume
221 changes, the biomass concentration and the CO₂ production rate varied by less than 4.5 % over
222 at least another 2 volume changes.

223

224 **Laboratory evolution**

225 Repeated batch cultivation (SBRs) of strains *S. cerevisiae* IMZ636 and IMZ696 was initiated by
226 serial transfers in shake flasks (5 to 11 transfers). Shake flask cultures were grown in an
227 anaerobic chamber with 20 mL SMS in 30 mL shake flasks incubated at 30 °C and 200 rpm. After
228 this initial phase, evolution was continued in N₂-sparged reactors of 500 mL total volume (Infors
229 HT Multifors 2, Infors AG, Switzerland) with 100 mL SMS (50 mL min⁻¹ N₂ gas, 400 rpm, 30 °C).
230 The 100 mL working volume was possible due to manufacturer's special modifications on the
231 vessel and jacket size. For strain IMZ636, three evolution lines were carried out in parallel. For
232 IMZ696, two parallel evolution lines were performed. Culture pH was maintained at 5.0 by

233 automatic addition of 2 mol L⁻¹ KOH. Growth rate was estimated from each batch based on the
234 variation of CO₂ concentration in the off-gas. In yeast, CO₂ production is directly proportional to
235 cell concentration in the reactor since the production of this gas is linked to glycolysis activity.
236 Empty-refill cycles were programmed using the Iris 6 bioprocess software (Infors AG,
237 Switzerland). When the off-gas CO₂ concentration achieved 0.4 %, the empty-refilling sequence
238 was started. Such CO₂ concentration was lower than the maximum of 0.9 % that would be
239 produced at the end of the exponential growth phase (this value was measured before starting
240 the empty-refill cycles to better adjust evolution settings), which guarantees that a new cycle
241 was started before the cells entered stationary phase, which otherwise could delay the
242 evolutionary process. In each cycle, 90 % of the medium was substituted by fresh medium. For
243 evolution of strain IMZ696, the cultivation method was changed from SBRs to accelerostat
244 cultivation (Bracher et al., 2017), after the growth rate did not increase further with the SBR
245 strategy. Accelerostat cultivation, which was conducted in the same reactors used for the SBR
246 cultivations, were continuously fed with SMS containing 25 g/L sugar, while culture liquid was
247 removed to keep the working volume constant. The dilution rate, which was initially set at 0.09
248 h⁻¹, was automatically increased in response to the CO₂ concentration in the off-gas. This means
249 that the speed of the feed pump (thus the dilution rate) was increased or decreased if the off-gas
250 CO₂ profile showed consistent increase or decrease over a period of 24 h. Four single-colony
251 isolates from each evolution line were obtained by restreaking thrice on plates containing
252 selective SMS medium, which were incubated anaerobically at 30 °C. To prepare frozen stock
253 cultures of sucrose-evolved strains under selective conditions, cells from a single colony were
254 inoculated in 20 mL liquid SMS medium in 30 mL shake flasks, which were incubated under
255 anaerobic conditions (Bactron X anaerobic chamber, Shell Lab, Cornelius, OR, 200 rpm, 30 °C).
256 Based on the anaerobic growth profile of the four single colony isolates on SMS (e.g. shortest lag
257 phase and/or highest growth rate), one colony was selected and named as representative of the
258 correspondent evolution line. Stocks in SMD were also prepared, to be used in characterization
259 experiments, such as measurement of the growth rate on sucrose-based medium.

260 Analytical methods

261 Optical density of cultures at 660 nm was monitored using a Libra S11 spectrophotometer
262 (Biochrom, Cambridge, United Kingdom). Culture dry weight measurements and rapid quenching
263 of culture samples were carried out as described by Marques et al. (2018). HPLC analysis of the
264 supernatant and residual sugar determination were performed as described previously (de Kok
265 et al., 2011; Marques et al., 2017). Off-gas CO₂ concentrations were measured with an NGA 2000
266 Rosemount gas analyser (Emerson, St. Louis, MO).

267

268 Proton translocation assay

269 Cells used in proton translocation assays were harvested from aerobic sucrose-limited
270 chemostat cultures. The same culture conditions as described for anaerobic chemostats above
271 were used, with the following changes: reactors were sparged with 500 mL min⁻¹ air, the sucrose
272 concentration in the medium vessel was decreased to 7.5 g/L, the dilution rate was set at 0.03 h⁻¹
273 and Tween 80 and ergosterol were omitted from the medium. After 5 volume changes, when
274 CO₂ concentrations in the off-gas were stable, cells were harvested by centrifugation at 5 000 x *g*
275 for 5 min, at room temperature, washed with distilled water and resuspended in potassium
276 phthalate buffer (1.25 mM, pH 5) to a final concentration of 12 g dry weight L⁻¹. 5 mL of the cell
277 suspension were incubated at 30 °C in a magnetic stirred vessel with a S220 SevenCompact™
278 pH/Ion electrode attached (Mettler Toledo, Greifensee, Switzerland). After stabilization of the
279 pH signal, 100 µL of a 1 mol L⁻¹ sugar (sucrose, maltose, glucose or fructose) solution was added
280 (final concentration 20 mM) and changes in the pH were recorded using the LabX™ pH Software
281 (Mettler Toledo, Greifensee, Switzerland). Calibration was performed by addition of 5 µL
282 aliquots of 10 mM NaOH to the cell suspensions.

283

284 RESULTS

285

286 **Increased growth rates of *PvSUF1*-dependent yeast strains after laboratory evolution in a**
287 **sucrose-based medium**

288 In the engineered *S. cerevisiae* strain IMZ636 (*malΔ mphΔ suc2Δ imaΔ sga1Δ LmSPase PvSUF1*),
289 sucrose metabolism is strictly dependent on uptake via the plant transporter *PvSuf1* and
290 subsequent intracellular cleavage by a bacterial sucrose phosphorylase. In anaerobic shake flask
291 cultures, strain IMZ636 grew on SMS (synthetic medium with 20 g/L sucrose as sole carbon
292 source) at a specific growth rate of 0.05 h⁻¹ (**Table 3**). This growth rate is much lower than that
293 of the reference strain CEN.PK113-7D (0.29 h⁻¹, **Table 3**). To select for faster growth on sucrose,
294 strain IMZ636 was grown in anaerobic sequencing-batch bioreactors (SBR). In three
295 independent evolution lines, the specific growth rate increased from 0.05 h⁻¹ to 0.10-0.17 h⁻¹
296 (estimated from off-gas CO₂ profiles) after 56-80 cycles (approximately 150 generations, **Figure**
297 **2**). When no further increase in the growth rate was observed, four single colonies were isolated
298 from each experiment and characterized in anaerobic shake-flask cultures on SMS. Differences in
299 growth rate among the four colonies isolated from each evolution line indicate heterogeneity in
300 the evolved population (results not shown). The fastest isolate of each evolution line displayed
301 specific growth rates of 0.07 h⁻¹ (IMS644 from reactor "A"), 0.09 h⁻¹ (IMS646 from reactor "B")
302 and 0.08 h⁻¹ (IMS647 from reactor "C") (**Table 3**). These growth rates were consistently higher
303 than those of the unevolved IMZ636 strain, but differed slightly from those estimated from CO₂
304 profiles in the SBR experiments. These differences may have been caused by the different
305 methods used to access growth rate (optical density measurements versus on-line CO₂ data;
306 shake flasks versus bioreactors). Additionally, the shake-flask experiments were performed with
307 single cell lines while the specific growth rates estimated from the SBR experiments represented
308 growth of an evolving and probably heterogeneous population.

309 The anaerobic specific growth rates on sucrose of the evolved, IMZ636-derived strains were still
310 3-4 fold lower than that of the congeneric reference strain CEN.PK113-7D (**Table 3**). Marques et
311 al. (2018) showed that overexpression of phosphoglucomutase (*PGM2*) in a unevolved *PvSUF1*-
312 *SPase*-expressing strain increased its growth rate from 0.05 to 0.07 h⁻¹ (IMZ696). To investigate

313 whether in the laboratory evolution experiments, specific growth rates were limited by
314 phosphoglucomutase levels, a new sequential batch cultivation evolution was initiated with a
315 *PGM2*-expressing strain (IMZ696-*PvSUF1*, *SPase*, *PGM2*). In two independent evolution lines, the
316 specific growth rate on sucrose increased from 0.07 to 0.15-0.20 h⁻¹ (calculated from the off-gas
317 CO₂ concentration from each reactor) after 52 cycles (ca. 120 generations in reactor A and 190
318 generations in reactor B, **Figure 3A** and **3B**). Since no further increase in growth rate was
319 observed in this reactor's configuration, laboratory evolution was continued using accelerostat
320 cultivation, with an initial dilution rate of 0.09 h⁻¹. In accelerostats, the selective pressure is on
321 growth rate but also on substrate affinity (Bracher et al., 2017). The feed rate was automatically
322 increased or decreased based on on-line analysis of the CO₂ concentration in the off-gas. After
323 20-30 days (approximately 130 generations), the dilution rate in the two reactors had increased
324 to 0.25 h⁻¹ and 0.17 h⁻¹ (**Figures 3C** and **3D**), while the residual sucrose concentration had
325 decreased from 8 g/L to approximately 2.5 g/L after accelerostat selection (results not shown).
326 These dilution rates were close to the anaerobic specific growth rate on sucrose reported for a
327 *LmSPase*-dependent strain expressing the native *MAL11* sucrose symporter (0.23 h⁻¹, Marques et
328 al. 2018). Single colony isolates from each reactor (IMS648 from reactor shown in **Figure 3A**
329 and IMS649 from reactor shown in **Figure 3B**) exhibited specific growth rates of 0.19 h⁻¹ and
330 0.23 h⁻¹, respectively (**Table 3**). These specific growth rates were close to that of IMZ709
331 (*MAL11*, *SPase*, *PGM2*), and almost three times higher than that of the unevolved parental strain
332 IMZ696 (**Table 3**).

333

334 **Sucrose/H⁺ symport activity of evolved *PvSuf1* transporters as revealed by proton-uptake** 335 **studies and biomass yields on sucrose**

336 Although *PvSUF1* has been reported to encode a sucrose uniporter (Zhou et al., 2007), a recent
337 study (Marques et al. 2018) on the expression of *PvSUF1* in *S. cerevisiae* casts doubt on the actual
338 mechanism of sucrose transport: sucrose-dependent proton uptake rates of $8.2 \pm 2.2 \mu\text{mol H}^+$ (g
339 biomass)⁻¹ min⁻¹ were measured with cell suspensions of *S. cerevisiae* strain IMZ696 (*PvSUF1*,

340 *SPase, PGM2*) grown in aerobic, sucrose-limited chemostat cultures (**Figure 4**, data extracted
341 from Marques et al. 2018). This measured uptake rate would in principle be more than sufficient
342 to account for all sucrose uptake in the aerobic chemostat cultures. If we considered the residual
343 sucrose concentration in the bioreactor negligible, a specific sucrose uptake rate of 3.7 ± 0.1
344 $\mu\text{mol sucrose (g biomass)}^{-1} \text{ min}^{-1}$ would be calculated. In replicate chemostat experiments with
345 strain IMZ696, different mutations were found in *PvSUF1* (*PvSuf1*^{T302I} in one culture and
346 *PvSuf1*^{E308K V323F} in the other). Since the frozen stock cultures from which the chemostats were
347 inoculated did not contain mutations in *PvSUF1*, these mutations probably conferred a selective
348 advantage during sucrose-limited chemostat cultivation (Marques et al. 2018).

349 All evolved strains (IMS644, IMS646, IMS647, IMS648 and IMS649) obtained in the present
350 study displayed sucrose-induced H⁺ uptake (**Figure 4**). Strain IMS644 showed an initial H⁺
351 uptake rate that was close to that of the unevolved parental strain IMZ696 ($13 \pm 3.7 \mu\text{mol H}^+$
352 $\text{min}^{-1} (\text{g cell})^{-1}$ for IMS644 vs. $8 \pm 2.2 \mu\text{mol H}^+ \text{min}^{-1} (\text{g cell})^{-1}$ for IMZ696) (**Figure 4**), but all other
353 strains showed an at least three-fold higher H⁺ uptake rate compared to IMZ696 ($25\text{-}40 \mu\text{mol H}^+$
354 $\text{min}^{-1} (\text{g cell})^{-1}$ vs. $8 \pm 2.2 \mu\text{mol H}^+ \text{min}^{-1} (\text{g cell})^{-1}$ for IMZ696) (**Figure 4**). These high rates of
355 sucrose-dependent proton uptake approached those of a reference strain expressing the *S.*
356 *cerevisiae* Mal11 proton symporter ($52 \pm 8.7 \mu\text{mol H}^+ \text{min}^{-1} (\text{g cell})^{-1}$ for IMZ709, data from
357 Marques et al. (2018) (**Figure 4**). The evolved *PvSUF1*-expressing strains exhibited similar
358 proton uptake rates with maltose as with sucrose, while no proton uptake was observed upon
359 addition of fructose or glucose. Consistent with literature reports (Wieczorke et al., 1999) the
360 control strain IMZ709 (*MAL11, SPase*) exhibited glucose-dependent proton uptake (data from
361 Marques et al. 2018) (**Figure 4**).

362 An alternative way to investigate energy coupling of disaccharide uptake in *S. cerevisiae* is to
363 measure biomass yields in anaerobic, disaccharide-limited chemostat cultures (de Kok, 2012). If
364 sucrose uptake occurs via symport with a single proton, one ATP molecule has to be consumed
365 by the plasma membrane H⁺/ATPase Pma1 to expel the symported proton. On the contrary, if
366 sucrose uptake is passive, no ATP will be consumed. This difference of 1 ATP has a high impact

367 on the biomass yield on sugar under anaerobic conditions, which can be precisely determined in
 368 anaerobic chemostats (de Kok et al., 2011; Marques et al., 2018; Verduyn, Postma, Scheffers, &
 369 van Dijken, 1990).

370 The strains used in this study cleaved sucrose intracellularly via phosphorolysis. In such strains,
 371 expression of a sucrose/H⁺ symporter should result in a net generation of 4 mol ATP per mol
 372 sucrose under anaerobic conditions. If sucrose uptake occurred by uniport, this ATP yield would
 373 change to 5 ATP/sucrose, a 25% increase (Marques et al., 2018). In sucrose-limited, anaerobic
 374 chemostat cultures, a 25% increase of the ATP yield from sucrose dissimilation should result in a
 375 25% increase in the biomass yield on sucrose (Verduyn et al., 1990). Two of the evolved strains
 376 (IMS646 from the evolution started with IMZ636 and IMS649 from the evolution started with
 377 IMZ696) were characterized in chemostat cultures and their biomass yields were compared to
 378 those displayed by reference strains (IMZ665 and IMZ709), which both expressed *MAL11*
 379 instead of *PvSUF1*. No differences in biomass yield were observed between a *MAL11* expressing
 380 strains and strains evolved with *PvSUF1*: the observed biomass yield of strain IMZ665 (*MAL11*,
 381 *SPase*) was 0.086 ± 0.002 g (g glucose equivalent)⁻¹ while that of IMS646 (*PvSUF1*, *SPase*) was
 382 0.082 ± 0.004 g (g glucose equivalent)⁻¹ (**Table 4** and **Table S2**). Similarly, for the strains
 383 expressing *PGM2* (IMZ709 and IMS649) the biomass yield was not higher in cultures of the
 384 evolved *PvSUF1*-expressing strain (0.087 ± 0.000 g (g glucose equivalent)⁻¹ for IMS649) than in
 385 cultures of the *MAL11*-expressing strain (0.091 ± 0.006 g (g glucose equivalent)⁻¹ for IMZ709)
 386 (**Table 4** and **Table S2**).

387

388 **Evolved strains contain mutations in *PvSUF1***

389 Non-conservative single-nucleotide mutations were detected in the *PvSUF1* open reading frames
 390 of all *PvSuf1*-dependent strains evolved for faster growth on sucrose (IMS644, IMS646, IMS647,
 391 IMS648 and IMS649; **Figure 5**). No mutations were found in the promoter (*TEF1*) or in the
 392 terminator (*CYC1*) regions of the *PvSUF1* expression cassettes. Strains IMS644 and IMS647,
 393 which were independently evolved from strain IMZ636 (*PvSUF1,SPase*), contained the same

394 mutation (*PvSUF1*^{YAAGSFSG-duplication}): a tandem duplication of 8 amino acids that, based on amino-
 395 acid hydrophobicity plots (*Protter* algorithm, (Omasits, Ahrens, Mu, & Wollscheid, 2014)), was
 396 predicted to be localized partially in the extracellular surface of trans-membrane domain 5
 397 (TM5) and in the loop connecting TM5 to TM6 (loop 5/6) (**Figure 5** and **Figure 6**). Strain
 398 IMS646, which was also evolved from IMZ636 (*PvSUF1*, *SPase*), contained a mutation that
 399 resulted in a substitution of glycine 326 (TM8) for a cysteine (G326C) (**Figure 5**). Evolved strain
 400 IMS649, derived from the IMZ696 strain (*PvSUF1*, *SPase*, *PGM2*), carried a combination of the
 401 abovementioned *PvSUF1*^{G326C} mutation and two additional mutations. One of these caused an
 402 I209F substitution in the loop connecting TM5 to 6 (loop 5/6) at the extracellular surface while
 403 the other led to a C265F substitution, positioned in loop 6/7 on the cytosolic side (**Figure 5** and
 404 **Figure 6**). The *PvSUF1* allele of the remaining strain, which was evolved from IMZ696 (*PvSUF1*,
 405 *SPase*, *PGM2*), IMS648, contained three mutations, leading to Y128C, C228G, and G457D
 406 substitutions (**Figure 5**). In this strain, the *PvSUF1*- and *PGM2*-expression cassettes were no
 407 longer located on the original 2 μ -expression vector. Instead, both cassettes were found to be
 408 integrated into chromosomal DNA. Accordingly, strain IMS655, which was obtained by curing
 409 the 2 μ -plasmid from strain IMS648, retained its ability to grow on SMS (**Figure S1**). The other
 410 four evolved strains did not grow on sucrose after plasmid removal (IMS652, IMS653, IMS654
 411 and IMS656). No mutations were found in the *PGM2* or *SPase* expression cassettes of any of the
 412 evolved strains.

413

414 **Independently evolved strains in a sucrose-based medium show common whole-** 415 **chromosome and segmental aneuploidies**

416 Besides the mutations found in *PvSUF1*, the evolved strains also showed duplication of
 417 chromosomes and/or chromosomal segments. Whole-genome sequencing revealed duplication
 418 of the right arm of chromosome 14 (after position ~500,000) in three independently evolved
 419 strains: IMS644, IMS646 and IMS649 (**Table 5**, **Figure S2**). Similarly, a segmental duplication of
 420 chromosome 16 (right arm, after position ~800,000) was found in strains IMS646 and IMS649.

421 A central region of chromosome 13 (position ~300,000 to ~350,000) was triplicated in strains
422 IMS644 and IMS647. Strain IMS644 showed a complete duplication of chromosome 1, as well as
423 of the right arm of chromosome 2 (after position ~600,000; **Table 5, Figure S2**). To investigate
424 whether these mutations contribute to the phenotype, reverse engineering of the mutated
425 *PvSUF1* alleles was carried out both in an unevolved and in an evolved strain background, as
426 detailed in the next section.

427

428 **Reverse engineering of evolved *PvSUF1* alleles enables fast growth on sucrose**

429 To investigate whether the faster growth on sucrose observed after evolution of *PvSUF1*-
430 expressing strains could be exclusively attributed to the mutations found in *PvSUF1*, *PvSUF1*
431 expression cassettes (*TEF1p-PvSUF1-CYC1t*) were first isolated from each of the evolved strains
432 (IMS646, IMS647 and IMS649) and cloned into a 2- μ m plasmid. Two versions were constructed,
433 one carrying an additional *PGM2*-expressing cassette and another one without it. The resulting
434 plasmids were transformed into an unevolved background strain, IMZ630 (*mal Δ mph Δ suc2 Δ*
435 *ima Δ SPase*), resulting in strains IMZ712-715 and IMZ729 (**Table 6**). Strain IMZ712 (expressing
436 *PvSUF1*^{G326C}) grew anaerobically on sucrose at 0.05 h⁻¹ (**Table 6**), which corresponds to the
437 specific growth rate displayed by the unevolved parental strain (IMZ636, **Table 3**). Co-
438 expression of *PGM2* did not lead to a higher growth rate (IMZ714, $\mu = 0.05$ h⁻¹, **Table 6**).
439 Overexpression of a *PvSUF1*^{YAAGSFSG-duplication} variant that encoded the 8 amino-acid duplication
440 described above (from strain IMS647) in the unevolved background also, by itself, did not result
441 in an increased specific growth rate (IMZ713 $\mu = 0.05$ h⁻¹) (**Table 6**). However, when this
442 mutation was combined with the overexpression of *PGM2* (IMZ715), a specific growth rate of
443 0.10 h⁻¹ was observed (**Table 6**). The *PvSUF1* allele derived from strain IMS649, the evolved
444 strain that grew at 0.20 h⁻¹ (**Table 3**), supported high specific growth rates upon introduction in
445 a unevolved background when combined with *PGM2* overexpression (strain IMZ729, $\mu = 0.17 \pm$
446 0.02 h⁻¹) (**Table 6**).

447 To investigate the possible impact of mutations outside *PvSUF1*, the *PvSUF1* expression
448 plasmid was cured from the fastest growing evolved strain IMS649. The resulting strain
449 (IMS656) was then directly transformed with the vectors extracted from the evolved strains
450 carrying *PvSUF1* variants (*PvSUF1*^{G326C}, *PvSUF1*^{YAAGSFSG-duplication} and *PvSUF1*^{I209F C265F G326C}) yielding
451 strains IMZ724, IMZ725 and IMZ727, respectively. The growth rates of these strains (0.07 h⁻¹,
452 0.06 h⁻¹ and 0.17 h⁻¹, respectively) were similar to that of the strains expressing evolved *PvSUF1*
453 in an unevolved background (**Table 6**). This result shows that the improved growth of strain
454 IMS649 was predominantly due to mutations in *PvSUF1* and that other chromosomal alterations
455 had at most a small impact on the strain's specific growth rate on sucrose (**Table 6**).

456

457 DISCUSSION

458 Optimal expression of heterologous transporters in yeast can be hampered by protein
459 misfolding, incorrect sorting, cell toxicity due to protein accumulation in intracellular
460 compartments (unfolded protein response - UPR), rapid endocytosis and turnover, among other
461 phenomena (Bassham & Raikhel, 2000; Froissard et al., 2006; Hernández, 2005; Nielsen, 2013).
462 In this study, genetic factors involved in the efficient expression of a putative plant sucrose
463 uniporter (*PvSuf1*) in *S. cerevisiae* were identified by a combination of laboratory evolution,
464 whole genome sequencing, reverse engineering of mutations observed in evolved strains and
465 physiological analysis of evolved and reverse engineered strains.

466 Laboratory evolution of yeast dependent on sucrose uptake via *PvSuf1* resulted in faster
467 growing strains – when compared to the parental ones – which contained mutations in the
468 *PvSUF1* allele (**Figure 5**). Besides faster growth, the mutations may also be responsible for
469 lowering the residual sucrose concentration in the accelerostat reactor (from 8 to 2.5 g/L, see
470 Results section). Chromosomal duplications were also found in the genome of the evolved
471 strains (**Table 5, Figure S2**) affecting genes that encode proteins involved in transporter
472 sorting, ubiquitination and degradation: *COS10*, *SEC12* and *SIS1*, which are present on
473 chromosome 14 (Luke, Sutton, & Arndt, 1991; Macdonald et al., 2015; Nakano, Brada, &

474 Schekman, 1988); *CUR1* and *SEC23* from chromosome 16 (Alberti, 2012) and *UBC7* found on
475 chromosome 13 (Hiller, Finger, Schweiger, & Wolf, 1996). However, these genomic alterations
476 are unlikely to be the main cause of the improved specific growth rate of the evolved strains
477 since reverse engineering of a mutated version of *PvSUF1* in unevolved *S. cerevisiae* sufficed to
478 generate a strain that grew as fast as the fastest-growing evolved strain (**Table 6**). Among the
479 mutations found in *PvSUF1*, those involving cysteine residues predominated (*PvSUF1*^{G326C},
480 *PvSUF1*^{G457D Y128C C228G}, *PvSUF1*^{I209F C265F G326C}; **Figure 5**). Correct formation of disulfide bonds has
481 previously been reported to be important for inter- and intramolecular interactions of SUT
482 (sucrose transporter) proteins and sucrose uptake activity (Krügel et al., 2008, 2012). *PvSuf1*
483 contains only 3 out of the 4 cysteine residues conserved in all other plant sucrose transporters
484 (**Figure 5**) (Lemoine, 2000). Since the *PvSUF1*^{G326C} mutation was found in two independently
485 evolved strains (IMS649 and IMS646), presence of a cysteine in position 326 of *PvSuf1* may be
486 important for protein folding and oligomerization in yeast, but further research is clearly needed
487 to test this hypothesis.

488 Sugar-proton symport assays showed that all *PvSUF1*-expressing *S. cerevisiae* strains tested
489 (including those that were not subjected to laboratory evolution) displayed at least some
490 sucrose/H⁺ symport activity, which was high enough to explain their observed rates of sucrose
491 uptake (**Figure 4**). While, based on heterologous expression, we cannot draw definitive
492 conclusions on energy coupling of wild type *PvSuf1*-mediated sucrose transport, our results
493 warrant reinvestigation of *PvSuf1*'s mechanism in a plant context.

494 The present study shows that laboratory evolution is a robust methodology to improve the
495 expression of sucrose transporters in yeast and that mutations on the transporter itself are
496 sufficient for its efficient expression meaning that alterations in the host genome are not
497 necessary. Four out of six observed amino acid substitutions encoded by evolved *PvSUF1* alleles
498 removed or introduced a cysteine residue, this demonstrates that engineering cysteine residues
499 might be a rational way to improve the expression of plant sugar transporters in yeast. Study of
500 the mutations identified in this work in different transporters, preferably combined with

501 structural information, will identify underlying mechanisms and increase the predictability of
502 functional expression of heterologous transporters in yeast.

503

504

505

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514

515 **Conflict of interest.** None declared

516

517 **REFERENCES**

518 Alberti, S. (2012). Molecular mechanisms of spatial protein quality control. *Prion*, 6(5), 437–442.
519 <http://doi.org/10.4161/pri.22470>

520 Badotti, F., Dário, M. G., Alves, S. L., Cordioli, M. L. a, Miletti, L. C., de Araujo, P. S., & Stambuk, B. U.
521 (2008). Switching the mode of sucrose utilization by *Saccharomyces cerevisiae*. *Microbial*
522 *Cell Factories*, 7, 4. <http://doi.org/10.1186/1475-2859-7-4>

523 Bassham, D. C., & Raikhel, N. V. (2000). Plant cells are not just green yeast. *Plant Physiology*,
524 122(April), 999–1001.

525 Boeke, J. D., La Croute, F., & Fink, G. R. (1984). A positive selection for mutants lacking orotidine-
526 5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Molecular and*
527 *General Genetics MGG*, 197(2), 345–346. <http://doi.org/10.1007/BF00330984>

528 Bracher, J. M., de Hulster, E., Koster, C. C., van den Broek, M., Daran, J.-M. G., van Maris, A. J. A., &
529 Pronk, J. T. (2017). Laboratory evolution of a biotin-requiring *Saccharomyces cerevisiae*
530 strain for full biotin prototrophy and identification of causal mutations. *Applied and*
531 *Environmental Microbiology*, 83(16), e00892–17. <http://doi.org/10.1128/AEM.00892-17>

- 532 Chen, L.-Q. (2014). SWEET sugar transporters for phloem transport and pathogen nutrition. *New*
533 *Phytologist*, 201(4), 1150–1155. <http://doi.org/10.1111/nph.12445>
- 534 De Kok, S. (2012). *Metabolic engineering of free-energy (ATP) conserving reactions in*
535 *Saccharomyces cerevisiae*. Delft University of Technology.
- 536 De Kok, S., Kozak, B. U., Pronk, J. T., & van Maris, A. J. a. (2012). Energy coupling in *Saccharomyces*
537 *cerevisiae*: selected opportunities for metabolic engineering. *FEMS Yeast Research*, 12, 387–
538 397. <http://doi.org/10.1111/j.1567-1364.2012.00799.x>
- 539 De Kok, S., Yilmaz, D., Suir, E., Pronk, J. T., Daran, J.-M., & van Maris, A. J. A. (2011). Increasing
540 free-energy (ATP) conservation in maltose-grown *Saccharomyces cerevisiae* by expression
541 of a heterologous maltose phosphorylase. *Metabolic Engineering*, 13(5), 518–26.
542 <http://doi.org/10.1016/j.ymben.2011.06.001>
- 543 Doidy, J., Grace, E., Kühn, C., Simon-Plas, F., Casieri, L., Wipf, D., ... Wipf, D. (2012). Sugar
544 transporters in plants and in their interactions with fungi. *Trends in Plant Science*, 17(7),
545 413–422. <http://doi.org/https://doi.org/10.1016/j.tplants.2012.03.009>
- 546
- 547 Entian, K.-D. D., & Kötter, P. (2007). 25 yeast genetic strain and plasmid collections. In *Methods in*
548 *Microbiology* (Vol. 36, pp. 629–666). [http://doi.org/10.1016/S0580-9517\(06\)36025-4](http://doi.org/10.1016/S0580-9517(06)36025-4)
- 549 Froissard, M., Belgareh-Touzé, N., Buisson, N., Desimone, M., Frommer, W. B., & Haguenaue-
550 Tsapis, R. (2006). Heterologous expression of a plant uracil transporter in yeast:
551 improvement of plasma membrane targeting in mutants of the Rsp5p ubiquitin protein
552 ligase. *Biotechnology Journal*, 1(3), 308–320. <http://doi.org/10.1002/biot.200500034>
- 553 Gietz, B. R. D., & Woods, R. A. (2002). Transformation of yeast by lithium acetate/single-stranded
554 carrier DNA/polyethylene glycol method. *Methods in Enzymology*, 350(2001), 87–96.
- 555 Goldberg, R. N., Tewari, Y. B., & Ahluwalia, J. C. (1989). Thermodynamics of the hydrolysis of
556 sucrose. *The Journal of Biological Chemistry*, 264(17), 9901–4. Retrieved from
557 <http://www.ncbi.nlm.nih.gov/pubmed/2722882>
- 558 He, Y., Wang, K., & Yan, N. (2014). The recombinant expression systems for structure
559 determination of eukaryotic membrane proteins, 5(9), 658–672.
560 <http://doi.org/10.1007/s13238-014-0086-4>
- 561 Hernández, D. H. (2005). The yeast *Saccharomyces cerevisiae* is not an efficient tool for in vivo
562 studies of plant vacuolar sorting receptors. *The Plant Cell*, 17(May), 1339–1342.
563 <http://doi.org/https://doi.org/10.1105/tpc.105.032276>
- 564 Hiller, M. M., Finger, A., Schweiger, M., & Wolf, D. H. (1996). ER degradation of a misfolded
565 luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science*, 273(5282), 1725
566 LP – 1728. <http://doi.org/10.1126/science.273.5282.1725>
- 567 Kim, H., Lee, W., Galazka, J. M., Cate, J. H. D., & Jin, Y. (2014). Analysis of cellodextrin transporters
568 from *Neurospora crassa* in *Saccharomyces cerevisiae* for cellobiose fermentation, 1087–
569 1094. <http://doi.org/10.1007/s00253-013-5339-2>

- 570 Krügel, U., He, H. X., Gier, K., Reins, J., Chincinska, I., Grimm, B., ... Kühn, C. (2012). The potato
571 sucrose transporter StSUT1 interacts with a DRM-associated protein disulfide isomerase.
572 *Molecular Plant*, 5(1), 43–62. <http://doi.org/10.1093/mp/ssr048>
- 573 Krügel, U., Veenhoff, L. M., Langbein, J., Wiederhold, E., Liesche, J., Friedrich, T., ... Kühn, C.
574 (2008). Transport and sorting of the solanum tuberosum sucrose transporter SUT1 is
575 affected by posttranslational modification. *The Plant Cell*, 20(9), 2497–513.
576 <http://doi.org/10.1105/tpc.108.058271>
- 577 Kuijpers, N. G. a, Solis-Escalante, D., Bosman, L., van den Broek, M., Pronk, J. T., Daran, J.-M., &
578 Daran-Lapujade, P. (2013). A versatile, efficient strategy for assembly of multi-fragment
579 expression vectors in *Saccharomyces cerevisiae* using 60 bp synthetic recombination
580 sequences. *Microbial Cell Factories*, 12, 47. <http://doi.org/10.1186/1475-2859-12-47>
- 581 Lemoine, R. (2000). Sucrose transporters in plants: update on function and structure. *Biochimica
582 et Biophysica Acta - Biomembranes*, 1465(1-2), 246–262. [http://doi.org/10.1016/S0005-
583 2736\(00\)00142-5](http://doi.org/10.1016/S0005-2736(00)00142-5)
- 584 Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler
585 transform. *Bioinformatics*, 25(14), 1754–1760.
586 <http://doi.org/10.1093/bioinformatics/btp324>
- 587 Lin, I. W., Sosso, D., Chen, L.-Q., Gase, K., Kim, S.-G., Kessler, D., ... Frommer, W. B. (2014). Nectar
588 secretion requires sucrose phosphate synthases and the sugar transporter SWEET9.
589 *Nature*, 508(7497), 546–9. <http://doi.org/10.1038/nature13082>
- 590 Luke, M. M., Sutton, A., & Arndt, K. T. (1991). Characterization of Sis1, a *Saccharomyces
591 cerevisiae* homologue of bacterial dnaJ proteins. *The Journal of Cell Biology*, 114(4), 623 LP
592 – 638. <http://doi.org/10.1083/jcb.114.4.623>
- 593 Macdonald, C., Payne, J. A., Katzmann, D. J., Piper, R. C., Macdonald, C., Payne, J. A., ... Piper, R. C.
594 (2015). A family of tetraspans organizes cargo for sorting into multivesicular bodies.
595 *Developmental Cell*, 33(3), 328–342. <http://doi.org/10.1016/j.devcel.2015.03.007>
- 596 Maiorella, B. L., Blanch, H. W., & Wilke, C. R. (1984). Economic evaluation of alternative ethanol
597 fermentation processes. *Biotechnology and Bioengineering*, 26(9), 1003–25.
598 <http://doi.org/10.1002/bit.260260902>
- 599 Mans, R. (2017) Strategies and genetic tools for engineering free-energy conservation in yeast.
600 Doctoral thesis, Delft University of Technology.
601 [https://www.narcis.nl/publication/RecordID/oai:tudelft.nl:uuid%3Ad99fcde3-4b01-
602 4077-9a1f-a8b6eda6a5e7](https://www.narcis.nl/publication/RecordID/oai:tudelft.nl:uuid%3Ad99fcde3-4b01-4077-9a1f-a8b6eda6a5e7)
- 603 Marques, W. L., Mans, R., Marella, E. R., Cordeiro, R. L., Broek, M. Van den, Daran, J.-M. G., ... Maris,
604 A. J. A. van. (2017). Elimination of sucrose transport and hydrolysis in *Saccharomyces
605 cerevisiae*: a platform strain for engineering sucrose metabolism. *FEMS Yeast Research*,
606 (November 2016), 1–11. <http://doi.org/10.1093/femsyr/fox006>
- 607 Marques, W. L., Raghavendran, V., Stambuk, B. U., & Gombert, A. K. (2016). Sucrose and
608 *Saccharomyces cerevisiae*: a relationship most sweet. *FEMS Yeast Research*, 16(October
609 2016), 1–16. <http://doi.org/10.1093/femsyr/fov107>

- 610 Marques, W. L., Robert, M., Pronk, J. T., van Maris, A. J. A., Mans, R., Henderson, R. K., ... Maris, A. J.
611 A. Van. (2018). Combined engineering of disaccharide transport and phosphorolysis for
612 enhanced ATP yield from sucrose fermentation in *Saccharomyces cerevisiae*. *Metabolic*
613 *Engineering*, 45(November 2017), 121–133. <http://doi.org/10.1016/j.ymben.2017.11.012>
- 614 Nakano, A., Brada, D., & Schekman, R. (1988). A membrane glycoprotein, Sec12p, required for
615 protein transport from the endoplasmic reticulum to the golgi apparatus in yeast. *The*
616 *Journal of Cell Biology*, 107(3), 851–863. Retrieved from
617 <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2115266/>
- 618 Nielsen, J. (2013). Production of biopharmaceutical proteins by yeast: advances through
619 metabolic engineering. *Bioengineered*, 4(4), 207–11. <http://doi.org/10.4161/bioe.22856>
- 620 Nijkamp, J. F., van den Broek, M., Datema, E., de Kok, S., Bosman, L., Luttkik, M. a, ... Daran, J.-M.
621 (2012). De novo sequencing, assembly and analysis of the genome of the laboratory strain
622 *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology.
623 *Microbial Cell Factories*, 11(1), 36. <http://doi.org/10.1186/1475-2859-11-36>
- 624 Omasits, U., Ahrens, C. H., Mu, S., & Wollscheid, B. (2014). Sequence analysis Protter: interactive
625 protein feature visualization and integration with experimental proteomic data.
626 *Bioinformatics (Oxford, England)*, 30(6), 884–886.
627 <http://doi.org/10.1093/bioinformatics/btt607>
- 628 Peters, S., Rose, T., & Moser, M. (2010). Sucrose: a prospering and sustainable organic raw
629 material. *Carbohydrates in Sustainable Development I*, 1–11. <http://doi.org/10.1007/128>
- 630 Salazar, A. N., Vries, A. R. G. De, Broek, M. Van Den, Wijsman, M., De al Torre Cortés, P.,
631 Brickwedde, A., ... Abeel, T. (2017). Nanopore sequencing enables near-complete de novo
632 assembly of *Saccharomyces cerevisiae* reference strain. *FEMS Yeast Research*,
633 17(September), 1–11. <http://doi.org/10.1093/femsyr/fox074>
- 634 Salerno, G. L., & Curatti, L. (2003). Origin of sucrose metabolism in higher plants: when, how and
635 why? *Trends in Plant Science*, 8(2), 63–9. [http://doi.org/10.1016/S1360-1385\(02\)00029-8](http://doi.org/10.1016/S1360-1385(02)00029-8)
- 636 Stambuk, B. U., Silva, M. A., Panek, A. D., & Araujo, P. S. De. (1999). Active α -glucoside transport in
637 *Saccharomyces cerevisiae*, 170, 73–78.
- 638 Ton, V., & Rao, R. (2004). Functional expression of heterologous proteins in yeast: insights into
639 Ca^{2+} signaling and Ca^{2+} -transporting ATPases. *Cell Physiology*, 287(32), 580–589.
640 <http://doi.org/10.1152/ajpcell.00135.2004>
- 641 Van Leeuwen, C. C., Weusthuis, R. A., Postma, E., Van den Broek, P. J., & Van Dijken, J. P. (1992).
642 Maltose/proton co-transport in *Saccharomyces cerevisiae*. Comparative study with cells and
643 plasma membrane vesicles. *Biochemical Journal*, 284(Pt 2), 441–445. Retrieved from
644 <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1132658/>
- 645 Verduyn, C., Postma, E., Scheffers, W. A., & van Dijken, J. P. (1990). Physiology of *Saccharomyces*
646 *cerevisiae* in anaerobic glucose-limited chemostat cultures. *Microbiology*, 136(3), 395–403.
647 <http://doi.org/10.1099/00221287-136-3-395>
- 648 Verduyn, C., Postma, E., Scheffers, W. A., & van Dijken, J. P. (1992). Effect of benzoic acid on
649 metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and

- 650 alcoholic fermentation. *Yeast (Chichester, England)*, 8(7), 501–17.
651 <http://doi.org/10.1002/yea.320080703>
- 652 Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., ... Earl, A. M. (2014).
653 Pilon: An Integrated Tool for Comprehensive Microbial Variant Detection and Genome
654 Assembly Improvement. *PLoS ONE*, 9(11), e112963.
655 <http://doi.org/10.1371/journal.pone.0112963>
- 656 Weimberg, R., & Doudoroff, M. (1954). Studies with three bacterial sucrose phosphorylases.
657 *Journal of Bacteriology*, 68(3), 381–388.
- 658 Weusthuis, R. a, Lamot, I., van der Oost, J., & Sanders, J. P. M. (2011). Microbial production of bulk
659 chemicals: development of anaerobic processes. *Trends in Biotechnology*, 29(4), 153–8.
660 <http://doi.org/10.1016/j.tibtech.2010.12.007>
- 661 Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C. P., & Boles, E. (1999).
662 Concurrent knock-out of at least 20 transporter genes is required to block uptake of
663 hexoses in *Saccharomyces cerevisiae*. *FEBS Letters*, 464(3), 123–128.
664 [http://doi.org/10.1016/S0014-5793\(99\)01698-1](http://doi.org/10.1016/S0014-5793(99)01698-1)
- 665 Zhou, Y., Grof, C. P., & Patrick, J. W. (2014). Proof of concept for a novel functional screening
666 system for plant sucrose effluxers. *Journal of Biological Methods*, 1(2), 1–6.
667 <http://doi.org/10.14440/jbm.2014.27>
- 668 Zhou, Y., Qu, H., Dibley, K. E., Offler, C. E., & Patrick, J. W. (2007). A suite of sucrose transporters
669 expressed in coats of developing legume seeds includes novel pH-independent facilitators.
670 *The Plant Journal: For Cell and Molecular Biology*, 49(4), 750–64.
671 <http://doi.org/10.1111/j.1365-313X.2006.03000.x>
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- 673

674 **Table 1: *S. cerevisiae* strains used in this study.**

Strain	Relevant genotype	Parental strain	Source
CEN.PK113-7D	MATa <i>URA3 LEU2 MAL2-8^c SUC2</i>		Entian and Kötter, 2007
IMZ630	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase</i>		Marques et al 2018
IMZ730	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE690 (URA3 PGM2)</i>	IMZ630	This study
IMZ636	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE413 (URA3 PvSUF1)</i>	IMZ630	Marques et al. 2018
IMS644	Single colony isolate from evolution line "A"	IMZ636	This study
IMS646	Single colony isolate from evolution line "B"	IMZ636	This study
IMS647	Single colony isolate from evolution line "C"	IMZ636	This study
IMZ696	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE486 (URA3 PvSUF1 PGM2)</i>	IMZ630	Marques et al. 2018
IMS648	Single colony isolate from evolution line "A"	IMZ696	This study
IMS649	Single colony isolate from evolution line "B"	IMZ696	This study
IMS652	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase Plasmid cured</i>	IMS644	This study
IMS653	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase Plasmid cured</i>	IMS646	This study
IMS654	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase Plasmid cured</i>	IMS647	This study
IMS655	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase PvSUF1::PGM2* Plasmid cured</i>	IMS648	This study
IMS656	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase Plasmid cured</i>	IMS649	This study
IMZ712	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE565 (URA3 PvSUF1^{G326C})</i>	IMZ630	This study
IMZ713	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE566 (URA3 PvSUF1^{YAAGSFSG-duplication})</i>	IMZ630	This study
IMZ714	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE567 (URA3 PvSUF1^{G326C::PGM2})</i>	IMZ630	This study
IMZ715	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE568 (URA3 PvSUF1^{YAAGSFSG-duplication::PGM2})</i>	IMZ630	This study
IMZ729	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE691 (URA3 PvSUF1^{I209F-C265F-G326C} PGM2)</i>	IMZ630	This study
IMZ724	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE545 (URA3 PvSUF1^{G326C})</i>	IMS656	This study
IMZ725	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE546 (URA3 PvSUF1^{YAAGSFSG-duplication})</i>	IMS656	This study
IMZ727	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE560 (URA3 PvSUF1^{I209F-C265F-G326C} PGM2)</i>	IMS656	This study
IMZ665	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE432 (URA3 MAL11)</i>		Marques et al 2018
IMZ709	MATa <i>ura3-52 LEU2 MAL2-8^c malΔ mphΔ suc2Δ ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ sga1Δ::LmSPase pUDE496 (URA3 MAL11 PGM2)</i>		Marques et al 2018

675 *"*PvSUF1::PGM2*" expression cassette migrated from the plasmid to a chromosome. The exact site of integration was not
676 investigated in this work.
677
678

679 **Table 2: Plasmids used in this study**

Name	Relevant characteristics	Origin
pUDE413	<i>2μ URA3 pTEF1-PvSUF1-tCYC1</i>	Marques et al. 2018
pUDE486	<i>2μ URA3 pTEF1-PvSUF1-tCYC1 pTPI1-PGM2-tTEF1</i>	Marques et al. 2018
pUDE690	<i>2μ URA3 pTPI1-PGM2-tTEF1</i>	This study
pUDE544	Evolved plasmid from IMS644	This study
pUDE545	Evolved plasmid from IMS646	This study
pUDE546	Evolved plasmid from IMS647	This study
pUDE559	Evolved plasmid from IMS648	This study
pUDE560	Evolved plasmid from IMS649	This study
pUDE565	<i>2μ URA3 pTPI1-PvSUF1^{G326C}-tCYC1</i>	This study
pUDE566	<i>2μ URA3 pTPI1-PvSUF1^{YAAGSFSG-duplication}-tCYC1</i>	This study
pUDE567	<i>2μ URA3 pTPI1-PvSUF1^{G326C}-tCYC1 pTPI1-PGM2-tTEF1</i>	This study
pUDE568	<i>2μ URA3 pTPI1-PvSUF1^{YAAGSFSG-duplication}-tCYC1 pTPI1-PGM2-tTEF1t</i>	This study
pUDE691	<i>2μ URA3 pTPI1-PvSUF1^{I217F, C265F, G326C}-tCYC1 pTPI1-PGM2-tTEF1</i>	This study

680

681

682 **Table 3:** Specific growth rates of unevolved and evolved *S. cerevisiae* strains grown in shake
 683 flask cultures containing 20 mL SMS (initial pH 6, 30 °C, 200 rpm) in an anaerobic chamber.
 684 Averages and mean deviations were obtained from duplicate experiments. *SPase* was integrated
 685 in the genome (*SGA1* locus), while *PvSUF1* was expressed in a 2 μ -plasmid with or without *PGM2*.

Strain	Relevant characteristics	Specific growth rate (h ⁻¹)
CEN.PK113-7D	<i>SUC2</i>	0.29 ± 0.00
IMZ636	<i>SPase, PvSUF1</i>	0.05 ± 0.01
IMS644	<i>SPase, PvSUF1</i> , evolved "A"	0.07 ± 0.00
IMS646	<i>SPase, PvSUF1</i> , evolved "B"	0.09 ± 0.01
IMS647	<i>SPase, PvSUF1</i> , evolved "C"	0.08 ± 0.01
IMZ696	<i>SPase, PvSUF1-PGM2</i> , parental strain	0.07 ± 0.01
IMS648	<i>SPase, PvSUF1*-PGM2</i> , evolved "A"	0.18 ± 0.01
IMS649	<i>SPase, PvSUF1-PGM2</i> , evolved "B"	0.19 ± 0.01

686 * *PvSUF1* was found in the genome of this strain, not in the plasmid as in the parental IMZ696.

687

688 **Table 4:** Biomass yields on sucrose and rates of sucrose uptake by *S. cerevisiae* strains grown in
 689 anaerobic sucrose-limited chemostat cultures (pH 5, 30 °C, 800 rpm, 500 mL N₂/min). A dilution
 690 rate of 0.07 h⁻¹ was used for strains IMZ665 (*MAL11*, *SPase*) and IMS646 (*PvSUF1*, *SPase*), while
 691 0.15 h⁻¹ was used for strains IMZ709 (*MAL11*, *SPase*, *PGM2*) and IMS649 (*PvSUF1*, *SPase*, *PGM2*).
 692 Cultures were grown on SMS with 25 g/L sucrose in the feeding medium. Averages and mean
 693 deviations were obtained from duplicate experiments. Additional physiological data are
 694 provided in **Table S2**.

Strain	IMZ665 (control)	IMS646 (evolved)	IMZ709 (control)	IMS649 (evolved)
Relevant genotype	<i>MAL11</i> <i>LmSPase</i>	<i>PvSUF1</i> <i>LmSPase</i>	<i>MAL11</i> <i>LmSPase</i> <i>PGM2</i>	<i>PvSUF1</i> <i>LmSPase</i> <i>PGM2</i>
Actual dilution rate (h ⁻¹)	0.070 ± 0.000	0.071 ± 0.001	0.147 ± 0.001	0.152 ± 0.001
Biomass yield (g g glucose equivalent ⁻¹)	0.086 ± 0.002	0.082 ± 0.004	0.091 ± 0.006	0.087 ± 0.000
q _{sucrose} (mmol/g biomass/h)	-2.26 ± 0.06	-2.40 ± 0.06	-4.5 ± 0.3	-4.83 ± 0.04
Residual sucrose (g/L)	0.08 ± 0.02	1.90 ± 1.18	2.03 ± 0.15	4.33 ± 0.80
Carbon recovery (%)	101 ± 1	95 ± 4	105 ± 7	102 ± 1

695

696

697 **Table 5:** Summary of whole-chromosome and segmental aneuploidies found in *PvSUF1*-
 698 expressing strains evolved on sucrose-based medium relative to the unevolved reference strain
 699 IMZ630. "Chr": chromosome. "+": presence of copy number variation. Chromosome positions are
 700 shown between brackets.

Strain	Chr1 - Entirely duplicated	Chr2 - Duplication of right arm (600,000 - telomere)	Chr13 - Triplication of short region (300,000 - 350,000)	Chr14 - Duplication of right arm (500,000 - telomere)	Chr16 - Duplication of right end (800,000 - telomere)
IMS644	+	+	+	+	
IMS646				+	+
IMS647			+		
IMS648					
IMS649				+	+

701

702

703 **Table 6:** Specific growth rates on sucrose in anaerobic shake flask cultures on SMS of *S.*
 704 *cerevisiae* strains (*malΔ mphΔ suc2Δ imaΔ SPase*) expressing different evolved *PvSUF1* alleles.
 705 Averages and mean deviations were derived from two biological replicates for each strain.

Strain	Parental strain	<i>PvSUF1</i> from evolved strain	Relevant characteristic	Specific growth rate (h ⁻¹)
IMZ712	IMZ630	IMS646	<i>SPase, PvSUF1</i> ^{G326C}	0.05 ± 0.01
IMZ713	IMZ630	IMS647	<i>SPase, PvSUF1</i> ^{YAAGSFSG-duplication}	0.05 ± 0.01
IMZ714	IMZ630	IMS646	<i>SPase, PvSUF1</i> ^{G326C} <i>PGM2</i>	0.05 ± 0.01
IMZ715	IMZ630	IMS647	<i>SPase, PvSUF1</i> ^{YAAGSFSG-duplication} <i>PGM2</i>	0.10 ± 0.01
IMZ729	IMZ630	IMS649	<i>SPase, PvSUF1</i> ^{I209F C265F G326C} <i>PGM2</i>	0.17 ± 0.02
IMZ724	IMS656	IMS646	<i>SPase, PvSUF1</i> ^{G326C}	0.07 ± 0.01
IMZ725	IMS656	IMS647	<i>SPase, PvSUF1</i> ^{YAAGSFSG-duplication}	0.06 ± 0.01
IMZ727	IMS656	IMS649	<i>SPase, PvSUF1</i> ^{I209F C265F G326C} <i>PGM2</i>	0.18 ± 0.01

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707

708 **LEGENDS TO FIGURES**

709

710 **Figure 1:** Strains construction scheme. Each blue box represents a different strain. Plasmids
 711 used are indicated on the top of the corresponding strain. The three main methods of generating
 712 strains are shown in the panels: "Evolution", "Plasmid removal" and "Reverse engineering".

713

714 **Figure 2:** Laboratory evolution in sequential batch cultures of *S. cerevisiae* IMZ636 (*PvSUF1*,
 715 *SPase*). After 5-11 serial transfers in shake flask cultures, incubated in an anaerobic chamber,
 716 evolution was continued in sequential batch bioreactors sparged with N₂ gas. Evolution was
 717 conducted independently in triplicate (panels **A**, **B** and **C**). Specific growth rates were estimated
 718 from the corresponding CO₂ profiles (not shown here) in the off-gas. 100 mL of SMS were used
 719 (pH 5.0, 30 °C, 250 rpm). Evolution was stopped after approximately 160, 170 and 150
 720 generations (panels **A**, **B** and **C**).

721

722 **Figure 3:** Laboratory evolution of *S. cerevisiae* IMZ696 (*PvSUF1*, *SPase*, *PGM2*) for faster
 723 anaerobic growth on sucrose. After eleven serial transfers in shake flasks in an anaerobic
 724 chamber, evolution was continued in bioreactors sparged with N₂ gas, first operated as
 725 sequential batch reactors (SBRs) (panels **A** and **B**) and subsequently as accelerostats (panels **C**
 726 and **D**) as described in the Methods section. **C**, **D** (accelerostat): feed and effluent pumps were
 727 turned on at 1 % of the maximum speed (equivalent to 0.09 h⁻¹ dilution rate). Feed pump speed
 728 (black line) was increased automatically based on the CO₂ concentration in the off-gas (blue
 729 line). A maximum pump speed corresponding to 0.25 h⁻¹ dilution rate was reached in one
 730 reactor (**C**) and of 0.17 h⁻¹ in the other one (**D**). Laboratory evolution in bioreactors was
 731 performed with 100 mL of synthetic medium containing 20 g/L sucrose (batches) and 25 g/L
 732 (accelerostat), pH 5, 30 °C, 250 rpm). Panels **A** and **C** correspond to one evolution line
 733 independent from another replicate (panels **B** and **D**). Both evolution lines were started with the
 734 same parental strain.

735

736 **Figure 4:** Proton uptake rate of unevolved *S. cerevisiae* IMZ696 (*PvSUF1*, *SPase*, *PGM2*), evolved
 737 strains (IMS644, IMS646, IMS647, IMS648 and IMS649) and the control strain IMZ709 (*MAL11*,
 738 *SPase*, *PGM2*). Cells were harvested from aerobic sucrose-limited chemostat cultures ($D = 0.03 \text{ h}^{-1}$,
 739 30 °C, pH 5.0), washed and immediately tested for proton uptake upon addition of sucrose
 740 (black bar), maltose (grey bar), fructose (*: H⁺ uptake not detected) or glucose (white bar, H⁺

741 uptake induced only in strain IMZ709). 20 mM sugar (final concentration) was added to a K-
742 phthalate suspension (pH 5.0) containing 2.5 g/L cells (30 °C). Average and mean deviation were
743 obtained from two biological replicates with three experimental replicates each. Calibration was
744 performed as described in the Methods section. Results from strains IMZ696 and IMZ709 were
745 previously published by Marques et al. (2018).

746

747 **Figure 5:** Mutations found in *PvSUF1* after laboratory evolution on sucrose-based medium.
748 Mutated amino acids are highlighted in red and the corresponding strain numbers are indicated.
749 "AA": amino acids. Arrows indicate the location of four conserved cysteine residues in plant
750 sucrose transporters. The cysteine at loop 2/3 is substituted by a serine in the wild-type *PvSuf1*
751 protein. Membrane insertion of *PvSuf1* was predicted with the *Protter* algorithm (Omasits et al.,
752 2014).

753

754 **Figure 6:** Model of the tertiary structure of wild-type *PvSuf1*. Extracellular loops are shown at
755 the top and intracellular loops at the bottom of the figure "A". View from the extracellular space
756 is shown in "B". The 8 amino-acid region in loop 5/6 that was duplicated in strains IMS644 and
757 IMS647 is shown in red. In the same loop, isoleucine 209, which was mutated to phenylalanine
758 (I209F) in strain IMS649 is highlighted in yellow. Images were prepared with PyMOL™ (version
759 1.7.4.5 Educational Product, Schrodinger, LLC).

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