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Engineering topology and kinetics of sucrose metabolism in *Saccharomyces cerevisiae* for improved ethanol yield

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ABSTRACT

Sucrose is a major carbon source for industrial bioethanol production by *Saccharomyces cerevisiae*. In yeasts, two modes of sucrose metabolism occur: (i) extracellular hydrolysis by invertase, followed by uptake and metabolism of glucose and fructose, and (ii) uptake via sucrose-proton symport followed by intracellular hydrolysis and metabolism. Although alternative start codons in the *SUC2* gene enable synthesis of extracellular and intracellular invertase isoforms, sucrose hydrolysis in *S. cerevisiae* predominantly occurs extracellularly. In anaerobic cultures, intracellular hydrolysis theoretically enables a 9% higher ethanol yield than extracellular hydrolysis, due to energy costs of sucrose-proton symport. This prediction was tested by engineering the promoter and 5′ coding sequences of *SUC2*, resulting in predominant (94%) cytosolic localization of invertase. In anaerobic sucrose-limited chemostats, this i*SUC2*-strain showed an only 4% increased ethanol yield and high residual sucrose concentrations indicated suboptimal sucrose-transport kinetics. To improve sucrose-uptake affinity, it was subjected to 90 generations of laboratory evolution in anaerobic, sucrose-limited chemostat cultivation, resulting in a 20-fold decrease of residual sucrose concentrations and a 10-fold increase of the sucrose-transport capacity. A single-cell isolate showed an 11% higher ethanol yield on sucrose in chemostat cultures than an isogenic *SUC2* reference strain, while transcriptome analysis revealed elevated expression of *AGT1*, encoding a disaccharide-proton symporter, and other maltose-related genes. After deletion of both copies of the duplicated *AGT1*, growth characteristics reverted to that of the unevolved *SUC2* and i*SUC2* strains. This study demonstrates that engineering the topology of sucrose metabolism is an attractive strategy to improve ethanol yields in industrial processes.

1. Introduction

Mainly used as an automotive fuel, bioethanol is the single largest product of industrial biotechnology, with an estimated global production of 87×10^9 litres in 2010 (Renewable Fuels Association, 2011). Currently, the predominant feedstocks for bioethanol production are corn starch or sugar-cane sucrose. The sugars derived from these agricultural crops are fermented under anaerobic conditions by the yeast *Saccharomyces cerevisiae*. While a huge research effort is underway to unlock additional, lignocellulosic biomass feedstocks (Parachin et al., 2011; Weber et al., 2010), improvement of sugar-cane sucrose-based ethanol production needs to continue due to the important role of feedstock cost in the overall process economics (up to 70% of the ethanol production cost (Maiorella et al., 1984)). Given the large volumes of industrial ethanol production, even small increases in the ethanol yield on sugar are economically significant.

S. cerevisiae can metabolize sucrose, the major sugar in cane juice and in molasses, in two ways. In the first and predominant mechanism, sucrose is hydrolyzed by an extracellular invertase encoded by the *SUC2* gene. Hydrolysis yields glucose and fructose, which enter into the cell by facilitated diffusion via hexose transporters encoded by members of the *HXT* gene family (Lagunas, 1993). In the second mechanism, sucrose can be actively transported into the cells by a proton-symport mechanism and hydrolyzed intracellularly (Batista et al., 2004; Santos et al., 1982; Stambuk et al., 2000). Both extra- and intracellular

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invertase are encoded by the same gene (*SUC2*), which has two different start codons, leading to synthesis of active invertase isoforms of 532 and 512 amino acids, respectively (Carlson and Botstein, 1982; Carlson et al., 1983; Gascón et al., 1968; Taussig and Carlson, 1983). The larger and predominant Suc2p isoform, which is glycosylated, is exported across the plasma membrane, partially retained in the cell wall and partially released in the extracellular medium. The shorter and non-glycosylated isoform of invertase is retained in the cytosol where it, together with intracellular maltase (encoded by the *MALx2* genes), contributes to the intracellular sucrose-hydrolyzing capacity of *S. cerevisiae* (Badotti et al., 2008; Carlson and Botstein, 1982; Gascón and Lampen, 1968). Already in 1982, it was shown that wild-type *S. cerevisiae* has a low capacity for sucrose uptake via a sucrose-proton symporter (Santos et al., 1982), an activity that was later attributed to the *AGT1* (*MAL11*) gene product (Stambuk et al., 2000). However, in sucrose-grown cultures of wild-type *S. cerevisiae* strains, sucrose hydrolysis occurs almost exclusively extracellularly (Batista et al., 2004; Burger et al., 1961; Carlson and Botstein, 1982; Gascón and Lampen, 1968; Sutton and Lampen, 1962).

Anaerobic fermentation of glucose or fructose via the yeast glycolytic pathway yields 2 mol ATP per mole of hexose. Therefore, 4 mol ATP are formed per mole of sucrose when this disaccharide is fermented by the extracellular hydrolysis pathway. When sucrose is metabolized by the intracellular hydrolysis pathway, the single proton that is taken up along with sucrose (Santos et al., 1982; Stambuk et al., 2000) has to be expelled by the plasma membrane ATPase (Pma1p) to maintain the proton motive force across this membrane and to prevent intracellular acidification. In *S. cerevisiae* and *Neurospora crassa*, the stoichiometry of the plasma membrane ATPase is 1 H^+ per ATP (Perlin et al., 1986; Serrano, 1977; Van Leeuwen et al., 1992; Weusthuis et al., 1993). This reduces the net ATP yield of anaerobic sucrose fermentation via the intracellular hydrolysis pathway to only 3 mol ATP per mole of sucrose.

During fermentative growth on sugars, part of the carbon source is used for the production of yeast biomass and glycerol, which is needed to reoxidize the 'excess' NADH formed in biosynthetic reactions (van Dijken and Scheffers, 1986). Biomass and glycerol production from glucose/fructose require a net input of free energy (ATP), which is provided by alcoholic fermentation of the remainder of the feedstock. Therefore, in growing cultures, a decrease in the ATP yield from alcoholic fermentation will result in a larger fraction of the sugar being converted to ethanol, with a concomitant lower biomass yield. Consequently, sucrose fermentation via the intracellular pathway (3 mol ATP per mole of sucrose) should enable a higher ethanol yield on sucrose than its fermentation via the extracellular pathway (4 ATP per sucrose).

Weusthuis et al. (1993) studied the impact of disaccharide-proton symport on biomass and product yields by comparing anaerobic growth of *S. cerevisiae* on maltose, which is transported by a maltose-proton symporter and intracellularly hydrolyzed by maltase, and glucose. They demonstrated that, consistent with model predictions, biomass and ethanol yields in anaerobic maltose-limited cultures were 25% lower and 8% higher, respectively, than in glucose-limited cultures. Similar yield differences are theoretically expected between anaerobic sucrose-limited cultures of *S. cerevisiae* utilizing sucrose via the intracellular pathway and via the extracellular pathway.

The goal of the present study was to investigate whether a relocation of sucrose hydrolysis from the extracellular space to the cytosol can be used to improve ethanol yields on sucrose and which additional steps may be required to improve sucrose utilization by strains that only express intracellular invertase. Growth and product formation by a strain with a modified *SUC2* gene were compared with that of the parental strain in anaerobic sucrose-limited chemostat cultures. Subsequently, evolutionary engineering was used to improve sucrose uptake kinetics and an evolved strain was characterized for growth and product formation in chemostat cultures. Transcriptome analysis and gene deletion studies were used to identify genetic changes in the evolved strain that contribute to its improved sucrose-uptake kinetics.

2. Materials and methods

2.1. Yeast strains and maintenance

The *S. cerevisiae* strains used in this study (Table 1) are congenic members of the CEN.PK family (Entian and Kotter, 2007; Van Dijken et al., 2000). Stock cultures were grown in shake flasks containing synthetic medium (Verduyn et al., 1992) with 20 g l^{-1} glucose as carbon source. After overnight growth, 20% (v/v) glycerol was added and 1 ml aliquots were stored at −80 °C.

2.2. Strain construction

All transformations were carried out as described previously (Gietz and Woods, 2002). Primers iSUC2 Repl Fw and iSUC2 Repl Rv (Table 2) were used to amplify a replacement cassette from plasmid pFA6a-*TRP1*-P_{ADH1} (DeMarini et al., 2001), carrying the *TRP1* marker gene and the *ADH1* promoter. The resulting replacement cassette contained homology to regions immediately up- and downstream of the second start codon of the *SUC2* gene after 60 bp. Integration of this cassette thereby introduced the strong constitutive *ADH1* promoter in front of the second start codon of *SUC2* and removed the 20 N-terminal amino acids of the resulting protein (Carlson and Botstein, 1982; Carlson et al., 1983). Transformation and selection for tryptophan prototrophy resulted in strain BSY021-34B. Correct integration was verified via diagnostic PCR with primers iSUC2 Ctrl Fw and iSUC2 Ctrl Rv (Table 2). To restore the uracil prototrophy that could interfere with chemostat characterization (Basso et al., 2010), the 523 bp *Nde*I/*Stu*I fragment of *URA3* from pRS406 (Sikorski and Hieter, 1989) was

Table 1
Saccharomyces cerevisiae strains used in this study.

Strain	Relevant genotype	Source
CEN.PK113-7D	MATa *URA3 TRP1 SUC2*	P. Kötter, Germany
BY4741 *agt1*Δ	MATa *agt1::loxP-KanMX4-loxP*	Euroscarf, Germany
BSY021-34B	MATa *ura3-52 trp1-289 TRP1*-P_{ADH1}::i*SUC2*	This study
IMI056	MATa *URA3 trp1-289 TRP1*-P_{ADH1}::i*SUC2*	This study
IMM007	IMI056 evolved	This study
IMM008	IMI056 evolved *agt1::loxP-KanMX4-loxP*	This study
IMM009	IMI056 evolved *agt1::loxP-KanMX4-loxP agt1::loxP-hphNT1-loxP*	This study

Table 2
Primers used in this study.

Name	Sequence (5′→3′)
iSUC2 Repl Fw	TTTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCATCAGAATTCGAGCTCGTTTAAAC
iSUC2 Repl Rv	GTGTGAAGTGGACCAAAGGTCTATCGCTAGTTTCGTTTGTCATTGTATATGAGATAGTTG
iSUC2 Ctrl Fw	CTCCCCCGTTGTTGTCTCAC
iSUC2 Ctrl Rv	GGCACTGTACTCCCAGTT
AGT1 KO Fw A	GCGAGTTGCAAGAATCTCTACG
AGT1 KO Rv A	GATGACGACCACATGGGTTTG
AGT1 Ko Fw B	TCATTTCATTGGTAAGCAAGAAGAAGGCTGCCTCAAAAAATGAGGATAAAAACATCAGCTGAAGCTTCGTACGC
AGT1 KO Rv B	CATTTATCAGCTGCATTTAATTCTCGCTGTTTTATGCTTGAGGACTGACTGATACGCATAGGCCACTAGTGGATCTG
AGT1 Ctrl Fw	GCCTCTTTCCACCACTTTG
AGT1 Ctrl Rv	ACGAGGACTGTCAGACCATTG
KanMX4 Ctrl Fw	TCGTATGTGAATGCTGGTCG
KanMX4 Ctrl Rv	CGCACGTCAAGACTGTCAAG
hphNT1 Ctrl Fw	ACGCGGATTTCGGCTCCAAC
hphNT1 Ctrl Rv	AGACGTCGCGGTGAGTTCAG

used to transform strain BSY021-34B (i*SUC2 ura3-52*), resulting in IMI056 (i*SUC2 URA3*) (Table 1). For deletion of *AGT1* in IMM007, a knockout cassette with the *KanMX4* marker gene was amplified from BY4741 *agt1*Δ genomic DNA using primers AGT1 KO Fw A and AGT1 KO Rv A (Table 2). Transformation and selection on agar plates containing 200 mg l^{-1} G418 (Invivogen, San Diego, USA) resulted in IMM008. Correct integration of the knockout cassette was verified via diagnostic PCR with primer pairs AGT1 Ctrl Fw/KanMX4 Ctrl Rv and KanMX4 Ctrl Fw/AGT1 Ctrl Rv (Table 2). For deletion of the second copy of *AGT1* in IMM008, a knockout cassette with the *hphNT1* marker gene was amplified from pUG-*hphNT1* (De Kok et al., 2011) using primers AGT1 KO Fw B and AGT1 KO Rv B (Table 2). Transformation and selection on agar plates containing 200 mg l^{-1} hygromycin B (Invivogen, San Diego, USA) resulted in IMM009. Correct integration of the knockout cassette was verified via diagnostic PCR with primer pairs AGT1 Ctrl Fw/hphNT1 Rv and hphNT1 Fw/AGT1 Ctrl Rv (Table 2). Molecular biology techniques were performed as described previously (De Kok et al., 2011).

2.3. Medium and cultivation

Precultures for chemostat experiments were grown until the mid-exponential growth phase at 30 °C in 500 ml shake flasks containing 100 ml synthetic medium (Verduyn et al., 1992) with 20 g l^{-1} sucrose in an Innova incubator shaker (New Brunswick Scientific, Edison, USA) set at 200 rpm. A synthetic medium with 25 g l^{-1} sucrose was used for all chemostat experiments (Verduyn et al., 1992). Sucrose was filter-sterilized as a 50% (w/v) solution before being transferred to the medium. The medium was supplemented with the anaerobic growth factors ergosterol (10 mg l^{-1}) and Tween80 (420 mg l^{-1}) dissolved in ethanol. Antifoam Emulsion C (Sigma, St. Louis, USA) was autoclaved separately (120 °C) as a 20% (w/v) solution and added to a final concentration of 0.2 g l^{-1}. Anaerobic chemostat fermentations were run in 2 l laboratory bioreactors (Applikon, Schiedam, The Netherlands) at 800 rpm and 30 °C as described previously (Wisselink et al., 2010). The working volume was kept at 1.0 l using an effluent pump, which was controlled by an electric level sensor. The exact working volume was measured at the end of each experiment. The pH was kept at 5.0 via automatic addition of 2 M KOH. Cultures were sparged with 500 ml min^{-1} nitrogen (<10 ppm oxygen). To minimize oxygen diffusion into the system, bioreactors were equipped with Norprene tubing and Viton O-rings and the medium vessels were flushed with nitrogen gas. Chemostat cultivations were preceded by batch cultivations under the same conditions. After sucrose exhaustion, which was indicated by a rapid decrease in CO_2 production, the cultivations were switched to continuous mode at a dilution rate of 0.10 h^{-1}. Culture purity was routinely monitored by phase contrast microscopy.

2.4. Analytical methods

Cultures were assumed to be in steady-state when, after at least five volume changes, the culture dry weight and the specific carbon dioxide production rate varied less than 2% over 2 volume changes. Culture dry weights were determined via filtration of 20 ml samples over dry preweighed nitrocellulose filters (Gelman laboratory, Ann Arbor, USA) with a pore size of 0.45 μm. After removal of the medium, the filters were washed twice with demineralized water, dried in a microwave oven for 20 min at 360 W and weighed. Supernatants were obtained after centrifugation of culture broth. Residual sugars were sampled as described previously (Mashego et al., 2003). Supernatants and media were analyzed via HPLC using an Aminex HPX-87H ion exchange column (BioRad, Richmond, USA) at 60 °C with 5 mM H_2SO_4 as the mobile phase at 0.6 ml min^{-1}. Ethanol, glycerol, succinate and lactate were detected by a Waters 2410 refraction index detector. Pyruvate and acetate were detected by a Waters 2487 UV detector at 214 nm. Ethanol concentrations were corrected for evaporation as described previously (Guadalupe Medina et al., 2010). Sucrose, glucose and fructose concentrations were analyzed via an enzymatic assay (10716260035, Boehringer Mannheim, Mannheim, Germany) according to manufacturer's instructions. Exhaust gas from the bioreactor was cooled in a condenser (2 °C) to minimize ethanol evaporation and dried in a Perma Pure Dryer (Permapure, Toms River, USA). CO_2 concentrations in the off-gas were analyzed with a Rosemount NGA 200000 gas analyzer (Rosemount Analytical, Orrvile, USA).

2.5. Invertase assay

For invertase activity measurements cells were collected from chemostats, washed twice and resuspended in cold water. Extracellular invertase activity was determined by measuring glucose formation at 30 °C in 50 mM Tris-succinate buffer (pH 5.0) with 150 mM sucrose using cells pre-incubated with 50 mM sodium fluoride (Silveira et al., 1996). To measure total invertase activity, cells were permeabilized as described previously (Stambuk, 1999) with ethanol, 10% (v/v) Triton X-100 and toluene (1:4:1), washed twice and then incubated at 30 °C with 100 mM sucrose in 50 mM Tris-succinate buffer (pH 5.0). Glucose formation was analyzed via an enzymatic assay as described above. All assays were carried out in triplicate with a standard deviation of $<10\%$.

2.6. Sucrose-proton symport assay

Proton symport during sucrose uptake was determined by recording pH changes in yeast suspensions (Stambuk et al., 2000). Yeast cells were suspended to a cell density of 25 g l^{-1} in 25 mM K-phtalate buffer pH 5.0 and placed in a water-jacketed vessel in a total volume of 5 ml. The suspension was mixed with a magnetic stirrer at 30 °C. Changes of pH triggered by the addition of sucrose (70 mM final concentration) were monitored with a pH sensor. To calculate the rate of proton uptake, a calibration curve was obtained by the addition of 100–500 nmol of NaOH to the cell suspension. Initial rates of sucrose-induced proton uptake were calculated from the slope of the first 10–20 s after sucrose addition, subtracting the basal rate of proton uptake observed before addition of sucrose. All assays were carried out at least in triplicate with standard deviations of $<15\%$.

2.7. Microarray processing and analysis

DNA microarray analyses were performed with the S98 Yeast GeneChip arrays (Affymetrix, Santa Clara, USA) as previously described (Daran-Lapujade et al., 2004; Piper et al., 2002). Cells were transferred directly from chemostats into liquid nitrogen and processed according to the manufacturer's instructions (Affymetrix, Santa Clara, USA) with the following modifications: double-stranded cDNA synthesis was carried out using 15 μg total RNA and the components of the One Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, USA). Double-stranded cDNA was purified using the GeneChip Sample Cleanup Module (Qiagen, Hilden, Germany) before in vitro transcription and labeling with the GeneChip IVT Labeling Kit (Affymetrix, Santa Clara, USA). Finally, labeled cRNA was purified using the GeneChip Sample Cleanup Module prior to fragmentation and hybridization of 15 μg biotinylated cRNA (De Nicola et al., 2007). Acquisition and quantification of array images and data filtering were performed using Affymetrix GeneChip Operating Software, version 1.2. The Significance Analysis of Microarrays (SAM) (version 2.23A) (Tusher et al., 2001) add-in to Microsoft Excel was used for comparison of replicate array experiments of IMI056 and IMM007 under sucrose limitation conditions. Transcript data have been deposited in the Genome Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE30535. Groups of corresponsive genes were consulted for enrichment of functional annotation according to Gene Ontology (Ashburner et al., 2000) as previously described (Knijnenburg et al., 2007).

3. Results

3.1. Topology of sucrose metabolism: theoretical analysis of impacts on biomass and ethanol yield

A theoretical analysis of the impact of the envisaged metabolic engineering strategy was based on the assumption that biomass composition, ATP requirement for maintenance and assimilatory pathways in *S. cerevisiae* are independent of the topology of sucrose metabolism. As discussed above, the energy requirement for sucrose transport limits the net ATP yield from anaerobic sucrose metabolism via intracellular hydrolysis to 1.5 ATP per hexose equivalent, as compared to 2 ATP per hexose equivalent for glucose metabolism and for sucrose metabolism via extracellular hydrolysis. As a consequence, compared to a scenario where sucrose hydrolysis occurs extracellularly, one additional sucrose molecule must be fermented to ethanol for every three sucrose molecules entering the cell, leading to a 25% lower biomass yield on sucrose. The same difference is expected for the glycerol yield since, in the absence of osmotic stress, glycerol production by anaerobic *S. cerevisiae* cultures is strictly coupled to growth (van Dijken and Scheffers, 1986).

The additional sucrose consumed to energize sucrose-proton symport is completely converted to ethanol and carbon dioxide and not to biomass or glycerol. Therefore, the ethanol yield on sucrose in cells using the intracellular pathway should be higher than in cells using the extracellular pathway. The increase in ethanol yield of a strain that exclusively employs the intracellular hydrolysis pathway relative to a strain using the extracellular pathway can therefore be predicted according to Eq. (1):

$$Y_{SucE,intra} = 0.75 Y_{SucE,extra} + 0.25 Y_{SucE,max} \quad (1)$$

In Eq. (1), $Y_{SucE,intra}$ and $Y_{SucE,extra}$ (mol ethanol mol $sucrose^{-1}$) are the ethanol yields on sucrose of strains that exclusively hydrolyze sucrose intracellularly and extracellularly, respectively. $Y_{SucE,max}$ is the maximum ethanol yield obtained upon complete dissimilation of sucrose via alcoholic fermentation. After substitution of 4 mol ethanol mol $sucrose^{-1}$ for $Y_{SucE,max}$, the relative ethanol yield of a strain using the intracellular sucrose hydrolysis pathway ($R = Y_{SucE,intra}/Y_{SucE,extra}$) is described by Eq. (2):

$$R = 0.75 + 1/Y_{SucE,extra} \quad (2)$$

In anaerobic sucrose-limited chemostat cultures (dilution rate, 0.10 h^{-1}) of the reference strain *S. cerevisiae* CEN.PK113-7D, the ethanol yield on sucrose was 2.96 mol mol^{-1} (Table 3). Assuming that the contribution of intracellular sucrose hydrolysis in this strain is negligible (Carlson and Botstein, 1982; Gascón and Lampen, 1968) and that, therefore, this ethanol yield corresponds to $Y_{sucE,extra}$, this predicts that a switch to intracellular sucrose hydrolysis enables an 8.7% increase of the ethanol yield on sucrose (Eq. (2)). This increase is virtually the same as the 8% difference in ethanol yields between anaerobic chemostat cultures grown on maltose, which is taken up by proton symport and hydrolyzed intracellularly, and on glucose (transported by facilitated diffusion), as predicted and demonstrated by Weusthuis et al. (1993).

3.2. Engineering of the SUC2 gene results in relocation of invertase to the cytosol

To verify the predicted effect of pathway topology on biomass and ethanol yields in sucrose-grown cultures, the localization of invertase in *S. cerevisiae* was changed by genetic modification. The *S. cerevisiae SUC2* gene encodes both the intracellular non-glycosylated invertase and the secreted glycosylated form of the enzyme (Carlson and Botstein, 1982; Carlson et al., 1983). To constitutively and exclusively express the intracellular form of invertase, the strong, constitutive *ADH1* promoter was used to replace the native, inducible *SUC2* promoter. Moreover, the transformation cassette containing the *ADH1* promoter was designed such that its integration removed the first 60 nucleotides of the *SUC2* gene, which encode the 20-amino-acid N-terminal signal peptide for extracellular targeting (Carlson et al., 1983).

Localization of invertase in the engineered '*iSUC2*' strain IMI056 and in the reference strain CEN.PK113-7D (*SUC2*) was analyzed by comparing total and extracellular sucrose-hydrolyzing activity. In the reference strain, 90% of the total invertase activity was found extracellularly (Table 3). In the *iSUC2* strain IMI056, 94% of the invertase activity was found intracellularly (Table 3). The total invertase activity in the *iSUC2* strain was higher than in the reference strain, probably as a result of the use of the strong *ADH1* promoter used for expression of the

Table 3
Physiology of *S. cerevisiae* strains CEN.PK113-7D (*SUC2*), IMI056 (i*SUC2*), IMM007 (i*SUC2* evolved) and IMM009 (i*SUC2* evolved *agt1*Δ/*agt1*Δ) in anaerobic sucrose-limited chemostat cultures at a dilution rate of 0.10 h^{-1}. Averages and mean deviations were obtained from independent duplicate experiments.

Strain	CEN.PK113-7D	IMI056	IMM007	IMM009
Relevant genotype	*SUC2*	i*SUC2*	i*SUC2* evolved	i*SUC2* evolved *agt1*Δ/*agt1*Δ
Actual dilution rate (h^{-1})	0.103 ± 0.001	0.101 ± 0.001	0.101 ± 0.000	0.102 ± 0.001
Sucrose consumption rate (mmol g DW^{-1} h^{-1})	3.11 ± 0.03	3.15 ± 0.02	4.25 ± 0.05	2.92 ± 0.02
Biomass yield (g mol $sucrose^{-1}$)	33.9 ± 0.4	31.7 ± 0.4	23.8 ± 0.7	34.2 ± 0.4
Relative biomass yield[a]	1.00	0.94 ± 0.02	0.70 ± 0.02	1.01 ± 0.02
Ethanol yield (mol mol $sucrose^{-1}$)	2.96 ± 0.01	3.09 ± 0.06	3.30 ± 0.06	2.97 ± 0.02
Relative ethanol yield[a]	1.00	1.04 ± 0.02	1.11 ± 0.02	1.01 ± 0.01
Glycerol yield (mol mol $sucrose^{-1}$)	0.25 ± 0.00	0.22 ± 0.00	0.20 ± 0.01	0.24 ± 0.00
Relative glycerol yield[a]	1.00	0.88 ± 0.00	0.80 ± 0.00	0.96 ± 0.00
Residual sucrose (g l^{-1})	< 0.1	1.8 ± 0.2	< 0.1	1.5 ± 0.1
Invertase activity (mmol g DW^{-1} h^{-1})				
Total	128 ± 7	284 ± 35	265 ± 22	N.D.
Extracellular	114 ± 4	17 ± 3	25 ± 3	N.D.
Sucrose: H^+ transport capacity (mmol g DW^{-1} h^{-1})	0.43 ± 0.09	1.74 ± 0.03	20.8 ± 0.1	< 0.3
Carbon recovery (%)[b]	96.2 ± 0.2	97.4 ± 0.8	100.1 ± 0.8	97.4 ± 1.4

N.D.: not determined.

[a] Yield relative to that of reference strain CEN.PK113-7D.
[b] Calculations of the carbon recovery were based on a carbon content of biomass of 48% (w/w).

engineered i*SUC2* gene. The low activity recovered in the extracellular fraction of the i*SUC2* strain IMI056 (Table 3) may be due to release of some intracellular invertase (e.g. by lysis) or a low activity of another, unknown extracellular sucrose-hydrolyzing enzyme. Since analysis of invertase activity was based on measurement of glucose release, this activity might also reflect release from the cytosol via Hxt transporters of some glucose originating from intracellular sucrose hydrolysis (Boles and Hollenberg, 1997; Jansen et al., 2002).

3.3. Cytosolic expression of invertase has a minor impact on growth stoichiometry

To analyze the impact of the retargeting of invertase to the cytosol on biomass and product formation, growth of the i*SUC2* strain IMI056 was studied in anaerobic, sucrose-limited chemostat cultures (D=0.10 h^{-1}) and compared to that of the isogenic reference strain CEN.PK113-7D (*SUC2*).

Biomass and ethanol yields on sucrose monohydrate (0.094 g biomass g sucrose H_2O^{-1} and 0.38 g ethanol g sucrose H_2O^{-1}, respectively, Table 3) of the reference strain were comparable to previously published data on growth of this strain in anaerobic glucose-limited chemostats (0.09 g biomass g $glucose^{-1}$ and 0.40 g ethanol g $glucose^{-1}$, respectively (Abbott et al., 2007)). The sucrose-proton symport capacity assayed in samples from chemostat cultures of the reference strain was 7-fold lower than the sucrose-consumption rate in these cultures (Table 3). Proton-symport activities were assayed at a near-saturating sucrose concentration of 70 mM. Given the estimated K_m of high-affinity sucrose-proton symport in *S. cerevisiae* of 5–8 mM (Stambuk et al., 2000), *in situ* transport activities in the chemostat cultures, in which the residual sucrose concentration was below 0.3 mM (Table 3), are expected to be at least an order of magnitude lower. These observations are consistent with a predominantly extracellular hydrolysis of sucrose in the *SUC2* reference strain CEN.PK113-7D.

The biomass yield of the i*SUC2* strain was only 6 ± 2% lower than that of the *SUC2* reference strain (Table 3). This difference is much smaller than the 25% difference that was anticipated in case of a completely intracellular hydrolysis of sucrose. Consistent with the small difference of the biomass yields of the two strains, only a minor increase of the ethanol yield was observed in the engineered strain (Table 3). Strikingly, residual concentrations of sucrose in the chemostat cultures of the engineered strain IMI056 (1.8 g l^{-1}) were much higher than those in cultures of the reference strain CEN.PK113-7D (< 0.1 g l^{-1}, Table 3). This observation suggested that suboptimal kinetics of sucrose transport across the plasma membrane prevented efficient sucrose metabolism via intracellular hydrolysis. Indeed, although 4-fold higher than in the reference strain, the sucrose-proton symport capacity of the i*SUC2* strain (1.74 mmol g $biomass^{-1}$ h^{-1}) was twofold lower than the sucrose consumption rate in the chemostat cultures (3.15 mmol g $biomass^{-1}$ h^{-1}). Based on these observations, we hypothesized that, due to the suboptimal kinetics of sucrose transport, alternative pathways, involving (an) unknown extracellular sucrose-hydrolyzing enzyme(s) and/or (a) low-affinity facilitated transporter(s) for sucrose, successfully competed with the intracellular hydrolysis pathway. We therefore attempted to improve the kinetics of sucrose-proton symport in *S. cerevisiae*.

3.4. Laboratory evolution in chemostat cultures improves sucrose transport kinetics and ethanol yield

Prolonged nutrient-limited growth of micro-organisms in chemostat cultures exerts a strong selective pressure for spontaneous mutants with an improved affinity for that limiting nutrient (μ_{max}/K_s (Button, 1991)). For example, prolonged sugar-limited chemostat cultivation of wild-type and engineered *S. cerevisiae* strains was shown to lead to improved transport kinetics for glucose (Brown et al., 1998; Ferea et al., 1999; Jansen et al., 2005), maltose (Jansen et al., 2004) and xylose (Kuyper et al., 2005). To investigate whether this approach is applicable to improve sucrose-uptake kinetics, the i*SUC2* strain IMI056 was grown in long-term anaerobic, sucrose-limited chemostat cultures.

Over 90 generations of sucrose-limited growth, the residual sucrose concentration in the chemostat cultures decreased from 2 g l^{-1} to around 0.1 g l^{-1} (Fig. 1). Over the same period, the sucrose-proton symport capacity increased by an order of magnitude (Fig. 1). A single-cell isolate (referred to as the 'evolved i*SUC2* strain' IMM007) was obtained from one of the chemostat cultures by plating on non-selective glucose-containing agar plates and used to inoculate fresh chemostat cultures. Within 8 volume changes, these cultures had reached a low residual sucrose concentration (< 0.1 g l^{-1}) and a very high sucrose-proton

symport capacity (20.8 ± 0.1 mmol g DW^{-1} h^{-1}), as was observed for the long-term chemostat cultures (Fig. 1 and Table 3). The observation that the improved sucrose uptake kinetics were retained during non-selective growth on glucose plates indicated that the observed changes were the result of (a) mutation(s) in the genome of the i*SUC2* strain rather than of a physiological adaptation.

Analysis of transport kinetics using sucrose-grown shake flask cultures revealed that the K_m of sucrose transport was unchanged (6 ± 1 mM for both IMI056 and IMM007). These similar K_m values are consistent with an improvement of sucrose uptake kinetics that was solely caused by an increased capacity (V_{max} values for IMI056 and IMM007 were 1.74 ± 0.03 and 20.8 ± 0.1 mmol g DW^{-1} h^{-1}, respectively, Table 3) of the previously described high-affinity sucrose transport system (Stambuk et al., 2000).

The biomass yield of the evolved i*SUC2* strain *S. cerevisiae* IMM007 on sucrose was 30 ± 2% lower and its ethanol yield on sucrose was 11 ± 2% higher than that of the *SUC2* reference strain CEN.PK113-7D (Table 3). These differences were in good agreement with the anticipated changes in case of a shift from extracellular hydrolysis to intracellular hydrolysis (25% decrease of biomass yield, 9% increase of ethanol yield; see above).

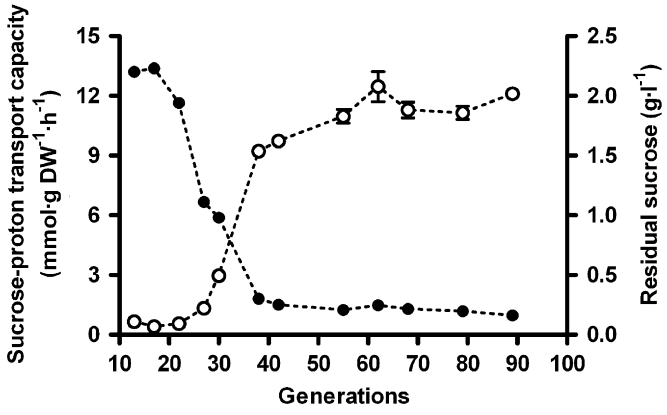

Fig. 1. Long-term cultivation of *S. cerevisiae* IMI056 (i*SUC2*) in anaerobic, sucrose-limited chemostat cultures at a dilution rate of 0.10 h^{-1}. Symbols: ●: residual sucrose concentration; ○: sucrose-proton symport activity assayed in culture samples. An independent replicate experiment yielded similar results.

3.5. AGT1 plays a key role in the improved sucrose transport kinetics of the evolved iSUC2 strain

To investigate the molecular basis of the improved sucrose-uptake kinetics in the evolved i*SUC2* strain IMM007, its transcriptome was compared with that of the non-evolved i*SUC2* strain IMI056. Genome-wide transcriptome analysis was carried out with cell samples of both strains grown in anaerobic sucrose-limited chemostat at a dilution rate of 0.10 h^{-1}. The average coefficient of variation of the transcriptome data derived from independent at least duplicate cultures did not exceed 20%. The level of *ACT1*, *ALG9*, *TAF10*, *TFC1* and *UBC6* transcripts, which are commonly used loading standards for Northern analysis (Teste et al., 2009), varied by less than 13% over the situations tested. A pairwise comparison of the two strains yielded a total of 85 genes that were differentially transcribed based on the statistical criteria applied in this study (absolute fold difference ≥ 2; false discovery rate 0.5%, see Section 2). Out of the 85 differentially expressed genes 84 showed a higher expression level in the evolved i*SUC2* strain IMM007 relative to its parental non-evolved strain IMI056 and only one gene (YGR234W) exhibited a lower expression level.

Genes involved in carbohydrate metabolism and transport and, albeit less pronounced, in stress and stimulus responses were overrepresented among the differentially expressed genes (Table 4). In particular, 9 out of 11 genes involved in maltose metabolism showed a strongly increased transcript level in the evolved strain (Table 4). Comparison of the relative transcript levels of the differentially expressed 'carbohydrate metabolism and transport' genes between the non-evolved and evolved i*SUC2* strain, showed higher transcript levels of genes involved in carbohydrate metabolism and, in particular, of maltose metabolism in the evolved strain. This general deregulation resembled the relative transcript levels in aerobic chemostat cultures of the reference strain CEN.PK113-7D grown under glucose and maltose limitation, respectively (Fig. 2).

Among the genes with an increased transcript level in the evolved i*SUC2* strain, *AGT1* encodes an α-glucoside-proton symporter whose substrates include both maltose and sucrose (Stambuk et al., 1999). To investigate the involvement of *AGT1* in the improved sucrose-transport kinetics, it was deleted in the evolved *iSUC2* strain IMM007. A transformant that showed

Table 4
Gene Ontology functional categories overrepresented in the set of genes differentially expressed (FC $\geq |2|$, FDR=0.5%) in the evolved i*SUC2* strain *S. cerevisiae* IMM007 relative to the unevolved i*SUC2* strain IMI056.

GO complete category	*k* in *n*[a]	*p*-Value[b]	Genes
Maltose metabolic process	9 out of 11	1.7×10^{-11}	*MPH2*, *MPH3*, *FSP2*, *IMA5*, *AGT1*, *MAL31*, *MAL12*, *MAL32*, *MAL13*
Carbohydrate metabolic process	20 out of 244	1.6×10^{-9}	*MPH2*, *MPH3*, *FSP2*, *IMA5*, *AGT1*, *MAL31*, *MAL12*, *MAL32*, YHR210C, *GRE3*, *SOL4*, *AMS1*, *MIG2*, *GIP2*, *GLC3*, *TPS2*, *TPS1*, *GPH1*, *TSL1*, *MAL13*
Disaccharide metabolic process	12 out of 24	1.5×10^{-13}	*MPH2*, *MPH3*, *FSP2*, *IMA5*, *AGT1*, *TPS2*, *MAL31*, *MAL12*, *MAL32*, *TPS1*, *TSL1*, *MAL13*
Trehalose metabolic process	3 out of 10	2.5×10^{-4}	*TPS2*, *TPS1*, *TSL1*
Trehalose biosynthetic process	3 out of 7	7.4×10^{-5}	*TPS2*, *TPS1*, *TSL1*
Carbohydrate transport	5 out of 41	1.8×10^{-4}	*MPH2*, *MPH3*, *AGT1*, *MAL31*, *HXT2*
Cellular carbohydrate metabolic process	18 out of 199	4.3×10^{-9}	*MPH2*, *MPH3*, *FSP2*, *IMA5*, *GRE3*, *AGT1*, *SOL4*, *AMS1*, *GIP2*, *GLC3*, *TPS2*, *MAL31*, *MAL12*, *MAL32*, *TPS1*, *GPH1*, *TSL1*, *MAL13*
Disaccharide biosynthetic process	3 out of 7	7.4×10^{-5}	*TPS2*, *TPS1*, *TSL1*
Response to stimulus	26 out of 911	4.7×10^{-4}	*AHP1*, *MNN4*, *UGA2*, *GRE3*, *DOG1*, *DOG2*, *ASK10*, *STF2*, *MIG2*, *HSP12*, *TSA2*, *TPS2*, *HSP30*, *FRM2*, *SSE2*, *TPS1*, *HSP26*, *SSA3*, *DDR2*, *GRE2*, *HUG1*, *DDR48*, *ALD2*, *ALD3*, *RAD52*, *TSL1*
Response to stress	24 out of 621	1.1×10^{-5}	*AHP1*, *MNN4*, *UGA2*, *GRE3*, *DOG1*, *DOG2*, *ASK10*, *STF2*, *HSP12*. *TSA2*, *TPS2*, *HSP30*, *SSE2*, *TPS1*, *HSP26*, *SSA3*, *DDR2*, *GRE2*, *HUG1*, *DDR48*, *ALD2*, *ALD3*, *RAD52*, *TSL1*

[a] *k* represents the number of gene from a GO category in the differentially expressed genes and *n* represents the number of genes from the same GO category in the whole genome.
[b] Enrichment analysis *p*-value according to Fischer exact statistics.

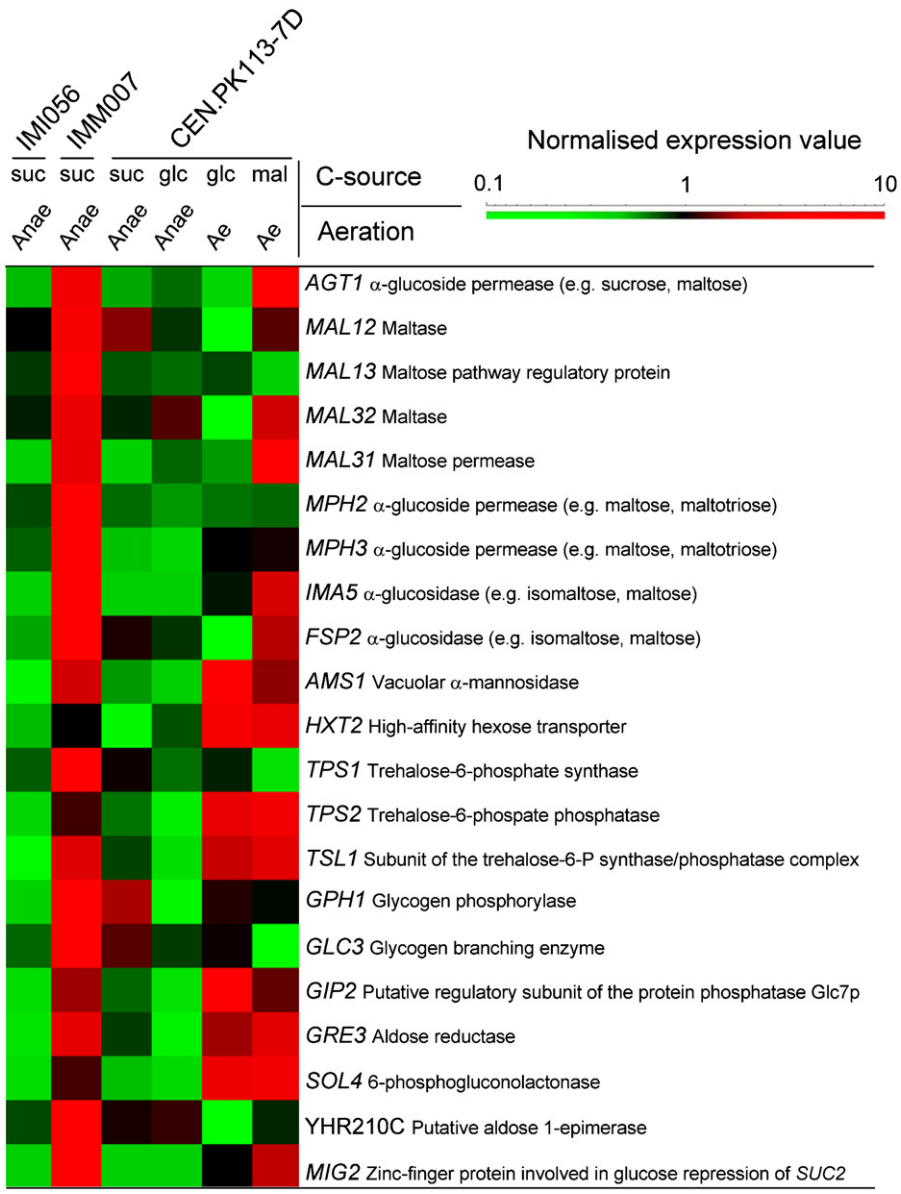

Fig. 2. Heat map of genes in GO categories related to carbohydrate metabolism with significantly higher transcript level in the evolved i*SUC2* strain *S. cerevisiae* IMM007 as compared to its parental strain IMI056 (Table 4). Transcript levels are compared to those of the reference strain CEN.PK113-7D (*SUC2*) grown in anaerobic sucrose-limited cultures (this study), in anaerobic glucose-limited chemostat cultures (Tai et al., 2005), and in aerobic glucose- and maltose-limited chemostat cultures (Daran-Lapujade et al., 2004).

correct integration of the *AGT1* gene replacement cassette still exhibited about half of the sucrose-proton symport capacity of the evolved strain (data not shown). Diagnostic PCR on genomic DNA showed that a duplication of the *AGT1* gene had occurred in the evolved i*SUC2* strain (data not shown). When both copies of *AGT1* in the evolved i*SUC2* strain were deleted, a drastic decrease of its sucrose-proton symport activity was observed (Table 3).

When both *AGT1* copies in the evolved i*SUC2* strain IMM007 were deleted, the resulting strain IMM009 exhibited the same yields of biomass, ethanol and glycerol as the *SUC2* reference strain CEN.PK113-7D (Table 3). This correspondence is consistent with an essential role of the sucrose transporter Agt1p in enabling intracellular sucrose hydrolysis and thus efficient sucrose fermentation (Badotti et al., 2008; Batista et al., 2004). Residual sucrose concentrations in chemostat cultures of the *agt1*Δ/*agt1*Δ strain IMM009 were much higher than those in the *SUC2* reference strain CEN.PK113-7D and comparable to those in the unevolved i*SUC2* strain IMI056 (Table 3). The difference with the *SUC2* reference strain can be attributed to the high extracellular invertase activity in the latter.

Sequencing of *AGT1* in the original i*SUC2* strain IMI056, and in the evolved i*SUC2* strains IMM007 and IMM008 (*agt1*Δ) revealed no mutations, neither in its coding region nor in an 800 bp upstream region. Since a duplication of *AGT1* is, by itself, unlikely to result in a 6-fold higher mRNA level, this observation suggest that the increased expression of *AGT1* was due to a second-site mutation, for example in a transcriptional regulator gene (e.g. *MALx3*). Such a mutation could then also be responsible for the upregulation of other genes involved in disaccharide metabolism in the evolved i*SUC2* strain (Table 4, Fig. 2). In view of anticipated problems in the assembly of the multiple *MAL* loci from short-read sequencing data and in the absence of a completely assembled and annotated reference sequence of *S. cerevisiae*

CEN.PK113-7D, we have refrained from resequencing the genome of the evolved i*SUC2* strain.

4. Discussion

4.1. Topology, kinetics and energetics of sucrose metabolism in yeast

Relocation of the *SUC2*-encoded invertase to the cytosol was, in itself, not sufficient to achieve the changes in growth stoichiometry that were predicted for intracellular sucrose hydrolysis by *S. cerevisiae*. This could be explained by the existence of a low extracellular sucrose-hydrolyzing activity of unknown identity that competed with an insufficient activity of the *AGT1*-encoded sucrose-proton symporter. Subsequent laboratory evolution in sucrose-limited chemostats did not lead to an increased activity of extracellular sucrose hydrolysis but, instead, led to improved kinetics of sucrose-proton symport (Table 3, Fig. 1). Apparently, under sucrose limitation, the selective advantage of a higher substrate affinity, in this case conveyed by increased transport capacity, outweighed the net ATP loss from active transport of sucrose. It is interesting to investigate whether the same evolutionary engineering strategy can be applied to improve uptake kinetics of engineered *S. cerevisiae* strains for other industrially relevant disaccharides, such as lactose and cellobiose (Domingues et al., 2010; Galazka et al., 2010; Sadie et al., 2011). In the latter case, a high affinity of cells for the disaccharide may also help to alleviate cellobiose inhibition of cellulases (Lynd et al., 2002) during simultaneous saccharification and fermentation processes.

In principle, improved kinetics of sucrose transport can also be achieved by targeted overexpression of *AGT1* or heterologous genes encoding sucrose-proton symporters, for example from yeasts that naturally hydrolyze sucrose intracellularly (Kaliterna et al., 1995). Irrespective of the method by which the transport kinetics of disaccharide metabolism are engineered, care should be taken to avoid an overcapacity as this may cause substrate-accelerated death when the yeast cells are exposed to high sugar concentrations, as demonstrated for *S. cerevisiae* strains that overexpress the maltose-proton symporters (Jansen et al., 2004).

Maltase (α-glucosidase, EC 3.1.2.20) has been shown to be responsible for intracellular sucrose hydrolysis in some naturally occurring sucrose-metabolizing yeasts (Kaliterna et al., 1995). In a separate study, we have shown that expression of maltase (Malx2p), combined with the deletion of *SUC2*, provides an alternative strategy for changing the topology of sucrose metabolism in *S. cerevisiae* (Dario et al., unpublished results).

4.2. From academic proof of principle to industrial application

This study provides an academic proof of principle for improvement of ethanol yields in anaerobic, sucrose-grown cultures of *S. cerevisiae* by engineering the topology of sucrose metabolism. The observed increase in ethanol yield in the evolved i*SUC2* strain was consistent with the theoretical prediction (9%, Eq. (2)). The yield improvement that can be achieved in industry with the i*SUC2* strategy depends on the specific growth rate and biomass yield. Application of cell recycling to reduce yeast growth, which is already applied in large-scale Brazilian bioethanol processes (Basso et al., 2008), may conceivably lead to specific growth rates lower than the value of 0.10 h^{-1} used in this study. In such cases, a larger fraction of the sucrose will already be dissimilated via alcoholic fermentation due to the increased impact of maintenance-energy requirement at low specific growth rates (Boender et al., 2009; Pirt, 1975). This would leave less room for improvement of the ethanol yield by changing the topology of sucrose metabolism (i.e. a higher $Y_{SucE,extra}$ in Eq. (2)). However, at a specific growth rate as low as 0.025 h^{-1}, still a significant increase of the ethanol yield of 3% is predicted (based on an $Y_{SucE,extra}$ of 3.56 mol mol sucrose^{-1}, derived from an ethanol yield of 1.78 mol mol glucose^{-1} (Boender et al., 2009)).

Also in anaerobic batch cultures of the evolved i*SUC2* strain, ethanol yields on sucrose were consistently higher (up to 20%, which exceeded theoretical predictions) than in cultures of the reference strain (data not shown). However, probably due to the impact of sampling and the dynamic nature of batch cultivation, carbon recovery in several cultures exceeded 100%. Our interpretation of the impact of the i*SUC2* genotype on product yields was therefore entirely based on the statistically robust data from the chemostat cultures.

In addition to improved ethanol yields, intracellular sucrose hydrolysis may have other advantages. For example, production of extracellular invertase can allow growth on glucose and fructose of other micro-organisms that lack invertase, including invertase-negative yeasts (Gore et al., 2009; Greig and Travisano, 2004). Moreover, extracellular accumulation of fructose can cause problems in industrial processes due to slower fructose utilization by industrial *S. cerevisiae* strains, resulting in residual fructose at the end of the cultivation and reduced ethanol yields (Berthels et al., 2004). Indeed, duplicate anaerobic batch cultures on sucrose of the evolved i*SUC2* strain showed a drastic reduction of extracellular glucose and fructose accumulation as compared to the *SUC2* reference strain (data not shown).

In view of the high impact of feedstock costs on the process economy of bioethanol production (Maiorella et al., 1984), industrial implementation of the i*SUC2* strategy offers an attractive perspective. Hitherto, implementation of genetically modified yeast strains in cane-sugar based ethanol production still presents challenges, for example due to regulatory demands for containment of genetically engineered strains. Furthermore, stricter measures to guarantee asepsis may be required to avoid contamination with competing micro-organisms. However, as ever more strain engineering approaches become available to improve key yeast characteristics, such as reduction of byproduct formation (Bro et al., 2006; Guadalupe Medina et al., 2010; Guo et al., 2010; Nissen et al., 2000) and improvement of ethanol tolerance (Yang et al., 2011), the incentive for introduction of engineered strains will grow. Further research in our groups will therefore focus on implementation of intracellular sucrose hydrolysis in industrial ethanol production strains and evaluation of their performance under industrial process conditions (Fischer et al., 2008; Nicolaou et al., 2010).

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