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Engineering sucrose metabolism in *Saccharomyces cerevisiae* for improved ATP yield.

Dissertation

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by

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Cover: "Sugar cane", 2018
Wesley Marques
Linocut, 19 x 30 cm. Print 4 of 4.

Dedicated to the memory of my beloved grandparents
Aparecida Cavalari and José Marques,
and to my friend Bruno Vaz.

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Summary

Contemporary society heavily depends on fossil sources. The energy and materials derived from fossil reserves were major contributors to the acceleration and intensification of agriculture and industry over the past 100 years. Such reserves are finite, hence, after expanding geographically, our economy is now consuming natural reserves that should not just support our generation but also those of the future. This unsustainable scenario becomes even more concerning when environmental impacts are taken into account. Even in the most — and probably unrealistic — optimistic climate scenarios, which assume no further increase in CO_2 emissions in the next decades, the global temperature would still raise by 2 °C at the end of this century with respect to pre-industrial era, which could already have a negative impact on, for instance, food security.

Depletion of limited reserves and concerns about climate change necessitate development of sustainable means of production, with low carbon footprints. Conversion of wind and solar power into electricity is already a reality in diverse countries. Microbial biotechnology — the field of research and industrial production in the context of which the research in this thesis has been performed — has the potential to contribute to the sustainable production of liquid and gaseous energy carriers (e.g. ethanol and methane) and a multitude of other chemicals (e.g. plastics and solvents) from renewable substrates. The renewable substrates for microbial fermentation can be diverse: sugars produced from crops such as sugar cane or corn, agricultural residues and even waste water. This thesis focuses on fermentation of sucrose, the predominant sugar in sugar cane, by baker's yeast (*Saccharomyces cerevisiae*). As discussed in **Chapter 1**, sucrose is a cheap substrate for industrial fermentations because it is abundantly available from sugar cane juice, without the necessity of enzymatic pretreatment of the raw material. *S. cerevisiae* has a long history of safe use by humans, it is robust under many industrially relevant conditions and accessible to modern techniques for 'genome editing' (i.e., advanced genetic modification). These characteristics

and the fact that this "sugar fungus" (from the Latin "*saccharum",* sugar and "*myces",* fungus) can: *i*) efficiently consume sucrose, *ii*) grow under acidic conditions (pH 3 - 4) and *iii*) grow without the necessity of expensive nutritional supplements; make it an important microbe for the production of industrial compounds.

Some molecules that can potentially be produced by *S. cerevisiae*, however, demand metabolic energy for their synthesis from sugar and/or for their export out of the yeast cell. For products whose synthesis requires a net input of metabolic energy in the form of ATP, production under anaerobic conditions — which is less costly than the use of aerobic production processes — is not currently possible. The key limitation in these cases is the small amount of ATP (4 molecules of ATP per molecule of sucrose) generated during anaerobic dissimilation of sucrose by *S. cerevisiae*. Increasing the free-energy conservation from sucrose metabolism is, therefore, a highly relevant challenge in yeast biotechnology. The research described in this thesis aims to address this challenge by contributing with new metabolic engineering tools and strategies for increasing energy conservation during anaerobic sucrose metabolism in yeast.

Chapter 1 sets the scene for the experimental work described in the other Chapters by reviewing the industrial relevance, molecular biology and physiological aspects of sucrose metabolism in *S. cerevisiae*.

Chapter 2 describes the construction of a novel experimental platform strain for research on disaccharide metabolism in *S. cerevisiae*. To generate a strain background in which alternative configurations of sucrose metabolism can be experimentally evaluated, all native transporters and hydrolases involved in sucrose consumption by *S. cerevisiae* were identified and, for the first time, completely eliminated in a single *S. cerevisiae* strain. The resulting strain provided a unique chassis for metabolic engineering not only of sucrose metabolism — as illustrated in the subsequent Chapters of this thesis — but, potentially, also of other industrially relevant oligosaccharides such as maltose, isomaltose and maltotriose. To construct this chassis strain, it was necessary to delete an entire gene family that encoded the isomaltase-

metabolizing genes. This goal was accomplished by CRISPR-Cas9-mediated genome editing, in which multiple genes were simultaneously targeted with the same shared Cas9 guide-RNA. As a result, six isomaltase-encoding genes were deleted in a single transformation step. The genome editing approach described in this Chapter has the potential to be applied to other gene families in yeasts and other organisms.

The goal of increasing the ATP yield from sucrose metabolism was pursued in **Chapter 3**. The native sucrose metabolism of *S. cerevisiae* predominantly occurs via its extracellular hydrolysis to glucose and fructose. This hydrolysis reaction releases about 29 kJ per mol of sucrose and the oxidation of the resulting monossacharides via the classical Embden-Meyerhof-Parnas pathway results in a net production of 4 molecules of ATP per sucrose molecule. Several anaerobic bacteria conserve the free energy from the sucrose-splitting reaction by expressing a sucrose phosphorylase (SPase). SPases cleave sucrose into fructose and glucose-1-phosphate while consuming one inorganic phosphate, thereby saving 1 ATP since activation of glucose by hexokinase is bypassed. In **Chapter 3,** expression of a bacterial SPase in *S. cerevisiae* was combined with the expression of heterologous membrane transporter proteins that should, in theory, function as sucrose uniporters. Replacing the native sucrose-proton symport mechanism of *S. cerevisiae* by an energy-independent sucrose uniporter is important. Without this modification, SPase-expressing strains would not conserve more energy than wild-type *S. cerevisiae*, as 1 ATP would be consumed by H^+ pumps (e.g. Pma1) for each proton that enters the cell via the native sucrose/H^+ symporters. Out of five heterologous transporters tested in **Chapter 3**, only the putative Sucrose Facilitator 1 from *Phaseolus vulgaris* (*Pv*Suf1) supported growth of the disaccharide-negative chassis strain described in **Chapter 2**, when expressed together with a bacterial SPase. The ATP yield of sucrose fermentation in this *PvSUF1-SPase* strain, estimated from the biomass yields of anaerobic sucrose-limited chemostat cultures, was 8% higher than that of a reference strain in which sucrose fermentation yielded 4 ATPs/sucrose. Further experiments were performed to investigate why the observed

increase in energy conservation was lower than the expected increase of 25 % (5 instead of 4 ATP per sucrose). The results of this analysis indicated that, in the yeast context, *Pv*Suf1 was not (only) a sucrose facilitator but clearly exhibited a sucrose-proton symport activity.

Chapter 4 describes a laboratory evolution study on the functional expression of the plant sucrose transporter *Pv*Suf1 in *S. cerevisiae*. The research described in this Chapter had three primary objectives: *i*) increase the specific growth rate in anaerobic, sucrose-grown cultures of the previously constructed *Pv*Suf1-dependent strains, *ii*) investigate the ATP yield of the evolved, faster growing strains and *iii*) identify causal mutations for the observed faster growth of evolved strains. After laboratory evolution, growth kinetics on sucrose were strongly improved. In independently evolved strains, mutations were identified in the native yeast genome as well as in the *PvSUF1* coding region. Via reverse engineering, it was shown that some mutations from *PvSUF1* were sufficient to enable fast growth on sucrose without the necessity of additional mutations in native yeast genes. The mutated *PvSUF1* alleles obtained after laboratory evolution all exhibited sucrose/H^+ symport activity. Biomass yield estimates from chemostats were in line with the active mechanism of transport observed for the mutated *Pv*Suf1s. Although, based on these heterologous expression study, no definitive conclusions could be drawn on the energy coupling of *Pv*Suf1 in its natural context, the results presented in **Chapters 3** and **4** do warrant an *in planta* reexamination of proton symport by this transporter.

Metabolic engineering of sucrose metabolism and evolutionary engineering were used in this thesis to increase the ATP yield from sucrose metabolism and to improve the kinetics of sucrose uptake through plant transporters overexpressed in yeast. The strains and knowledge generated provide a valuable basis for further research to lower costs of yeast-based processes in which ATP is required for product synthesis. The key contributions are: *i)* succesful replacement of sucrose hydrolysis by phosphorolysis and the demonstration of the impacts of such modification to cell ATP yield; *ii)* demonstration of simultaneous-multiple-gene-disruption in

yeast mediated by a single shared *Cas9* guide RNA; *iii*) a new platform *S. cerevisiae* strain that completely lacks activity of transporters and hydrolases required for metabolism of sucrose (and potentially other disaccharides); *iv*) a set of mutations that improve functional expression of the plant-transporter gene *PvSUF1* in yeast cells. Further exploration of the mutations identified in this thesis (mainly in *Pv*Suf1 protein), screening of new sugar channels, and transporter engineering should guide further research aimed at delivering more energy-efficient strains to industry.

Resumo

Uma parcela importante da energia e materiais consumidos em nossa sociedade provém de fontes fósseis. Graças ao acesso a esses recursos, a indústria e a agricultura intensificaram-se consideravelmente nos últimos cem anos. No entanto, após expandir-se geograficamente em busca de tais recursos, está em curso uma expansão temporal, ou seja, recursos que deveriam ser utilizados no futuro já estão sendo explorados. Esse cenário é ainda mais insustentável quando os impactos ambientais atrelados ao uso de reservas fósseis são contabilizados. Mesmo no cenário mais otimista, e provavelmente irreal, em que as emissões de CO_2 deixassem de aumentar nas próximas décadas, a temperatura global ainda assim deverá aumentar em 2 °C até 2100 (em relação à média global anterior à era pré-industrial), o que já seria suficiente para prejudicar consideravelmente a produção de alimentos.

Alternativas ao uso de recursos fósseis existem. A energia solar e a eólica já representam parte importante da matriz energética de vários países. Microbiologia industrial — o campo de pesquisa em que se insere essa tese — pode contribuir para a produção sustentável de combustíveis líquidos e gasosos (ex: etanol e metano) e de uma imensa variedade de produtos químicos de interesse industrial (ex: polímeros e solventes) a partir de substratos renováveis. Dentre tais substratos pode-se citar: açúcares obtidos a partir de cana-de-açúcar e milho, resíduos da agricultura e até mesmo efluentes líquidos. O foco desta tese está na fermentação de sacarose, açúcar predominante em cana-de-açúcar, por levedura (*Saccharomyces cerevisiae*). Tal como discutido no **Capítulo 1**, sacarose é um substrato barato, abundante e prontamente disponível, i. e., dispensa qualquer pré-tratamento da biomassa. *S. cerevisiae* tem uma longa história de uso pelos seres humanos, é robusta em várias condições industriais e acessível para modernas técnicas de edição gênica. Essas características e o fato de que esse "fungo do açúcar" (do Latim "*saccharum*", açúcar e "*myces*", fungo) pode: *i)* consumir sacarose eficientemente, *ii)* crescer em condições ácidas (pH 3 - 4) e *iii)* não exigir

nutrientes caros para seu cultivo; fazem do mesmo um importante microrganismo industrial.

Algumas moléculas que potencialmente podem ser produzidas por *S. cerevisiae*, no entanto, demandam energia para a sua síntese a partir de açúcares e/ou para serem transportadas para o meio extracelular. Para compostos desse tipo, ou seja, cuja síntese requer o consumo de energia celular na forma de ATP, a produção em anaerobiose — que é mais barata na indústria em relação a processos aeróbicos — não é atualmente possível. A limitação principal nesses casos é a insuficiente quantidade de ATP gerada durante a dissimilação anaeróbica da sacarose, ou seja, 4 ATPs/sacarose. Aumentar a conservação de energia a partir do metabolismo da sacarose é, portanto, um desafio altamente relevante em biotecnologia de leveduras. Por conseguinte, os estudos apresentados nessa tese tiveram como objetivo central desenvolver novas estratégias de engenharia metabólica para aumentar o rendimento de ATP do metabolismo de sacarose por levedura em condições de anaerobiose.

O **Capítulo 1** consiste numa revisão bibliográfica que serve para contextualizar o trabalho experimental apresentado nos demais capítulos. Discute-se neste capítulo a relevância do binômio *S. cerevisiae* e sacarose para a indústria, bem como aspectos fisiológicos e genéticos do metabolismo desse substrato em levedura.

No **Capítulo 2** descreve-se a construção de uma linhagem que serve de plataforma para a investigação do metabolismo de dissacarídeos em levedura. Para gerar tal linhagem, todos os transportadores e hidrolases nativos, envolvidos no consumo de sacarose, foram identificados e, pela primeira vez, completamente eliminados. Essa linhagem, que é incapaz de transportar e de hidrolisar sacarose, serve de chassis para engenharia metabólica não apenas do metabolismo de sacarose — como ilustrado nos capítulos subsequentes dessa tese — mas potencialmente também de outros sacarídeos como maltose, isomaltose e maltotriose. Para construir essa linhagem (ou chassis), foi necessário deletar, entre outros, uma família gênica que codifica as enzimas isomaltases (Ima). Essas deleções foram realizadas empregando-se

uma técnica baseada no sistema *CRISPR-Cas9*, de modo que vários genes foram simultaneamente deletados usando-se apenas um único RNA-guia. Como resultado, a família gênica dos *IMA*s foi eliminada em apenas uma rodada de transformação. A estratégia na qual um único RNA-guia serve para deletar mais de um gene pode ser aplicada a diversas outras famílias gênicas em levedura e em outros organismos.

Aumentar o rendimento de ATP do metabolismo de sacarose foi o objetivo do trabalho descrito no **Capítulo 3**. O metabolismo nativo de sacarose em *S. cerevisiae* ocorre predominantemente através de sua hidrólise extracelular resultando em glicose e frutose. A hidrólise da sacarose libera 29 kJ por mol de dissacarídeo e a oxidação dos monossacarídeos resultantes pela via clássica de Embden-Meyerhof-Parnas produz 4 ATP por molécula de sacarose. Várias bactérias anaeróbicas conservam a energia da clivagem da sacarose através da atividade da enzima sacarose fosforilase (SPase). A SPase cliva sacarose em frutose e glicose-1-fosfato, consumindo um fosfato inorgânico. Essa estratégia dispensa o consumo de um ATP para ativar a glicose pela atividade da hexoquinase. No **Capítulo 3**, a expressão de uma SPase bacteriana em *S. cerevisiae* foi combinada com a expressão de um transportador de sacarose que, de acordo com estudos anteriores, funciona como facilitador. A substituição dos simportadores de sacarose/H^+ por transportadores passivos é importante. Sem essa modificação, uma linhagem expressando SPase não conservaria mais ATP que a selvagem, pois os prótons internalizados junto com a sacarose seriam expulsos da célula através de bombas de H^+ (ex: Pma1), cuja atividade consumiria um ATP por próton transportado. Em outras palavras: o ATP economizado pela atividade da SPase seria gasto pela Pma1 e o rendimento energético do consumo da sacarose, portanto, permaneceria inalterado. Foram testados cinco facilitadores de sacarose encontrados em plantas. Destes, apenas um, o Facilitador de Sacarose 1 de *Phaseolus vulgaris* (*Pv*Suf1) permitiu o crescimento da linhagem-chassis incapaz de consumir dissacarídeos (descrita no **Capítulo 2**), quando expresso junto com uma SPase bacteriana. O rendimento em ATP dessa "linhagem *PvSUF1-SPase*", que é inferido a partir do rendimento em biomassa do cultivo

dessa linhagem em modo quimiostato limitado por sacarose em anaerobiose, foi 8% maior que o rendimento da linhagem referência, a qual gera 4 ATPs/sacarose. Experimentos complementares foram realizados para investigar porque o aumento no rendimento de ATP não foi de 25%, como seria esperado, ou seja, 5 em vez de 4 ATPs por sacarose. Os resultados destes experimentos indicam que, quando expresso em levedura, o *Pv*Suf1 não atua (apenas) como um facilitador de sacarose, mas também exibe atividade de simportador com prótons.

No **Capítulo 4** é descrita a evolução em laboratório de *S. cerevisiae* expressando *Pv*Suf1. Os objetivos do trabalho descrito neste capítulo foram: *i)* aumentar a velocidade de crescimento de linhagens dependentes do *Pv*Suf1 para o transporte de sacarose em anaerobiose; *ii)* medir o rendimento em ATP dos mutantes obtidos e *iii)* identificar as mutações que explicassem os fenótipos evoluídos. Após evolução em laboratório, a velocidade de crescimento da linhagem "*PvSUF1-SPase*" aumentou consideravelmente. Através de engenharia reversa, ficou provado que um dos alelos evoluídos do gene *PvSUF1*, quando expresso numa linhagem não evoluída, é suficiente para desencadear crescimento tão rápido quanto aquele da linhagem evoluída. Todos os alelos mutados de *PvSUF1* obtidos após a evolução exibiram atividade de simporte de sacarose/H^+. Estimativas de rendimento em biomassa (equivalente ao rendimento em ATP) dos mutantes obtidos estão de acordo com a atividade de simporte medida. Apesar de não ser possível tirar conclusões sobre o mecanismo de transporte do *Pv*Suf1 em seu contexto natural, já que os dados aqui apresentados foram obtidos a partir da expressão heteróloga em levedura, os **Capítulos 3** e **4** alertam para a necessidade de se reexaminar o mecanismo desse transportador *in planta*.

Nesta tese, engenharia metabólica e evolução em laboratório foram usadas com o intuito de aumentar o rendimento em ATP a partir do metabolismo de sacarose em levedura, em anaerobiose. As linhagens e o conhecimento aqui gerados servirão de base para futuros esforços de redução de custos da produção industrial de compostos cuja biossíntese requer ATP. As principais contribuições dessa tese foram, portanto: *i)* a substituição da

hidrólise da sacarose pela fosforólise e a demonstração dos impactos dessa modificação para a conservação de energia celular; *ii)* a deleção simultânea via CRISPR/Cas9 de vários genes utilizando-se apenas um único RNA-guia; *iii)* a construção de uma linhagem-plataforma de *S. cerevisiae* que não apresenta nenhuma atividade de transporte e nem de hidrólise de sacarose (e potencialmente de outros dissacarídeos) e *iv*) um conjunto de mutações que melhoram a expressão do transportador de planta *Pv*Suf1 em leveduras. A investigação de tais mutações; a identificação de outros facilitadores de dissacarídeos e a modificação racional dos transportadores conhecidos irão guiar os futuros trabalhos que objetivam entregar para a indústria microrganismos com metabolismo energético mais eficiente.

Samenvatting

Translation: Mark Bisschops

De huidige maatschappij is sterk afhankelijk van fossiele grondstoffen. Tijdens de afgelopen honderd jaar hebben energie en materialen gewonnen uit fossiele reserves enorm bijgedragen aan de ontwikkeling en intensivering van landbouw en industrie. Deze reserves zijn echter eindig, en op dit moment worden er in onze economie, na eerst geografisch te zijn uitgebreid, niet alleen de natuurlijke grondstoffen van huidige, maar ook die van toekomstige generaties verbruikt. Dit onhoudbare scenario is nog zorgwekkender wanneer de invloeden op het milieu in ogenschouw worden genomen. Zelfs in de meest optimistische, en waarschijnlijk onrealistische, klimaatscenario's, die er vanuit gaan dat de CO_2 uitstoot niet verder toeneemt in de komende decennia, warmt de aarde met 2 ˚C op tegen het eind van deze eeuw. Zo'n minimale toename kan al een negatieve invloed hebben op, bijvoorbeeld, voedselzekerheid.

De uitputting van eindige reserves en zorgen over klimaatverandering dwingen de ontwikkeling van duurzame productiemethodes met kleinere ecologische voetafdrukken af. De omzetting van wind en zonne-energie in elektriciteit is al realiteit in verschillende landen. Microbiële biotechnologie – het onderzoeks- en toepassingsgebied waarbinnen het onderzoek beschreven in dit proefschrift is uitgevoerd – heeft de potentie om bij te dragen aan de duurzame productie van vloeibare en gasvormige brandstoffen (bijv. ethanol en methaan) en een veelvoud aan andere chemicaliën, zoals kunststoffen en oplosmiddelen, van hernieuwbare grondstoffen.

De mogelijke hernieuwbare grondstoffen voor deze microbiële fermentaties zijn divers: suikers gewonnen uit planten zoals suikerriet en maïs, agrarische reststromen en zelfs afvalwater. Dit proefschrift richt zich op de fermentatie van sacharose, de voornaamste suiker in suikerrietsap, door bakkersgist (*Saccharomyces cerevisiae*). Zoals besproken in Hoofdstuk 1, is sacharose een goedkoop substraat voor industriële fermentaties aangezien het ruim beschikbaar is in suikerrietsap, zonder dat enzymatische

voorbehandeling van het ruwe materiaal noodzakelijk is. *S. cerevisiae* wordt reeds lang veilig toegepast door de mens, is robuust onder veel industrieel relevante omstandigheden en er bestaan moderne technieken om het genoom te 'editen', met andere woorden, geavanceerde genetische modificaties zijn mogelijk. Deze eigenschappen en het feit dat deze "suikerschimmel" (Latijn: "*saccharum*", suiker en "*myces*", schimmel) daarnaast i) efficiënt sacharose kan consumeren, ii) kan groeien onder zure condities (pH 3 – 4) en iii) groeit zonder dat dure voedingstoffen hoeven te worden toegevoegd, maken het tot een belangrijk organisme voor de productie van industriële verbindingen.

Sommige moleculen waarvoor *S. cerevisiae* als productie-organisme overwogen wordt, vereisen echter metabole energie voor de synthese en/of export uit de gistcel. Voor zulke producten, waarvoor voor de synthese een netto input van metabole energie in de vorm van ATP nodig is, is productie onder anaërobe condities – hetgeen goedkoper is dan aërobe productieprocessen – op dit moment niet mogelijk. De voornaamste beperking in deze gevallen is de kleine hoeveelheid ATP (4 moleculen ATP per molecuul sacharose) die gevormd wordt tijdens de anaërobe dissimilatie van sacharose door *S. cerevisiae*. Het verhogen de conservering van vrije energie tijdens sacharosestofwisseling is daarom een zeer relevante uitdaging in de gist-biotechnologie. Het onderzoek beschreven in dit proefschrift heeft als doel om deze uitdaging aan te gaan door nieuwe technieken en strategieën voor metabole aanpassingen te ontwikkelen om het energiebehoud te vergroten tijdens de omzetting van sacharose in gist.

Hoofdstuk 1 schetst de achtergrond voor het experimentele werk dat wordt omschreven in de andere hoofdstukken, door een overzicht te geven van de industriële relevantie, moleculaire biologie en fysiologische aspecten van sacharosestofwisseling in gist.

Hoofdstuk 2 beschrijft de constructie van een nieuwe experimentele platform-giststam om onderzoek te doen naar disacharidestofwisseling in *S. cerevisiae.* Om een stam achtergrond te maken waarin alternatieve routes van sacharosestofwisseling experimenteel geëvalueerd kunnen worden, zijn alle transporters en hydrolases in de gist die betrokken kunnen zijn bij

sacharoseconsumptie geïdentificeerd en, voor de eerste keer, volledig verwijderd in een enkele *S. cerevisiae*-stam. De uiteindelijke stam vormt een uniek "chassis" voor het gericht aanpassen van de stofwisseling, ook wel "metabolic engineering" genoemd. Niet alleen voor de stofwisseling van sacharose – zoals geïllustreerd in de volgende hoofdstukken van dit proefschrift – maar ook van andere industrieel relevante oligosachariden zoals maltose, isomaltose en maltotriose. Om deze chassis-stam te construeren was het noodzakelijk om een complete "gen-familie" van isomaltose-stofwisselingsgenen te verwijderen. Dit doel werd bereikt met CRISPR-Cas9-technologie, waarbij er op meerdere genen tegelijk gericht werd met hetzelfde Cas9-gids-RNA. Met als resultaat, dat 6 isomaltase-coderende genen in een enkele transformatiestap werden verwijderd. De technologie om het genoom te veranderen zoals beschreven in dit Hoofdstuk, heeft de potentie om te worden toegepast op andere gen-families in gisten en andere organismen.

Het doel om de ATP-opbrengst van sacharosestofwisseling te vergroten werd nagestreefd in **Hoofdstuk 3**. De oorspronkelijke sacharosestofwisseling in *S. cerevisiae* verloopt voornamelijk via de extracellulaire hydrolyse in glucose (druivensuiker) en fructose (vruchtensuiker). Bij deze hydrolyse komt ongeveer 29 kJ per mol sacharose vrij en de daaropvolgende afbraak resulteert in een nettoproductie van 4 moleculen ATP per sacharose- molecuul. Verschillende anaërobe bacteriën conserveren de vrij energie van de sacharose-splitsingsreactie door middel van de expressie van een sacharose fosforylase (SPase). SPases splitsen sacharose in fructose en glucose-1-fosfaat, waarbij anorganisch fosfaat gebruikt wordt. Op die manier wordt 1 ATP bespaard, aangezien de activatie van glucose door hexokinase niet meer nodig is. In **Hoofdstuk 3** wordt de expressie van een bacterieel SPase in *S. cerevisiae* gecombineerd met de expressie van heterologe membraan-transporteiwitten die, in theorie, functioneren als sacharose-uniporters. Het vervangen van het oorspronkelijke sacharose-proton-symport mechanisme van *S. cerevisiae* door een energie-onafhankelijke sacharose- uniporter is belangrijk. Zonder deze aanpassing

zullen stammen die SPase tot expressie brengen namelijk niet meer energie conserveren dan wildtype *S. cerevisiae*, aangezien 1 ATP geconsumeerd wordt voor elk proton dat de cel binnenkomt via de oorspronkelijke sacharose/H^+ symporters. Van de vijf heterologe transporteiwitten getest in **Hoofdstuk 3**, maakte alleen de mogelijke Sacharose Facilitator 1 van *Phaseolus vulgaris* (*Pv*Suf1) groei mogelijk van de disacharide-negatieve chassisstam omschreven in **Hoofdstuk 2**, indien samen met een bacterieel SPase tot expressie gebracht. De geschatte ATP-opbrengst van sacharose- fermentatie in deze *PvSUF1-SPase* stam, gebaseerd op biomassa-opbrengsten in anaërobe sacharose-gelimiteerde chemostaatcultures, lag 8% hoger dan die van een referentiestam, waarin sacharose formatie 4 ATP/sacharose opbracht. Vervolgexperimenten werden uitgevoerd om te onderzoeken waarom de waargenomen toename in energiebehoud lager was dan de verwachte 25% toename (5 in plaats van 4 ATP per sacharose). De resultaten van deze experimenten gaven aan dat, in gist, *Pv*Suf1 niet (alleen) als sacharose-facilitator functioneert, maar ook duidelijk sacharose-proton-symportactiviteit toont.

Hoofstuk 4 beschrijft een laboratorium-evolutiestudie voor de functionele expressie van de plantaardige sacharosetransporter *Pv*Suf1 in *S. cerevisiae*. Het onderzoek beschreven in dit Hoofdstuk had 3 hoofddoelen: i) het verhogen van de specifieke groeisnelheid in anaërobe, cultures op sacharose van de eerder geconstrueerde *Pv*Suf1-afhankelijke stammen, ii) het onderzoeken van de ATP-opbrengst in de geëvolueerde, sneller groeiende stammen en iii) het identificeren van oorzakelijke mutaties voor de geobserveerde snellere groei van geëvolueerde stammen. Na evolutie in het laboratorium, was de groeikinetiek op sacharose sterk verbeterd. In onafhankelijk van elkaar geëvolueerde stammen werden mutaties geïdentificeerd in zowel het oorspronkelijke gistgenoom als in de coderende regio van *PvSUF1*. Via reverse engineering werd aangetoond dat enkele mutaties in *PvSUF1* genoeg waren om snelle groei op sacharose mogelijk te maken, zonder bijkomende mutaties in het gistgenoom. De gemuteerde *PvSUF1*- allelen, verkregen na laboratoriumevolutie, hadden allemaal

sacharose/H^+ symport activiteit. Schattingen van de biomassa-opbrengst in chemostaten waren in lijn met het actieve transportmechanisme waargenomen voor de gemuteerde *PvSUF1* allelen. Hoewel, op basis van deze heterologe expressiestudie, geen definitieve conclusies getrokken konden worden over de energiekoppeling van *Pv*Suf1 in zijn natuurlijke context, rechtvaardigen de resultaten gepresenteerd in de **Hoofdstukken 3** en **4** een nieuw onderzoek naar proton-symport door deze transporter *in planta*.

"Metabolic engineering" en "evolutionary engineering" zijn in dit proefschrift gebruikt om de ATP-opbrengst van sacharosestofwisseling te verhogen en om de kinetiek van sacharose-opname via planttransporters in gist te verbeteren. De ontwikkelde stammen en kennis vormen een waardevolle basis voor vervolgonderzoek om de kosten te verlagen van processen waarin gist gebruikt wordt om producten te maken die ATP vereisen. De hoofdbijdrages zijn: *i)* het succesvol vervangen van sucrose hydrolyse door fosforolyse en het laten zien van de effecten van deze verandering op cellulaire ATP-opbrengst; *ii)* demonstratie van een complete gen-familie-verwijdering in gist door middel van een enkel Cas9-gids-RNA; *iii)* een nieuwe *S. cerevisiae* platformstam zonder enige activiteit van transporters en hydrolases nodig voor de stofwisseling van sacharose (en mogelijk andere disachariden); *iv)* een set mutaties die de functionele expressie van de plantaardige transporter *PvSUF1* vergroten in gistcellen. Verdere bestudering van de mutaties gevonden in dit proefschrift (voornamelijk in *Pv*Suf1), het onderzoeken en identificeren van nieuwe suiker-kanalen, en het aanpassen van transporters kunnen bijdragen aan vervolgonderzoek om energie-efficiëntere stammen voor de industrie te leveren.

1

General Introduction

Sucrose and *Saccharomyces cerevisiae*: a relationship most sweet.

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Abstract

Sucrose is an abundant, readily available and inexpensive substrate for industrial biotechnology processes and its use is demonstrated with much success in the production of fuel ethanol in Brazil. *Saccharomyces cerevisiae*, which naturally evolved to efficiently consume sugars such as sucrose, is one of the most important cell factories due to its robustness, stress tolerance, genetic accessibility, simple nutrient requirements and long history as an industrial workhorse. This minireview is focused on sucrose metabolism in *S. cerevisiae*, a rather unexplored subject in the scientific literature. An analysis of sucrose availability in nature and yeast sugar metabolism was performed, in order to understand the molecular background that makes *S. cerevisiae* consume this sugar efficiently. A historical overview on the use of sucrose and *S. cerevisiae* by humans is also presented considering sugar cane and sugarbeet as the main sources of this carbohydrate. Physiological aspects of sucrose consumption are compared with those concerning other economically relevant sugars. Also, metabolic engineering efforts to alter sucrose catabolism are presented in a chronological manner. In spite of its extensive use in yeast-based industries, a lot of basic and applied research on sucrose metabolism is imperative, mainly in fields such as genetics, physiology and metabolic engineering.

INTRODUCTION

Yeasts are the major producers of biotechnology products worldwide, exceeding production by any other group of industrial microorganisms. In this scenario, *Saccharomyces cerevisiae* is the principal cell factory, which is mainly due to: a long history of safe use, and consequently its Generally Regarded As Safe (FDA, USA) status; an extensive understanding of its physiology; and the availability of genetic systems for cloning and expression (Demain, Phaff and Kurtzman 2011). *S. cerevisiae* was the first eukaryotic cell that had its complete genome sequenced (Goffeau *et al*. 1996) and also the first eukaryote for which an in silico genome scale metabolic model was reconstructed (Förster *et al*. 2003).

Besides its use in the food and beverage markets, *S. cerevisiae* is also applied for the production of heterologous proteins, pharmaceuticals, bulk and fine chemicals (Bekatorou, Psarianos and Koutinas 2006; Hensing *et al*. 1995; Ro *et al*. 2006). A frequent bottleneck in these bioprocesses is substrate cost, which can overshadow product advantage, especially when petroleum-derived products are the competitors (Abbott *et al*. 2009).

In tropical countries, such as Brazil, sucrose obtained from sugar cane has been used as a substrate in biorefineries for several decades. The Brazilian fuel ethanol industry successfully demonstrates the cost effectiveness of cane sugar (UNICA 2013). Despite recent progress in second generation fuel ethanol, in which lignocellulosic hydrolysates are used as a substrate, sucrose still remains as a preferred and abundant carbon and energy source for yeast fermentations, in great part due to its low price, when compared to other substrates (Maiorella *et al.* 2009; Gombert and van Maris 2015). Nevertheless, there are still a number of scientific challenges in sucrose fermentation that remain to be addressed. These challenges and the recent scientific achievements in this field constitute the focus of this minireview, as well as a brief history of sucrose and yeast usage by humankind.

SUCROSE IN NATURE AND IN HUMAN SOCIETY

Natural occurrence of sucrose and its role in nature

Sucrose (α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside) is the most abundant free low molecular weight carbohydrate in the world (Peters, Rose and Moser 2010). It can be synthesized by a wide range of organisms including some prokaryotes (photosynthetic proteobacteria, cyanobacteria, planctomycetes and firmicutes) (Khmelenina *et al*. 2000; Reed and Stewart 1985; MacRae and Lunn 2012) and eukaryotes (single-celled photosynthetic protists and green plants) (Porchia and Salerno, 1996). Two enzymes are essential for sucrose biosynthesis: sucrose-phosphate synthase (SPS, EC 2.4.1.14) and sucrose phosphatase (SPP, EC 3.1.3.24) (**Figure 1a**). SPS synthesizes sucrose 6-phosphate from fructose 6-phosphate (an intermediate from the Calvin-Benson cycle) and a nucleoside-diphosphoglucose (usually UDP-glucose, which can be obtained from fructose 6-phosphate). Next, SPP hydrolyses sucrose 6-phosphate into orthophosphate and sucrose (MacRae and Lunn 2012). Besides SPS and SPP, there is another enzyme that can synthesize sucrose called Sucrose synthase (SuSy; EC 2.4.1.13). SuSy catalyses the reversible synthesis of sucrose from NDP-glucose and fructose (**Figure 1b**). However, in general, this enzyme acts towards sucrose cleavage without major impacts for photosynthetic sucrose synthesis (Geigenberger and Stitt 1993; Ruan Y-L, 2014).

The main roles played by sucrose in biological systems are related to osmoregulation, tolerance to temperature and desiccation, cell signalling and carbon transport and storage (MacRae and Lunn 2008). Mutant cyanobacteria that are unable to synthesize sucrose are still viable. However, in green plants, sucrose biosynthesis is a prerequisite for life (Salerno and Curatti 2003). This is the reason why sucrose is widespread in Viridiplantae (green algae and the land plants which evolved from them). It can be found in green algae (e.g. Chlorophyceae and Ulvophyceae) (Winkenbach, Grant and Bidwell 1972; Salerno, 1985a; 1985b, Kolman 2015) and in Streptophyta (e.g. Charales and

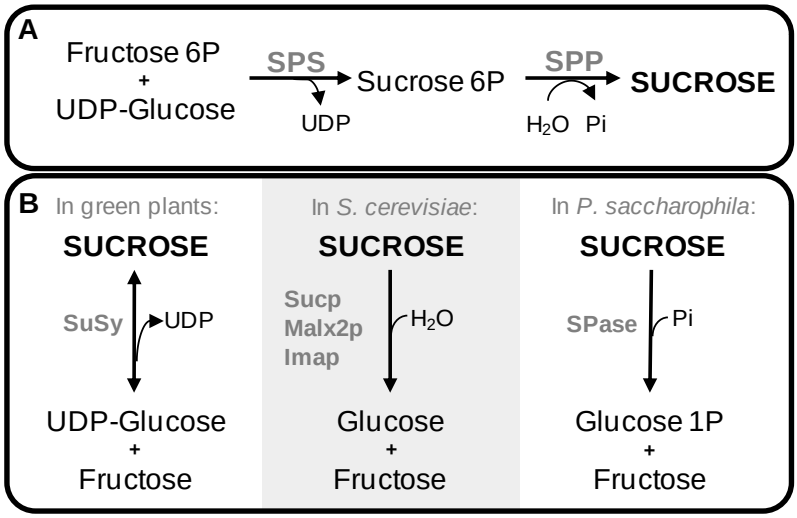

Figure 1: Sucrose biosynthesis and cleavage. **A**) Biosynthesis: two enzymes are essential: SPS and SPP. These enzymes probably originated in bacteria and were transferred to plants through the cyanobacterial ancestor of chloroplasts (MacRae and Lunn 2012). **B**) Sucrose cleavage via sucrose synthase (SuSy) in green plants; via hydrolases in *S. cerevisiae* (e.g. Sucp; Malx2p and Imap) and via phosphorolysis in bacteria such as *P. saccharophila* (SPase: sucrose phosphory- lase).

Embryophytes) (Macrae and Lunn 2012). In Bryophytes, for instance, this disaccharide protects the organism against desiccation (Smirnoff, 1992).

Among Tracheophyta, Monilioformopses (ferns and their allies) have genes related to sucrose synthesis (Hawker and Smith 1984). Also, sucrose metabolism in Gymnosperms is barely studied, a notable exception being the conifers where sucrose synthesis and degradation are tightly related to seasonal changes (Egger *et al*. 1996). On the other hand, studies in Angiosperms have revealed sucrose as the major form of carbon transport among plant tissues (Ayre 2011; Macrae and Lunn 2012). The physicochemical properties of sucrose could be the reason for this preference (Kühn *et al*. 1999). The viscosity of sucrose solutions is low even in highly concentrated solutions (e.g. phloem sap, 200 to 1600 mM), allowing high translocation rates (0.5 to 3 m h^{-1}). Since sucrose is a non-reducing sugar, it can be accumulated in high amounts inside the cells, without reacting with

proteins or other molecules, as do reducing sugars such as maltose, glucose or fructose. One possible disadvantage could be the size of the molecule, i.e. only a few carbon atoms are transported, since sucrose is a disaccharide and not a larger polymer. However, this is compensated by the high osmotic potential created at similar weight/volume ratios, thereby increasing phloem transport efficiency (Lang 1978; Kühn *et al*. 1999; van Bell, 1999). A more detailed review on sucrose biosynthesis is well described by MacRae and Lunn (2008; 2012).

Sucrose and human society

According to Shaffer (2001), sugar crystallization started around 350 AD in India. Originally from Southeast Asia, sugar cane (*Saccharum spp.)* was the first sucrose source utilized by humans and its domestication started about 8000 BC in New Guinea (Roach and Daniels 1987). Recent sugar cane varieties can accumulate up to 12–20% (w/w) sucrose in the internods (Linglea *et al*. 2009). In addition to its applications in cooking, sucrose was also used as a medicine to treat intestine, stomach and bladder pains by Greeks (UCLA, 2002). Later, during the Arab agricultural revolution in the 7th century, sugar production increased due to the advent of sugar mills and larger plantations (Watson 1974). During the crusades in the 11th century, sugar was brought to Europe, where it supplemented honey, the only sweetener available at that time. However, sucrose remained as a luxury product until its price decay was caused by the extensive and cheaper production in the New World in the 16th century (Mintz 1986).

In 1747, sucrose was first crystallized from sugar beet (*Beta vulgaris*) by the German scientist Andreas Marggraf (Marggraf 1747). Soon after, his student, Franz K. Achard, built the first sugar factory based on this temperate-climate crop (Achard 1799; Wolff 1953). The sucrose content of sugar beet is about 16-19% (w/w) and the world average yield of harvested sugar beet is around 60 metric tons per hectare. Each hectare produces approximately 10-12 tonnes of sugar (Hoffmann 2010; FAO 2015a; CEFS 2013). The biggest sugar beet producer is the Russian Federation (39.3 million metric tons

harvested in 2013). By region, the European Union is the main producer of this cultivar with approximately 167 million metric tons harvested in 2013 (67.9 % of the world production) (FAO 2015a). Besides the edible sugar market, about 10% of the aforementioned amount is destined for the production of ethanol (ARD 2012). Despite the high ethanol yield (7,000 litres per hectare from sugar beet, compared to 5,000 litres/hectare from sugar cane and 3,000 litres/hectare from corn) (Nersesian 2010), sugar beet use in the ethanol industry remains "not promising" due to its costly and energy intensive processing, when compared to other European alternatives such as wheat and other cereals (Nersesian 2010; ARD 2012).

Sugar cane and sugarbeet constitute the main sources of edible sugar currently produced, with sugar cane accounting for approximately 80% of the world sugar production (ARD 2013). Besides these sources, date palm (*Phoenixdactylifera*), sorghum (*Sorghum vulgare*) and the sugar maple (*Acer saccharum*) are other minor commercial sugar crops (van Putten, Dias and de Jong 2013).

Brazil is the world leader in sugar and sugar cane production with more than 653 million tons harvested in the crop year 2013/2014, twice the amount produced by India, the second largest producer (Brazilian Sugar cane Industry Association 2015a; FAO 2015b). For the crop season 2015/2016, an increment of 18 million tons is expected due to more favourable rainy conditions. In Brazil, around 50% of the harvested sugar cane is used for producing edible sugar, and the rest is employed for fuel ethanol production (Brazilian Sugar cane Industry Association 2015b). Brazilian sugar cane plantations yield approximately 70-80 metric tons per hectare (Sugar cane Technology Center 2011). Concerns about the use of sugar cane to produce biofuels/biochemicals instead of food are still real and somewhat polemical. To assuage the critics, it is important to highlight that only 1.1% (≈ 9 million hectares) of the Brazilian territory is currently used for sugar cane plantation (UNICA 2013) and the latest national agro-ecological zoning reports the existence of additional 65 million hectares available for sugar cane culture, without making use of protected areas (e.g. Amazon forest) (Manzatto *et al*.

2009). Although Brazilian intellectual property regulations still require substantial improvements, Brazil has become a hot-spot for biotech industries due to the low cost of feedstock (mainly sucrose from sugar cane) by the well-established sugar cane crushing industry (Nielsen 2012).

NATURAL OCCURRENCE AND IMPORTANCE OF *Saccharomyces cerevisiae* IN HUMAN HISTORY

The Latin word "*Saccharomyces*" literally means "sugar fungus" and clarifies that this ascomycetous genus is preferentially found in sugar-rich environments (Gerke, Chen and Cohen 2006). *S. cerevisiae* in particular is characterized by a long history of co-existence with *Homo sapiens* due to its role in the manufacture of bread, wine, sake and beer, among others ("*cerevisiae*" is a Latin word for "of beer") (Schneiter 2004). Humans have gradually incorporated yeast in their diet, and *Bacteriodes thetaiotaomicron* (and a limited number of other *Bacteroidetes*) present in the human gut microbiota have evolved a complex machinery to metabolise the highly complex yeast cell-wall mannans. While most of the gut microbes target the components derived from the human diet, *Bacteriodes* digests the human domesticated and ingested yeasts, thereby contributing to the overall activity of the human microbiota and, consequently, to human health (Cuskin *et al*. 2015).

The DNA of *S. cerevisiae* was found in wine jars from the tomb of the King Scorpion, in Abydos (3,150 BC) (Cavalieri *et al*. 2003) and the earliest evidence for winemaking dates back to 7,000–5,500 BC from pots found in China (McGovern *et al*. 2004). This long history of domestication led to the concept that natural isolates of *S. cerevisiae* would be 'refugees from human-associated cultures' instead of truly "wild" exemplars (Mortimer, 2000; Plech, De Visser and Korona 2014). However, recent genomics studies provide strong evidence for the presence of "wild" *S. cerevisiae* in nature (Fay and Benavides 2005; Liti *et al*. 2009; Wang *et al*. 2012; Cromie *et al*. 2013; Leducq 2014; Plech, De Visser and Korona 2014). Wang *et al*. (2012) isolated *S.*

cerevisiae from environments close and far from human activity, and added eight new lineages (named CHN I to CHN VIII) to the five previously known "wild" strains (Liti *et al*. 2009). They show evidence that indicates primeval forests, situated in Far Eastern Asia, as the origin of the *S. cerevisiae* species. For instance, the oldest lineage CHN I and other basal ones (CHN II-V) were only found in China. In other words, these authors present evidence that any *S. cerevisiae* lineage associated to human activity worldwide was originated from wild lineages from China (Wang *et al*. 2012). In nature, *Saccharomyces cerevisiae* species can be isolated from a vast range of habitats such as oak and beech bark, plant exudates, soil underneath trees (e.g. forest and orchard soil) (Bowles and Lachance 1983; Sniegowski, Dombrowski and Fingerman 2002; Fay and Benavides 2005; Sampaio and Gonçalves 2008); in fruits (e.g. fig, Lychee), in flower nectars (e.g. from Bertram palm; Liti *et al.* 2009), in rotten wood (Wang *et al*. 2012), in stromata from the obligate tree parasite ascomycetes (e.g. *Cyttaria hariotii*, since their fructifying body is rich in sugars; Libkind *et al*. 2011), in the intestines of insects (Stefanini *et al*. 2012), in human infections (Wheeler *et al*. 2003; Muller *et al.* 2011), etc. According to Goddard and Greig (2015), this vast range of habitats points towards a nomad model to understand yeast ecology. In support to this model is the highly diverse tolerance spectrum of yeasts, towards, for instance, pH, osmolarity and temperature (Serrano *et al*. 2006; Petrovska, Winkelhausen and Kuzmanova 1999; Salvado *et al*. 2011), as well as the low density of *S. cerevisiae* in habitats such as fruits and oak barks, which contradicts the idea that these might be the species' niche (Taylor *et al*. 2014; Kowallik, Miller and Greig 2015). Therefore, it is perfectly possible that *S. cerevisiae* is a "nomad, able to survive as a generalist at low abundance in a vast ranges of habitats" (Goddard and Greig 2015).

S. cerevisiae, as other strains in the same genus, is capable of consuming several different substrates as carbon sources (e.g. sucrose, maltose, glycerol, ethanol, etc) (Samani *et al*. 2015). Opulente *et al*. (2013) compared patterns of sugar consumption and structure of metabolic pathways in 488 different *Saccharomyces* strains. Based on this, the authors were able to

"partially predict" the substrate specificity of a strain based on the environment from which it was isolated (Opulente *et al*. 2013). Because *S. cerevisiae* has the metabolic capacity for sucrose consumption (Grossmann and Zimmermann 1979), one of the main questions that arise is: where, in nature, does *S. cerevisiae* feed on sucrose? Experiments with plants show accumulation of sucrose in wounded tissues, rather than other sugars such as glucose and fructose (Shmidt *et al*. 2009; van Dam and Oomen 2008). During certain periods, when glucose sources such as fruits and flower nectar are not available, yeasts could grow on sucrose present in plant exudates (e.g. as a consequence of insect damage), as speculated by Lemaire *et al*. (2004). Furthermore, it is also possible that *S. cerevisiae* spores remain dormant until the environmental conditions get favourable again. According to Neiman (2011), the ecological role of sporulation might be related to yeast dispersion via insects as vectors.

SUCROSE AS AN IMPORTANT INDUSTRIAL SUBSTRATE FOR *Saccharomyces cerevisiae*

Besides its use as sweetener, sucrose has been explored by humans as an industrial substrate for the microbial production of different compounds/products or, in some cases, the yeast itself is the desired product. Around 400 million kilograms of yeast biomass are produced each year worldwide (Gómez-Pastor, Pérez-Torrado and Matallana 2011). Industrial production and commercialisation of yeast started at the end of the 19th century, after being intensively studied by Louis Pasteur, who first demonstrated the role of yeast in alcoholic fermentation (Pasteur 1857). Today, yeast cells (in different formulations) are used as animal feed, in the bakery and fermentation industries (brewing, beverages, biofuels, pharmaceutical, enzymes and chemicals) (Bekatorou, Psarianos and Koutinas 2006; Swanson and Fahey 2004).

More than 4000 years ago, in ancient Egypt, yeast fermentation was already employed to leaven bread (Sugihara 1985). Today, *S. cerevisiae* is

employed in the bakery industries all over the world. In many cases, sucrose is added to the dough up to 30 % (w/w), causing a collateral osmotic stress (Sasano *et al*. 2012). Besides osmotolerance, other important traits of yeast in bread making processes have been the object of intensive research in the recent years, such as rapid fermentation rates, capacity to endure freeze-thawing stress and production of large amounts of CO_2 (Randez-Gil, Córcoles-Sáez and Prieto 2013).

The main advantages of *S. cerevisiae* as a host for the production of heterologous enzymes are correct protein folding, post-translational modifications and efficient protein secretion (Mattanovich *et al*. 2012; Nielsen 2013), as demonstrated in the production of insulin by Novo Nordisk. Despite the advantages mentioned above, the following disadvantages could limit its extensive use as protein factory: *i*) high-mannose type N-glycosylation, which results in a reduced half-life of the glycoprotein *in vivo*, which prejudices its therapeutic use (Nielsen, 2013); *ii*) retention of the exported protein in the periplasmic space; *iii*) *S. cerevisiae* metabolism is preferentially fermentative (Crabtree effect, discussed further below), which prejudices biomass propagation (Nevoigt 2008).

Besides its use in the production of recombinant proteins, *S. cerevisiae* is also an attractive industrial host for fine and bulk chemicals production. Compared to chemical synthesis or extraction from nature, industrial microbiology requires less energy input; has decreased generation of toxic wastes and, most importantly, is based on renewable feedstock utilization (Demain, Phaff and Kurtzman 2011). Lactic acid production, for instance, is carried out using fermentation with lactic acid bacteria. However, pH control represents a considerable manufacturing cost in these processes (Bozell and Petersen 2010). Due to its higher physiological activity in acidic conditions, *S. cerevisiae* is a great alternative for the production of lactic and other organic acids (van Maris *et al*. 2004; Abbott *et al*. 2009). Another example is succinic acid, which has a market size around US$ 7 billion and recently started to be produced with engineered *S. cerevisiae* to compete with petroleum

counterparts (Jansen, van de Graaf and Verwaal 2012; Reverdia 2012; Myriant 2012).

Fuel ethanol production is, by far, the largest industrial activity that uses sucrose as a substrate for yeast fermentation (at least in Brazil). Sugar cane juice contains by weight 8-20% sucrose and 0.3-2.5% of reducing sugars, e.g. glucose and fructose (Basso, Basso and Rocha 2011; OECD 2011). Despite the high sugar concentration, sugar cane juice is deficient in phosphorous and nitrogen. The composition varies depending on the sugar cane variety and maturity, the soil composition, and the climate, as well as juice processing conditions (Curtin 1973; OECD 2011). In Brazilian industrial mills, sugar cane juice is also used for edible sugar production, which generates a sugar rich byproduct called "molasses". Molasses is composed of 45-60 % (w/w) sucrose, 5-20 % (w/w) glucose and fructose, low levels of phosphorus and high levels of minerals such as potassium and calcium, and some yeast growth inhibitors (Basso *et al*. 2011; OECD 2011). Molasses is diluted in water to a final sugar concentration of about 14-18% and added to the fermentation reactor in addition to sugar cane juice (Amorim *et al*. 2011).

Another example of a sucrose rich substrate already used in industry is sugar beet, which can be converted into ethanol (ARD 2012). According to Ogbonna, Mashima and Tanaka (2001), sugar beet juice (16.5% sucrose, w/w) is complete in nutrients required for *S. cerevisiae* growth and ethanol production, and inhibitory compounds are not present in detrimental levels.

MOLECULAR BACKGROUND OF SUCROSE CONSUMPTION IN *S. cerevisiae*

One key step in sucrose metabolism in *S. cerevisiae* is its cleavage by invertase (β-fructofuranosidase, EC 3.2.1.26) into glucose and fructose (**Figure 1b** and **Table 1**). Other organisms can cleave sucrose in different ways. Besides the reaction carried out by plant Sucrose Synthase (mentioned before, **Figure 1b**), some bacteria (e.g. *Pseudomonas saccharophila*) express Sucrose Phosphorylase, an enzyme that converts sucrose and inorganic

Table 1: *Saccharomyces cerevisiae* enzymes that hydrolyse sucrose.

Enzyme	K_{cat} (s^{-1})	K_m (mM)	K_{cat}/K_m (mM^{-1} s^{-1})	Reference & assay conditions
Invertase (Suc2p) [a]	≈ 943.33	26.10 ± 2.00	≈ 36.14	Reddy & Maley (1996) pH 5.0 at 37 ºC
		≈26.58		Sanjay & Sugunan (2005) pH 5.0 at 30 ºC
Mal12p/Mal32p[b]	≈ 0.45	11.96 ± 0.6	≈ 0.04	Voordeckers *et al*. (2012) pH 6.0 at 30 ºC
Ima1p	51 ± 4	144 ± 26	0.35 ± 0,09	
Ima2p	55 ± 4	147 ± 24	0.38 ± 0.09	Deng *et al.* (2014)
Ima3p[c]	25 ± 1	116 ± 12	0.22 ± 0.03	pH 7.0 at 30 ºC
Ima5p	3.8 ± 0.2	191 ± 24	0.20±0.004	

[a] The authors do not specify which invertase(s), i.e. which gene-encoded proteins, were assayed. It maybe *SUC2* and/or its paralogs depending on the yeast strain used.
[b] Mal12p and Mal32p have similar hydrolytic parameters because they are 99.7% identical atthe aminoacid level (Voordeckers *et al*., 2012).
[c] *IMA3's* ORF is 100 % identical to *IMA4's* at the nucleotide level (Teste *et al*., 2010).

phosphate into fructose and glucose 1-phosphate (**Figure 1b**) (Weimberg and Doudoroff 1954; Goedl *et al.* 2010).

S. cerevisiae's invertase was already studied more than 100 years ago and was the enzyme used by Michaelis and Menten for their classic paper "*Die Kinetik der Invertinwirkung*" (Berthelot 1860; Brown 1902; Michaelis and Menten 1913; Johnson and Goody 2011). This enzyme is named invertase because the hydrolysis of sucrose causes an inversion of optical rotation in the sugar solution, from positive to negative. The easiness of optical rotation determination is the reason why invertase was already studied during the early 20th century. Besides sucrose, invertase can also hydrolyse raffinose (α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside) producing fructose and melibiose (α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside), and the polysaccharide inulin (linear chains of β-2,1-linked D-fructofuranose molecules terminated by a glucose residue) (Gascón and Lampen 1968; Wang and Li 2013; Yang *et al*. 2015). Yeast invertase has also a low transfructosylating activity, allowing the synthesis of fructo-oligosaccharides from sucrose (Lafraya *et al*. 2011).

In *S. cerevisiae* sucrose consumption starts with its hydrolysis by invertase in the periplasmic space (outside of the cells, between the cell wall and the cytoplasmic membrane). Subsequently, the monosaccharides (glucose and fructose) enter the cells by facilitated diffusion and become available for their intracellular phosphorylation by gluco- and hexokinases, which corresponds to the first enzymatic step in the classical Embden-Meyerhof-Parnas glycolytic pathway.

Yeast invertase is encoded by the so-called *SUC* genes, which constitute a gene family originally identified by Winge and Roberts (1952) and later confirmed by Hawthorne (1955). Nine *SUC* genes (*SUC1-SUC5, SUC7-SUC10*) have been already found in telomeric *loci* in different chromosomes and *S. cerevisiae* strains (Korshunova, Naumova and Naumov 2005; Naumov and Naumova 2010). *SUC2* is the only one positioned in a sub-telomeric region (left end of chromosome IX; however, this position can vary according to the strain, Naumov and Naumova 2011), and is postulated as the ancestral gene since it can be found in every *S. cerevisiae* strain, as well as in other *Saccharomyces* yeasts, such as *S. paradoxus* (Carlson and Botstein 1983; Naumov *et al*. 1996). Nevertheless, there is significant sequence variation in the *SUC2* gene from these *Saccharomyces* yeasts, and this sequence variation has been proposed as a method to identify different yeast strains (Oda *et al.* 2010).

The molecular characterization of five *SUC* genes (*SUC1-SUC5*) present in different *S. cerevisiae* strains revealed that all these genes encode functional invertases (Grossmann and Zimmermann 1979; Hohmann and Zimmermann 1986). Regarding the other *SUC* genes (*SUC7-SUC10*), they have been only studied at the genetic level (chromosomal location and gene nucleotide sequence). The results published recently by Naumova and co-workers (2014) show that while the sequences of *SUC2* from 17 different *S. cerevisiae* strains have 98.9-100% similarity, in the case of the other telomeric invertase genes the one closer to *SUC2* is *SUC1* (95.4-95.6% identity), while the other *SUC* genes (*SUC3-SUC5* and *SUC7-SUC10*) are 99.4-100% identical to each other and have a similarity of 92.3-95.6% to *SUC2*. Their data also show that *SUC3*

and *SUC5* have identical nucleotide sequences. All other *Saccharomyces* yeasts (*S. arboricola*, *S. bayanus*, *S. cariocanus*, *S. paradoxus*, *S. kudriavzevii*, and *S. mikatae*) seem to have a single invertase gene with an overall 88.0-99.8% identity. All these *SUC* genes seem to encode functional invertases, since most nucleotide polymorphisms are silent (Naumova *et al*. 2014).

Baker's, brewer's and distiller´s yeasts were found to contain multiple copies of *SUC* genes, and it was postulated that this reflects an adaptation to sucrose-rich broths (Codón, Benítez and Korhola 1998; Naumova *et al*. 2013). However, the Brazilian industrial fuel ethanol yeast strains (e.g. BG-1, CAT-1, PE-2, SA-1, and VR-1, Stambuk *et al.* 2009; Babrzadeh *et al*. 2012) and wine strains contain only one copy of *SUC2*, such as the laboratory strains S288c and those from the CEN.PK family (Carlson and Botstein 1983). According to Stambuk *et al.* (2009), invertase activity in these sugar cane industrial strains is probably not a limiting step in sucrose catabolism.

Besides secreted invertases, *S. cerevisiae* also produces cytosolic forms of invertase. The *SUC2* gene can be transcribed into two different mRNAs that differ in their 5' ends, with lengths 1.9 Kb and 1.8 Kb, respectively. The longer one includes the coding sequence for a signal peptide (20 amino acids) that directs the protein into the secretory pathway (Carlson and Botstein, 1982; Perlman, Halvorson and Cannon 1982; Hohmann and Gozalbo 1988). Both invertase types behave similarly with respect to pH and temperature, with optima in the range of pH 4.6-5.0 and 35-50 ºC (Gascón and Lampen 1968). The intracellular form is a homodimer that weighs about 120-135 kDa. The extracellular form is also a homodimer, which aggregates into tetramers, hexamers and/or octamers. Glycosylation occurs only in the extracellular form and contributes to 50% of the protein mass, which is about 240-270 kDa for the homodimer (Gascón and Lampen 1968; Gascón, Neumann and Lampen 1968; Trimble and Maley 1977; Deryabin *et al*. 2014). Glycosylation renders invertase resistant to attack by proteases, allows proper protein oligomerization, and traps this enzyme between the plasma membrane and the cell wall (Esmon *et al*. 1987; Tammi *et al*. 1987; Reddy *et al*. 1988).

Invertase belongs to family 32 of the glycoside hydrolases (GH32) that includes inulinases, levanases and transglycosylases with fructose transferase activity (Cantarel *et al*. 2009). GH32 enzymes have a characteristic N-terminal 5-fold β–propeller catalytic domain surrounding a central negatively charged active site cavity, and an additional β–sandwich domain appended to the catalytic domain. An aspartate located close to the N-terminus acts as the catalytic nucleophile and a glutamate acts as the general acid/base catalyst. Despite the long history of research on yeast invertase, the high degree of glycosylation of this enzyme challenged the determination of the crystal structure of the protein (Sainz-Polo *et al.* 2012, 2013). The molecular mass of the purified intracellular invertase (expressed in *E. coli*) is 428 kDa, consistent with an octamer association which is best described as a tetramer of dimers that oligomerize by inter-subunit extension of the two β–sheets that end in the β–sandwich domain within each subunit. The intracellular enzyme has two classes of dimers ("open" and "closed") located at opposite vertices of the octameric rectangle. The "closed" dimers form a more narrowed pocket at the active site (when compared to the "open" domains), and are unable to accommodate oligosaccharides with more than 3 or 4 sugar units. Interestingly, the model for the extracellular invertase predicts an octameric aggregate of only "closed" dimers, which may explain its predominant invertase (and not inulinase) character at the molecular level (Sainz-Polo *et al.* 2012, 2013).

The utilization of sucrose by *S. cerevisiae* was also a nice model to unravel the complex regulation of glucose repressible genes in yeast. Mutants defective in sucrose utilization were isolated by Carlson and co-workers in 1981, and besides mutations in the *SUC2* gene, these authors were able to isolate also several new *snf*- (*s*ucrose *n*on-*f*ermenting) mutants (Carlson, Osmond and Botstein 1981; Neigeborn and Carlson 1984) that were shown to play key roles in glucose repression, including *SNF1*, a protein kinase required for transcription of glucose-repressed genes and several other metabolic functions in yeast (Celenza and Carlson 1984); *SNF2*, *SNF5* and *SNF6* that are part of the chromatin remodeling complex involved in transcriptional

regulation (Laurent, Treitel and Carlson 1991); *SNF3*, which encodes for a low affinity glucose sensor, with homology to sugar transporters, that regulates *HXT* gene expression (Özcan *et al*. 1996); and *SNF4*, part of the Snf1p kinase complex (Celenza, Eng and Carlson 1989).

Indeed, transcriptional regulation of *SUC2* is complex. Intracellular invertase is expressed constitutively at low levels, while extracellular invertase is subjected to glucose repression (Carlson and Botstein 1982). The repressors that have been shown to bind to the *SUC2* promoter are Rgt1 (inactivated through phosphorylation by Snf3/Rgt2 in the presence of glucose), Mig1/Mig2 (inactivated through phosphorylation by Snf1 under low glucose concentration); Sfl1 (inactivated through phosphorylation by Tpk2 under low glucose concentration); and, less important, there is Sko1, which weakly binds to the *SUC2* promoter. Sko1 is inactivated through phosphorylation, at the end of the HOG pathway, only under high glucose concentration. Sko1 represses the glucose transporter gene *HXT1* in the absence or at low glucose. The mentioned repressors have to bind Cyc8-Tup1 to be active, besides extensive chromatin remodelling carried out by the SWI/SNF complex (Belinchón and Gancedo 2007; Trumbly 1992; Gancedo 1992; 1998; 2008; Bendrioua *et al.* 2014; Geng and Laurent 2004; Weinhandl *et al.* 2014). The role played by low amounts of glucose in the inactivation of some of these repressors is in agreement with data reported by Özcan *et al*. (1997). Their experiments show that *SUC2* expression is about 5-10 fold higher in the presence of low glucose or fructose concentration (0.1% w/v), than in the absence of these sugars. Although an activator of *SUC* genes is predicted in all models of gene regulation, up to now the identity of such transcriptional activator is still unknown (Belinchón and Gancedo 2007). Dynamic regulation of gene expression using sucrose is much desired in industries where sugar cane is the feedstock. To address this issue, Williams *et al*. (2015a) identified four genes which are differentially regulated by sucrose. Employing a heterologous RNA interference module, overexpression/repression of promoter-GFP fusion was achieved using sucrose as an inducer (Williams *et al.* 2015b).

From an ecological point of view, *SUC2* regulation is a classic example of an optimised strategy for the efficient consumption of mixed substrates. When glucose is not abundant or even absent in nature, *SUC2* expression is probably at a basal level (**Figure 2**). In an environment rich in sucrose, this basal invertase activity could be sufficient to create a low concentration of glucose/fructose around the cells which may cause maximum expression of *SUC2*. Besides the absolute concentration of glucose/fructose, temporal changes in concentration are also connected to *SUC2* expression. In other words, *S. cerevisiae* only maximises the induction of genes related to glucose/fructose consumption if it is able to utilise them (Özcan *et al*. 1997; Bendrioua *et al.* 2014). On the contrary, when glucose/fructose accumulate above a certain threshold (2.5-3.2 g/L), *SUC2* is repressed leading to the consumption of the hexoses already available (Meijer *et al*. 1998; Elbing *et al*. 2004). These opposite effects exerted by glucose balance invertase levels and optimise sugar consumption in *S. cerevisiae* (Özcan *et al*. 1997).

The extracellular hydrolysis of sucrose has been extensively studied as an interesting model for social microbial behaviour, its dynamics and evolution. The secretion of a public good (invertase) by cooperators (e.g. a *SUC2* yeast strain) allows the hydrolysis of sucrose, producing glucose and fructose that diffuses away from the cooperator cell and can be consumed by other cells of the population, including cheaters (e.g. a *suc2*Δ yeast strain) that will not have the metabolic cost of synthesizing the enzyme (Greig and Travisano 2004; Gore, Youk and van Oudenaarden 2009). Consequently, in well mixed batch cultures, cheaters can exploit the public good and invade populations of cooperators, depending on factors such as cell density and frequency, spatial population expansion, presence of other species of cheaters (e.g. *E. coli* bacteria), presence of environmental stresses, and sucrose concentration (Greig and Travisano 2004; MacLean and Brandon 2008; Gore, Youk and van Oudenaarden 2009; MaClean *et al.* 2010; Koschwanez, Foster and Murray 2011; Celiker and Gore 2012; Dai *et al.* 2012; Damore and Gore 2012; Dai, Korolev and Gore 2013; Van Dyken *et al.* 2013; Sanchez and Gore 2013). However, the real significance of this “social trait” in natural

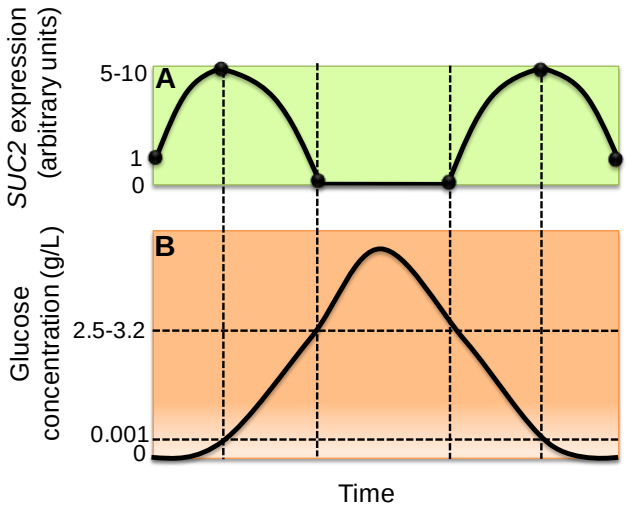

Figure 2. Dynamics of invertase expression **(A)** according to the amount of glucose released extracellularly **(B)** in a sucrose-rich environment. Initially, when glucose is zero, *SUC2* is expressed at a basal level causing glucose release. *SUC2* achieves its maximum expression when extracellular hexoses are around 0.001 g/L. At an extracellular glucose concentration higher than 2.5-3.2 g/L, *SUC2* is repressed. When the concentration of hexoses decreases again, *SUC2* repression is relieved and may return to its maximum expression levels, if hexose concentration decreases to very low levels (<0.001 g/L). Based on values reported by Ozcan *et al*.(1997) and Meijer *et al*. (1998).

populations of *Saccharomyces* has been recently challenged since a survey of over one hundred wild yeast isolates (80 strains of *S. paradoxus* and 30 strains of *S. cerevisiae*) revealed no cheater strains (Bozdag and Greig 2014). All the strains had significantly high levels of invertase activity; all *S. paradoxus* strains had only the *SUC2* gene in chromosome IX, while from the *S. cerevisiae* wild yeasts only 3 strains (isolated from sucrose-rich palm nectars) had additional *SUC* genes (*SUC3*, *SUC8* and *SUC9*), besides *SUC2*, which was present in all strains. Therefore, there is no evidence to support the idea that nonproducing cheaters may occur among wild *Saccharomyces* yeasts (Bozdag and Greig 2014).

Nevertheless, *S. cerevisiae* strains lacking invertase activity (*suc2* mutants) are still able to consume sucrose (Badotti *et al*. 2006; 2008), suggesting the existence of alternative genes allowing sucrose consumption. Known native *S. cerevisiae* hydrolases that act on sucrose are listed in **Table 1**. Since sucrose is also an α–glucoside, maltases (Mal12p and Mal31p) are as active on sucrose as they are on maltose (Zimmermann, Khan and Eaton 1973), but their catalytic efficiency (K_{cat}/K_m) is not as high as that of invertase (Reddy and Maley 1996). Isomaltases (Ima1-Ima5p), which share the same ancestry with maltases, can also hydrolyse sucrose (Brown, Murray and Verstrepen 2010; Voordeckers *et al*. 2012). Interestingly, Ima proteins are inhibited by high isomaltose concentrations, a phenomenon that does not occur when sucrose is the substrate (Deng *et al*. 2014).

Since maltases and isomaltases are intracellular proteins, sucrose must be transported into the cytoplasm to be hydrolysed by these enzymes. Stambuk *et al*. (1999) first demonstrated that a maltose proton symporter (encoded by the *AGT1* gene) can also transport sucrose, besides other α-glucosides (Han *et al*. 1995). Later, Stambuk, Batista and De Araujo (2000) determined the kinetics of active sucrose transport in *S. cerevisiae*, revealing the presence of a high-affinity (K_m= 7.9 ± 0.8 mM) sucrose transport activity mediated by Agt1p, and a low-affinity (K_m= 120 ± 20 mM) transport activity by the maltose transporters encoded by *MALx1* genes (*x* refers to the locus number). Each *MALx1* is located at a different telomere-associated *MAL* locus in the *S. cerevisiae* genome (Chow, Sollitti and Marmur 1989; Cheng and Michels 1991; Needleman, 1991; Naumov, Naumova and Michels 1994; Duval *et al*. 2010). According to the known regulation of *MAL* genes, no sucrose transport can be observed without addition of the inducer maltose to the medium, or the strain needs to be *MAL* constitutive to express the transporters and enzymes that will allow sucrose utilization (Badotti *et al*. 2006; 2008).

PHYSIOLOGY OF *S. cerevisiae* DURING GROWTH ON SUCROSE

Growth of *S. cerevisiae* on sucrose compared to other carbon sources

In industrial processes, *S. cerevisiae* is exposed to a variety of sugars other than sucrose. Grape must, for instance, is mainly composed by glucose and fructose (Fleet and Heard 1993). In the case of beer wort, maltose and maltotriose are also present, besides sucrose (Bamforth 2003). Even in sugar cane juice, which is predominantly composed by sucrose, glucose and fructose are also present (Wheals *et al*. 1999). Due to glucose repression, sugars other than glucose are only consumed after the depletion of this monosaccharide. Glucose activates signalling cascades that repress the transcription of genes necessary for the metabolism of other carbon sources (e.g. sucrose, maltose, galactose, ethanol, glycerol) (Trumbly 1992; Verstrepen *et al*. 2004; Kim *et al*. 2013). The presence of glucose in the medium is responsible for the so-called Crabtree effect in *S. cerevisiae* (De Deken 1966), meaning that under such conditions, even when the oxygen supply is abundant, cells perform fermentation instead of (or together with) respiration, which is a consequence of both glucose repression (described above) and insufficient respiratory capacity, also termed overflow metabolism at the level of pyruvate (Fiechter and Gmünder 1989). In spite of the lower amount of ATP obtained per mole of substrate consumed, the 'Crabtree' effect offers, at least, the following potential advantages to *S. cerevisiae*: *i*) consumption of glucose at higher rates, meaning that the sugar becomes less available for competing organisms in the same niche; *ii*) accumulation of ethanol to toxic levels, meaning that competing organism may be killed (*S. cerevisiae*'s tolerance to ethanol is one of its hallmarks) and that the accumulated ethanol may be later used by *S. cerevisiae* as a carbon and energy source, as long as oxygen is available for respiration (Pfeiffer, Schuster and Bonhoeffer 2001; Verstrepen *et al*. 2004; Piškur *et al*. 2006; Hagman *et al*. 2013).

Depending on the carbon source and strain, yeast physiology can vary. The specific growth rate of strain CEN.PK122 (diploid) on glucose ($\mu_{glucose} \approx 0.38\ h^{-1}$) is slightly lower than on sucrose ($\mu_{sucrose} \approx 0.41\ h^{-1}$) or maltose

($\mu_{maltose} \approx 0.40\ h^{-1}$), determined using shake-flask cultures (van Dijken *et al*. 2000). Accordingly, anaerobic batch cultures of the CEN.PK113-7D strain (haploid) also reveal faster growth on sucrose ($\mu_{sucrose} = 0.35 \pm 0.00\ h^{-1}$; T.O. Basso, unpublished data) than on glucose ($\mu_{glucose} = 0.30 \pm 0.01\ h^{-1}$; van Hoek, van Dijken and Pronk 2000). The major difference between sucrose and glucose metabolism relies on the extracellular hydrolysis of sucrose by invertase, but since the CEN.PK strains are *MAL* constitutive, the active sucrose transport described above might be responsible for the increased growth rate on sucrose, when compared to glucose.

Another probable and maybe more accurate explanation can be related to the G protein coupled receptor *GPR1*, which activates the cAMP signalling pathway, thereby increasing the glycolytic flux (Tamaki *et al*. 2007). Lemaire *at al*. (2004) demonstrated that this receptor has a higher affinity for sucrose than for glucose. In accordance with the above explanation, Badotti *et al*. (2008) also suggest the influence of *GPR1* on the faster growth of yeast on sucrose, as compared to glucose. From an ecological point of view, *GPR1* can be associated to *S. cerevisiae's* feast/famine cycles in nature. This receptor can be activated by low sucrose concentration in famine periods, and serve for the detection of high glucose concentration during periods of feast, when fruits and flower nectar are available (Lemaire *et al*. 2004).

Growth of *S. cerevisiae* in a medium containing both glucose and sucrose can be divided in four phases. In the first phase, glucose is fermented and no sucrose is consumed, the respiratory quotient (RQ = moles of CO_2 produced/moles of O_2 consumed) is high (RQ ≈ 9) because there is no significant consumption of O_2 (Raamsdonk *et al*. 2001). The second phase starts after glucose depletion and is characterised by sucrose fermentation, which slightly decreases the RQ value (RQ ≈ 6) because the glucose repression effect becomes less intense. Next, after sucrose depletion, the ethanol produced in the previous phases is consumed by respiration. In this stage, no fermentation takes place and the RQ drastically drops to 0.6, in agreement to the stoichiometry of ethanol respiration. At last, when ethanol is exhausted, the acetate previously produced is consumed increasing RQ to 1 accordingly

to acetate respiration stoichiometry (Raamsdonk *et al*. 2001; Dynesen *et al.*1998).

In agreement with the classical Embden–Meyerhof–Parnas pathway coupled to ethanolic fermentation, 2 ATPs are produced from each glucose converted into ethanol and CO_2 by *S. cerevisiae*. This would lead to an ATP yield of 4 ATPs per sucrose consumed. However, in the case of sugars actively transported into the cells, the real yield is only 3 ATPs, since 1 ATP is consumed by H^+-ATPase pumps to extrude the proton imported together with the disaccharide (Weusthuis *et al*. 1993). This difference in 1 ATP (25 % less than the 4 ATP/sucrose yield) can be detected in anaerobic cultures through the biomass yield on substrate, a parameter proportional to cell free-energy yield (Verduyn *et al*. 1990; De Kok *et al*. 2011). For instance, when the CEN.PK113-7D strain is cultivated in maltose-limited anaerobic chemostats, a 25 % smaller biomass yield ($Y_{x/s\ (MALTOSE)}$ = 0.072 ± 0.000 g g gluc eq^{-1}) is observed, when compared to glucose- ($Y_{x/s\ (GLUCOSE)}$ = 0.095 ± 0.002 g g gluc eq^{-1}) (De Kok *et al*. 2012) or sucrose-limited chemostats ($Y_{x/s\ (SUCROSE)}$ = 0.09 ± 0.01 g g gluc eq^{-1}) (Basso *et al.* 2011).

Compared to growth rate values observed when *S. cerevisiae* is grown on glucose, sucrose or maltose as the sole carbon and energy source, growth on galactose is much slower. *S. cerevisiae* strain CEN.PK122 grows with $\mu_{galactose} \approx$ 0.28 h^{-1} on galactose as the sole carbon source in aerobic shake-flasks (van Dijken *et al*. 2000). This is industrially relevant since galactose is present in cheese whey (Siso 1996) and in lignocellulosic hydrolysates (De Bari *et al.* 2014). The reasons for this slower growth of yeast on galactose can be related to the galactose uptake rate, which is around three times slower than glucose uptake (Ostergaard *et al*. 2000). Bro *et al*. (2005) showed that *PGM2* (phosphoglucomutase) expression limits fluxes through the Leloir pathway, which is one of the first steps in galactose metabolism (Frey, 1996).

S. cerevisiae's physiology in sucrose-limited chemostats

Surprisingly, only very limited data are available describing the growth of *S. cerevisiae* in sucrose-limited chemostat cultivations, either under

aerobiosis or under anaerobiosis. Results reported by Diderich *et al*. (1999), Abbott *et al.* (2008) and Basso *et al*. (2010; 2011) can be directly compared, since the yeast strain, medium composition and chemostat parameters employed were identical.

Under anaerobiosis, the specific sucrose consumption rate is higher than during aerobiosis (**Table 2**). Because the energetic yield is lower under fermentative metabolism (when compared to respiratory metabolism), the glycolytic flux is higher, in order to guarantee enough ATP supply for cell growth and maintenance. A similar behavior is observed for the situation in which glucose is the limiting substrate (**Table 2**).

Another parameter drastically influenced by oxygen availability in sugar-limited chemostats of *S. cerevisiae* is the biomass yield ($Y_{x/s}$). $Y_{x/s}$ with sucrose or glucose as the sole carbon source is about 5-fold higher under aerobiosis compared to anaerobiosis (**Table 2**). As mentioned before, biomass yield is directly proportional to cell ATP yield, which is higher under respiratory metabolism than in fermentative assimilation (Verduyn *et al.* 1990). Besides this, it is not possible to detect differences in the $Y_{x/s}$ values, when sucrose-limited chemostat cultures are compared to glucose-limited cultivations. This can easily be explained by the fact that the metabolism of both sugars results in the same amount of ATP per hexose-equivalent consumed. Besides the biomass yield, the ethanol yield ($Y_{e/s}$) is also similar, when the two situations are compared.

Under aerobic conditions, at a dilution rate of 0.1 h^{-1}, no ethanol is produced (**Table 2**). Under these low growth rate conditions, the so-called 'Crabtree' effect (De Deken 1966) is not observed and in connection with the very low residual substrate concentration, every substrate molecule is oxidised by respiration. Above dilution rates of around 0.3 h^{-1} (the exact value depends on the strain), the 'Crabtree' effect sets in, leading to respiro-fermentative metabolism even in fully aerated glucose-limited chemostats (Diderich *et al*. 1999; Kock, Preez and Kilian 2000).

Table 2: Physiology of *Saccharomyces cerevisiae* in aerobic and anaerobic carbon-limited chemostat cultures at a dilution rate of 0.1 h^{-1}.

Oxygen availability	Anaerobiosis [a]	Anaerobiosis [b]	Aerobiosis [d]	Aerobiosis [c]
Carbon source in the medium vessel (w/v)	Glucose (2.5%)	Sucrose (2.5%)	Glucose (0.75%)	Sucrose (1%)
Specific substrate consumption rate(mmol g DW^{-1} h^{-1})	6.03 ± 0.10	3.11 ± 0.003	1.1 ± 0.1	0.56 ± 0.03
Biomass yield (g $g_{gluc\ eq}^{-1}$)	0.09 ± 0.00	0.09 ± 0.00	0.50 ± 0.01	0.51± 0.00
Ethanol yield (g g_{gluceq}^{-1})	0.40 ± 0.01	0.38 ± 0.00	0	0
Glycerol yield (g g_{gluceq}^{-1})	0.07 ± 0.00	0.06 ± 0.00	N. D.	0
Residual substrate [e] (g l^{-1})	< 0.1	< 0.1	< 0.1	0.18± 0.01
Carbon recovery (%)	99.4 ± 0.8	96.2 ± 0.2	N. D.	95.0 ± 5.1

*Cultivation conditions: 1L working volume; 30 ºC; pH 5.0; dissolved oxygen above 60 % for aerobic cultures; synthetic medium according to Verduyn *et al.,*(1992). Averages and mean deviations were obtained from duplicate experiments.
N.D.: Not determined.
Conversion of values presented in the cited references was carried out in order to establish unit uniformity.
Reactor stirrer speed is slightly different, 700 rpm for Basso *et al*., (2010) and 800 rpm for the other authors.
[a]Data extracted from Abbott *et al*.(2008). The authors used *S. cerevisiae* CEN.PK113-7D and give averages ± standard deviations for three independent cultures.
[b]Data extracted from Basso *et al*.(2011). The authors used *S. cerevisiae* CEN.PK113-7D.
[c]Data extracted from Diderich *et al*.(1999). The authors used *S. cerevisiae* CEN.PK113-7D.
[d]Data extracted from Basso *et al*.(2010). The authors used auxotrophic *S. cerevisiae* CEN.PK113-5D and then supplemented with uracil in the medium.
[e]The referred 'residual substrate' is glucose or sucrose depending on the 'carbon source in the medium vessel' used.

Similar to the behavior of ethanol, in sucrose-limited chemostats at 0.1 h^{-1}, glycerol is only produced by *S. cerevisiae* under anaerobiosis (**Table 2**). Glycerol is known as a "redox valve", the role of which is the regeneration of NAD^+. Unlike sugar conversion to ethanol (and accompanying CO_2), which is a redox neutral process, sugar conversion into biomass results in a net generation of NADH, which mainly takes place during the oxidative decarboxylation reactions related to amino acid and lipid biosyntheses. In this context, the NADH-dependent reduction of dihydroxyacetone phosphate to glycerol-3-phosphate (G3P), which is subsequently dephosphorylated to

glycerol, is crucial to maintain yeast redox balance (van Dijken and Scheffers 1986; Bakker *et al*. 2001).

The last parameter shown on **Table 2**, residual sugar substrate, is low for all the conditions presented, which shows that high affinity hexose transporters (e.g. *HXT*) are involved in the uptake of the residual sugars (Diderich *et al*. 1999; Abbott *et al.* 2008; Basso *et al*. 2010; 2011).

To conclude, the physiology of *S. cerevisiae* during sucrose-limited chemostats at 0.1 h^{-1} seems to be highly similar to that observed on glucose, at least when the scarce available data are inspected. More quantitative data are required, in order to verify whether this behavior holds for different strains and for different dilution rates. The use of more precise/sensitive analytical methods could aid in making these comparisons more solid. It will be interesting to see whether the critical dilution rate (which corresponds to the dilution rate value at which alcoholic fermentation sets in in aerobic sugar-limited chemostat cultivations carried out at increasing dilution rates) for sucrose-limited chemostat cultivations will or not be the same as the corresponding value observed for glucose-limited chemostat cultivations, when a particular strain is evaluated. Also, the physiology of yeast under other nutrient limitations (e.g. nitrogen) using sucrose-based media and chemostat cultivations is an unexplored area that has the potential to deliver results different from those obtained using glucose- or maltose-based media, due to the different degrees of glucose repression that is expected to take place when these different sugars are used.

ENGINEERING SUCROSE UTILISATION BY *S. cerevisiae*

Prior to the advent of metabolic engineering, many studies achieved tremendous success in elucidating the mechanisms of sucrose consumption by yeasts (Zimmermann, Khan and Eaton 1973; Santos *et al*. 1982; Oda and Ouchi 1991a,b). A breakthrough study was carried out by Batista *et al*. (2004), who investigated the uptake of sucrose by a *S. cerevisiae* strain which is devoid of hexose transport. In this unique background, the authors could determine the contribution of active sugar uptake for sucrose metabolism. When the high

affinity sucrose-H^+ symporter gene *AGT1* was deleted in the *hxt*-null background, the resulting strain could no longer grow on sucrose, confirming the role of *AGT1* in active sucrose uptake (**Table 3**) (Batista *et al.* 2004). In another study, the *AGT1* permease gene was deleted from a laboratory strain that lacks invertase activity, but can still cleave sucrose intracellularly through cytoplasmic α-glucosidases and also transport sucrose through low-affinity (for sucrose) *MALx1* maltose permeases; a decreased sugar uptake was observed, with increased respiratory metabolism, leading to 1.5 to 2 fold more biomass as compared to the reference strain, with a concomitant decrease in ethanol production. The phenotype achieved (**Table 3**) is economically relevant for biomass-related applications in which ethanol is an undesired by-product (Badotti *et al.* 2008).

Later, the same research group engineered a laboratory strain of *S. cerevisiae*, with the aim of increasing the ethanol yield on sugar. The signal peptide encoding sequence was deleted from the *SUC2* gene, in order to abolish extracellular invertase activity. In this engineered strain, sucrose has to be internalised by proton symporters, which leads to the indirect expenditure of one ATP per sucrose molecule taken up, because of the energetic cost involved in proton extrusion by Pma1p, necessary to keep intracellular pH homeostasis. In such a strain, the carbon flux towards ethanol increases, resulting in a higher ethanol yield compared to the reference strain (**Table 3**) (Basso *et al*. 2011; Stambuk *et al*. 2011). Moreover, using an evolutionary engineering approach, Basso *et al.* (2011) submitted the 'i*SUC2* strain' from Stambuk *et al*. (2011) to a long-term sucrose-limited anaerobic chemostat cultivation. The evolved lineage could produce approximately 0.42 $g_{ethanol} g_{glucose\ eq}^{-1}$, which is around 11 % higher than the yield obtained with the reference strain CEN.PK113-7D. These authors also observed that a duplication of the *AGT1* gene was involved in the observed phenotype (**Table 3**), which is in agreement with transport assays that confirmed that the limiting step for efficient sucrose metabolism was sucrose transport, namely transport capacity (Basso *et al*., 2011).

Table 3: Previous works on the metabolic engineering of sucrose consumption in *S. cerevisiae*.

Parental strain|Relevant genotype	Selected achievement	Reference
MC966A | MATa *MAL2 MEL SUC2 hxt1Δhxt2Δ hxt3Δ hxt4Δ hxt5Δ hxt6Δ hxt7Δ gal2Δ agt1Δ*::*kanMX6*	Hexose transporter-null strain is still able to ferment sucrose through active uptake by Agt1p and intracellular hydrolysis by maltases and invertase	Batista *et al.,* 2004
1403-7A | MATa *MAL4^{C} MGL3 suc-gal3 gal4 trp1 ura3 agt1Δ*::*kanMX6*	1.5- to 2-fold more biomass during aerobic batch growth on sucrose compared to reference strain due to reduced overflow metabolism	Badotti *et al*., 2008
CEN.PK2-1C | MATa *mal13 AGT1 MAL12 MAL2-8c TRP1-*P_{ADH1}::i*SUC2 ura3-52 trp1-289*	$Y_{ethanol/sucrose} \approx 0.38$ g $g_{gluc\ eq}^{-1}$ 5 % increase [a]	Stambuk *et al*., 2011
CEN.PK113-5D | MATa *URA3 trp1-289 TRP1*-P_{ADH1}::i*SUC2* (evolved)[b]	$Y_{ethanol/sucrose} \approx 0.42$ g g_{gluceq}^{-1} 11 % increase [a]	Basso *et al*., 2011

[a] Compared to CEN.PK113-7D, which is the *S. cerevisiae* reference strain. Decreased accumulation of extracellular fructose, glucose and glycerol was also observed
[b] This strain was obtained by evolution in prolonged anaerobic sucrose-limited chemostats.

An evolutionary engineering approach was also used by Koschwanez and co-workers to select evolved yeast strains in low (1 mM) sucrose media. The analysis of more than 12 evolved populations (which grew better and outcompeted the parental strain in low sucrose concentrations) revealed that none of them increased sucrose transport activity, which unfortunately may reflect the genetic *MAL* negative phenotype of the W303 yeast, which they used in their experiments. Nevertheless, when *AGT1* was overexpressed through a strong promoter, the cells could grow in 1 mM sucrose (Koschwanez, Foster and Murray 2013). A total of 10 evolved clonal populations had increased (3-21 fold) invertase expression, and in most cases the expression of *HXT* sugar transporters was also increased to facilitate hexose (from sucrose hydrolysis) uptake by the cells. However, the most predominant phenotype found in almost all the evolved strains was the ability to form multicellular clumps due to a failure in cell separation (Koschwanez, Foster and Murray 2013). In such clumps of cells the hexoses produced by one cell hydrolysing sucrose can be efficiently taken up by the adjacent cell, which

will not occur if the two cells were separate and distant one from the other (Koschwanez, Foster and Murray 2011, 2013). Indeed, sucrose has also been described as a potent inducer of yeast filamentation and/or pseudohyphal growth (Van de Velde and Thevelein 2008), which may explain why flocculant yeasts are the predominant type of yeasts isolated from the industrial production of fuel ethanol from sugar cane in Brazil (Basso *et al.* 2008).

CONCLUDING REMARKS

Sucrose has been with humans since time immemorial. To eat for the sheer pleasure is a human trait and food with sugar (desserts) is a delectable treat. For the biotechnology industry, sucrose is an abundant, readily available and inexpensive substrate, mainly in tropical areas, such as in Brazil. *S. cerevisiae*, which naturally evolved to efficiently consume sugars such as sucrose, is currently one of the most important cell factories due to its robustness, stress tolerance, inexpensive nutrient requirements and genetic accessibility. For these reasons, this review focused on sucrose metabolism by *S. cerevisiae*, a surprisingly unexplored subject in the scientific literature, when compared to the knowledge accumulated on the metabolism of sugars that occur more frequently in temperate climate crops, such as maltose. Thus, it can be concluded that sucrose has been a "neglected" sugar or carbon-source by the research community. As described in this review, sucrose transporters and hydrolases are vast in yeast, which makes the construction of sucrose knockout strains still a challenge. Not much information is available on the physiology of *S. cerevisiae* grown on sucrose-based media in chemostat cultivations (only a few datasets from sucrose-limited chemostats at a dilution rate of 0.1 h^{-1} are available, for instance). The number of published works exploring the engineering of sucrose utilization in *S. cerevisiae* is rather low. Some of the key issues to be addressed in the coming years are: 1) what are the similarities and differences in the physiology and regulation of metabolism of *S. cerevisiae*, when growth on sucrose is compared to growth on glucose or maltose? The following aspects can be considered of particular importance:

the identification of all genes that should be eliminated to render a yeast strain incapable of thriving on sucrose and the degree of glucose repression to which cells are exposed during the release of glucose provoked by sucrose hydrolysis via invertase and/or via other enzymes or even chemical hydrolysis; 2) how can this knowledge be employed to improve sucrose-based industrial processes? 3) how can this knowledge lead us to a better understanding of the original habitat of *S. cerevisae*, before it started being in close contact with human societies?

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Scope of this thesis

The goal of this thesis was to engineer sucrose metabolism in *S. cerevisiae* in order to increase cell free-energy conservation. Under anaerobic conditions, replacement of native sucrose hydrolases by bacterial phosphorylases combined with replacement of native sucrose/H^+ symporters by uniporters has the potential to increase by 25 % the ATP yield on sucrose (from 4 to 5 ATP/sucrose). The use of strains that conserve more energy as expression-hosts for product pathways decreases the demand for oxygen during microbial cultivation or, in some instances, it may be possible to completely eliminate aeration. Use of anaerobic production processes can significantly reduce costs in industrial settings. In the path to construct such strains, it was necessary to identify and implement all gene deletions necessary for abolishing native sucrose transport and hydrolysis in *S. cerevisiae*. This challenge was addressed in **Chapter 2** of this thesis. Sucrose and *S. cerevisiae* have been subject of research for more than a century. Yet, scientists had not succeeded in completely abolishing sucrose hydrolysis in *S. cerevisiae* via rational gene deletions. With the aim to identify all relevant sucrose hydrolases, laboratory evolution of strains carrying deletions in known sucrose-hydrolase genes was followed up by whole genome sequencing. Candidate genes were then deleted using a CRISPR/Cas9 approach that had not yet been tried in yeast: use of a single Cas9 target-site to eliminate an entire gene family in only one transformation step.

In a yeast chassis strain that was completely unable to consume sucrose, five putative plant sucrose-facilitators were expressed in combination with a sucrose phosphorylase from *Leuconostoc mesenteroides* (*Lm*SPase) (**Chapter 3**). As mentioned above, the goal was to increase the ATP yield by 25% relative to that in the wild type reference strain. To access differences in ATP conservation of the constructed strains, biomass yield were estimated from cells growing in anaerobic chemostats, in which biomass and ATP yields on sugar are proportional.

Because the metabolic engineering strategy applied in **Chapter 3** to increase energy conservation was highly dependent on the efficient expression of plant sucrose uniporters in yeast, it was investigated (**Chapter 4**) which mutations in a heterologous transporter gene and/or in native *S. cerevisiae* genes contribute to efficient, functional expression of a plant transporter in the yeast plasma membrane. For this purpose, a strain whose growth depended on functional expression of a plant sucrose transporter was subjected to laboratory evolution and subsequent DNA sequencing. Reverse engineering was then applied to check which mutations contributed to improve growth on sucrose.

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2

Elimination of sucrose transport and hydrolysis in *Saccharomyces cerevisiae*: a platform strain for engineering sucrose metabolism

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Abstract

Many relevant options to improve efficacy and kinetics of sucrose metabolism in *Saccharomyces cerevisiae* and, thereby, the economics of sucrose-based processes remain to be investigated. An essential first step is to identify all native sucrose-hydrolysing enzymes and sucrose transporters in this yeast, including those that can be activated by suppressor mutations in sucrose-negative strains. A strain in which all known sucrose-transporter genes (*MAL11, MAL21, MAL31, MPH2, MPH3*) were deleted did not grow on sucrose after 2 months of incubation. In contrast, a strain with deletions in genes encoding sucrose-hydrolysing enzymes (*SUC2, MAL12, MAL22, MAL32*) still grew on sucrose. Its specific growth rate increased from 0.08 to 0.25 h^{-1} after sequential batch cultivation. This increase was accompanied by a 3-fold increase of *in vitro* sucrose-hydrolysis and isomaltase activities, as well as by a 3- to 5-fold upregulation of the isomaltase-encoding genes *IMA1* and *IMA5*. One-step Cas9-mediated deletion of all isomaltase-encoding genes (*IMA1-5*) completely abolished sucrose hydrolysis. Even after 2 months of incubation, the resulting strain did not grow on sucrose. This sucrose-negative strain can be used as a platform to test metabolic engineering strategies and for fundamental studies into sucrose hydrolysis or transport.

INTRODUCTION

Sucrose is a cheap substrate for industrial fermentation that is extensively used for ethanol production (Maiorella, Blanch and Wilke 1984; Della-Bianca *et al.* 2013; Gombert and van Maris 2015) and can be used for a wide range of other yeast-based industrial processes (Marques *et al.* 2016; Meadows *et al.* 2016). The main sources of sucrose are sugar cane in the tropics and sugar beet in temperate regions. Extraction of sucrose from feedstocks is inexpensive and no enzymatic pre-treatment is necessary prior to microbial fermentation (Maiorella, Blanch and Wilke 1984; Marques *et al.* 2016). Furthermore, the use of sucrose for industrial biotechnology does not have to compete with food production. For example, in the Brazilian ethanol industry, food and biofuel production can coexist (Mitchell 2008). Therefore, diversifying the range of products that can be produced from sucrose fermentation will have a positive impact on sustainable industrial production of fuels and chemicals (Meadows *et al.* 2016).

Sucrose-based ethanol production employs bakers' yeast (*Saccharomyces cerevisiae*), which efficiently consumes sugars and can withstand industrially relevant stresses such as high ethanol and acid concentrations (Della-Bianca *et al.* 2014). To convert sucrose into hexoses, *S. cerevisiae* secretes invertase (β-fructosidase, sucrose hydrolase; Suc) (Winge and Roberts 1952; Hawthorne 1958). Invertase hydrolyses sucrose into the monomers, glucose and fructose, which subsequently enter the cell through facilitated diffusion via hexose transporters and are metabolized via the classical Embden-Meyerhof-Parnas glycolytic pathway (Lagunas 1993). Alternatively, sucrose can be imported by a proton symporter (Mal11, formerly known as Agt1) and hydrolysed in the cytosol (Stambuk *et al.* 1999). Cytosolically localised invertase and maltases (Malx2) then hydrolyse sucrose intracellularly. *In vitro* studies have shown that isomaltases can also hydrolyse sucrose, but their contribution to *in vivo* sucrose metabolism remains to be addressed (Carlson and Botstein 1982; Stambuk *et al.* 1999; Badotti *et al.* 2008; Brown, Murray and Verstrepen 2010; Voordeckers *et al.* 2012; Deng *et*

al. 2014). Even closely related yeast strains exhibit significant diversity in the identity and number of representatives from each of the three disaccharide hydrolase-encoding gene families (*SUC*, *MALx2*, *IMA*) (Carlson and Botstein 1983; Brown, Murray and Verstrepen 2010). For example, the genome of the haploid laboratory strain *S. cerevisiae* CEN.PK113-7D contains 5 different *IMA* genes on sub-telomeric regions: *IMA1* (three copies: CHRIII, CHRVII, CHRXI), *IMA2* (CHRXV), *IMA3* (CHRIX), *IMA4* (CHRX) and *IMA5*(CHRX) (Teste, François and Parrou 2010; Nijkamp *et al.* 2012), while the related strain CEN.PK102-3A, which was used in the present study, does not contain the *IMA1* copy at CHRXI (de Kok *et al.* 2011).

Detailed complete knowledge of sucrose transport and hydrolysis is important for both industry and academia. Functional analysis of eukaryotic sucrose transporters and hydrolytic or phosphoroclastic sucrose-cleaving enzymes is often done in *S. cerevisiae*. An example is the development of a functional screening system for plant sucrose exporters (Zhou, Grof and Patrick 2014). In this system, functional expression of plant sucrose exporters is analysed by monitoring conversion of glucose into sucrose and subsequent sucrose efflux from an *S. cerevisiae* strain that heterologously expresses sucrose-phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP) genes. In industry, engineering of sucrose metabolism can contribute to increased product yields on substrate. In a proof-of-principle study with a laboratory strain of *S. cerevisiae*, Basso *et al.* (2011) demonstrated an increase in the ethanol yield of *S. cerevisiae* on sucrose of 11%, when compared to the reference strain, by deleting the secretion signal sequence from the invertase coding gene (*SUC2*). This modification resulted in a predominant intracellular localisation of invertase activity, thereby creating a necessity for sucrose uptake via proton symport. To avoid intracellular acidification, protons entering the cell via the sucrose-proton symporter have to be expelled by the plasma-membrane H^+-ATPase (Pma1). This export mechanism costs one ATP per proton, thus reducing the ATP yield of alcoholic fermentation from 4 to 3 ATP per sucrose molecule. This change in energy coupling was shown to lead

to a decrease of the biomass yield and an increase of the ethanol yield of the engineered strain on sucrose.

In metabolic engineering, incomplete knowledge of the targeted pathway can delay strain construction due to the activity of competing pathways or activation of such pathways by suppressor mutations. Although mutants that are unable to grow on sucrose have been reported (Carlson, Osmond and Botstein 1981), it has not yet been investigated whether these strains can regain the ability to grow on sucrose after laboratory evolution (Marques *et al.* 2016), for instance through recruitment of any of the numerous glycoside hydrolases in *S. cerevisiae* (Yamamoto *et al.* 2004; Naumoff and Naumov 2010; Naumoff 2011). To address this question, *S. cerevisiae* strains in which either the genes encoding known sucrose transporters or those encoding disaccharide-hydrolysing enzymes were inactivated were subjected to laboratory evolution and mutations underlying the acquired phenotypes were identified by whole-genome resequencing.

MATERIAL AND METHODS

Strains and maintenance

S. cerevisiae strains used in this work (**Table 1**) belong to the CEN.PK family (Entian and Kötter 2007; Nijkamp *et al.* 2012). To prepare stock cultures, yeast strains were grown in 50 ml shake flasks containing 10 ml of either Yeast-Peptone-Dextrose (YPD) medium or, for strains carrying plasmids with auxotrophic marker genes, synthetic medium (SM). YPD contained 10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone and 20 g L^{-1} glucose in demineralized water. SM, supplemented with vitamins, trace elements and 20 g L^{-1} glucose, was prepared according to Verduyn *et al.* (1992). Glycerol was added to growing cultures (final concentration 30 % v/v) and 1 mL aliquots were stored at -80 ºC.

Table 1: Strains used in this study

Name	Relevant genotype	Parental strain	Origin
CEN.PK113-7D	*MATa MAL1x MAL2x MAL3x MAL4x MAL2-8^C SUC2 LEU2 URA3*		P.Kötter, Germany
CEN.PK102-3A	*MATa MAL1x MAL2x MAL3x MAL4x MAL2-8^C SUC2 leu2-112 ura3-52*		P.Kötter, Germany
IMK291	CEN.PK102-3A *MATa leu2-112 ura3-52 MAL2-8^C Mal11-mal12::loxP mal21-mal22::loxP mal31-32::loxP mph2/3::loxP mph2/3::loxP-hphNT1-loxP suc2::loxP-kanMX-loxP*	IMK289	This study
IMX469	*malΔ mphΔ suc2Δ MAL11-LEU2*	IMK291	This study
IMU048	*malΔ mphΔ suc2Δ MAL11-LEU2 URA3*	IMX469	This study
IMU055	*malΔ mphΔ suc2Δ MAL11-LEU2 MAL12-URA3*	IMX469	This study
IMX470	*malΔ mphΔ suc2Δ LEU2*	IMK291	This study
IMU051	*malΔ mphΔ suc2Δ LEU2 URA3*	IMX470	This study
IMU054	*malΔ mphΔ suc2Δ LEU2 MAL12-URA3*	IMX470	This study
IMZ571	*malΔ mphΔ suc2Δ MAL11-LEU2* cas9-*URA3*	IMX469	This study
IMK700	*malΔ mphΔ suc2Δ MAL11-LEU2* cas9-*URA3 ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ amdSYM*	IMZ571	This study
IMS422	*malΔ mphΔ suc2Δ MAL11-LEU2 URA3* (single colony isolate from evolution, replicate 1)	IMU048	This study
IMS423	*malΔ mphΔ suc2Δ MAL11-LEU2 URA3* (single colony isolate from evolution, replicate 2)	IMU048	This study
IMS424	*malΔ mphΔ suc2Δ MAL11-LEU2 URA3* (single colony isolate from evolution, replicate 3)	IMU048	This study
IMS517	*malΔ mphΔ suc2Δ MAL11-LEU2*	IMS422	This study
IMS518	*malΔ mphΔ suc2Δ MAL11-LEU2*	IMS423	This study
IMS519	*malΔ mphΔ suc2Δ MAL11-LEU2*	IMS424	This study
IMS604	*malΔ mphΔ suc2Δ MAL11-LEU2 cas9-URA3*	IMS517	This study
IMS605	*malΔ mphΔ suc2Δ MAL11-LEU2 cas9-URA3*	IMS518	This study
IMS606	*malΔ mphΔ suc2Δ MAL11-LEU2 cas9-URA3*	IMS519	This study
IMK716	*malΔ mphΔ suc2Δ MAL11-LEU2 cas9-URA3 ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ*	IMZ604	This study
IMK717	*malΔ mphΔ suc2Δ MAL11-LEU2 cas9-URA3 ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ*	IMZ605	This study
IMK718	*malΔ mphΔ suc2Δ MAL11-LEU2 cas9-URA3 ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ*	IMZ606	This study
IMK743	*malΔ mphΔ suc2Δ MAL11-LEU2 ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ amdSYM*	IMK700	This study
IMZ620	*malΔ mphΔ suc2Δ MAL11-LEU2 ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ amdSYM IMA1-URA3*	IMK743	This study
IMZ621	*malΔ mphΔ suc2Δ MAL11-LEU2 ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ amdSYM IMA2-URA3*	IMK743	This study
IMZ622	*malΔ mphΔ suc2Δ MAL11-LEU2 ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ amdSYM IMA3,4-URA3*	IMK743	This study
IMZ623	*malΔ mphΔ suc2Δ MAL11-LEU2 ima1Δ ima2Δ ima3Δ ima4Δ ima5Δ amdSYM IMA5-URA3*	IMK743	This study

Molecular biology techniques

Diagnostic PCR was performed using DreamTaq™ DNA Polymerase (Thermo Fisher Scientific, Waltman, MA). PCR amplification for cloning and sequencing purposes was performed with Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Both enzymes were used according to the manufacturer's instructions. Primers and oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO). DNA purification from PCR reaction mixtures was done using GenElute™ PCR Clean-up Kit (Sigma-Aldrich). Separation of DNA fragments was performed in 1% (w/v) agarose gel (Thermo Fisher Scientific) in TAE buffer (40 mM Tris-acetate pH 8.0 and 1 mM EDTA). DNA fragments were purified from gels using the Zymoclean Gel DNA Recovery kit (ZymoResearch, Irivine, CA). Plasmid assembly was done with the Gibson Assembly™ Master Mix (New England Biolabs, Beverly, MA) according to manufacturer's protocol. Restriction endonucleases (Thermo Fisher Scientific) and T4 DNA ligase (Promega Corporation, Madison, WI) were used accordingly to manufacturer's instructions. Plasmids were isolated from *E. coli* using GenElute™ HP Plasmid Miniprep Kit (Sigma-Aldrich) according to the provided protocol. Yeast genomic DNA was extracted using YeaStar Genomic kit (Zymo Research).

Plasmid construction

Plasmid pUDC156 (**Table 2**) was assembled by *in vivo* homologous recombination of a plasmid backbone and an insert fragment containing a yeast expression cassette for the *cas9* gene from *Streptococcus pyrogenes* (Kuijpers *et al.* 2013). The plasmid backbone was amplified from MB4917 (Zelle *et al.* 2010) with primers 7833 & 4697 and the *cas9*-expression cassette was amplified from p414-TEF1p-cas9-CYC1t (DiCarlo *et al.* 2013) with primers 1768 & 7236 (**Table S1**). Both fragments contain 60 bp overlaps with each other and were assembled by co-transformation to *S. cerevisiae* strain IMX469 resulting in IMZ571. Plasmid pUDR127 (**Figure 1A; Table 2**) contains two gRNA cassettes: one for deletion of *IMA5* (*gRNA-IMA5*) and one targeting-

Table 2: Plasmids used in this study

Name	Relevant characteristics	Origin
pUG6	loxP-*KanMX4*-loxP	Gueldener *et al.* 2002
p426-GPD	*2μ URA3 pTDH3-tCYC1*	Mumberg, Müller and Funk 1995
p414-TEF1p-Cas9-CYC1t	*CEN6/ARS4 ampR TRP1p TEF1-cas9-tCYC1*	DiCarlo *et al.* 2013
MB4917	*CEN6 ampR URA3*	Zelle *et al.* 2010
pROS11	*2μ amdSYM gRNA-CAN1.Y gRNA-ADE2.Y*	Mans *et al.* 2015
pUDC156	*CEN6 URA3pTEF1-cas9-tCYC1*	This study
pUDR127	*2μ amdSYM gRNA-IMA5 gRNA-IMA1,2,3,4*	This study
pUDI035	*Integration plasmid LEU2* P_{TDH3}*-MAL11-*T_{CYC1}	de Kok *et al.* 2011
pUDI084	*Integration plasmid LEU2 empty vector*	This study
pUDE044	*2μ URA3* P_{TDH3}*-Mal12-*T_{ADH1}	de Kok *et al.* 2011
pUDE260	*2μ URA3 empty vector*	This study
pUDE427	*2μ URA3* P_{TDH3}*-IMA1-*T_{CYC1}	This study
pUDE428	*2μ URA3* P_{TDH3}*-IMA2-*T_{CYC1}	This study
pUDE429	*2μ URA3* P_{TDH3}*-IMA3,4-*T_{CYC1}	This study
pUDE430	*2μ URA3* P_{TDH3}*-IMA5-*T_{CYC1}	This study

sequence shared by *IMA1*, *IMA2*, *IMA3* and *IMA4* (*gRNA-IMAs*). This plasmid was constructed via Gibson assembly of three fragments: two gRNA cassettes overlapping with each other in the *2μm* replicon and a plasmid backbone. The plasmid backbone was obtained via PCR amplification, using pROS11 (Mans *et al.* 2015) as template with primer 6005 (**Table S1**). The gRNA cassette *gRNA-IMA5* was obtained via PCR from pROS11 with primers 8761 and 5975 and cassette *gRNA-IMAs* was obtained via PCR from pROS11 with primers 8759 and 5974 (**Table S1**). Plasmid pUDI084 was made by removal of the *MAL11* coding sequence from pUDI035 (de Kok *et al.* 2011) using *Spe*I and *Nhe*I restriction sites and subsequent re-circularization of the plasmid using T4 DNA ligase (**Table 2**). Similarly, pUDE260 was constructed by removal of *PGMβ* from pUDE063 (de Kok *et al.* 2011) using *Pvu*II sites and subsequent re-circularization of the plasmid (**Table 2**). The *IMA*-reinsertion plasmids (pUDE427 to pUDE430) were made by Gibson assembly of an *IMA* expression cassette and a plasmid backbone. The latter was obtained via PCR with p426-GPD (Mumberg, Müller and Funk 1995) as a template, using primer pairs 7823 & 5975 and 5974 & 7812, which amplify the backbone in two parts to

minimize chances of plasmid re-circularization (**Table S1**). Templates for the *IMA* expression cassettes were PCR amplified using genomic DNA from strain IMU048 with primers that are specific to each *IMA* (primers 8607 & 8611 for *IMA1*; 8608 & 8612 for *IMA2*; 8609 & 8613 for *IMA3,4* and 8610 & 8614 for *IMA5*). From these templates, the coding sequence of each *IMA* could be individually amplified using primer pairs 9302 & 9305 (*IMA1*), 9303 & 9306 (*IMA2* and *IMA3,4*) and 9304 & 9307 (*IMA5*). Plasmids were transformed to *E. coli* for storage and amplification, where necessary after isolation of *in vivo* assembled plasmids from yeast cultures .

Strain construction

S. cerevisiae transformations were carried out according to Gietz and Woods (2002) using 1 µg DNA, unless specified otherwise below. Transformants were selected on agar plates containing SM with 20 g L^{-1} glucose. The following components were added when necessary: G418 200 mg L^{-1}; uracil 0.15 g L^{-1}; leucine 0.5 g L^{-1}. Cells expressing the *amdSYM* marker were selected on plates according to Solis-Escalante *et al.* (2013). Strain IMK289 (de Kok *et al.* 2011) was transformed with a deletion cassette containing loxP-*KanMX4*-loxP marker. This cassette was amplified from plasmid pUG6 using primers 1482 & 1483 that has homology to the sequence outside the *SUC2* open reading frame (ORF). The resulting strain was named IMK291. To construct *MAL11*-expressing strains, vector pUDI035 (**Table 2**) was linearized with *BstE*II (restriction site at *LEU2* marker) and transformed into IMK291, resulting in strain IMX469 (**Table 1**). Transformation of pUDE260 and pUDE044 into IMX469 resulted in IMU048 and IMU055, respectively. Linearization of pUDI084 (**Table 2**) with *BstE*II and transformation into IMK291 resulted in IMX470. Transformation of pUDE260 and pUDE044 into IMX470 resulted in IMU051 and IMU054, respectively. IMK700 was made via co-transformation of IMZ571 with 1 µg of plasmid pUDR127 together with 4 µg of each dsDNA repair fragment: one for *IMA5* and another one for all the other *IMA genes* (**Figure 1**). Repair fragments were obtained by annealing two complementary PAGE purified single-stranded

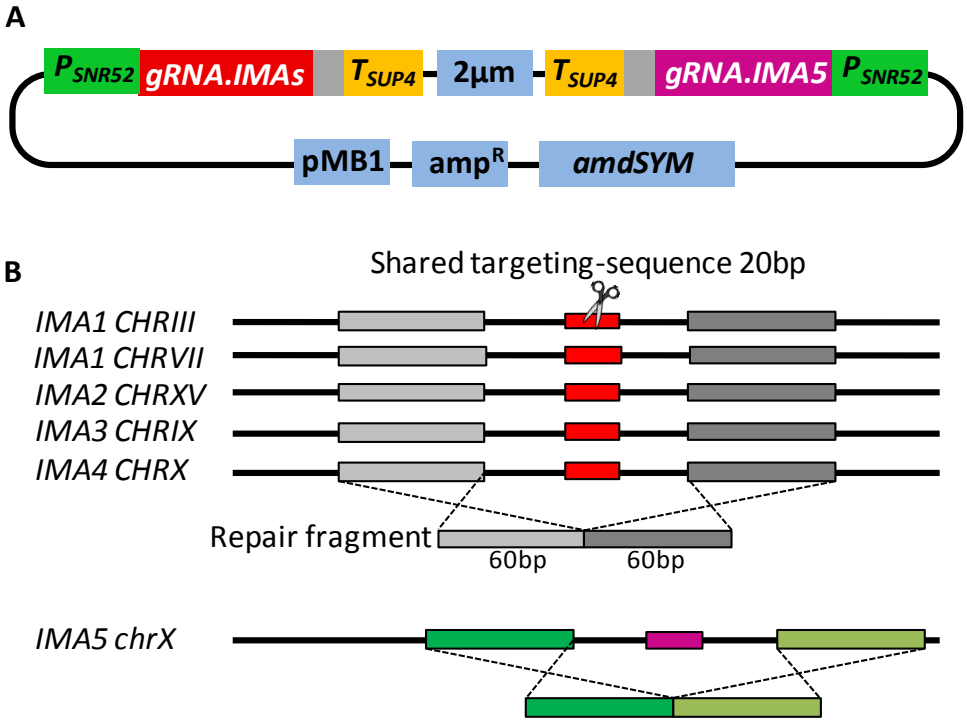

Figure 1: Strategy for deletion of the *IMA* genes using a shared Cas9 target site. **A)** Plasmid pUDR127, that contains two gRNA-cassettes: '*gRNA-IMAs*' (in red) with a target sequence to cleave all *IMA genes* except *IMA5* and "*gRNA-IMA5*", in purple. The SNR52 promoter and SUP4 terminator are shown in green and yellow, respectively. Sequences encoding structural RNAs are shown in grey. 2µm: origin of replication in yeast; pMB1: origin of replication in bacteria; amp^R: cassette for ampicillin resistance in *E. coli*; amdSYM: dominant marker that allows use of acetamide as nitrogen source. **B)** Illustration of the shared Cas9 target sequences among the *IMA genes*. The coding sequence of each *IMA* is represented by black lines. The red boxes corresponds to the shared target sequence. The dark- and light-grey fragments are the homology regions (60 bp) up- and downstream of the target site where the repair fragment (120 bp) can recombine. In view of its sequence divergence from the other *IMA* genes, an exclusive target sequence, in purple, and repair sites, in dark and light green, were chosen for inactivation of *IMA5*.

evolved on sucrose – IMS422, IMS423 and IMS424– pUDE260 was cured from these strains by cultivation on synthetic medium plates with 20 g L^{-1} glucose and 1 g L^{-1} 5'-fluoroorotic acid (Boeke, La Croute and Fink 1984), resulting in strains IMS517, IMS518 and IMS519, respectively. Transformation of pUDC156 into these three strains yielded strains IMS604, IMS605 and IMS606, respectively. Finally, transformation of pUDR127 and repair fragments for IMA5 and IMA1-4 (as mentioned above) into these three strains yielded strains IMK717, IMK718 and IMK719. For re-insertion of the IMA genes on multi-copy plasmids, pUDC156 was cured from IMK700 resulting in IMK743. Then, each IMA overexpression plasmid (named pUDE427-pUDE430, Table 2) was transformed into IMK743 resulting in strains IMZ620-623.

Medium and cultivation

Shake-flask cultures were performed in 500 mL shake flasks containing 100 mL of SM with 20 g L^{-1} sucrose, in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ) set at 200 rpm and at 30 ºC under an air atmosphere. For growth rate determinations, cells were inoculated in SM with 20 g L^{-1} glucose from a frozen stock culture. After reaching stationary phase, the culture was transferred to SM with 20 g L^{-1} sucrose (initial OD_{660nm} = 0.2) and incubated until exponential growth was observed. Exponentially growing cultures were then transferred to fresh medium (initial OD_{660nm} = 0.2) and samples were taken hourly until stationary phase was reached. Optical density at 660 nm was measured with a Libra S11 spectrophotometer (Biochrom, Cambridge, UK). Specific growth rates were calculated from at least 5 data points.

Laboratory evolution

Sequential batch cultivation of strain IMU048 was performed in 10 mL SM (Verduyn *et al.* 1992) with 20 g L^{-1} sucrose, in 50 mL polypropylene tubes (Greiner Bio One, Frickenhausen, BW) in an Innova incubator shaker (New Brunswick Scientific), at 200 rpm and at 30 ºC. At the end of each cultivation

cycle (OD_{660nm} between 3 - 4.2), 0.1 mL of culture was transferred to fresh medium to start a next cycle. In total, 30 transfers were carried out in 70 days, which accounts for approximately 188 generations. One single-colony isolate from each evolution line was obtained by restreaking three times on non-selective medium (YPD) plates.

Sucrose and glucose determination

Concentrations of sucrose and glucose in culture supernatants were analysed by high-performance liquid chromatography (Agilent 1100 HPLC, Agilent Technologies, Santa Clara, CA), using an Aminex HPX-87H ion exchange column (BioRad, Richmond, CA) coupled to a refractive-index detector and eluted with 0.5 mM H_2SO_4 at 0.8 mL min^{-1} and at 40 °C. This temperature was used instead of the regularly applied temperature of 60 °C to avoid sucrose hydrolysis during analysis.

Enzyme activity assays

For enzyme activity assays, shake-flask cultures on SM with 20 g L^{-1} sucrose were harvested during exponential growth (OD660nm = 3 – 4). For strains IMU054, IMU055, IMZ620, IMZ621, IMZ622 and IMZ623, ethanol (20 g L^{-1}) was used as carbon source instead of sucrose. For strains IMK700, IMK716, IMK717 and IMK718, 20 g L^{-1} ethanol was used as carbon source plus 20 g L^{-1} sucrose as inducer. Samples were harvested, washed and prepared for sonication according to Postma *et al.* (1989). Cell extracts were prepared by sonication with 0.7 mm glass beads at 0°C for 2 min at 0.5 min intervals with an MSE sonicator (150 W output; 8 µm peak-to-peak amplitude). Unbroken cells and debris were removed by centrifugation (4 °C, 20 min, 47,000 × g). The supernatant was used for enzyme activity assays. For measurement of extracellular enzyme activity, 100 mL of exponentially growing cells (OD_{660nm} = 3 – 4) was centrifuged (4 °C, 10 min, 5000 × g), the supernatant was concentrated up to 200 times with a Vivaspin® 20 filter with a 10000 MW cut-off (Sartorius Stedim, Aubagne, France) and dialyzed overnight against 10 mM potassium-phosphate buffer (pH7.5) at 4 °C. Protein

levels in extracellular samples and cell extracts were determined with the Lowry assay (Lowry *et al.* 1951). Sucrose or isomaltose hydrolytic activity was measured at 30 ºC by monitoring the reduction of $NADP^+$ at 340 nm in a 1 ml reaction mixture containing 50 mM imidazole-HCl (pH 7.0), 1 mM $NADP^+$, 12.5 mM $MgCl_2$, 1 mM ATP, 3.5 units hexokinase, 3.5 units glucose-6-phosphate dehydrogenase and 10 – 40 µl cell extract. The reaction was started by the addition 100 mM of substrate. An extinction coefficient of 6.3 mM^{-1} was used for NADPH.

Real-time quantitative PCR

Exponentially growing cultures (OD_{660nm} = 3 – 4) in 100 mL SM (Verduyn *et al.* 1992) with 20 g L^{-1} of sucrose were harvested as described previously (Piper *et al.* 2002). RNA was extracted using the hot-phenol method (Schmitt, Brown and Trumpower 1990) and RNA quality was assayed by electrophoresis using an Agilent BioAnalyzer 2100 (Agilent Technologies). Genomic DNA elimination was performed from 2 µg of total RNA in a 28 µL reaction using the QuantiTec Reverse Transcription kit (Qiagen, Düsseldorf, Germany). Reagents for cDNA synthesis from the same kit were added up to a final volume of 40 µL following the manufacturer's instructions. 6 µL of cDNA solution diluted 150 times was used in 20 µL qPCR mix that additionally included 10 µL of Rotor-Gene SYBR Green PCR Master Mix (Qiagen) and forward and reverse primers (1 µM each) (**Table S1**). A Rotor-Gene Q (Qiagen) was used with the following sequence: denaturation at 95 ºC for 5 min followed by 40 cycles of denaturation at 95 ºC for 5 s, annealing at 60 ºC for 10 s and extension at 72 ºC for 20 s. A melting curve up from 60 ºC to 95 ºC was performed to verify primer specificity. PCR efficiency of each primer was determined by a dilution series using a pool of cDNA from different samples. Transcript levels of the 'housekeeping' gene *UBC6* (Teste *et al.* 2009) were used for data normalization. Threshold cycles (CT) were exported from Rotor-Gene Q software (version 2.0.2) and analysed using the REST-2009 algorithm (Pfaffl, Horgan and Dempfle 2002). One of the two biological replicates of CEN.PK113-7D was chosen as reference condition (expression ratio = 1).

Sanger and whole-genome sequencing

Sanger sequencing of PCR products of each *IMA* gene was performed at BaseClear BV (Leiden, The Netherlands). The PCR products were obtained with genomic DNA extracted from strains IMS422, IMS423 and IMS424 and primers: 8607 & 8611 (*IMA1*), 8608 & 8612 for (*IMA2*), 8609 & 86013 for (*IMA3,4*) and 8610 & 8614 (*IMA5*) (Table S1). *IMA3* and *IMA4* were treated as a single gene (*IMA3,4*) since their coding sequences are identical (Teste, François and Parrou 2010). DNA for whole genome sequencing was extracted using Qiagen 100/G kit following the manufacturers protocol (Qiagen, Hilden, Germany). Whole genome sequencing was performed by Novogene Beijing, China). A library of 350-bp genomic fragments was created and sequenced paired-end (150-bp reads). A minimum data quantity of 4000 MB was generated per strain, representing a minimum 330-fold coverage. The data analysis was performed as described in van den Broek *et al*. (2015). The sequencing data of the parental strain, IMU048, and of the three evolved isolates, IMS422, IMS423 and IMS424, were deposited at NCBI under the BioProject ID: PRJNA353914.

RESULTS

Experimental design to eliminate non-biological sucrose hydrolysis

Although sucrose is generally stable when dissolved in water, under certain conditions it can be hydrolysed into fructose and glucose, such as for instance by acid catalysis in low-pH solutions (Krieble 1935; Wolfenden and Yuan 2008). To investigate possible artefacts created by such non-biological hydrolysis of sucrose, strain IMU051 (*malΔ mphΔ suc2Δ*), which cannot grow on sucrose, but can grow on hexoses, was incubated in synthetic medium shake-flasks at pH values between 2 and 7 (**Figure 2**). At pH values of 5-7, no

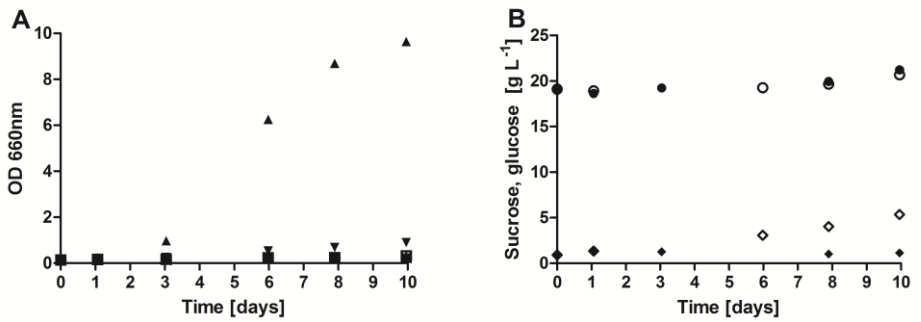

Figure 2: Non-biological hydrolysis of sucrose caused by medium acidity. **A**) growth of *Saccharomyces cerevisiae* strain IMU051 (*malΔ mphΔ suc2Δ*) in synthetic medium with 20 g L^{-1} sucrose as sole carbon source with initial pH set at different values: pH 2, ■; pH 3, ▲; pH 4, ▼; pH 5, □; pH 6, "**X**" and pH 7, "**+**". The symbols ■, □, X and + are overlapping. **B**) Sugar concentrations in flasks with synthetic medium at an initial pH of 3. Closed symbols; glucose (♦) and sucrose (●) concentrations in flasks inoculated with IMU051. Open symbols; glucose (◊) and sucrose (○) concentrations in flasks without inoculum. At day 0.00 and 1.06: ● overlapping with ○ and ♦ overlapping with ◊. Data were not corrected for evaporation to keep the experimental setup identical to that used for growth studies. The experiment was conducted with two independent replicates, of which one representative replicate is shown. 500 mL shake flasks containing 100 mL synthetic medium with sucrose 20 gL^{-1} were incubated at 30° C and at 200 rpm.

growth was observed (**Figure 2A**). At pH 3, linear growth was observed (**Figure 2A**), which was consistent with the occurrence of sucrose hydrolysis in a sterile culture at this acidic pH (**Figure 2B**). Whilst sucrose hydrolysis also occurred at pH 2 (**Figure S2**, Supporting Information), no growth was observed at this pH value, since *S. cerevisiae* CEN.PK113-7D cannot grow at this low pH (Della-Bianca *et al.* 2014). To avoid a contribution of non-biological hydrolysis of sucrose to the initiation of growth, all further growth experiments in this study were done in cultures with an initial pH of 6. For those cultures that did show growth, the pH eventually decreases to values below 3. However, since this drop only occurs towards the end of the exponential growth, this did not influence the initiation of growth.

Residual sucrose hydrolysis encoded by unknown genes

To investigate what is needed to completely abolish both sucrose transport and hydrolysis in *S. cerevisiae* CEN.PK102-3A, a strain was constructed in which the following genes were deleted: *SUC2* (encoding invertase), *MAL12*, *MAL22* and *MAL32* (encoding maltases) and *MAL11*, *MAL21*, *MAL31*, *MPH2*, *MPH3* (encoding transporters). The resulting strain (IMU051; *malΔ mphΔ suc2Δ*), did not show any growth over a period of up to 2 months in synthetic medium with sucrose as the sole carbon source (**Table 3**). However, for future metabolic engineering strategies concerning sucrose metabolism, it is important to understand whether this inability to grow is caused by lack of transport or lack of sucrose hydrolysis. To investigate this, three new strains were constructed: one expressing the transporter Mal11 (IMU048; *malΔ mphΔ suc2Δ MAL11*), one expressing the α-glucosidase Mal12 (IMU054; *malΔ mphΔ suc2Δ MAL12*) and a control strain expressing both genes (IMU055; *malΔ mphΔ suc2Δ MAL11 MAL12*). The maltase Mal12 was chosen for intracellular cleavage of sucrose rather than the invertase Suc2, since previous work has shown that extracellular Suc2 activity can be detected even if *SUC2* is expressed without the secretion signal sequence (Basso *et al.* 2011).

Combined expression of *MAL11* and *MAL12* resulted in a maximum specific growth rate of 0.19 h^{-1} for the control strain IMU055 (**Table 3**). Even after prolonged incubation of up to 2 months, expression of only the hydrolysing enzyme Mal12 (IMU054) did not enable growth in synthetic medium with sucrose as the sole carbon source, indicating that there is no residual sucrose transport activity sufficient to allow growth and that this phenotype is stable. In contrast, the strain that only expressed the transporter Mal11 (IMU048; *malΔ mphΔ suc2Δ MAL11*) grew at a maximum specific growth rate of 0.08 h^{-1} in synthetic medium with sucrose, after a lag phase of about 4 days (**Table 3**). All shake flasks cultivations were performed under an air atmosphere. In line with this observation, an activity of sucrose hydrolysis of 0.48 ± 0.03 μmol mg $protein^{-1}$ min^{-1} was detected in cell extracts of this

Table 3: Maximum specific growth rates of *Saccharomyces cerevisiae* strains grown in shake flasks containing synthetic medium (initial pH 6.0) with 20 g L^{-1} sucrose as sole carbon source. For enzyme-activity assays, strains were grown in synthetic medium with 20 g L^{-1} sucrose as sole carbon source. For enzymatic-activity determination, IMU054 and IMK700, that cannot consume sucrose, and strain IMU055, that overexpresses Mal12, were grown in synthetic medium with 20 g L^{-1} ethanol as sole carbon source. Averages and mean deviations were obtained from duplicate experiments.

Strain	Relevant genotype	Growth on sucrose (h^{-1})	Intracellular sucrose hydrolysis (μmol mg protein^{-1} min^{-1})	Extracellular sucrose hydrolysis (μmol mg protein^{-1} min^{-1})	Intracellular isomaltose hydrolysis (μmol mg protein^{-1} min^{-1})
CEN.PK113-7D	*IMAx MALxx SUC2*	0.37 ± 0.01	0.43 ± 0.01	5.40 ± 0.04	0.13 ± 0.01
IMU051	*malΔ mphΔ suc2Δ*	No growth*	N.D.	N.D.	N.D.
IMU054	*malΔ mphΔ suc2Δ MAL12*	No growth*	2.04± 0.07	N.D.	N.D.
IMU055	*malΔ mphΔ suc2Δ MAL11 MAL12*	0.19 ± 0.01	1.67 ± 0.08	N.D.	N.D.
IMU048	*malΔ mphΔ suc2Δ MAL11*	0.08 ± 0.01	0.48 ± 0.03	B.D.	1.05 ± 0.04
IMK700	*malΔ mphΔ suc2Δ MAL11 imaΔ cas9*	No growth*	B.D.	N.D.	B.D.
IMS422	IMU048 evolved #1	0.25 ± 0.02	1.10 ± 0.02	B.D.	2.31 ± 0.36
IMS423	IMU048 evolved #2	0.25 ± 0.02	1.57 ± 0.08	B.D.	1.59 ± 0.13
IMS424	IMU048 evolved #3	0.26 ± 0.02	1.10 ± 0.11	B.D.	2.16 ± 0.07

*Incubation period: 10 days.
B.D.: Below detection limit, i.e. < 0.03 μmol mg protein^{-1} min^{-1}.
N.D.: Not determined.

strain. Although lower than the activity of 1.67 ± 0.08 μmol mg protein^{-1} min^{-1} observed in the reference strain IMU055, this activity was sufficient to sustain growth on sucrose (**Table 3**).

Laboratory evolution enables sucrose hydrolysis and increased *IMA* expression

To investigate which genes encode the enzymes responsible for residual sucrose hydrolysis activity in strain IMU048 (*malΔ mphΔ suc2Δ MAL11*), this strain, which grows slowly in a sucrose-based medium (μ = 0.08 h^{-1}, **Table 3**), was subjected to sequential batch cultivation in synthetic

medium with sucrose 2% (w/w) as sole carbon source. After 30 transfers (70 days, corresponding to approximately 180 generations), single-colony isolates (IMS422, IMS423 and IMS424), were obtained from three independent evolution experiments. In addition to a 3-fold increase in maximum specific growth rate (from 0.08 h^{-1} to 0.25 - 0.26 h^{-1}, **Table 3**), the lag phase of these strains was shortened from 4 days to 1 day.

Enzyme-activity assays were conducted to investigate whether the increased growth rate of the evolved strains on sucrose correlated with sucrose hydrolytic activity. Indeed, intracellular sucrose hydrolysis activities in the evolved strains were 2 - 3 fold higher (1.10 - 1.57 μmol mg $protein^{-1}$ min^{-1}) than in the non-evolved parental strain IMU048 (0.48 μmol mg $protein^{-1}$ min^{-1}). No extracellular sucrose-hydrolysing activity was detected in cultures of these strains. In contrast, in cultures of the reference strain CEN.PK113-7D, extracellular sucrose activity was 5.40 μmol^{-1} mg $protein^{-1}$ min^{-1} while intracellular activity was only 0.43 μmol^{-1} mg $protein^{-1}$ min^{-1} (**Table 3**). Activity of isomaltases, which are known to also hydrolyse sucrose (Deng *et al.* 2014) were measured to investigate a possible contribution of these enzymes to the observed evolved phenotype. Indeed, isomaltase activity was about 1.5 - 2 times higher in the evolved strains (1.59 - 2.31 μmol mg $protein^{-1}$ min^{-1}) compared to IMU048 (1.05 μmol mg $protein^{-1}$ min^{-1}) (**Table 3**). In CEN.PK113-7D, isomaltase activity was only 0.13 μmol mg $protein^{-1}$ min^{-1} (**Table 3**). Since isomaltases cannot hydrolyse maltose (Deng *et al.* 2014), it was checked if the evolved strains could consume this sugar. After 2 months of incubation in synthetic medium with maltose, no growth was observed.

Quantitative real-time PCR was performed to investigate if the differences in sucrose and isomaltose hydrolysis observed among reference, unevolved and sucrose-evolved strains could be explained by differences in gene expression. Expression of *IMA2*, *IMA3* and *IMA4* was analysed with a single primer pair since the sequences of these genes are highly similar (**Figure 3**). No difference in expression ratio was observed for the pool consisting of *IMA2*, *IMA3* and *IMA4* transcripts among the strains tested (**Figure 3C**). In contrast, *IMA1* and *IMA5* expression was significantly higher in

IMU048 (*malΔ mphΔ suc2Δ MAL11*) than in CEN.PK113-7D. Expression of these genes in the evolved strains was even 3 to 5 fold higher than in the unevolved strain IMU48 (**Figure 3A** and **B**). In all analysed strains, the relative expression level of *IMA5* was approximately 5-10 times higher than that of *IMA1* (**Figure 3A** and **B**).

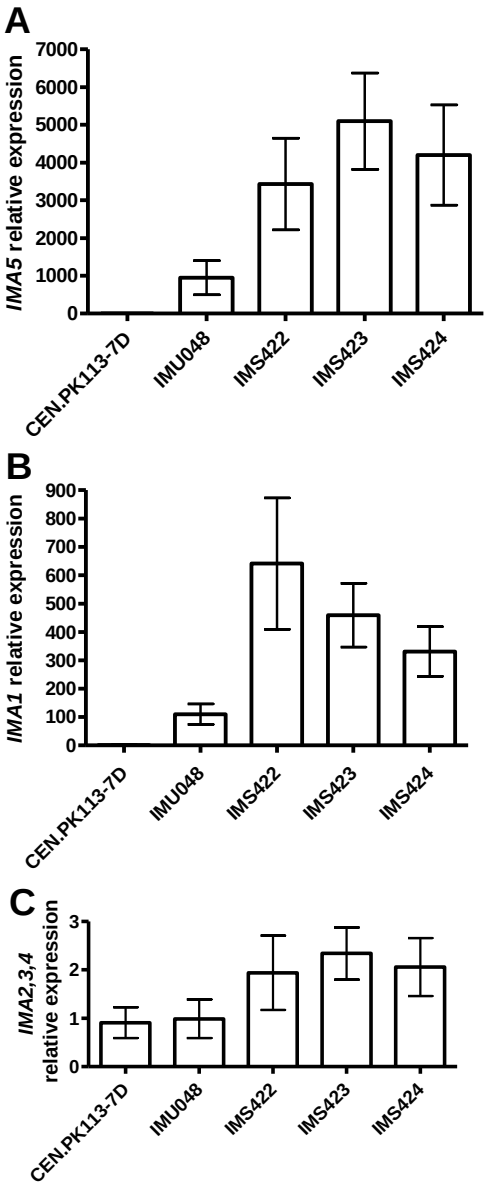

Figure 3: Relative expression of *IMA1-5* in *Saccharomyces cerevisiae* strains evolved for faster growth on synthetic medium with sucrose as the sole carbon source. Relative expression of *IMA5* **(A)**; *IMA1* **(B)** and *IMA2*, *IMA3* and *IMA4* **(C)**. Expression of *IMA2*, *IMA3* and *IMA4* was assayed by a single primer pair since these genes are highly similar in nucleotide sequence. CEN.PK113-7D: reference strain (*IMAx MALxx MPHx SUC2)*; IMU048: unevolved parental strain (genotype: *malΔ mphΔ suc2Δ MAL11*) and three independently evolved strains (IMS422, IMA423 and IMS424). CEN.PK113-7D was used as calibrator sample (expression set to 1). Averages and standard errors were obtained from independent duplicate biological experiments and three technical replicates. Cells were harvested at mid-exponential phase (OD_{660nm}= 3) from shake-flask cultures on

synthetic medium with sucrose as sole carbon source.

Deletion of *IMA genes* is required and sufficient to completely eliminate sucrose hydrolysis.

Based on the results shown above, we tested whether deletion of the *IMA* genes would be sufficient to abolish sucrose consumption in the evolved strains (IMS422, IMS423 and IMS424) and in an unevolved strain (IMZ571; *malΔ mphΔ suc2Δ MAL11-LEU2* cas9-*URA3*) (**Table 1**). All 6 copies of *IMA* genes present in strain IMZ571 and IMS422-424, all derived from strain CEN.PK102-3A (**Table 1**) (de Kok *et al.* 2011; Nijkamp *et al.* 2012) were deleted in a single transformation step using only two gRNAs cloned in a single plasmid. One of these gRNAs targeted *IMA5* while the other simultaneously targeted all other *IMA* genes. None of the resulting strains (named IMK716, IMK717, IMK718 and IMK700, respectively) grew in synthetic medium with sucrose as sole carbon source after 10 days of incubation (**Table 4**) or after a further 2-months incubation. Both the sucrose- and isomaltose hydrolysis activities in the resulting strains were below the detection limit (**Table 4**).

***IMA1* and *MAL23-C* copy number increase in evolved strains.**

Sanger sequencing of the *IMA* genes, including their promoter regions did not reveal any mutations (*IMA* genes from the evolved strains in comparison to that of the parental). Whole genome sequencing of the parental strain IMU048 revealed that, upon sequential cycle of deletions using loxP sites, the strain became aneuploidy for CHRIII and CHRVII. A region from CHRVII located between retrotransposons (from position 70,700 to 82,000) was found duplicated already in the parental strain and, in the evolved strains, such duplication was lost. Regarding CHRIII, the parental strain gained an extra copy after deletions using loxP sites, truncated of the majority of the left arm. Sequencing of three independently evolved isolates indicated a further increase in copy number of the right arm of CHRIII (**Figure 4**). Relative to the

Table 4: Maximum specific growth rates of *Saccharomyces cerevisiae* strains grown in shake flasks containing synthetic medium (initial pH 6.0) with 20 g L^{-1} sucrose as sole carbon source. For enzymatic activity determination, strains were grown in synthetic medium with 20 g L^{-1} ethanol as sole carbon source (sucrose was not used since strains IMK716-18 cannot grow on this substrate). Averages and mean deviations were obtained from duplicate experiments.

Strain	Relevant genotype	Growth on sucrose (h^{-1})	Intracellular sucrose hydrolysis (μmol mg protein^{-1} min^{-1})	Intracellular isomaltose hydrolysis (μmol mg protein^{-1} min^{-1})
IMK716	IMU048 evolved #1 *cas9 imaΔ*	No growth	B.D.	B.D.
IMK717	IMU048 evolved #2 *cas9 imaΔ*	No growth	B.D.	B.D.
IMK718	IMU048 evolved #3 *cas9 imaΔ*	No growth	B.D.	B.D.
IMZ620	*malΔ mphΔ suc2Δ MAL11 imaΔ IMA1*	0.19 ± 0.01	2.38 ± 0.03	4.60 ± 0.54
IMZ621	*malΔ mphΔ suc2Δ MAL11 imaΔ IMA2*	0.19 ± 0.01	2.88 ± 0.28	1.95 ± 0.03
IMZ622	*malΔ mphΔ suc2Δ MAL11 imaΔ IMA3,4*	0.17 ± 0.01	0.92 ± 0.03	0.64 ± 0.14
IMZ623	*malΔ mphΔ suc2Δ MAL11 imaΔ IMA5*	0.04 ± 0.01	0.17 ± 0.03	6.11 ± 0.18

*Incubation period: 10 days.
B.D.: Below detection limit, i.e. < 0.03 μmol mg protein^{-1} min^{-1}.

left arm of the same chromosome (until approximately position 90,000), the region between positions 90,000 and 150,000, which includes the centromere, showed an approximately two-fold increase in read depth. The right-arm distal end (beyond position 150,000) even showed a three-fold higher increase in read depth in all three evolved strains relative to the distal end of the left arm (**Figure 4**). Retrotransposons (e.g. YCLWΔ15 and YCRCΔ6) located near positions 90,000 and 150,000 may have contributed to translocation/duplication events at these loci. The duplicated region in the parental strain IMU048 and triplicated in the evolved isolates harboured *IMA1* and *MAL23-C*. To investigate the impact of an increased expression of *IMA* genes during growth on sucrose, they were individually overexpressed in a strain in which all known sucrose-hydrolysing activities were inactivated (IMK743; *malΔ mphΔ suc2Δ MAL11 imaΔ*) (**Table 1**). All four constructed strains grew in a sucrose-based medium: strains IMZ620 and IMZ621 (overexpression of *IMA1* and *IMA2*, respectively) both grew at specific growth

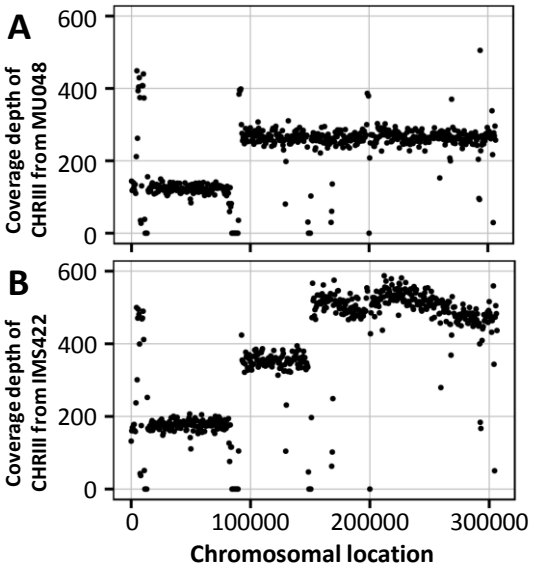

Figure 4: Depth of coverage analysis of Chromosome III (CHRIII) from unevolved strain IMU048 **(A)** and the evolved strain IMS422 **(B)**. Sequencing reads from the IMU048 and IMS422 have been mapped onto the CEN.PK113-7D genome sequence (Nijkamp *et al.* 2012). Read count data represent the average coverage of non overlapping 500 bp window. These data are representative of the other two evolved strains (IMS423 and IMS424).

rate of 0.19 h^{-1}. Accordingly, sucrose and isomaltose hydrolysis activities in these strains were ca. 2 µmol mg protein^{-1} min^{-1} (**Table 4**). Strain IMZ622, which overexpressed *IMA3/4*, grew at 0.17 h^{-1}, while IMZ623 (*IMA5* overexpressed) grew at 0.04 h^{-1}. Sucrose hydrolysis activities in cell extracts of strains IMZ622 and IMZ623 were 0.92-0.17 µmol mg protein^{-1} min^{-1}, respectively (**Table 4**). No maltose hydrolysis was detected in any of the *IMA*-overexpressing strains.

DISCUSSION

This study confirms the power of laboratory evolution in identifying suppressor mutations of seemingly essential genes (Liu *et al.* 2015). Prolonged incubation of the *malΔ mphΔ suc2Δ MAL11 imaΔ* strain for up to two months

demonstrated that deletion of the *IMA*-genes is both required and sufficient to eliminate growth on sucrose in a strain lacking invertase and maltase activities. Deletion of the disaccharide transporter genes *MAL11*, *MAL21*, *MAL31*, *MPH2* and *MPH3* similarly resulted in a sucrose-negative phenotype that was stable for over two months incubation in a sucrose-containing medium. The phenotypic stability of the sucrose-negative strains generated in this study makes them a suitable platform for use in diverse applications: *i)* screening heterologous disaccharide transporters (e.g. characterization of plant sucrose transporters in yeast; study maltose/maltotriose transporters relevant for beer brewing (Alves *et al.* 2008); *ii)* screening and characterization of disaccharide hydrolases and phosphorylases; *iii)* metabolic engineering of disaccharide metabolism for improving the production of biofuels and other bio-based chemicals. Before laboratory evolution for improved growth on sucrose, strain IMU048 (*malΔ mphΔ suc2Δ MAL11*) already showed a much higher expression of *IMA1* and *IMA5* compared to CEN.PK113-7D. A main difference between IMU048 and CEN.PK113-7D is the location of sucrose hydrolysis. In the reference strain CEN.PK113-7D, sucrose is predominantly hydrolysed extracellularly (Basso *et al.* 2011), whereas in IMU048 sucrose is imported via Mal11 (proton symporter) and hydrolysed in the cytosol. The presence of intracellular sucrose could possibly activate transcription factors such as the *MAL*-activators *MALx3* (Alamäe *et al.* 2003; Weinhandl *et al.* 2014). It is important to clarify that the deletions carried out in this study included maltases and *MAL*-transporters but not the *MAL*-activators (*MAL13*, *MAL23-C* and *MAL33*). *MAL23-C* (known as *MAL2-8^{c}*) is constitutively expressed (Gibson *et al.* 1997) but might undergo posttranslational inhibition by chaperones, as has been shown for Mal63, for which the inhibition is relieved when maltose is present (Bali *et al.* 2003; Ran, Bali and Michels 2008). Teste, François and Parrou (2010) showed that the *IMA1* and *IMA5* promoter regions contain 1 and 3 binding sites for *MALx3,* respectively. The other *IMA*-promoters do not contain binding sites for *MALx3* (Teste, François and Parrou 2010). The same authors showed that maltose induces higher expression levels of *IMA5* than of *IMA1*, and has no effect on

the expression levels of *IMA2*, *IMA3* and *IMA4*. The strongly increased transcript levels of *IMA1* (110 fold) and *IMA5* (950 fold) in IMU048, relative to those in CEN.PK113-7D (**Figure 3A** and **B**), but not of *IMA2,3,4* (**Figure 3C)**, is in line with the hypothesis that sucrose activates the *MAL*-activators. Extracellular glucose and fructose released in cultures of the strain CEN.PK113-7D by the action of Suc2 might further contribute to the repression of the *MAL*-activators (Hu *et al.* 1995; Horák 2013) in this reference strain.

As mentioned above, the complement of genes belonging to the disaccharide hydrolase gene families varies greatly among *Saccharomyces cerevisiae* strains. With even closely related strains showing a high degree of genetic variation, it is important to have access to the genome sequence of the immediate parental strain that is used as a platform on which a metabolic engineering strategy will be implemented. The importance of strain-dependent gene contents is exemplified by a literature debate on the number of binding sites for *MALx3* in the promoters of *IMA1* and *IMA5*. In contrast to Teste, François and Parrou (2010), Pougach *et al.* (2014) stated that the *IMA5* promoter only has a single binding site for *MALx3*. In our study, Sanger sequencing of the promoter region of *IMA5* confirmed the presence of three *MALx3* binding sites previously identified by Teste, François and Parrou (2010): the motif CGGN{9}CGG was found at position -141 and -543 and motif MGCN{9}MGS at position -516 (Sirenko, Ni and Needleman 1995). This confirmation does not exclude the possibility that the data of Pougach *et al.* (2014) are correct, since they used a different *S. cerevisiae* strain (KV5000, which originates from BY4741, a S288c derived strain) whilst Teste, François and Parrou (2010) used the CEN.PK113-7D strain.

After laboratory evolution, expression of both *IMA1* and *IMA5* increased by 3-fold compared to the unevolved IMU048. Whole-genome sequencing showed that the strain subjected to evolution (IMU048) already contains a duplication of a large part of CHRIII, that starts before the centromere and extends until the right end of the chromosome **(Figure 4)**. Likewise, CHRVII of IMU048 contains a duplicated region between

retrotransposon sites that was lost after the evolution. Therefore, genes located in this region might not contribute to sucrose consumption. Such aneuploidies goes often undetected, but this mechanism has been identified in a fraction of deletion mutants generated by the Saccharomyces genome deletion consortium (Winzeler *et al.* 1999; Hughes *et al.* 2000). Several cases reported that the extra chromosome harboured a close homologue of the gene deleted, in our study the deletion of *MAL31* and *MAL32* may have led to the aneuploidy of CHRIII that harbours the *MAL2* locus. Additionally both unevolved (IMU048) and evolved strains (IMS422-4) carried structural variant of the CHRIII. Such rearrangement, especially at chromosome 3, has been extensively reported in the literature as result of recombination between retrotransposons located around position 90,000 and 150,000 (Mieczkowski, Lemoine and Petes 2006; de Kok *et al.* 2012) (Mieczkowski, Lemoine and Petes 2006; de Kok *et al.* 2012). In all the three evolved strains obtained in this study, a third copy of the mentioned region was found (**Figure 4**). This region not only contains *IMA1*, but also *MAL23-C*. If, as discussed above, sucrose indeed activates *MAL23-C*, *IMA1* expression level could be increased by increase in copy number of the gene encoding its activator, *MAL23-C*. Whilst in this study overexpression of *IMA1* was shown to result in faster growth on sucrose, *IMA5* was shown to be predominantly an isomaltase and overexpression resulted in only very slow growth and low intracellular sucrose hydrolysis activity (**Table 4**) (Deng *et al.* 2014). It therefore seems likely that the increased expression of *IMA5* is collateral to the upregulation of *MAL23-C*.

In addition to the scientific value of the sucrose-negative strain platform, this study demonstrates how a single CRISPR targeting-sequence can be used to simultaneously delete multiple genes in *S. cerevisiae*. This strategy allows the deletion and/or modification of entire gene families in a single transformation event.

AKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIALS

Table S1: Primers used in this study.

Primer code	Name	Sequence (5'→ 3')
Primers for *SUC2* deletion		
1482	KO Fw SUC2	CCATTATGAGGGCTTCCATTATTCCCCGCATTTTTATTACTCTGAAC AGGCAGCTGAAGCTTCGTACGC
1483	KO Rv SUC2	CTTTAGAATGGCTTTTGAAAAAAATAAAAAAGACAATAAGTTTTAT AACCGCATAGGCCACTAGTGGATCTG
Primer for plasmid construction		
7833	Rv pUC19 5P	GGTCATAGCTGTTTCCTGTGTG
4697	c BPGM-URA fw	CGAGTCATGTAATTAGTTATGTCACG
1768	LacZ Ctrl Rv	GCTTCCGGCTCCTATGTTG
7236	ADH universal ampwith p426 hom re	CGGTTAGAGCGGATGTGGGG
5974	2mu insidefw	TACTTTTGAGCAATGTTTGTGGA
5975	2mu inside rv	AACGAGCTACTAAAATATTGCGAA
8759	IMA_targetRNA#2F	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATG ATC**TTCTTCTGGAGACCTCCTAA**GTTTTAGAGCTAGAAATAGCAAG TTAAAATAAG
8761	IMA5_taretRNA_F	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAATG ATC**TGGCCAACCTGATTTCAATT**GTTTTAGAGCTAGAAATAGCAAG TTAAAATAAGGCTAGTCCGTTATCAAC
6005	p426 CRISP rv	GATCATTTATCTTTCACTGCGGAGAAG
9302	pTDH3_IMA1start	CTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAGAACTT AGTTTCGACGGATATGACTATTTCTTCTGCACATCCAGAGACAGAAC
9303	pTDH3_IMA234strt	CTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAGAACTT AGTTTCGACGGATATGACTATTTCTTCTGCACATC
9304	pTDH3_IA5star	CTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAGAACTT AGTTTCGACGGATATGACGATCATCCATAATCCTAAATGGTGGAAAG
9305	tCYC1_IMA1end	CGGTTAGAGCGGATGTGGGGGGAGGGCGTGAATGTAAGCGTGACAT AACTAATTACATGATCATTCGCTGATATATATTCTTCCTTC
9306	tCYC1_IMA234end	CGGTTAGAGCGGATGTGGGGGGAGGGCGTGAATGTAAGCGTGACAT AACTAATTACATGATCATTCAGATATGTAAATTCTGCCCTCCCATG
9307	tCYC1_IMA5end	CGGTTAGAGCGGATGTGGGGGGAGGGCGTGAATGTAAGCGTGACAT AACTAATTACATGATTACTTCAACAAGTAAAGTCTTCCTTCCCATGG CTGCAAGG
Repair fragments		
8592	IMA_repair oligo fw	CGACTCGCCACAAGATGATATGGGTTACGATATTGCCAACTACGAAA AGGTCTGGCCAACATGCTAGAACACCTATGCAATGGTCTCGTGAGGA GCCAAATGCTGGTTTTTCTGGTCCTA
8593	IMA_repair oligo rv	TAGGACCAGAAAAACCAGCATTTGGCTCCTCACGAGACCATTGCATA GGTGTTCTAGCATGTTGGCCAGACCTTTTCGTAGTTGGCAATATCGT AACCCATATCATCTTGTGGCGAGTCG
8763	IMA5_Repair_oligo_Fw	GCGAAAAACTACTGTTTCTTGATGACTATATAGAAAGTGCTCAATAT TGAAAAGAAAAACCGAGCACATTTAACATAAACGTACTGATCTTTCG AAACAAATAGTTATGCCGATATTTAT
8764	IMA5_Repair_oligo_Rv	ATAAATATCGGCATAACTATTTGTTTCGAAAGATCAGTACGTTTATG TTAAATGTGCTCGGTTTTTCTTTTCAATATTGAGCACTTTCTATATA GTCATCAAGAAACAGTAGTTTTTCGC

Primer code	Name	Sequence (5'→ 3')
Specific primers for *IMA* genes		
8607	IMA1-Diag-Fw	TTATGTCAGTACTTGGGATCG
8608	IMA2-Diag-Fw	gtcCATAGGCACGTAAAAG
8609	IMA3&4-Diag-Fw	CATTCGTAAGACGTCcacag
8610	IMA5-DiagFw	CCTCCGTGGAGAATCCTCG
8611	IMA1-Diag-Rv	ATACGGCCACCATCTCATTC
8612	IMA2-Diag-Rv	ATAGTAAATGAGATTGTACG
8613	IMA3&4-Diag-Rv	GTAGTAATTCTGAAATTG
8614	IMA5-Diag-Rv	AGCGTACAGCGTAAGTTAAG
SANGER-sequencing of *IMA* genes		
9045	SeqF_ima1_block1	CTCAAGATCCGGTTGGAACG
9046	SeqF_ima1_block2	GTGAACAACCGCTCAGCAC
9047	SeqF_ima2_block1	GCGGATGTATTATTAACACCTGAC
9048	SeqF_ima2_block2	AGTTTACGCTTCGTACTTTGTC
9049	SeqF_ima3_block1	ACCGGACATGATATTGGGTAAG
9050	SeqF_ima3_block2	CGGAGGCAATGCAATAATGG
9051	SeqF_imas_block3	GGGTTACGATATTGCCAACTAC
9052	SeqF_imas_block4	CATCAGAAACAGAGTGAAGGATGG
9053	SeqF_imas_block5	CAATGGTCTCGTGAGGAGCCAAATG
9054	SeqR_ima1_block1	TATCTACGGCGCAGTACAACG
9055	SeqR_ima2_block1	TACATAACTCATACCGCACTACC
9056	SeqR_ima3_block1	CACATACCCAATATCGCATTACC
9057	SeqR_imas_block2	TCACGAGACCATTGCATAGG
9058	SeqR_imas_block3	GCCATCCTTCACTCTGTTTC
9059	SeqR_imas_block4	GTTGGCAATATCGTAACCCATATC
9060	SeqR_ima1_block5	TGCTGAGCGGTTGTTCACAC
9061	SeqR_ima2_block5	GTAGTCCCACGACAAAGTACG
9062	SeqR_ima3_block5	GAGCGTAAACAACCCTCGATCC
9063	SeqF_ima5_block1	CAGCCAATGGCGCTTCCCAC
9064	SeqF_ima5_block2	TGTCTTTCATGCGAAGAAGC
9065	SeqF_ima5_block3	AACATGACGATCATCCATAATCC
9066	SeqF_ima5_block4	GACGTAGGCAGCATGTATTC
9067	SeqF_ima5_block5	ATATGCCGCCATTAAACGTG
9068	SeqR_ima5_block1	AGGGTGAGAATAGTCGAATGT
9069	SeqR_ima5_block2	CTTTGTGCTCCTTTCTAACTTG
9070	SeqR_ima5_block3	GGTGTGCTTGAAGTTGAACATC
9071	SeqR_ima5_block4	CCAGGTCAACAATAACCTTGAT
9072	SeqR_ima5_block5	CTCCGTCTAGGCATTTACGCTTTC
Primers for Real-Time quantitative PCR		
8765	IMA5_qPCR_Fw	TGAGCAAATCAAATGCGTTG
8766	IMA5_qPCR_Rv	ATGGTTTGGCATCCTTCGAG
8767	IMA1_qPCR_Fw	TTTGTTCCGTTACAACTTGG
8768	IMA1_qPCR_Rv	GTACCGGTCAAGGCACTTAG
8771	IMA2,3,4_qPCR_Fw	AAGTTCCGTCAAAATTTGAT
8772	IMA2,3,4_qPCR_Rv	CGCTCAGTGACACCAAC
8773	ALG9_qPCRref_Fw	CACGGATAGTGGCTTTGGTGAACAATTAC
8774	ALG9_qPCRref_Rv	TATGATTATCTGGCAGCAGGAAAGAACTTGGG
8775	UBC6_qPCRref_Fw	GATACTTGGAATCCTGGCTGGTCTGTCTC
8776	UBC6_qPCRref_Rv	AAAGGGTCTTCTGTTTCATCACCTGTATTTGC
8777	TFC1_qPCRref-Fw	GCTGGCACTCATATCTTATCGTTTCACAATGG
8778	TFC1_qPCRref-Rv	GAACCTGCTGTCAATACCGCCTGGAG

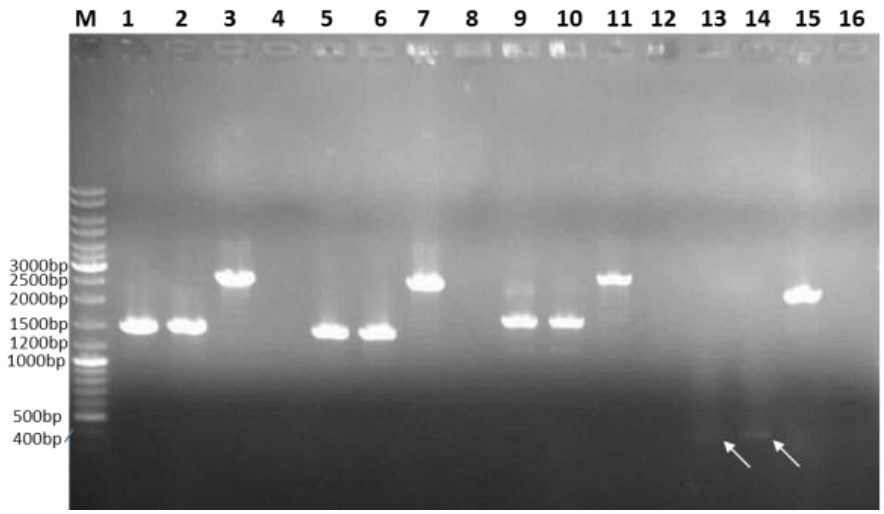

Figure S1: Diagnostic PCR of the deletion of the *IMA* genes. The deletion was carried out in strain IMK700 (lane 2, 6, 10 and 14). Lanes 1, 5, 9 and 13 correspond to a strain that is not under the scope of this paper. IMU048 was used for comparison since it carries all the *IMA* genes (lanes 3, 7, 11 and 15). No template control is shown on lanes 4, 8, 12 and 16. "M": GeneRuler™ DNA Ladder Mix. *Lane 2,3 and 4:* diagnosis of *IMA1* deletion with primers 8607 & 8611 (see Table S1). Expected fragment of 1448 bp (gene deleted) or 2531 bp (not deleted);*Lane 6, 7 and 8:* diagnosis of *IMA2* deletion with primers 8608 & 8612. Expected fragment of 1362 bp (gene deleted) or 2446 bp (not deleted);*Lane 10, 11 and 12:* diagnosis of *IMA3* and *IMA4* deletion with primers 8609 & 8613. Expected fragment of 1637 bp (gene deleted) or 2720 bp (not deleted);*Lane 14, 15 and 16:* diagnosis of *IMA5* deletion with primers 8610 & 8614. Expected fragment of 415 bp (gene deleted) or 2162 bp (not deleted); Both copies of *IMA1* (CHRIII and CHRVII) were not individually checked due to their similarity. The same applies for *IMA3* (CHRIX) and *IMA4* (CHRX). For this reason, rearrangement of between these chromosomes cannot be ruled out.

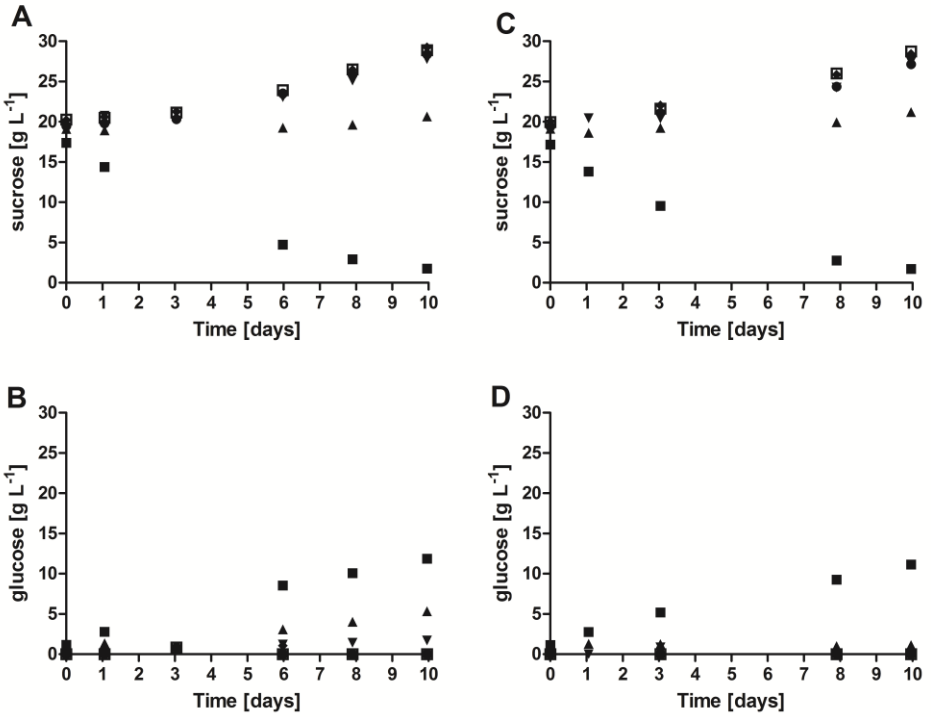

Figure S2: Non-biological hydrolysis of sucrose in synthetic medium set at different pH values. **A** and **B** show sucrose and glucose concentrations in the supernatant of shake-flasks without inoculum, respectively. **C** and **D** show sugar concentration in the presence of IMU051 (*malΔ mphΔ suc2Δ*), a strain that cannot consume sucrose but can grow on released monosaccharides if the pH is permissive (pH 2 is not). Symbols: ■, pH 2; ▲, pH 3; ▼, pH 4; ♦ pH 5; ●, pH 6 and □, pH 7. The experiment was conducted with two independent replicates, of which one representative is shown. 500 mL shake-flasks containing 100 mL synthetic medium with sucrose 20 g L^{-1} was incubated at 30° C and 200 rpm. This experimental setup does not prevent water evaporation, which explains the increase in sugar concentrations. No correction for evaporation has been performed to keep this experiment similar to growth assays carried out in this work.

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3

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

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Abstract

Anaerobic industrial fermentation processes do not require aeration and intensive mixing and the accompanying cost savings are beneficial for production of chemicals and fuels. However, the free-energy conservation of fermentative pathways is often insufficient for the production and export of the desired compounds and/or for cellular growth and maintenance. To increase free-energy conservation during fermentation of the industrially relevant disaccharide sucrose by *Saccharomyces cerevisiae*, we first replaced the native yeast α-glucosidases by an intracellular sucrose phosphorylase from *Leuconostoc mesenteroides* (*Lm*SPase). Subsequently, we replaced the native proton-coupled sucrose uptake system by a putative sucrose facilitator from *Phaseolus vulgaris* (*Pv*Suf1). The resulting strains grew anaerobically on sucrose at specific growth rates of 0.09 ± 0.02 h^{-1} (*LmSPase*) and 0.06 ± 0.01 h^{-1} (*PvSUF1*, *LmSPase*). Overexpression of the yeast *PGM2* gene, which encodes phosphoglucomutase, increased anaerobic growth rates on sucrose of these strains to 0.23 ± 0.01 h^{-1} and 0.08 ± 0.00 h^{-1}, respectively. Determination of the biomass yield in anaerobic sucrose-limited chemostat cultures was used to assess the free-energy conservation of the engineered strains. Replacement of intracellular hydrolase with a phosphorylase increased the biomass yield on sucrose by 31%. Additional replacement of the native proton-coupled sucrose uptake system by *Pv*Suf1 increased the anaerobic biomass yield by a further 8%, resulting in an overall increase of 41%. By experimentally demonstrating an energetic benefit of the combined engineering of disaccharide uptake and cleavage, this study represents a first step towards anaerobic production of compounds whose metabolic pathways currently do not conserve sufficient free-energy.

INTRODUCTION

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

Although many conversions of sugars into industrially relevant products are feasible based on thermodynamics and mass conservation, ATP formation by substrate-level phosphorylation in central metabolism can be insufficient to provide the energy required for product-formation pathways, product export, cellular growth and/or maintenance (de Kok *et al.* 2012; Cueto-Rojas *et al.* 2015). For example, in the conversion of glucose into lactic acid by *Saccharomyces cerevisiae*, all ATP formed by substrate-level phosphorylation in glycolysis is required for export of product (Abbott *et al*., 2009; Van Maris *et al*., 2004). In this specific example, increased free-energy (ATP) conservation could enable homofermentative, anaerobic lactate production.

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

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

S. cerevisiae does not conserve the free energy of sucrose hydrolysis (ΔG_0' = -29 kJ/mol). In some anaerobic microorganisms sucrose is cleaved by phosphorolysis instead of hydrolysis. In the latter cleavage process, sucrose phosphorylase (SPase) converts inorganic phosphate and sucrose into glucose-1-phosphate and fructose. Glucose-1-phosphate can subsequently be converted into glucose-6-phosphate by phosphoglucomutase. As this phosphorolytic cleavage circumvents the ATP-requiring hexokinase reaction, it enables higher overall free-energy conservation than sucrose hydrolysis (**Figure 1C**). Genes encoding SPase are known from various bacterial species (Kawasaki *et al.* 1996). Other disaccharide phosphorylases, such as maltose-

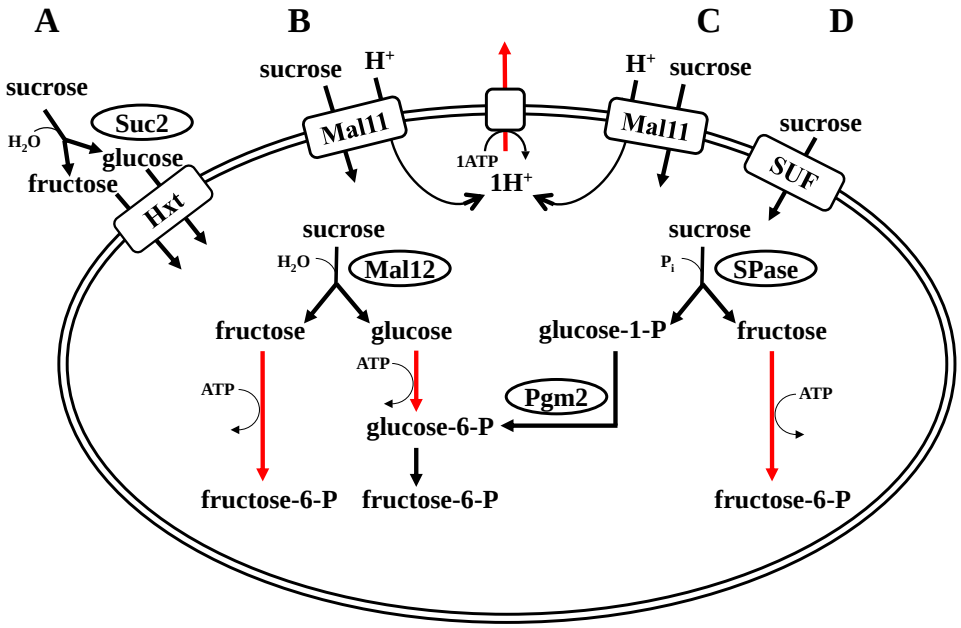

Figure 1. Schematic representation of different strategies for the uptake and cleavage of sucrose. **A**) Extracellular hydrolysis of sucrose, facilitated by the external invertase Suc2, followed by facilitated diffusion of the monosaccharides. **B**) Uptake of sucrose via the proton-symporter Mal11,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

and cellobiose phosphorylase, have previously been functionally expressed in *S. cerevisiae* (de Kok *et al.* 2011; Sadie *et al.* 2011).

While intracellular phosphorolysis theoretically enables a higher free-energy conservation (gain of 1 ATP per sucrose molecule), it requires transport of extracellular sucrose to the cytosol. However, in wild-type *S. cerevisiae*, uptake of sucrose via a proton-symporter (e.g. Mal11 (Stambuk *et al.* 1999)) and subsequent export of the proton via the H^+-ATPase results in a

net expense of 1 ATP (Weusthuis *et al.* 1993). Therefore, an improved free-energy conservation can be achieved when the proton-symport system is replaced by transport via facilitated diffusion (SUF, **Figure 1D**). Sucrose transporters from *Phaseolus vulgaris* and *Pisum sativum* have been functionally expressed in *S. cerevisiae* and were described as probable sucrose facilitators (SUFs) (Zhou *et al*. (2007)). Additionally, sucrose transporters from the SWEET family, e.g. from *Arabidopsis thaliana* and *Oryza sativa*, have also been proposed to catalyse facilitated diffusion (Chen *et al.* 2010, 2012; Lin *et al.* 2014).

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

MATERIALS AND METHODS

Strain storage and maintenance

The *S. cerevisiae* strains used in this study (**Table 1**) share the CEN.PK genetic background (Entian and Kötter 2007; Nijkamp *et al.* 2012) with the exception of BY4742 which is derived from S288C (Brachmann *et al.* 1998). Cultures were grown at 30°C in 500 mL shake flasks containing 100 mL synthetic medium (SM) (Verduyn *et al.* 1992) with 20 g/L glucose as a carbon source in an Innova incubator shaker (Eppendorf, Hamburg, Germany) set at 200 rpm. Frozen stocks were prepared by addition of glycerol (30% v/v) to exponentially growing shake-flask cultures of *S. cerevisiae* and stored aseptically in 1 mL aliquots at -80°C.

Plasmid construction

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

Table 1:The *Saccharomyces cerevisiae* strains that were used in this study.

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

with primers 6845 & 6846 and ligation was done via Gibson assembly.

Table 2: Plasmids used in this study

Name	Relevant characteristics	Origin
pUDC156	*ARS4-CEN6 URA3 pTEF1-cas9-tCYC1*	Marques *et al*., 2017
pUDR128	*2μ natNT2 gRNA-IMA5 gRNA-IMA1,2,3,4*	This study
pROS15	*2μ natNT2 gRNA-CAN1.Y gRNA-ADE2.Y*	Mans *et al*., 2015
pUD155	*attB1-LmSPase-attB2*	Baseclear B.V., Leiden, NL
pUDE262	*2μ URA3 pTDH3-LmSPase-tADH1*	This study
pUDE063	*2μ URA3 pTDH3-pgmB-tADH1*	de Kok *et al*., 2011
pUDE260	*2μ URA3 pTDH3-tADH1*	This study
p426TEF-*amdSYM*	*2μ amdSYM pTEF1-tCYC1*	This study
p426TEF	*2μ URA3 pTEF1-tCYC1*	Mumberg *et al*., 1995
pUG-*amdSYM*	*amdSYM*	Solis-Escalante *et al*., 2013
pUDE379	*2μ amdSYM pTEF1-MAL11-tCYC1*	This study
pUDI035	*Integrative plasmid, LEU2 pTDH3-MAL11-tCYC1*	de Kok *et al*., 2011
pUDE432	*2μ URA3 pTEF1-MAL11-tCYC1*	This study
p426GPD	*2μ URA3 pTDH3-tCYC1*	Mumberg *et al*., 1995
pUDE485	*2μ natNT1 pTPI1-PGM2-tTEF1*	This study
pUDE206	*2μ natNT1 pTPI1-I-SceI-tTEF1*	González-Ramos *et al*., 2016
pUDE496	*2μ URA3 pTEF1-MAL11-tCYC1 pTPI1-PGM2-tTEF1*	This study
pUD400	pMA-T *AtSWEET12*	GeneArt, Regensburg, Germany
pUD401	pMK-RQ *OsSWEET11*	GeneArt, Regensburg, Germany
pDR196-*PsSUF1*	*2μ URA3 pPMA1-PsSUF1-tADH1*	Zhou *et al.,* 2007
pDR196-*PsSUF4*	*2μ URA3 pPMA1-PsSUF4-tADH1*	Zhou *et al.,* 2007
pUDI085	*Integrative plasmid, LEU2 pTDH3-PsSUF1-tCYC1*	This study
pUDI086	*Integrative plasmid, LEU2 pTDH3-PsSUF4-tCYC1*	This study
pUC57-*PvSUF1*	*PvSUF1-codon optimized*	GenScript, Piscataway, NJ
pUDI087	*Integrative plasmid, LEU2 pTDH3-PvSUF1-tCYC1*	This study
pUDE367	*2μ amdSYM pTEF1-PsSUF1-tCYC1*	This study
pUDE368	*2μ amdSYM pTEF1-PsSUF4-tCYC1*	This study
pUDE369	*2μ amdSYM pTEF1-PvSUF1-tCYC1*	This study
pUDE370	*2μ amdSYM pTEF1-AtSWEET12-tCYC1*	This study
pUDE374	*2μ amdSYM pTEF1-OsSWEET11-tCYC1*	This study
pUDE413	*2μ URA3 pTEF1-PvSUF1-tCYC1*	This study
pUDE438	*2μ URA3 pTEF1-AtSWEET12-tCYC1*	This study
pUDE439	*2μ URA3 pTEF1-OsSWEET11-tCYC1*	This study
pUDE441	*2μ URA3 pTEF1-PsSUF1-tCYC1*	This study
pUDE442	*2μ URA3 pTEF1-PsSUF4-tCYC1*	This study
pFB001	*2μ URA3 YPet-tCYC1*	Bianchi *et al*., 2016
pRHA00	*2μ URA3 MAL11-YPet-tCYC1*	This study
pRS315	*ARS4-CEN6 LEU2*	Sikorski and Hieter, 1989
pRHA00L	*2μ LEU2 MAL11-YPet-tCYC1*	This study
pR151	*2μ LEU2 pTEF1-MAL11-YPet-tCYC1*	This study
pUDE453	*2μ URA3 pTEF1-MAL11-YPet-tCYC1*	This study
pUDE471	*2μ URA3 pTEF1-PvSUF1-YPet-tCYC1*	This study
pUDE486	*2μ URA3 pTEF1-PvSUF1-tCYC1 pTPI1-PGM2-tTEF1*	This study
pUDR119	*2μ amdSYM gRNA-SGA1*	van Rossum *et al*., 2016
pUDE044	*2μ URA3 pTDH3-MAL12-tADH1*	de Kok *et al*., 2011

Plasmid pUDE379 was constructed via Gibson assembly of the *MAL11* ORF amplified from pUDI035 (de Kok *et al.* 2011) using primers 8379 & 8380

and a vector backbone amplified from plasmid p426TEF-*amdSYM* using primers 7998 & 7999. Plasmid pUDE432 was constructed via Gibson assembly of a *MAL11* expression cassette, amplified from pUDE379 using primers 9043 & 9044 and a vector backbone linearized from p426GPD (Mumberg, Müller and Funk 1995) using KpnI and SacI restriction sites.

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

Sequences coding for *AtSWEET12* (NCBI gene ID: 832431) and *OsSWEET11* (NCBI gene ID: 4346153) were purchased from GeneArt (Regensburg, Germany) and were delivered in vectors pMA-T (*AtSWEET12*, plasmid named pUD400) and pMK-RQ (*OsSWEET11*, plasmid named pUD401). From pDR196-*PsSUF1* and pDR196-*PsSUF4* (Zhou *et al.* 2007), *PsSUF1* (NCBI accession number DQ221698.1) and *PsSUF4* (NCBI accession Number DQ221697.2) gene cassettes were obtained via digestion with XbaI and SalI. These cassettes were ligated into the backbone of pUDI035, which was obtained via digestion with the same enzymes, resulting in plasmids pUDI085 and pUDI086, respectively. *PvSUF1* (NCBI accession number DQ221700.1) was codon optimized for expression in *S. cerevisiae* with OptimumGene™ (GenScript, **Supplementary material**), purchased from GenScript USA Inc. (Piscataway, NJ) and delivered within pUC57-*PvSUF1*. From pUC57-*PvSUF1*, the *PvSUF1* ORF was obtained and subsequently cloned into pUDI035 via the SpeI and SalI restriction sites, replacing the *MAL11* ORF, resulting in pUDI087 (**Table 1**). Plasmids pUDE367, pUDE368, pUDE369, pUDE370 and pUDE374

were constructed by assembly of cassettes containing the ORF of the transporter gene and a plasmid backbone. The transporter expression cassettes containing the *PsSUF1*, *PsSUF4* and *PvSUF1* ORFs were amplified from pUDI085, pUDI086 and pUDI087 using primers 8018 & 8019, 8020 & 8021 and 8022 & 8023, respectively and *AtSWEET12* and *OsSWEET11* ORFs were amplified from pUD400 and pUD401 using primers 8000 & 8001, respectively. The vector backbone was amplified from plasmid p426TEF-*amdSYM* using primers 7998 & 7999 and the transporter genes were inserted in the vector backbone via Gibson assembly. Plasmids pUDE413, pUDE438, pUDE439, pUDE441 and pUDE442 were constructed via Gibson assembly of transporter expression cassettes amplified from pUDE369, pUDE370, pUDE374, pUDE367 and pUDE368 respectively with primers 9043 & 9044 and the p426GPD vector backbone digested with KpnI and SacI.

For fluorescent tagging of Mal11 and *Pv*Suf1 with the YPet fluorescent protein (Nguyen and Daugherty 2005), first plasmid pRHA00 was made by amplifying the backbone of pFB001 (Bianchi *et al.* 2016), using primers 5273 & 5274 and the *MAL11* gene from the genomic DNA of *S. cerevisiae* strain BY4742 (EUROSCARF, Accession No. Y10000 (Brachmann *et al.* 1998)) with primers 5271 & 5272 followed by *in vivo* assembly. *MAL11* from BY4742 is identical to *MAL11* from CEN.PK-derived strains. Then, the *URA3* marker was omitted from pRHA00 via PCR with primers 5437 & 5438 and replaced with the *LEU2* gene, amplified from pRS315 (Sikorski and Hieter 1989) with primers 5435 & 5436 via *in vivo* assembly. pR151 was then made by Gibson assembly of three fragments; the plasmid backbone and *MAL11* gene amplified from pRHA00L with primers 5959 & 6324 and 5961 & 5272 respectively and the *TEF1* promoter amplified from pUDE379 with primers 4995 & 5960. Plasmid pUDE453 was made via Gibson assembly of a *MAL11*-YPet expression cassette, amplified from pR151 using primers 6717 & 580 and vector backbone amplified from pUDE413 with primers 5921 & 7812. For construction of plasmid pUDE471 via Gibson assembly, the *MAL11* gene from pUDE453 was replaced by *PvSUF1* via amplification of the plasmid backbone, including the YPet-tag, from pUDE453 with primers 5921 & 9772 and

amplification of the *PvSUF1* ORF from pUDE413 with primers 6717 & 9763 before assembly of both fragments.

Strain construction

S. cerevisiae transformations were carried out according to Gietz and Woods (2002) using 1 µg of DNA per transformation, if not stated otherwise. Transformants were selected on 2% (w/v) agar plates containing synthetic medium (SM) (Verduyn *et al.* 1992) with 20 g/L glucose plus the following components when necessary: G418 (200 mg/L); uracil (0.15 g/L); L-leucine (0.5 g/L) (Pronk 2002). Cells expressing the *amdSYM* marker were selected on plates according to Solis-Escalante *et al.* (2013). Cells expressing the *natNT2* marker were selected on plates containing nourseothricin (100 mg/L) (Jena Bioscience, Jena, Germany) in SM with 1 g/L glutamic acid as sole nitrogen source. IMZ570 was made via *in vivo* assembly of plasmid pUDC156 in IMK291 (**Table 1**). IMZ570 was transformed with 1 µg of plasmid pUDR128, 4 µg dsDNA repair fragment for *IMA1-4* and 4 µg dsDNA repair fragment for *IMA5* (Mans *et al.* 2015; Marques *et al.* 2017). Subsequently, pUDR128 and pUDC156 were cured from IMZ570 by cultivation on YPD plates with 20 g/L glucose and 1 g/L 5'-fluoroorotic acid (Boeke, La Croute and Fink 1984), resulting in IMK698. The *LEU2* marker fragment was amplified from pUDI035 with primers 1742 & 1743 and integrated in IMK698 resulting in strain IMX935. IMZ616 was made via transformation of IMX935 with pUDC156. For the construction of IMZ627 and IMZ630, cassettes with homology to the *SGA1* locus were amplified from pUDE044 (de Kok *et al.* 2011) and pUDE262 with primers 9355 & 9356 resulting in expression cassettes containing *MAL12* and *LmSPase* respectively. IMZ616 was then transformed with 1 µg of pUDR119 (gRNA-*SGA1*) (van Rossum *et al.* 2016) together with either 1 µg *MAL12* expression cassette or 1 µg *LmSPase* expression cassette and subsequent removal of pUDR119 and pUDC156 resulted in strains IMZ627 (*MAL12* expression) and IMZ630 (*LmSPase* expression), respectively. IMZ636, IMZ666, IMZ667, IMZ671, IMZ672 and IMZ692 were made by transformation of plasmids pUDE413, pUDE439, pUDE438, pUDE442, pUDE441 and pUDE260

into IMZ630, respectively. IMZ633 was made via transformation of plasmid pUDE413 into IMZ627. pUDE432 was transformed into IMZ627 and IMZ630, resulting in IMZ664 and IMZ665, respectively. IMZ696, IMX1272 and IMX1273 were constructed via transformation of pUDE486, pUDE453 and pUDE471 into IMZ630, respectively. IMZ709 was made via *in vivo* assembly of pUDE469 in IMZ630. IMX935 was transformed with pUDE260, pUDE413, pUDE432, pUDE453 and pUDE471, resulting in IMX1274-1278 respectively.

Molecular biology techniques

PCR amplification for strain construction was performed with Phusion Hot Start II High Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions using PAGE-purified oligonucleotide primers (Sigma-Aldrich, St. Louis, MO). Diagnostic PCR was done via colony PCR on randomly picked yeast colonies, using DreamTaq (Thermo Fisher Scientific) and desalted primers (Sigma-Aldrich). The primers used in this study are listed in **Table S1**. Yeast genomic DNA was isolated using the YeaStar Genomic DNA kit (D2002, Zymo Research, Irvine, CA). DNA fragments obtained by PCR were separated by gel electrophoresis on 1% (w/v) agarose gels (Thermo Fisher Scientific,) in Tris-acetate-EDTA buffer (Thermo Fisher Scientific) at 100 V for 30 min. DNA fragments were excised from gel and purified by gel purification (D2004, Zymo Research). Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich) according to the supplier's manual and from yeast with the Zymoprep Yeast Plasmid Miniprep II kit (D2004, Zymo Research). Restriction analysis with enzymes was performed using FastDigest enzymes (Thermo Fisher Scientific) according to the manufacturer's manual. Plasmid assembly was performed using *in vivo* recombination in yeast (Kuijpers *et al.* 2013) , T4 DNA ligase (Thermo Fisher Scientific), Gibson Assembly Cloning Kit (New England Biolabs, Ipswich, MA) or NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). Assembly of plasmids was done according to the suppliers' protocols, but downscaled to a total volume of 5 µL for the Gibson and NEBuilder HiFi DNA Assembly Cloning kit. Ligation of plasmids was

performed using T4 DNA ligase according to manufacturer's instructions (New England Biolabs). *E. coli* DH5α (18258-012, Thermo Fisher Scientific) or XL1-Blue (GE Healthcare Life Sciences, Uppsala, Sweden) were used for chemical transformation (T3001, Zymo Research) or for electroporation. Chemical transformation of *E. coli* was done according to the supplier's instructions. Electroporation was done in a 2 mm cuvette (165-2086, BioRad, Hercules, CA) using a Gene PulserXcell Electroporation System (BioRad), following the manufacturer's protocol. Electrocompetent *E. coli* cells were prepared according to the BioRad protocol, except for the use of lysogeny broth (LB) (Bertani 1951) without NaCl when pre-growing the cells.

Media and cultivation

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

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

sucrose and incubated anaerobically. Exponentially growing cultures were washed and used as inoculum for anaerobic experiments.

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

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

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

Analytical methods

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

Determination of sucrose hydrolase, sucrose phosphorylase and phosphoglucomutase activities

For enzyme-activity assays, culture samples corresponding to 62.5 mg dry weight were harvested during the steady state of the chemostat cultures.

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

Microscopy of YPet tagged Mal11 and *Pv*Suf1

For fluorescence microscopy, samples were taken from aerobic, steady-state chemostat cultures with *S. cerevisiae* strains IMX1272 (*Mal11-YPet*, *LmSPase*) and IMX1273 (*PvSUF1-YPet*, *LmSPase*). Cells were then imaged by phase-contrast microscopy using a Zeiss D1 Imager with a 100x objective (EC Plan-Neofluar 100x/1.30 Oil Ph 3 M27), equipped with an AxioCamMR camera (Zeiss, Jena, Germany). For fluorescence microscopy a HAL100 fluorescent lamp and Filter set 10 (Ex 450-490 nm/Em 515-565 nm) (Zeiss, Jena, Germany) were used.

Proton-solute symport assays

Cells for proton-solute symport assays were harvested from aerobic, sucrose-limited chemostat cultures at a dilution rate of 0.030 h^{-1}. The reactors

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

Transport of radiolabelled sucrose

Yeast strains were grown aerobically on synthetic medium with 2% ethanol as carbon source. Cells from exponential cultures were harvested by centrifugation and then washed twice and resuspended in potassium citrate-phosphate (KCP) buffer at pH 5, containing 0.2% (v/v) ethanol. Cell suspensions in buffer were kept on ice for no longer than 4 hours before use. Transport assays were performed at 30°C using cell suspensions with an optical density (OD_{600}) of 8. Cells were incubated at 30°C for 5 min and then [U-^{14}C] sucrose (600 mCi/mmol; American Radiolabeled Chemicals, Inc.) was added to approximately 48100 Bq/mL (final sucrose concentration of 1 mM) to start the uptake reaction. After 20 min of uptake, 10 µM of the protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was added to the uptake reactions. At given time intervals, 50 µL samples of the uptake reaction mixture were mixed with 2 mL ice-cold KCP and then rapidly filtered using cellulose-nitrate filters with 0.45 µm pores (GE-

Healthcare, Little Chalfont, UK). Prior to filtration of the cell suspension, the filters were pre-soaked in KCP with 1 mM of sucrose to block non-specific adsorption of ^{14}C-sucrose. Filters were washed once with 2 mL KCP and dissolved in 2 mL scintillation solution (Emulsifier^{plus}, PerkinElmer, Waltham, MA, USA). The radioactivity on each filter paper was measured using a liquid scintillation counter (Tri-Carb 2800TR liquid scintillation analyzer, PerkinElmer). The amount of sucrose in each sample was normalized to 10^6 cells by counting cells using a Accuri C6 flow cytometer (BD AccuriTM, Durham, USA) and an estimate of 60 fL internal volume per cell was used to calculate the concentration of intracellular sucrose.

Flow cytometry

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

RESULTS

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

The first step towards improving the free-energy conservation of sucrose fermentation was to functionally replace the native yeast invertase with a sucrose phosphorylase (SPase). Deletion of the native sucrose hydrolysing enzymes and sucrose-proton symporters was achieved by targeted deletion of *SUC2*, the *MAL* loci, the α-glucoside permease genes *MPH2* and *MPH3* and the isomaltase genes *IMA1-5* (de Kok *et al.* 2011; Marques *et al.* 2017). The resulting strain IMZ616 (**Table 1**) was unable to grow on sucrose

over a period of up to 2 months and no sucrose hydrolysis activity could be detected in cell extracts (**Table 3**). Therefore, IMZ616 was used as a platform strain to express SPase. Chromosomal integration of an expression cassette carrying the *Leuconostoc mesenteroides* sucrose-phosphorylase gene (*LmSPase*), in combination with expression of the native sucrose-proton symporter *MAL11* from a multi-copy plasmid resulted in strain IMZ665. Functional expression of *Lm*SPase was confirmed via measurement of *in vitro* sucrose phosphorylase activity of 0.90 (μmol/min)/mg protein (**Table 3**). Under anaerobic conditions, strain IMZ665 was able to grow in synthetic medium with sucrose as the sole carbon source, at a specific growth rate of 0.09 ± 0.02 h^{-1} (**Table 3**). Even after prolonged incubation, strains with a similar genetic background that expressed only *MAL11* were unable to grow in medium with sucrose as the sole carbon source (Marques *et al.* 2017). Additionally, a reference strain expressing *MAL11* combined with a native glucosidase (*MAL12*) was constructed (IMZ664, **Table 1**). This strain grew anaerobically on sucrose, at a specific growth rate of 0.19 ± 0.01 h^{-1} (**Table 3**). The higher specific growth rate of this isogenic strain indicated that the sucrose transporter *MAL11* was not rate limiting for growth of IMZ665 (*MAL11*, *LmSPase*).

To investigate the impact of *Lm*SPase expression on the ATP yield from sucrose fermentation, biomass yields on sucrose were measured. Alcoholic fermentation of one sucrose molecule via the proton-coupled symporter *MAL11* and sucrose hydrolase *MAL12* generates 3 ATP (**Figure 1B**). Replacement of sucrose hydrolysis by sucrose phosphorylase increases the theoretical yield to 4 ATP per sucrose (**Figure 1C**). The anaerobic biomass yield on sucrose can be used as an *in vivo* read-out of the energetic difference between strains (Weusthuis *et al.* 1993; de Kok *et al.* 2011), where a strain yielding 4 ATP per sucrose is predicted to have a 33% higher biomass yield than a 3 ATP strain (Basso *et al.* 2011). To minimize effects of specific growth rate on the biomass yield, IMZ664 (*MAL11*, *MAL12*) and IMZ665 (*MAL11*, *LmSPase*) were investigated under identical conditions and at identical

Table 3: Specific growth rates on sucrose and enzyme activities of sucrose hydrolase and sucrose phosphorylase of *S. cerevisiae* strains expressing either the sucrose transporter Mal11 or *Pv*Suf1 in combination with either the sucrose hydrolase Mal12 or sucrose phosphorylase *Lm*SPase in anaerobic bioreactors. Average growth rates were determined from triplicate experiments and based on CO_2 production in anaerobic batch fermentations at pH 5.0 and 25 g/L sucrose. Enzyme activities represent the average of measurements on duplicate steady-state chemostat cultures at pH 5.0, 25 g/L sucrose and a dilution rate of 0.030 h^{-1} (IMZ664, IMZ665 and IMZ636) or 0.07 h^{-1} (IMZ709 and IMZ696). IMZ616 was incubated in aerobic shake flasks with 20 g/L sucrose for growth assessment and 20 g/L ethanol and 20 g/L sucrose for enzyme activity assays.

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

N.D. = not determined
B.D. = below detection (< 0.04 (µmol/min)/mg protein)
[a] Measured in duplicate
[b] No growth observed after 2 months

specific growth rates in anaerobic steady-state chemostat cultures. In view of the maximum anaerobic specific growth rate of IMZ665 on sucrose (µ = 0.09 ± 0.02 h^{-1}, **Table 3**), a dilution rate of 0.030 h^{-1} was chosen.

IMZ665 (*MAL11*, *LmSPase*) showed an increased biomass yield compared to the *MAL11*, *MAL12* expressing strain IMZ664 (0.069 ± 0.000 vs 0.053 ± 0.001 g/g glucose equivalent) (**Table 4**). This observed difference of 31% is close to the theoretical value of 33%. Increased free-energy conservation in the catabolic pathway dictates that less sucrose needs to be fermented to ethanol and CO_2 to provide the same amount of ATP required for

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

Strain	IMZ664	IMZ665	IMZ636	IMZ709	IMZ696
Relevant genotype	*MAL11 MAL12*	*MAL11 LmSPase*	*PvSUF1 LmSPase*	*MAL11 LmSPase PGM2*	*PvSUF1 LmSPase PGM2*
Biomass yield (g/g glucose equivalent)	0.053 ± 0.001	0.069 ± 0.000	0.075 ± 0.000	0.080 ± 0.001	0.087 ± 0.002
$q_{sucrose}$ (mmol/g biomass/h)	-1.59 ± 0.05	-1.21 ± 0.00	-1.11 ± 0.00	-2.25 ± 0.08	-2.27 ± 0.06
$q_{ethanol}$ (mmol/g biomass/h)	5.81 ± 0.15	4.17 ± 0.01	3.69 ± 0.06	7.17 ± 0.31	7.05 ± 0.29
q_{CO2} (mmol/g biomass/h)	5.73 ± 0.17	4.29 ± 0.13	3.79 ± 0.05	8.34 ± 0.20	8.35 ± 0.10
$q_{glycerol}$ (mmol/g biomass/h)	0.31 ± 0.00	0.29 ± 0.01	0.26 ± 0.01	0.57 ± 0.02	0.57 ± 0.02
$q_{lactate}$ (mmol/g biomass/h)	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.06 ± 0.00	0.02 ± 0.00
$q_{pyruvate}$ (mmol/g biomass/h)	B.D.[b]	B.D.	B.D.	0.01 ± 0.00	B.D.
$q_{acetate}$ (mmol/g biomass/h)	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Protein content (g/g biomass)	0.46 ± 0.01	0.44 ± 0.02	0.46 ± 0.02	N.D.	N.D.
Residual sucrose (g/L)	0.12 ± 0.02	0.07 ± 0.01	0.24 ± 0.01	0.14 ± 0.00	0.71 ± 0.17
Carbon recovery (%)	103 ± 1	103 ± 1	100 ± 1	101 ± 1	101 ± 1
Actual dilution rate (/h)	0.030 ± 0.002	0.030 ± 0.000	0.030 ± 0.000	0.065 ± 0.002	0.070 ± 0.001

N.D. = not determined
B.D. = below detection (< 0.01 mmol/g biomass/h)

biomass formation. Accordingly, the biomass specific uptake rate of sucrose was 32% lower (1.21 ± 0.00 vs 1.59 ± 0.05 mmol/g biomass/h) and rates of ethanol and CO_2 production were 39% (4.17 ± 0.01 vs 5.81 ± 0.15 mmol/g biomass/h) and 34% (4.17 ± 0.01 vs 5.81 ± 0.15 mmol/g biomass/h) lower in strain IMZ655 (*MAL11*, *LmSPase*) as compared to those in IMZ664 (*MAL11*, *MAL12*). Replacement of sucrose hydrolysis by phosphorolysis also resulted in a decrease in the residual sucrose concentration from 0.12 ± 0.02 g/L to 0.07 ± 0.01 g/L (**Table 4**).

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

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

Cellular localisation of both endogenous YPet-tagged Mal11 (IMX1272) and YPet-tagged *Pv*Suf1 (IMX1273) was investigated microscopically (**Figure 3**). Both strains showed a similar fluorescence at the periphery of cytosol, confirming that both Mal11 and *Pv*Suf1 were indeed targeted to the plasma membrane. However, distribution of the remainder of the fluorescence differed significantly between the two strains. Whereas intracellular fluorescence in strain IMX1272 (Mal11) was predominantly associated with vacuoles (**Figure 3A**), fluorescence of IMX1273 (*Pv*Suf1) appeared to be distributed over multiple smaller intracellular compartments (**Figure 3C**).

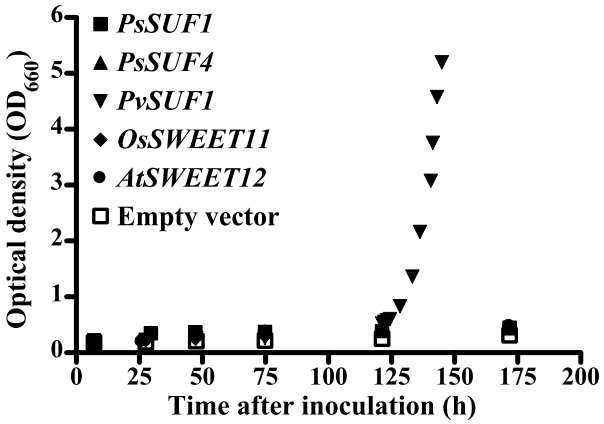

Figure 2. Growth curves of *S. cerevisiae* strains expressing *LmSPase* in combination with either *PsSUF1* (IMZ683 (■)), *PsSUF4* (IMZ682 (▲)), *PvSUF1* (IMZ636 (▼)), *OsSWEET11* (IMZ677 (◆)), *AtSWEET12* (IMZ678 (●)) or an empty vector (IMZ692 (□)) in aerobic shake flasks containing synthetic medium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

Lastly, cells from IMX1273 (*Pv*Suf1) appeared to form cell clusters and were slightly elongated.

In theory, facilitated uptake of sucrose via a uniporter, combined with phosphorolytic cleavage of sucrose via SPase and anaerobic alcoholic fermentation, should lead to the formation of 5 ATP per sucrose (**Figure 1D**). This increased ATP yield is predicted to result in a 25% increase in the anaerobic biomass yield when compared to a strain yielding 4 ATP per sucrose (Weusthuis *et al.* 1993; Basso *et al.* 2011; de Kok *et al.* 2011). To quantitatively investigate the impact of combined expression of *LmSPase* and *PvSUF1*, strain IMZ636 (*PvSUF1*, *LmSPase*) was grown in anaerobic sucrose-limited chemostat cultures at a dilution rate of 0.030 h^{-1}. The observed anaerobic biomass yield on sucrose was increased by 8% from 0.069 ± 0.000 g/g glucose equivalent for strain IMZ655 (*MAL11*, *LmSPase*) to 0.075 ± 0.000

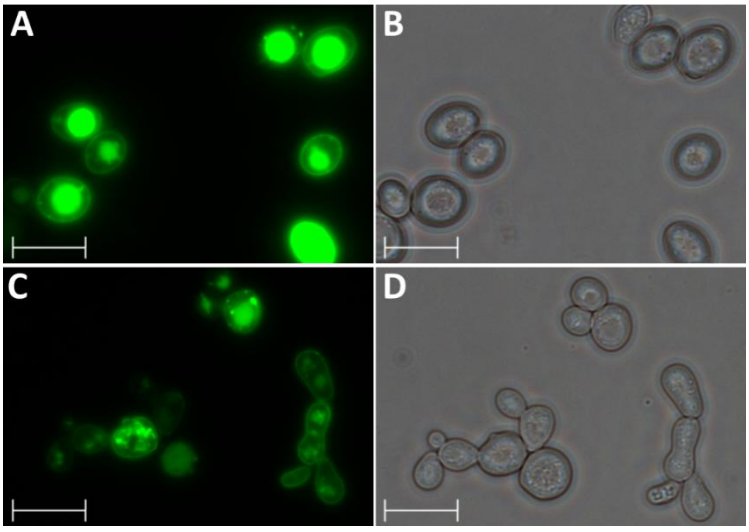

Figure 3. Fluorescent and phase-contrast pictures of *S. cerevisiae* strains IMX1272 (*MAL11-YPet, LmSPase*, **A & B**) and IMX1273 (*PvSUF1-YPet, LmSPase*, **C & D**). Cells were collected from aerobic, steady-state, sucrose-limited chemostat cultures grown at a dilution rate of 0.030 h^{-1}. The scale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

g/g glucose equivalent for IMZ636 (*PvSUF1*, *LmSPase*) (**Table 4**). In line with an increased ATP yield, the biomass specific sucrose uptake rate (1.11 ± 0.00 vs 1.21 ± 0.00 mmol/g biomass/h), specific ethanol production rate (3.69 ± 0.06 vs 4.17 ± 0.01 mmol/g biomass/h) and CO_2 production rate (3.79 ± 0.05 vs 4.29 ± 0.13 mmol/g biomass/h) decreased for IMZ636 (*PvSUF1*, *LmSPase*) compared to IMZ665 (*MAL11*, *SPase*). The observed 8% increase in the anaerobic biomass yield was lower than the predicted 25%. Mislocalization and/or increased protein turnover of *Pv*Suf1 could have increased the maintenance energy requirement and thereby resulted in a lower-than-expected increase in the biomass yield. The impact of the cellular maintenance energy requirements on the biomass yield decreases with an increase in the specific growth rate (Pirt 1965; Leuenberger 1971). Therefore testing of *PvSUF1*- and *MAL11*-expressing strains at higher dilution rates could provide insight into maintenance energy related effects on the biomass yield.

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

Increasing the maximum specific growth rate of the engineered strains on sucrose would benefit both the intended industrial applications of this strategy as well as further investigations into their physiology. To investigate whether the conversion of glucose-1-phosphate, the product of the SPase reaction, to glucose-6-phosphate by phosphoglucomutase was limiting growth, *PGM2* was overexpressed from a multi-copy plasmid. Introduction of this vector resulted in an approximately 20-fold increase of phosphoglucomutase activity in cell extracts (**Table 3**). Combined overexpression of *PGM2* and *LmSPase* with *MAL11* (IMZ709) or *PvSUF1* (IMZ696) increased the maximum specific growth rate in anaerobic, sucrose-grown cultures from 0.09 h^{-1} to 0.23 h^{-1} and from 0.06 h^{-1} to 0.08 h^{-1} respectively (**Table 3**). The higher maximum specific growth rates of *PGM2*-expressing strains allowed for a chemostat-based, quantitative evaluation of the impact of the combined expression of *Pv*Suf1 and *Lm*SPase at higher dilution rates and an additional set of steady-state chemostat cultures was performed at a dilution rate of 0.07 h^{-1}.

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

Sucrose uptake by *Pv*Suf1 expressing strains leads to alkalinisation of the extracellular environment.

While *Pv*Suf1 has been described as a sucrose facilitator or uniporter (Zhou *et al.* 2007), involvement of protons in sucrose uptake (partly coupled

transport (Lolkema and Poolman 1995; Poolman, Knol and Lolkema 1995), mediated by *Pv*Suf1, could explain the lower-than-expected anaerobic biomass yield of *PvSUF1*-expressing strains on sucrose. To investigate this possibility, IMZ696 (*PvSUF1*, *LmSPase*, *PGM2*) and the isogenic *MAL11*-expressing strain IMZ709 were subjected to a sucrose-proton symport assay (**Figure 4**). To minimize residual sucrose concentrations, cells for this assay were collected from aerobic, sucrose-limited bioreactors at a dilution rate of 0.030 h^{-1}. In the essay, an alkalinisation upon sucrose addition to the cell suspension indicates the presence of a proton-coupled sucrose-uptake mechanism (Stambuk, Batista and De Araujo 2000). Indeed, such an alkalinisation of the extracellular medium was observed upon addition of either sucrose or maltose to cell suspensions of the *MAL11*-expressing strain IMZ709 (**Figure 4B**). Surprisingly, alkalinisation of the extracellular medium was also observed upon disaccharide addition to cell suspensions of the *PvSUF1*-expressing strain IMZ696 (**Figure 4A**), although at a lower initial rate (8.2 ± 2.2 and 7.9 ± 1.0 μmol H^+/g biomass/min for sucrose and maltose respectively) than observed for IMZ709 (51.4 ± 8.6 and 42.6 ± 6.2 μmol H^+/g biomass/min for sucrose and maltose respectively). As a control, fructose and glucose were added to cell suspensions of both strains. In accordance with hexose uptake via facilitated diffusion mediated by the hexose transporters, no pH change upon addition of glucose or fructose to cell suspensions of IMZ696 (*PvSUF1*, *LmSPase*, *PGM2*) was observed. Addition of fructose to cell suspensions of IMZ709 (*MAL11*, *LmSPase*, *PGM2*) did not result in a pH change. In line with previous observations from Wieczorke *et al*., 1999, some alkalinisation of the extracellular medium was observed upon glucose addition to this strain. Resequencing of the *PvSUF1* genes at the end of both aerobic chemostat cultures revealed 2 point mutations (**Supplementary material**), leading to different amino acid substitutions in both cultures (H67G and T302I respectively). These mutations did not involve acidic residues making it unlikely that they affected proton coupling of sucrose transport (Lemoine 2000).

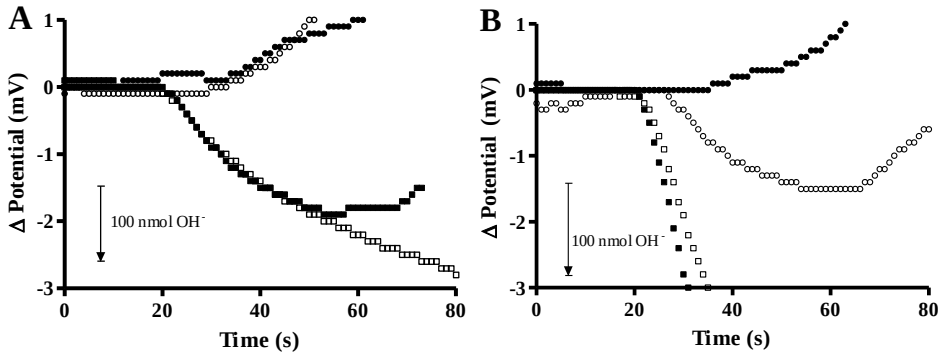

Figure 4. Proton uptake induced by addition of sugars to cell suspensions of two yeast strains. **(A)** Strain IMZ696 expressing *PvSUF1*, *LmSPase* and *PGM2*. **(B)** Strain IMZ709 expressing *MAL11*, *LmSPase* and *PGM2*. Cells were pre-grown in aerobic, sucrose-limited chemostat cultures, harvested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

If *Pv*Suf1 indeed exhibits (partial) proton-coupled sucrose transport, the additional driving force for sucrose uptake provided by the proton motive force can lead to intracellular sucrose accumulation in the absence of a catabolic pathway (Zhou *et al.* 2007). To investigate whether *Pv*Suf1 expression resulted in intracellular sucrose accumulation, strains were constructed that expressed either *Pv*Suf1 or Mal11 without a sucrose cleavage enzyme and used for radioactively labelled sucrose uptake measurements (**Figure 5**). As expected, sucrose accumulation was observed in the *MAL11*-expressing strain IMX1276 to an accumulation ratio of 45 (intracellular/extracellular sucrose concentration) before the protonophore

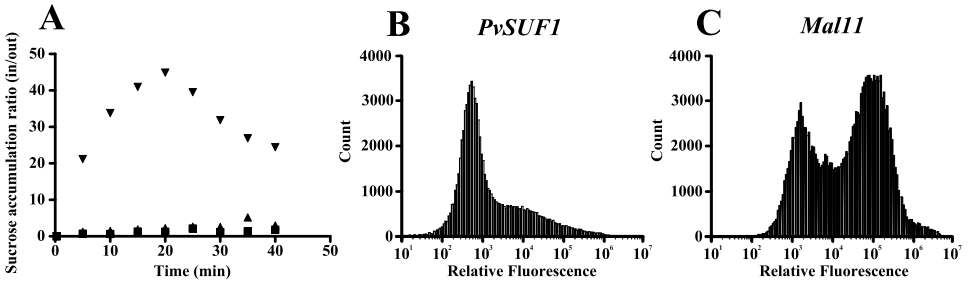

Figure 5:.(A) Transport of 1 mM ^{14}C-sucrose by *S. cerevisiae* IMX1274 (empty vector, ■), IMX1275 (*PvSUF1*, ▲), and IMX1276 (*MAL11*,▼). Cells were grown and prepared as described above. After 20 minutes of incubation, 10 µM FCCP was added to the reactions. **(B)** Flow cytometry analysis of *S.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

FCCP was added (**Figure 5A**). Upon addition of FCCP, the proton motive force was dissipated, which resulted in the efflux of sucrose following the sucrose concentration gradient. Uptake of sucrose by the *PvSUF1*-expressing strain IMX1275 was very close to that of the empty vector reference strain IMX1274 and no intracellular accumulation was observed, which is consistent with a uniporter mechanism or poorly coupled transporter. In line with the observed lower average fluorescence of the *PvSUF1*-expressing strain (**Figure 3**), also flow cytometry data from ethanol-grown isogenic strains expressing YPet-tagged transport proteins showed relatively low expression levels of *Pv*Suf1 compared to Mal11 (**Figure 5B & C**).

DISCUSSION

The native sucrose hydrolases of *S. cerevisiae* were functionally replaced by a prokaryotic phosphorylase. The resulting increase in the anaerobic biomass yield on sucrose (31%) is very close to the theoretically expected difference (33%, i.e. from 3 to 4 moles of ATP per mole of sucrose consumed), proving the energetic benefit of replacing sucrose hydrolysis by

phosphorolysis. Besides the energetic benefit for the cleavage of intracellular sucrose via SPase, also the kinetics of *L. mesenteroides* SPase, K_m = 5.7 mM and k_{cat} = 165 s^{-1} (Goedl *et al.* 2007), are better than those of Mal12, K_m = 12 mM and k_{cat} = 0.45 s^{-1} (Voordeckers *et al.* 2012), which could explain why strains expressing *PvSUF1* were only able to grow on sucrose when co-expressing *Lm*SPase (as opposed to *MAL11*) and could also explain the lower residual sucrose concentrations observed under nutrient limitation in *Lm*SPase expressing strains (**Table 4**). This provides an additional advantage for fed-batch or continuous industrial applications by lowering the fraction of unused substrate. Other potential benefits for fed-batch or continuous industrial applications ensue from changing extracellular hydrolysis to intracellular metabolism. In wild type *S. cerevisiae*, extracellular hydrolysis and subsequent alcoholic fermentation of the monosaccharides result in 4 ATP per sucrose (**Figure 1A**). An engineered strain using proton-coupled uptake (e.g. Mal11) and intracellular phosphorolysis has an identical ATP yield (**Figure 1C**), but has the potential to overcome two sucrose-associated challenges encountered in industry: *i*) accumulation of residual fructose at the end of cultivation processes due to preferred uptake of the released glucose over fructose by yeast (Beato *et al*., 2016; Berthels *et al*., 2004; Prijambada *et al*., 2013; Wu *et al*., 2010) and *ii*) competition for the extracellularly released monosaccharides by bacterial contaminants or wild yeasts (Greig and Travisano 2004; Gore, Youk and van Oudenaarden 2009; Celiker and Gore 2012; Reis *et al.* 2014). Although some of these benefits might also translate to industrial batch fermentations, further study would be required to investigate the impact of the composition of molasses together with glucose repression of, for instance, *MAL11*.

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

strains and co-expression of a phosphoglucomutase is essential for faster sucrose conversion.

Replacement of a hydrolase by a phosphorylase has previously been demonstrated for maltose and cellobiose (de Kok *et al.* 2011; Sadie *et al.* 2011). However, only by combining the energetic benefits of disaccharide uniport and phosphorolysis can the energetic efficiency of wild type strains be exceeded. In this study, combined expression of *Lm*SPase and *Pv*SUF1 resulted in an 8% increase in the biomass yield on sucrose compared to the Mal11/*Lm*SPase-based reference strain and 41% compared to the Mal11/Mal12-based reference strain (**Figure 1**, **Table 4**). Although promising, the observed 8% increase in biomass yield was below the theoretical prediction of 25%. Four parameters that could have contributed to this lower than anticipated increase of the biomass yield were evaluated in this study: increased cellular maintenance energy requirements in *PvSUF1*-expressing strains, a change in biomass composition, sucrose hydrolysis activity and/or (partially) proton-coupled sucrose uptake catalysed by *Pv*SUF1.

Cellular maintenance energy requirements have been shown to be growth rate independent in anaerobic, sugar-limited *S. cerevisiae* cultures (Boender *et al.* 2009). Especially at low specific growth rates, changes in genotype or experimental conditions that lead to changes in maintenance-energy requirements can strongly affect biomass yield (Leuenberger 1971). We hypothesized that the abundance of fluorescence originating from YPet-tagged *Pv*SUF1 observed in various intracellular compartments (**Figure 3C**), as well as the observed changes in morphology, could indicate increased protein turnover and/or maintenance in *PvSUF1*-expressing strains. However, the observation that the difference in the biomass yield between strains expressing *Lm*SPase with either Mal11 or *Pv*SUF1 was independent of the dilution rate (**Table 4**) indicated that a difference in maintenance energy requirements was probably not responsible for the lower-than-predicted increase in biomass yield. Secondly, a change in biomass composition could result in a lower biomass yield on sucrose in *Pv*SUF1 expressing strains. Since protein synthesis is the main contributor to the energetic cost of biomass

formation (Stouthamer 1973), the protein content of IMZ664 (*MAL11*, *MAL12*), IMZ665 (*MAL11*, *LmSPase*) and IMZ636 (*PvSUF1*, *LmSPase*) was determined in steady-state chemostat cultures at a dilution rate of 0.030 h^{-1}. No significant differences in cellular protein content were observed between the steady state chemostat cultures of the relevant strains grown at 0.030 h^{-1} (**Table 4**). Alternatively, sucrose hydrolytic activity catalysed by *Lm*SPase, which has previously been described (Goedl *et al.* 2010), has the potential to lower the biomass yield on sucrose due to competition with phosphorolytic cleavage. A low but significant (0.06 (µmol/min)/mg protein, **Table 3**) sucrose hydrolase activity was measured in cell extracts of IMZ696 (*PvSUF1*, *LmSPase*), grown in steady-state chemostat cultures at a dilution rate of 0.07 h^{-1}. Since sucrose hydrolytic activity was also observed in IMZ709 (*MAL11*, *LmSPase,* 0.07 (µmol/min)/mg protein, **Table 3**), and no activity could be detected in a strain void of either SPase or Mal12 (IMZ616, **Table 3**), this hydrolytic activity might originate from *Lm*SPase. Hydrolytic activity by *Lm*SPase is likely absent *in vivo* when expressed in *S. cerevisiae*, as it has been described to be ≥50 times slower than phosphorolysis, repressed in the presence of sucrose and glycerol (Goedl *et al.* 2010) and abolished in the presence of phosphate (Silverstein *et al.* 1967). Additionally, the fact that the benefit of solely replacing intracellular sucrose hydrolysis by phosphorolysis was very close to the theoretical prediction (31% vs 33%), makes it unlikely that this *in vitro* hydrolytic activity is responsible for the lower than predicted increase in the biomass yield in the combined strategy. Lastly, proton-coupled sucrose uptake catalysed by *Pv*SUF1, resulting in subsequent ATP-dependent proton extrusion, could lower the biomass yield compared to a sucrose uniporter. In contrast to a previous characterisation of *Pv*SUF1 in literature (Zhou *et al.* 2007), the presented study revealed proton-dependent sucrose uptake in cell suspensions of the *PvSUF1*-expressing strain IMZ696 (*PvSUF1*, *LmSPase*, *PGM2*) (**Figure 4A**). The stoichiometry between sucrose and proton uptake could not be determined due to the low activity of sucrose uptake (**Figure 5A**). Nonetheless, it seems likely that proton-coupled sucrose uptake is at least partly responsible for the lower-than-expected biomass yield in

PvSUF1-expressing strains. In this study, *Pv*SUF1 was the only one of five investigated heterologous transporters that supported growth on sucrose (**Figure 2**). Subsequent analysis of *Pv*Suf1, showed low protein levels (**Figure 5B**), partial localization to the plasma membrane (**Figure 3**) and a potential benefit from additional mutations for growth on sucrose. These observations illustrate the challenge of expressing heterologous transporters as part of metabolic engineering strategies.

The strategy of replacing extracellular hydrolysis with a combination of uptake through facilitated diffusion and intracellular phosphorolysis can also be applied to increase free-energy conservation for other oligosaccharides. Phosphorylases have been described for maltose, cellobiose, trehalose, lactose and cellodextrin (Kishore and Alexander 1967; Alexander 1968; Belocopitow and Maréchal 1970; De Groeve *et al.* 2009; de Kok *et al.* 2011; Sadie *et al.* 2011) and functional expression of maltose, cellobiose and cellodextrin phosphorylase has already been demonstrated in *S. cerevisiae* (de Kok *et al.* 2011; Sadie *et al.* 2011; Ha *et al.* 2013). In addition to sucrose facilitators, a putative maltose facilitator from *Arabidopsis thaliana* (Rost, Frank and Beck 1996; Niittyla *et al.* 2004; Reidel, Turgeon and Cheng 2008) has been described and a cellodextrin facilitator from *Neurospora crassa* has previously been expressed and evolved for efficient transport in *S. cerevisiae* (Lian *et al.* 2014).

Combined facilitated diffusion and intracellular phosphorolysis of oligosaccharides results in a lower requirement of carbon to provide ATP, thereby increasing product yields and improving the volumetric productivity for anabolic products. Furthermore, this concept may enable homofermentative production of fuels and chemicals whose formation currently has a very low, zero or negative ATP yield, improving process economics (Van Maris *et al.* 2004; de Kok *et al.* 2012; Cueto-Rojas *et al.* 2015). An additional advantage of engineering such a homofermentative pathway is that it directly couples cell growth to product formation. Evolutionary engineering of such a strain can be applied to select for energy-efficient mutants with a higher sucrose conversion rate and the resulting strains can

then be used as a platform for the production of other industrially relevant products.

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SUPPLEMENTARY MATERIAL

Table S1:Primers used in this study.

Primer	Sequence (5' → 3')	Purpose
3093	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATT CCGCCAGATCATCAATAGGCACCTTCGTACGCTGCAGG TCGAC	*amdSYM* amplification
3094	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCA TAGCCATGCCTTCACATATAGTGACGGATCGCTTGCCT GTAAC	*amdSYM* amplification
6845	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCA TAGCCATGCCTTCACATATAGTACAGGCAACACGCAGA TATAGG	p426TEF backbone amplification
6846	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATTC GACCAGCCTAAGAATGTTCAACGGCCCACTACGTGAAC CATC	p426TEF backbone amplification
8379	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGAT CCATGAAAAATATCATTTCATTGGTAAGCAAGAAG	*MAL11* ORF amplification
8380	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAGC TTCCTTAACATTTATCAGCTGCATTTAATTC	*MAL11* ORF amplification
7998	AAGCTTATCGATACCGTCGACC	p426TEF *amdSYM* backbone amplification
7999	GGATCCACTAGTTCTAGAAAACTTAGATTAG	p426TEF *amdSYM* backbone amplification
9043	TCACAGAGGGATCCCGTTACCCATCTATGCTGAAGATT TATCATACTATTCCTCCGCTCGGCGATCGCGTGTGGAA GAAC	*MAL11* expressing cassette amplification
9044	CGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGC TCGCCGCAAATTAAAGCCTTCG	*MAL11* expressing cassette amplification
10303	TTAAATCTATAACTACAAAAAACACATACATAAACTAA AAATGTCATTTCAAATTGAAACG	*PGM2* ORF amplification
10304	ACTACAATATAAAAAAACTATACAAATGACAAGTTCTT GATTAAGTACGAACCGTTGGTTCTTCAG	*PGM2* ORF amplification
6486	TTTTAGTTTATGTATGTGTTTTTTGTAGTTATAGATTT AAGCAAG	pUDE206 backbone amplification
9719	TCAAGAACTTGTCATTTGTATAG	pUDE206 backbone amplification
10305	GGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTG TTTAGTGTGAGCGGGATTTAAACTGTGAGG	*PGM2* expressing cassette amplification
10306	GTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAG GACAGTATAGCGACCAGCATTC	*PGM2* expressing cassette amplification
10307	AACAGCTATGACCATGATTA	pUDE432 or pUDE413 backbone amplification
10308	TCCTGTGTGAAATTGTTATC	pUDE432 or pUDE413 backbone amplification
8018	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGAT CCCTAGAATGGATAATCCTTCCACCAATG	Transporter expression cassette amplification
8019	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAGC TTCTAATGAAATCCACCCGCAATTGG	Transporter expression cassette amplification
8020	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGAT CCAATGCCGAATCCCGACTCTTC	Transporter expression cassette amplification
8021	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAGC TTGTCGACTCATACTGGGTTTCTAGG	Transporter expression cassette amplification
8022	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGAT CCATGGAAGCCCCATCCCCAAC	Transporter expression cassette amplification

Primer	**Sequence (5' → 3')**	**Purpose**
8023	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAGC TTACTTAATGAAAACCACCTGCTACCATAC	Transporter expression cassette amplification
8000	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTG	Transporter expression cassette amplification
8001	ACTAATTACATGACTCGAGGTCGACG	Transporter expression cassette amplification
1742	GGTCGCCTGACGCATATACC	*LEU2* integration (*LEU2* homology)
1743	TAAGGCCGTTTCTGACAGAG	*LEU2* integration (*LEU2* homology)
5271	CAAGGAGAAAAAACCCCGGATTCTAGAACTAGTGGATC CCCCATGAAAAATATCATTTCATTGGTAAG	*MAL11* amplification from BY4742
5272	GAATAATTCTTCACCTTTAGAACCTTGAAAATATAAAT TTTCCCCTCCACATTTATCAGCTGCATTTAATTC	*MAL11* amplification from BY4742 and pRHA00L
5273	GGAGGGGAAAATTTATATTTTCAAGGTTC	Backbone amplification from pFB001
5274	GGGGGATCCACTAGTTCTAGAATC	Backbone amplification from pFB001
5435	CGCATCTGTGCGGTATTTC	*LEU2* amplification from pRS315
5436	GGCTTAACTATGCGGCATC	*LEU2* amplification from pRS315
5437	TGCACTCTCAGTACAATCTGCTC	Backbone amplification from pRHA00
5438	TGCGGTGTGAAATACCGC	Backbone amplification from pRHA00
5959	AGGGGAAAATTTATATTTTCAAGG	Backbone amplification from pRHA00L
6324	TTGTTCCCTTTAGTGAGGG	Backbone amplification from pRHA00L
5961	TCTAGAACTAGTGGATCCCAAAATGAAAAATATCATTT CATTGG	*MAL11* amplification from pRHA00L
4995	AATTAACCCTCACTAAAGGG	*TEF1*-promoter amplification from pUDE379
5960	CATTTTGGGATCCACTAGTTCTAG	*TEF1*-promoter amplification from pUDE379
6717	CTCATTAGAAAGAAAGCATAGCAATC	Cassette amplification from pR151 or pUDE413
580	GAATGTAAGCGTGACATAAC	Cassette amplification from pR151
5921	AAAACTTAGATTAGATTGCTATGCTTTCTTTCTAATGA GC	pUDE413 backbone amplification or YPet-tag amplification from pUDE453
7812	TCATGTAATTAGTTATGTCACGCTTACATTC	pUDE413 backbone amplification
9772	CGAAGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTC ATGGAGGGGAAAATTTATATTTTCAAGGTTCTAAAG	YPet-tag amplification from pUDE453
9763	ATGAAAACCACCTGCTACCATAC	Cassette amplification from pUDE413
9355	TGTAAATATCTAGGAAATACACTTGTGTATACTTCTCG CTTTTCTTTTATTTTTTTTTGTAGTTTATCATTATCAA TACTCGCCATTTC	*LmSPase* or *MAL12* integration (*SGA1* homology)
9356	TTTACAATATAGTGATAATCGTGGACTAGAGCAAGATT TCAAATAAGTAACAGCAGCAAAGTGTGGAAGAACGATT ACAACAG	*MAL12* or *LmSPase* integration (*SGA1* homology)

***LmSPase* codon optimized:**

ATGGAAATCCAAAACAAGGCTATGTTGATCACTTACGCTGACTCTTTGGGTAAGAACTTGA
AGGACGTTCACCAAGTTTTGAAGGAAGACATCGGTGACGCTATCGGTGGTGTTCACTTGTT
GCCATTCTTCCCATCTACTGGTGACAGAGGTTTCGCTCCAGCTGACTACACTAGAGTTGAC
GCTGCTTTCGGTGACTGGGCTGACGTTGAAGCTTTGGGTGAAGAATACTACTTGATGTTCG
ACTTCATGATCAACCACATCTCTAGAGAATCTGTTATGTACCAAGACTTCAAGAAGAACCA
CGACGACTCTAAGTACAAGGACTTCTTCATCAGATGGGAAAAGTTCTGGGCTAAGGCTGGT
GAAAACAGACCAACTCAAGCTGACGTTGACTTGATCTACAAGAGAAAGGACAAGGCTCCAA
CTCAAGAAATCACTTTCGACGACGGTACTACTGAAAACTTGTGGAACACTTTCGGTGAAGA
ACAAATCGACATCGACGTTAACTCTGCTATCGCTAAGGAATTCATCAAGACTACTTTGGAA
GACATGGTTAAGCACGGTGCTAACTTGATCAGATTGGACGCTTTCGCTTACGCTGTTAAGA
AGGTTGACACTAACGACTTCTTCGTTGAACCAGAAATCTGGGACACTTTGAACGAAGTTAG
AGAAATCTTGACTCCATTGAAGGCTGAAATCTTGCCAGAAATCCACGAACACTACTCTATC
CCAAAGAAGATCAACGACCACGGTTACTTCACTTACGACTTCGCTTTGCCAATGACTACTT
TGTACACTTTGTACTCTGGTAAGACTAACCAATTGGCTAAGTGGTTGAAGATGTCTCCAAT
GAAGCAATTCACTACTTTGGACACTCACGACGGTATCGGTGTTGTTGACGCTAGAGACATC
TTGACTGACGACGAAATCGACTACGCTTCTGAACAATTGTACAAGGTTGGTGCTAACGTTA
AGAAGACTTACTCTTCTGCTTCTTACAACAACTTGGACATCTACCAAATCAACTCTACTTA
CTACTCTGCTTTGGGTAACGACGACGCTGCTTACTTGTTGTCTAGAGTTTTCCAAGTTTTC
GCTCCAGGTATCCCACAAATCTACTACGTTGGTTTGTTGGCTGGTGAAAACGACATCGCTT
TGTTGGAATCTACTAAGGAAGGTAGAAACATCAACAGACACTACTACACTAGAGAAGAAGT
TAAGTCTGAAGTTAAGAGACCAGTTGTTGCTAACTTGTTGAAGTTGTTGTCTTGGAGAAAC
GAATCTCCAGCTTTCGACTTGGCTGGTTCTATCACTGTTGACACTCCAACTGACACTACTA
TCGTTGTTACTAGACAAGACGAAAACGGTCAAAACAAGGCTGTTTTGACTGCTGACGCTGC
TAACAAGACTTTCGAAATCGTTGAAAACGGTCAAACTGTTATGTCTTCTGACAACTTGACT
CAAAACTAA

***PvSUF1* codon optimized:**

ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCACATTAT
CCGTAGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTCGCCGTTGCTTCAAT
AGCTGCAGGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGTTAACCCCATATGTCCAA
TTGTTAGGTGTACCTCATGCCGCTGCATCCTTTATATGGTTATGTGGTCCAATCAGTGGTT
TGGTTGTCCAACCTATCGTTGGTTACTACTCTGATAGATCTACTTCTAGATACGGTAGAAG
AAGACCTTTTATTTTAGGTGGTGCAGTCGCCGTAGCTATCGCAGTTTTCTTGATTGGTTAT
GCCGCTGATATTGGTTACTCAGCTGGTGACGACATAACCAAAAAGACTAGACCAAGAGCCG
TTGCTGTCTTCGTAATTGGTTTCTGGATCTTGGACGTTGCAAACAACATGTTGCAAGGTCC
TTGCAGAGCCTTTTTGGCTGATTTGGCAGCCGGAGATCAAAGAAAGACTAGAATCGCAAAC
GGTTTCTTTTCTTTCTTTATGGCCGTTGGTAACGTTTTAGGTTATGCTGCAGGTTCTTTTT
CAGGTTTGCACAAAATTTTCCCTTTTACTCAAACAAAGGCATGTGATGTTTTCTGCGCCAA
TTTGAAGTCTTGTTTCTTTTTCTCTATCTTGTTGTTGTTATTTTTGTCCACAGTTGCTTTG
ATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGCCGTTCAAGAAGACGCTCAACCTTCTT
GCTTCTTTCAATTGTTCGGTGCTTTGAAGGAATTGAAGAGACCAATGTGGATGTTGATGTT
AGTCACCGCAGTAAATTGGATTGGTTGGTTTCCTTATTTCTTGTTTGATACTGACTGGATG
GGTAGAGAAGTTTATGGTGGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAGAGTCG
GTTCCTTGGGTTTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAACC

ATTGGGTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTTAACTTCATCTTGGCT
ATCGGTTTCGGTATGACAGTCGTAATAACCAAAATGGCAGAACATCAAAGACACTTAAACC
CAGCAGCCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGGTATTCTTTGCTGT
ATTGGGTGTTCCATTAGCAATCACATTCTCTGTTCCTTTTGCTTTGGCATCCATCTATTCT
TCAGCAAGTGGTGCCGGTCAAGGTTTGTCATTAGGTGTTTTGAATTTGGCTATTGTTGTCC
CACAAATGGTAGTTTCCGCCTTAAGTGGTCCTTGGGACGCTTTATTCGGTGGTGGTAACTT
GCCAGCTTTTATGGTTGGTGCTGCAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTG
TTACCTACCCCTAAGCCAGCCGACGAAGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTC
ATTAA

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

ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCACATTAT
CCGTAGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTCGCCGTTGCTTCAAT
AGCTGCAGGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGTTAACCCCATATGTCCAA
TTGTTAGGTGTACCTCAGGCCGCTGCATCCTTTATATGGTTATGTGGTCCAATCAGTGGTT
TGGTTGTCCAACCTATCGTTGGTTACTACTCTGATAGATCTACTTCTAGATACGGTAGAAG
AAGACCTTTTATTTTAGGTGGTGCAGTCGCCGTAGCTATCGCAGTTTTCTTGATTGGTTAT
GCCGCTGATATTGGTTACTCAGCTGGTGACGACATAACCAAAAAGACTAGACCAAGAGCCG
TTGCTGTCTTCGTAATTGGTTTCTGGATCTTGGACGTTGCAAACAACATGTTGCAAGGTCC
TTGCAGAGCCTTTTTGGCTGATTTGGCAGCCGGAGATCAAAGAAAGACTAGAATCGCAAAC
GGTTTCTTTTCTTTCTTTATGGCCGTTGGTAACGTTTTAGGTTATGCTGCAGGTTCTTTTT
CAGGTTTGCACAAAATTTTCCCTTTTACTCAAACAAAGGCATGTGATGTTTTCTGCGCCAA
TTTGAAGTCTTGTTTCTTTTTCTCTATCTTGTTGTTGTTATTTTTGTCCACAGTTGCTTTG
ATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGCCGTTCAAGAAGACGCTCAACCTTCTT
GCTTCTTTCAATTGTTCGGTGCTTTGAAGGAATTGAAGAGACCAATGTGGATGTTGATGTT
AGTCACCGCAGTAAATTGGATTGGTTGGTTTCCTTATTTCTTGTTTGATACTGACTGGATG
GGTAGAGAAGTTTATGGTGGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAGAGTCG
GTTCCTTGGGTTTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAACC
ATTGGGTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTTAACTTCATCTTGGCT
ATCGGTTTCGGTATGACAGTCGTAATAACCAAAATGGCAGAACATCAAAGACACTTAAACC
CAGCAGCCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGGTATTCTTTGCTGT
ATTGGGTGTTCCATTAGCAATCACATTCTCTGTTCCTTTTGCTTTGGCATCCATCTATTCT
TCAGCAAGTGGTGCCGGTCAAGGTTTGTCATTAGGTGTTTTGAATTTGGCTATTGTTGTCC
CACAAATGGTAGTTTCCGCCTTAAGTGGTCCTTGGGACGCTTTATTCGGTGGTGGTAACTT
GCCAGCTTTTATGGTTGGTGCTGCAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTG
TTACCTACCCCTAAGCCAGCCGACGAAGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTC
ATTAA

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

ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCACATTAT
CCGTAGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTCGCCGTTGCTTCAAT
AGCTGCAGGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGTTAACCCCATATGTCCAA
TTGTTAGGTGTACCTCATGCCGCTGCATCCTTTATATGGTTATGTGGTCCAATCAGTGGTT
TGGTTGTCCAACCTATCGTTGGTTACTACTCTGATAGATCTACTTCTAGATACGGTAGAAG

AAGACCTTTTATTTTAGGTGGTGCAGTCGCCGTAGCTATCGCAGTTTTCTTGATTGGTTAT
GCCGCTGATATTGGTTACTCAGCTGGTGACGACATAACCAAAAAGACTAGACCAAGAGCCG
TTGCTGTCTTCGTAATTGGTTTCTGGATCTTGGACGTTGCAAACAACATGTTGCAAGGTCC
TTGCAGAGCCTTTTTGGCTGATTTGGCAGCCGGAGATCAAAGAAAGACTAGAATCGCAAAC
GGTTTCTTTTCTTTCTTTATGGCCGTTGGTAACGTTTTAGGTTATGCTGCAGGTTCTTTTT
CAGGTTTGCACAAAATTTTCCCTTTTACTCAAACAAAGGCATGTGATGTTTTCTGCGCCAA
TTTGAAGTCTTGTTTCTTTTTCTCTATCTTGTTGTTGTTATTTTTGTCCACAGTTGCTTTG
ATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGCCGTTCAAGAAGACGCTCAACCTTCTT
GCTTCTTTCAATTGTTCGGTGCTTTGAAGGAATTGAAGAGACCAATGTGGATGTTGATGTT
AGTCACCGCAGTAAATTGGATTGGTTGGTTTCCTTATTTCTTGTTTGATATTGACTGGATG
GGTAGAGAAGTTTATGGTGGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAGAGTCG
GTTCCTTGGGTTTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAACC
ATTGGGTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTTAACTTCATCTTGGCT
ATCGGTTTCGGTATGACAGTCGTAATAACCAAAATGGCAGAACATCAAAGACACTTAAACC
CAGCAGCCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGGTATTCTTTGCTGT
ATTGGGTGTTCCATTAGCAATCACATTCTCTGTTCCTTTTGCTTTGGCATCCATCTATTCT
TCAGCAAGTGGTGCCGGTCAAGGTTTGTCATTAGGTGTTTTGAATTTGGCTATTGTTGTCC
CACAAATGGTAGTTTCCGCCTTAAGTGGTCCTTGGGACGCTTTATTCGGTGGTGGTAACTT
GCCAGCTTTTATGGTTGGTGCTGCAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTG
TTACCTACCCCTAAGCCAGCCGACGAAGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTC
ATTAA

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4

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

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Abstract

Alteration of energy coupling of sugar transporters can improve product yields in industrial microbiology. Substitution of the native pathway for sucrose metabolism in *Saccharomyces cerevisiae* by uptake via a plant sucrose uniporter (*Phaseolus vulgaris* sucrose facilitator 1) combined with intracellular phosphorolysis (by *Leuconostoc mesenteroides* sucrose phosphorylase) could increase the ATP yield of anaerobic sucrose fermentation by 25 %. However, recent attempts to construct yeast strains in which sucrose metabolism was dependent on *PvSUF1* led to slow sucrose uptake. Here, such *PvSUF1*-dependent *S. cerevisiae* strains were evolved for faster growth. Of five independently evolved strains, two showed an approximately two-fold higher anaerobic growth rate on sucrose than the parental strain (μ = 0.19 h^{-1} and μ = 0.08 h^{-1}, respectively). All five mutants displayed sucrose-induced proton uptake (13-50 μmol H^+ (g biomass)$^{-1}$ min^{-1}). Their ATP yield from sucrose dissimilation, as estimated from biomass yields in anaerobic chemostat cultures, was the same as that of a congenic strain expressing the native sucrose symporter Mal11p. Four out of six observed amino acid substitutions encoded by evolved *PvSUF1* alleles removed or introduced a cysteine residue and may be involved in transporter folding and/or oligomerization. Expression of one of the evolved *PvSUF1* alleles in an unevolved strain enabled it to grow on sucrose at the same rate (0.19 h^{-1}) as the corresponding evolved strain. This study shows how laboratory evolution can be used to improve sucrose uptake via heterologous plant transporters and reveals relevant amino acid residues for the efficient heterologous expression of such transporters.

INTRODUCTION

Sucrose (α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside) is an intensively used carbon source in microbial biotechnology (Maiorella, Blanch and Wilke 1984; Peters, Rose and Moser 2010; Marques *et al.* 2016) and plays a vital role as a carbon and energy carrier in plants (Salerno and Curatti 2003). In plants, *s*ucrose exits source cells via plasmodesmata as well as via membrane transport mediated by sucrose uniporters (Doidy *et al.* 2012).

In *Saccharomyces cerevisiae*, a yeast employed in many different industrial bioprocesses, the predominant pathway for sucrose catabolism starts with its extracellular hydrolysis, catalysed by invertase. The free energy available from sucrose hydrolysis ($\Delta G_0'$ = -29 kJ/mol)(Goldberg, Tewari and Ahluwalia 1989) is not conserved by the yeast cells. The resulting monomers glucose and fructose are subsequently transported into the cells by facilitated diffusion. Alternatively, after its uptake via proton symporters, sucrose can also be hydrolysed in the cytosol (Stambuk *et al.* 1999; Marques *et al.* 2017).

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

not change relative to a wild-type strain: in both cases, anaerobic fermentation of sucrose yielded 4 mol ATP per mol of sucrose. To gain one additional mol of ATP per mol of sucrose consumed, the native sucrose transporters should be replaced by a transporter that mediates facilitated diffusion (de Kok *et al.* 2012). If the ATP yield of sucrose fermentation by *S. cerevisiae* could be increased to 5 mol of ATP per mole of sucrose, this could theoretically expand the range of products that can be made in anaerobic yeast-based processes. Such anaerobic production processes have considerable cost advantages relative to aerated processes (Weusthuis *et al.* 2011; de Kok 2012; Mans 2017).

Many studies have demonstrated functional expression of heterologous transporters in *S. cerevisiae* (Ton and Rao 2004; He, Wang and Yan 2014; Kim *et al.* 2014; Lin *et al.* 2014). However, efficient transporter sorting, folding and stability in yeast cells can be a major challenge (Hernández 2005; Froissard *et al.* 2006). Therefore, functional characteristics of plant transporters expressed in *S. cerevisiae* cannot always be derived from *in planta* results (Bassham and Raikhel 2000). Few previous studies have investigated the expression of plant sucrose uniporters in yeast (Zhou *et al.* 2007; Chen 2014; Lin *et al.* 2014; Zhou, Grof and Patrick 2014). Marques *et al*. (2018) expressed 5 candidate genes for sucrose uniporters in *S. cerevisiae*, of which only one, encoding the *Phaseolus vulgaris* Sucrose Facilitator 1 (*PvSUF1*), supported growth of a strain (after one week time) in which extracellular invertase and sucrose symport have been eliminated. However, the anaerobic specific growth rate on sucrose of this strain was only 0.05 h^{-1}, which was six-fold lower than that of the reference strain CEN.PK113-7D. Indeed, rates of uptake of radiolabelled sucrose by the *PvSUF1*-expressing strain were close to the detection limit. While some YPet-tagged *Pv*Suf1 was found at the yeast plasma membrane, it also accumulated in intracellular compartments, suggesting poor intracellular targeting and/or high turnover of heterologously expressed *Pv*Suf1 (Marques *et al.* 2018).

In view of the potential relevance of expressing *PvSUF1* and other plant sugar transporter genes in the metabolic engineering of *Saccharomyces*

cerevisiae, this study aimed at investigating genetic factors involved in optimal functional expression of *PvSUF1* in this yeast. To this end, we used laboratory evolution to select for *Pv*Suf1-dependent *S. cerevisiae* strains with improved sucrose-uptake kinetics and analysed causal mutations for improved sucrose consumption by evolved strains (Kasemets *et al.* 2003; Bachmann *et al.* 2013; Dragosits and Mattanovich 2013; Mans, Daran and Pronk 2018). To study the energy coupling of sucrose transport by evolved and non-evolved *Pv*Suf1 variants, we analysed sucrose-induced proton-uptake by reference and evolved strains and measured biomass yields of yeast strains expressing different *Pv*Suf1 variants in anaerobic, sucrose-limited chemostat cultures.

MATERIALS AND METHODS

Microbial strains and cultivation medium

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

Molecular biology techniques

PCR amplifications for strain construction were performed with Phusion Hot Start II High Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Diagnostic PCR was carried out using DreamTaq (Thermo Fisher Scientific). The primers used in this study (**Table S1)** where purchased from Sigma-Aldrich. Yeast genomic

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

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

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

DNA was isolated using the YeaStar Genomic DNA kit (D2002, Zymo Research, Irvine, CA). DNA fragments obtained by PCR were separated by gel electrophoresis using 1% (w/v) agarose gels (Thermo Fisher Scientific) in Tris-acetate-EDTA buffer (Thermo Fisher Scientific). DNA fragments were excised from the gels and purified by gel purification kit (D2004, Zymo Research). Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich) according to the supplier's manual and from *S. cerevisiae* using Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research). Restriction analysis was performed using FastDigest enzymes (Thermo Fisher Scientific) according to the manufacturer's manual. *E. coli* DH5α cells (18258-012, Thermo Fisher Scientific) were transformed via electroporation using a Gene Pulser Xcell Electroporation System (Bio-Rad), following the manufacturers protocol.

Sanger and whole-genome sequencing

Genome-integrated and episomal expression cassettes present in the evolved strains (IMS644, IMS646, IMS647, IMS648 and IMS649) were Sanger sequenced at BaseClear BV (Leiden, The Netherlands). Primers 6018&7822 (**Table S1**) were used to amplify the *SPase*-expression cassette for sequencing. Similarly, primers 5606&7827 were used to amplify the *PvSUF1* allele of strain IMS648, before Sanger sequencing. Plasmids expressing the *PGM2* and *PvSUF1* genes were extracted from yeast using Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research) and transformed into *E. coli* (DH5α cells, 18258-012, Thermo Fisher Scientific) via electroporation using a Gene Pulser Xcell Electroporation System (Bio-Rad) for propagation. After extraction from *E. coli* using the Sigma GenElute Plasmid kit (Sigma-Aldrich), plasmids were used as a template to sequence the *PGM2* and *PvSUF1* cassettes. Genes were sent for Sanger sequencing using the primers listed in **Table S1** resulting in a two times coverage of each base pair. The promoter and terminator regions sequenced were: 420 bp upstream and 280 bp downstream of the *PvSUF1* ORF, 500 bp upstream and 170 bp downstream of the *PGM2* ORF and 670 bp upstream and 370 bp downstream of the *LmSPase* ORF. Genomic DNA for

whole-genome sequencing was extracted using the Qiagen 100/G kit following the manufacturer's protocol (Qiagen, Hilden, Germany), from shake-flask cultures grown on SMD. Whole-genome sequencing was performed by Novogene (HK) Company Ltd (Hong Kong, China). A PCR-free insert library of 350-bp genomic fragments was created and sequenced paired end (150-bp reads). A minimum data quantity of 2600 MB was generated per strain, representing a minimum 216-fold coverage. Data analysis was performed by mapping the sequence reads to the CEN.PK113-7D reference (Salazar *et al.* 2017) using the Burrows-Wheeler alignment (BWA) tool (Li and Durbin 2009) and processed with Pilon (Walker *et al.* 2014). The sequencing data of the parental strain IMZ630 and of the evolved isolates (IMS644, IMS646, IMS647, IMS648 and IMS649) were deposited at NCBI under the BioProject ID: PRJNA471800.

Plasmid construction

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

Table 2: Plasmids used in this study

Name	Relevant characteristics	Origin
pUDE413	*2μ URA3 pTEF1-PvSUF1-tCYC1*	Marques *et al*. 2018
pUDE486	*2μ URA3 pTEF1-PvSUF1-tCYC1 pTPI1-PGM2-tTEF1*	Marques *et al*. 2018
pUDE690	*2μ URA3 pTPI1-PGM2-tTEF1*	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	*2μ URA3 pTPI1-PvSUF1*^{G326C}*-tCYC1*	This study
pUDE566	*2μ URA3 pTPI1-PvSUF1*$^{YAAGSFSG\text{-}duplication}$*-tCYC1*	This study
pUDE567	*2μ URA3 pTPI1-PvSUF1*^{G326C}*-tCYC1 pTPI1-PGM2-tTEF1*	This study
pUDE568	*2μ URA3 pTPI1-PvSUF1*$^{YAAGSFSG\text{-}duplication}$*-tCYC1 pTPI1-PGM2-tTEF1t*	This study
pUDE691	*2μ URA3 pTPI1-PvSUF1*$^{I217F,\ C265F,\ G326C}$*-tCYC1 pTPI1-PGM2-tTEF1*	This study

when pUDE413-backbone was used, and plasmids pUDE567 and pUDE568, respectively, when backbone from pUDE486 was used (**Table 2**). pUDE691 was constructed in the same way as pUDE567 and pUDE568 with the exception that, for construction of this plasmid, the *PvSUF1*-expression cassette was amplified from pUDE560 (**Table 2**).

Strain construction

Strain IMZ730 was constructed by transforming pUDE690 into IMZ630 (**Figure 1**). Plasmids present in the evolved strains, IMS644, IMS646, IMS647, IMS648 and IMS649, were removed by overnight cultivation on YPD medium followed by selection on SMD agar plates supplemented with 0.15 g/L uracil and 1 g/L 5'-fluoroorotic acid (Boeke, La Croute and Fink 1984), resulting in strains IMS652, IMS653, IMS654, IMS655 and IMS656, respectively (**Figure 1**). Expression cassettes (*TEF1*p-*PvSUF1-CYC1*t) were extracted from the evolved strains, cloned into a 2-μm plasmid (with and without *PGM2*) resulting in plasmids pUDE565, pUDE566, pUDE567, pUDE568 and pUDE691, which were subsequently transformed into an unevolved strain background

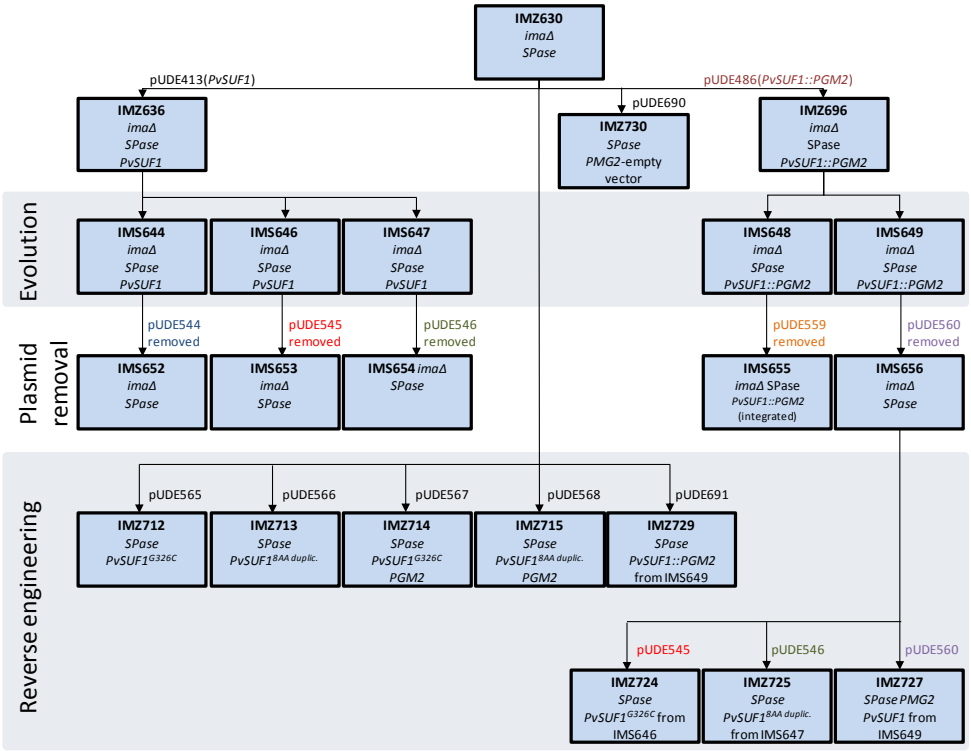

Figure 1: Strains' construction scheme. Each blue box represents a different strain. Plasmids used are indicated on the top of the corresponding strain. The three main methods of generating strains are shown in the panels: "Evolution", "Plasmid removal" and "Reverse engineering".

(IMZ630), resulting in strains IMZ712, IMZ713, IMZ714, IMZ715 and IMZ729, respectively. Similarly, the *PvSUF1*-containing plasmids extracted from the evolved strains (pUDE545, pUDE546 and pUDE560) were transformed into an evolved background, IMS656 (obtained after plasmid removal from strain IMS649), resulting in strains IMZ724, IMZ725 and IMZ727, respectively (**Figure 1**).

Cultivation conditions

Shake flask cultivations were carried out in 500 mL flasks containing 100 mL synthetic medium with 20 g/L initial sucrose (SMS) (if not stated,

sugar concentration in SMD or SMS was 20 g/L), in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ) set at 200 rpm and 30 ºC. For growth rate determinations, frozen stock cultures were first inoculated in a shake flask containing SMD. After reaching stationary phase, cultures were transferred to SMS (initial $OD_{660nm} \leq 0.2$) and incubated under an anaerobic atmosphere (5% H_2 6% CO_2 and 89% N_2) in a Bactron X anaerobic chamber (Shell Lab, Cornelius, OR) until exponential growth was observed. Inside the anaerobic chamber, exponentially growing cultures were then transferred to fresh SMS (initial $OD_{660nm} = 0.2$) and samples were taken hourly until stationary phase was reached. Specific growth rates were calculated from at least five OD measurements evenly distributed over the exponential growth phase. For anaerobic cultivations, synthetic medium was supplemented with 10 mg/L ergosterol and 420 mg/L Tween 80. Since stock solutions of these anaerobic growth factors were prepared with ethanol, the initial ethanol concentration in media for anaerobic growth was 0.67 g/L. Chemostat cultivations were performed in 1.5 L bioreactors (800 rpm, 30°C) (Applikon, Delft, The Netherlands) with 1 L SMS supplemented with 0.15 g/L Antifoam C (Sigma-Aldrich), which was autoclaved separately (120 °C for 20 min) (Verduyn *et al.* 1992). The culture pH was maintained at 5.0 by automated addition of 2 M KOH. For aerobic cultivation, 500 mL min^{-1} compressed air was sparged in the reactor. To maintain anaerobic conditions, the bioreactors were sparged with 500 mL N_2 min^{-1} (<5 ppm O_2) (also the medium vessels were sparged with N_2) and equipped with Norprene tubing to minimize oxygen diffusion. After the batch phase, medium pumps were switched on, resulting in the continuous addition of SMS containing 25 g/L sucrose to the cultures. The working volume was kept constant at 1.0 L using an effluent pump controlled by an electric level sensor, resulting in a constant dilution rate. The exact working volume and medium flow rate were measured at the end of each experiment. Chemostat cultures were assumed to be in steady state when, after five volume changes, the biomass concentration and the CO_2 production rate varied by less than 4.5 % over at least another 2 volume changes.

Laboratory evolution

Repeated batch cultivation (SBRs) of strains *S. cerevisiae* IMZ636 and IMZ696 was initiated by serial transfers in shake flasks (5 to 11 transfers). Shake flask cultures were grown in an anaerobic chamber with 20 mL SMS in 30 mL shake flasks incubated at 30 ºC and 200 rpm. After this initial phase, evolution was continued in N_2-sparged reactors of 500 mL total volume (Infors HT Multifors 2, Infors AG, Switzerland) with 100 mL SMS (50 mL min^{-1} N_2 gas, 400 rpm, 30 ºC). The 100 mL working volume was possible due to manufacturer's special modifications on the vessel and jacket size. For strain IMZ636, three evolution lines were carried out in parallel. For IMZ696, two parallel evolution lines were performed. Culture pH was maintained at 5.0 by automatic addition of 2 mol L^{-1} KOH. Empty-refill cycles were programmed using the Iris 6 bioprocess software (Infors AG, Switzerland). When the off-gas CO_2 concentration achieved 0.4 %, the empty-refilling sequence was started. Such CO_2 concentration was lower than the maximum of 0.9 % that would be produced at the end of the exponential growth phase (this value was measured before starting the empty-refill cycles to better adjust evolution settings), which guarantees that a new cycle was started before the cells entered stationary phase, which in turn could delay the evolutionary process. In each cycle, 90 % of the medium was substituted by fresh medium. For evolution of strain IMZ696, the cultivation method was changed from SBRs to accelerostat cultivation (Bracher *et al.* 2017), after the growth rate did not increase further with the SBR strategy. Accelerostat cultivation, which was conducted in the same reactors used for the SBR cultivations, were continuously fed with SMS containing 25 g/L sugar, while culture liquid was removed to keep the working volume constant. The dilution rate, which was initially set at 0.09 h^{-1}, was automatically increased in response to the CO_2 off-gas measurements. This means that the speed of the feed pump (thus the dilution rate) was increased or decreased if the off-gas CO_2 profile showed consistent increase or decrease over a period of 24 h. One single-colony isolate from each evolution line was obtained by restreaking thrice on plates containing selective SMS medium, which were incubated anaerobically at 30

°C. To prepare frozen stock cultures of sucrose-evolved strains under selective conditions, cells from a single colony were inoculated in 20 mL liquid SMS medium in 30 mL shake flasks, which were incubated under anaerobic conditions (Bactron X anaerobic chamber, Shell Lab, Cornelius, OR, 200 rpm, 30 °C). Stocks in SMD were also prepared, to be used in characterization experiments, such as measurement of the growth rate on sucrose-based medium.

Analytical methods

Optical density of cultures at 660 nm was monitored using a Libra S11 spectrophotometer (Biochrom, Cambride, United Kingdom). Culture dry weight measurements and rapid quenching of culture samples were carried out as described by Marques *et al*. (2018). HPLC analysis of the supernatant and sugar determination were performed as described previously (de Kok *et al.* 2011; Marques *et al.* 2017). Off-gas CO_2 concentrations were measured with an NGA 2000 Rosemount gas analyser (Emerson, St. Louis, MO).

Proton translocation assay

Cells used in proton translocation assays were harvested from aerobic sucrose-limited chemostat cultures. The same culture conditions as described for anaerobic chemostats above were used, with the following changes: reactors were sparged with 500 mL min^{-1} air, the sucrose concentration in the medium vessel was decreased to 7.5 g/L, the dilution rate was set at 0.03 h^{-1} and Tween 80 and ergosterol were omitted from the medium. After 5 volume changes, when CO_2 concentrations in the off-gas were stable, cells were harvested by centrifugation at 5 000 x *g* for 5 min, at room temperature, washed with distilled water and resuspended in potassium phthalate buffer (1.25 mM, pH 5) to a final concentration of 12 g dry weight L^{-1}. 5 mL of the cell suspension were incubated at 30 °C in a magnetic stirred vessel with a S220 SevenCompact™ pH/Ion electrode attached (Mettler Toledo, Greifensee, Switzerland). After stabilization of the pH signal, 100 µL of a 1 mol L^{-1} sugar

(sucrose, maltose, glucose or fructose) solution was added (final concentration 20 mM) and changes in the pH were recorded using the LabX™ pH Software (Mettler Toledo, Greifensee, Switzerland). Calibration was performed by addition of 5 µL aliquots of 10 mM NaOH to the cell suspensions.

RESULTS

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

In the engineered *S. cerevisiae* strain IMZ636 (*malΔ mphΔ suc2Δ imaΔ sga1Δ LmSPase PvSUF1*), sucrose metabolism is strictly dependent on uptake via the plant transporter *Pv*Suf1 and subsequent intracellular cleavage by a bacterial sucrose phosphorylase. In anaerobic shake flask cultures, strain IMZ636 grew on SMS (synthetic medium with 20 g/L sucrose as sole carbon source) at a specific growth rate of 0.05 h^{-1} (**Table 3**). This growth rate is much lower than that of the reference strain CEN.PK113-7D (0.29 h^{-1}, **Table 3**). To select for faster growth on sucrose, strain IMZ636 was grown in anaerobic sequencing-batch bioreactors (SBR). In three independent evolution lines, the specific growth rate increased from 0.05 h^{-1} to 0.10-0.17 h^{-1} (estimated from off-gas CO_2 profiles) after 56-80 cycles (approximately 150 generations, **Figure 2**). When no further increase in the growth rate was observed, four single colonies were isolated from each experiment and the fastest growing isolate of each evolution line was characterized in anaerobic shake-flask cultures on SMS. The fastest isolate of each evolution line displayed specific growth rates of 0.07 h^{-1} (IMS644 from reactor "A"), 0.09 h^{-1} (IMS646 from reactor "B") and 0.08 h^{-1} (IMS647 from reactor "C") (**Table 3**). These growth rates were consistently higher than those of the non-evolved IMZ636 strain, but differed slightly from those estimated from CO_2 profiles in the SBR experiments. These differences may have been caused by the different methods used to access growth rate (optical density measurements versus on-line CO_2 data; shake flasks versus bioreactors). Additionally, the shake-flask

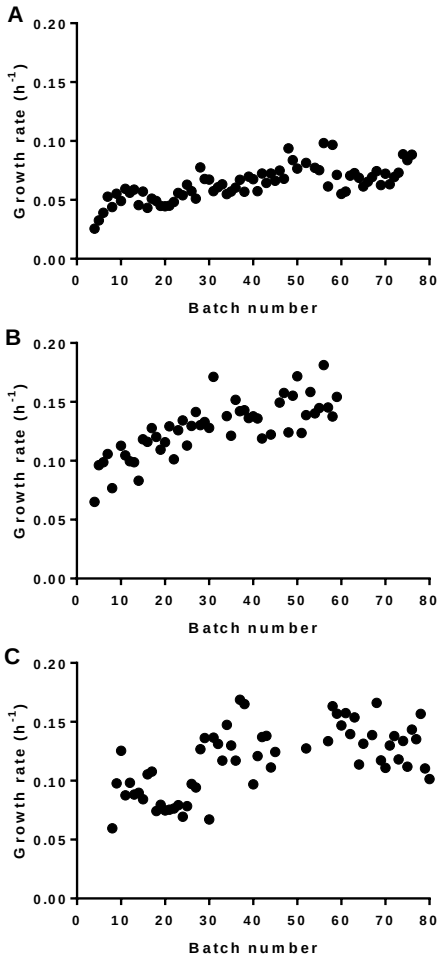

Figure 2: Laboratory evolution in sequential batch cultures of *S. cerevisiae* IMZ636 (*PvSUF1, SPase*). After four serial transfers in shake flask cultures, incubated in an anaerobic chamber, evolution was continued in sequential batch bioreactors sparged with N_2 gas. Evolution was conducted independently in triplicate (panels **A**, **B** and **C**). Specific growth rates were estimated from the corresponding CO_2 profiles (not shown) in the off-gas. 100 mL of SMS were used as working volume (pH 5.0, 30°C, 250 rpm). Evolution was stopped after approximately 160, 170 and 150 generations (panels **A**, **B** and **C**).

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

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

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

experiments were performed with single cell lines while the specific growth rates estimated from the SBR experiments represented growth of an evolving and probably heterogeneous population.

The anaerobic specific growth rates on sucrose of the evolved, IMZ636-derived strains were still 3-4 fold lower than that of the congenic reference strain CEN.PK113-7D (**Table 3**). Marques *et al*. (2018) showed that overexpression of phosphoglucomutase (*PGM2*) in a non-evolved *PvSUF1-SPase*-expressing strain increased its growth rate from 0.05 to 0.07 h^{-1} (IMZ696). To investigate whether in the laboratory evolution experiments, specific growth rates were limited by phosphoglucomutase levels, a new sequential batch cultivation evolution was initiated with a *PGM2*-expressing strain (IMZ696-*PvSUF1, SPase, PGM2*). In two independent evolution lines, the specific growth rate on sucrose increased from 0.07 to 0.15-0.20 h^{-1} (calculated from the off-gas CO_2 concentration from each reactor) after 52 cycles (ca. 120 generations in reactor A and 190 generations in reactor B, **Figure 3A** and **3B**). At this stage, laboratory evolution was continued using accelerostat cultivation, with an initial dilution rate of 0.09 h^{-1}. The feed rate was automatically increased or decreased based on on-line analysis of the CO_2

concentration in the off-gas. After 20-30 days (approximately 130 generations), the dilution rate in the two reactors had increased to 0.25 h^{-1} and 0.17 h^{-1} (**Figures 3C** and **3D**), while the residual sucrose concentration had decreased from 8 g/L to approximately 2.5 g/L after accelerostat selection (results not shown). These dilution rates were close to the anaerobic specific growth rate on sucrose reported for a *Lm*SPase-dependent strain expressing the native *MAL11* sucrose symporter (0.23 h^{-1}, Marques *et al*. 2018). Single colony isolates from each reactor (IMS648 from reactor shown in **Figure 3A** and IMS649 from reactor shown in **Figure 3B**) exhibited specific growth rates of 0.19 h^{-1} and 0.23 h^{-1}, respectively (**Table 3**). These specific growth rates were close to that of IMZ709 (*MAL11, SPase, PGM2*), and almost three times higher than that of the unevolved parental strain IMZ696 (**Table 3**).

Sucrose/H^+ symport activity of evolved *Pv*Suf1 transporters as revealed by proton-uptake studies and biomass yields on sucrose

Although *PvSUF1* has been reported to encode a sucrose uniporter (Zhou *et al.* 2007), a recent study (Marques *et al*. 2018) on the expression of *PvSUF1* in *S. cerevisiae* casts doubt on the actual mechanism of sucrose transport: sucrose-dependent proton uptake rates of 8.2 ± 2.2 μmol H^+ (g biomass)$^{-1}$ min^{-1} were measured with cell suspensions of *S. cerevisiae* strain IMZ696 (*PvSUF1, SPase, PGM2*) grown in aerobic, sucrose-limited chemostat cultures (**Figure 4**, data extracted from Marques *et al*. 2018). This measured uptake rate would in principle be more than sufficient to account for all sucrose uptake in the aerobic chemostat cultures. If we considered the residual sucrose concentration in the bioreactor negligible, a specific sucrose uptake rate of 3.7 ± 0.1 μmol sucrose (g biomass)$^{-1}$ min^{-1} would be calculated.

In replicate chemostat experiments with strain IMZ696, different mutations were found in *PvSUF1 (Pv*Suf1^{T302I} in one culture and *Pv*Suf1$^{E308K\ V323F}$ in the other). Since the frozen stock cultures from wich the chemostats were inoculated did not contain mutations in *PvSUF1*, these mutations

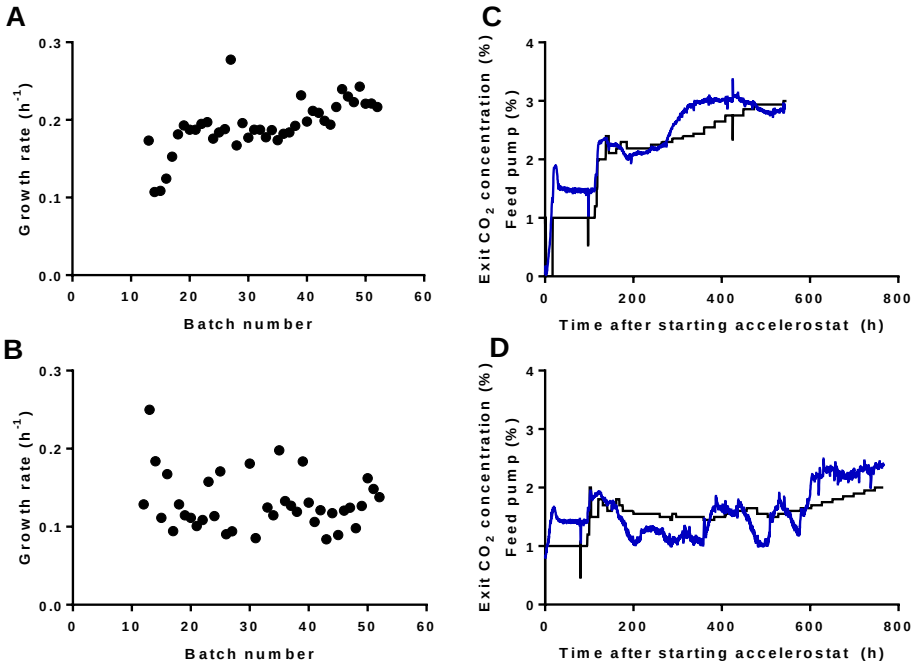

Figure 3: Laboratory evolution of *S. cerevisiae* IMZ696 (*PvSUF1, SPase, PGM2*) for faster anaerobic growth in sucrose. After eleven serial transfers in shake flasks in an anaerobic chamber, evolution was continued in bioreactors sparged with N_2 gas, first operated as sequential batch reactors (SBRs) (panels **A** and **B**) and subsequently as accelerostats (panels **C** and **D**) as described in the Methods section. **C**, **D** (accelerostat): feed and effluent pumps were turned on at 1 % of the maximum speed (equivalent to 0.09 h^{-1} dilution rate). Feed pump speed (black line) was increased automatically based on the CO_2 concentration in the off-gas (blue line). A maximum pump speed corresponding to 0.25 h^{-1} dilution rate was reached in one reactor (**C**) and of 0.17 h^{-1} in the other (**D**). Laboratory evolution in bioreactors was performed with 100 mL of synthetic medium containing 20 g/L sucrose (batches) and 25 g/L (accelerostat), pH 5, 30°C, 250 rpm). Panels **A** and **C** correspond to one evolution line independent from another replicate (panels **B** and **D**). Both evolution lines were started with the same parental strain.

probably conferred a selective advantage during sucrose-limited chemostat cultivation (Marques *et al*. 2018).

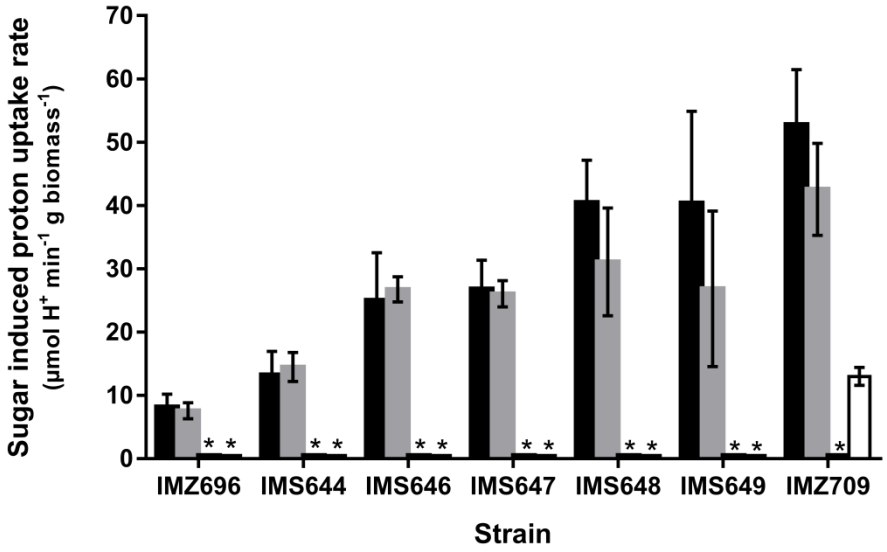

Figure 4: Proton uptake rate of unevolved *S. cerevisiae* IMZ696 (*PvSUF1,SPase,PGM2*), evolved strains (IMS644, IMS646, IMS647, IMS648 and IMS649) and the control strain IMZ709 (*MAL11, SPase, PGM2*). Cells were harvested from aerobic sucrose-limited chemostat cultures (D = 0.03 h^{-1}, 30°C, pH 5.0), washed and immediately tested for proton uptake upon addition of sucrose (black bar), maltose (grey bar), fructose (*: H^+ uptake not detected) or glucose (white bar, H^+ uptake induced only in strain IMZ709 and *: not detected). 20 mM sugar (final concentration) was added to a K-phtalate suspension (pH 5.0) containing 2.5 g/L cells (30°C). Average and mean deviation were obtained from two biological and three experimental replicates. Calibration was performed as described in the Methods section. Results from strains IMZ696 and IMZ709 were previously published by Marques *et al*. (2018).

All evolved strains (IMS644, IMS646, IMS647, IMS648 and IMS649) obtained in the present study displayed sucrose-induced H^+ uptake (**Figure 4**). Strain IMS644 showed an initial H^+ uptake rate that was close to that of the unevolved parental strain IMZ696 (13 ± 3.7 µmol H^+ min^{-1} (g cell)$^{-1}$ for IMS644

vs. 8 ± 2.2 μmol H^+ min^{-1} (g cell)$^{-1}$ for IMZ696) (**Figure 4**), but all other strains showed an at least three-fold higher H^+ uptake rate compared to IMZ696 (25-40 μmol H^+ min^{-1} (g cell)$^{-1}$ vs. 8 ± 2.2 μmol H^+ min^{-1} (g cell)$^{-1}$ for IMZ696) (**Figure 4**). These high rates of sucrose-dependent proton uptake approached those of a reference strain expressing the *S. cerevisiae* Mal11 proton symporter (52 ± 8.7 μmol H^+ min^{-1} (g cell)$^{-1}$ for IMZ709, data from Marques *et al*. (2018) **(Figure 4**). The evolved *PvSUF1*-expressing strains exhibited similar proton uptake rates with maltose as with sucrose, while no proton uptake was observed upon addition of fructose or glucose. Consistent with literature reports (Wieczorke *et al.* 1999) the control strain IMZ709 (*MAL11, SPase*) exhibited glucose-dependent proton uptake (data from Marques *et al*. 2018) (**Figure 4**).

An alternative way to investigate energy coupling of disaccharide uptake in *S. cerevisiae* is to measure biomass yields in anaerobic, disaccharide-limited chemostat cultures (de Kok 2012). If sucrose uptake occurs via symport with a single proton, one ATP molecule has to be consumed by the plasma membrane H^+/ATPase Pma1 to expel the symported proton. On the contrary, if sucrose uptake is passive, no ATP will be consumed. This difference of 1 ATP has a high impact on the biomass yield on sugar under anaerobic conditions, which can be precisely determined in anaerobic chemostats (Verduyn *et al.* 1990; de Kok *et al.* 2011; Marques *et al.* 2018).

The strains used in this study cleaved sucrose intracellularly via phosphorolysis. In such strains, expression of a sucrose/H^+ symporter should result in a net generation of 4 mol ATP per mol sucrose under anaerobic conditions. If sucrose uptake occurred by uniport, this ATP yield would change to 5 ATP/sucrose, a 25% increase (Marques *et al.* 2018). In sucrose-limited, anaerobic chemostat cultures, a 25% increase of the ATP yield from sucrose dissimilation should result in a 25% increase in the biomass yield on sucrose (Verduyn *et al.* 1990). Two of the evolved strains (IMS646 from the evolution started with IMZ636 and IMS649 from the evolution started with IMZ696) were characterized in chemostat cultures and their biomass yields were compared to those displayed by reference strains (IMZ665 and IMZ709),

which both expressed *MAL11* instead of *PvSUF1*. No differences in biomass yield were observed between a *MAL11* expressing strains and strains evolved with *PvSUF1*: the observed biomass yield of strain IMZ665 (*MAL11, SPase*) was 0.086 ± 0.002 g (g glucose equivalent)$^{-1}$ while that of IMS646 (*PvSUF1, SPase*) was 0.082 ± 0.004 g (g glucose equivalent)$^{-1}$ (**Table 4** and **Table S2**). Similarly, for the strains expressing *PGM2* (IMZ709 and IMS649) the biomass yield was not higher in cultures of the evolved *PvSUF1*-expressing strain (0.087 ± 0.000 g (g glucose equivalent)$^{-1}$ for IMS649) than in cultures of the *MAL11*-expressing strain (0.091 ± 0.006 g (g glucose equivalent)$^{-1}$ for IMZ709) (**Table 4** and **Table S2**).

Evolved strains contain mutations in *PvSUF1*

Non-conservative single-nucleotide mutations were detected in the *PvSUF1* open reading frames of all *Pv*Suf1-dependent strains evolved for faster growth on sucrose (IMS644, IMS646, IMS647, IMS648 and IMS649; **Figure 5**). No mutations were found in the promoter (*TEF1*) or in the terminator (*CYC1*) regions of the *PvSUF1* expression cassettes. Strains IMS644 and IMS647, which were independently evolved from strain IMZ636 (*PvSUF1,SPase*), contained the same mutation (*PvSUF1*$^{\text{YAAGSFSG-duplication}}$): a tandem duplication of 8 amino acids that, based on amino-acid hydrophobicity plots (*Protter* algorithm, (Omasits *et al*. 2014)), was predicted to be localized partially in the extracellular surface of trans-membrane domain 5 (TM5) and in the loop connecting TM5 to TM6 (loop 5/6) (**Figure 5** and **Figure 6**). Strain IMS646, which was also evolved from IMZ636 (PvSUF1, SPase), contained a mutation that resulted in a substitution of glycine 326 (TM8) for a cysteine (G326C) (**Figure 5**). Evolved strain IMS649, derived from the IMZ696 strain (PvSUF1, SPase, PGM2), carried a combination of the abovementioned PvSUF1$^{\text{G326C}}$ mutation and two additional mutations. One of these caused an I209F

Table 4: Biomass yields on sucrose and rates of sucrose uptake by *S. cerevisiae* strains grown in anaerobic sucrose-limited chemostat cultures (pH 5, 30 °C, 800 rpm, 500 mL N_2/min). A dilution rate of 0.07 h^{-1} was used for strains IMZ665 (*MAL11, SPase*) and IMS646 (*PvSUF1, SPase*), while 0.15 h^{-1} was used for strains IMZ709 (*MAL11, SPase, PGM2*) and IMS649 (*PvSUF1, SPase, PGM2*). Cultures were grown on SMS with 25 g/L sucrose in the feeding medium. Averages and mean deviations were obtained from duplicate experiments. Additional physiological data are provided in **Table S2**

Strain	IMZ665 (control)	IMS646 (evolved)	IMZ709 (control)	IMS649 (evolved)
Relevant genotype	*MAL11 LmSPase*	*PvSUF1 LmSPase*	*MAL11 LmSPase PGM2*	*PvSUF1 LmSPase PGM2*
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

substitution in the loop connecting TM5 to 6 (loop 5/6) at the extracellular surface while the other led to a C265F substitution, positioned in loop 6/7 on the cytosolic side (**Figure 5** and **Figure 6**). The PvSUF1 allele of the remaining strain, which was evolved from IMZ696 (PvSUF1, SPase, PGM2), IMS648, contained three mutations, leading to Y128C, C228G, and G457D substitutions (**Figure 5**). In this strain, the *PvSUF1*- and *PGM2*-expression cassettes were no longer located on the original 2µ-expression vector. Instead, both cassettes were found to be integrated into chromosomal DNA. Accordingly, strain IMS655, which was obtained by curing the 2µ-plasmid from strain IMS648, retained its ability to grow on SMS (**Figure S1**). The other four evolved strains did not grow on sucrose after plasmid removal (IMS652, IMS653, IMS654 and IMS656). No mutations were found in the *PGM2* or *SPase* expression cassettes of any of the evolved strains.

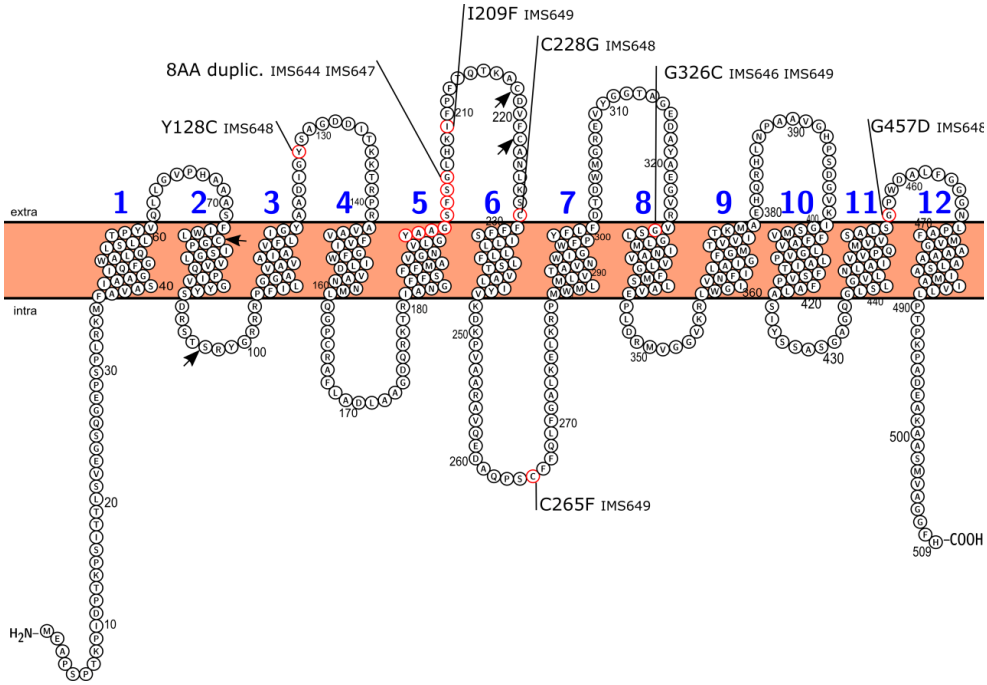

Figure 5: Mutations found in *PvSUF1* after laboratory evolution in sucrose-based medium. Mutated amino acids are highlighted in red and the codes of the corresponding strains are indicated. "AA": amino acids. Arrows indicate the location of four conserved cysteine residues in plant sucrose transporters. The cysteine at loop 2/3 is substituted by a serine in the wild-type *Pv*Suf1 protein. Membrane insertion of *Pv*Suf1 was predicted with the *Protter* algorithm (Omasits *et al.*, 2014).

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

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

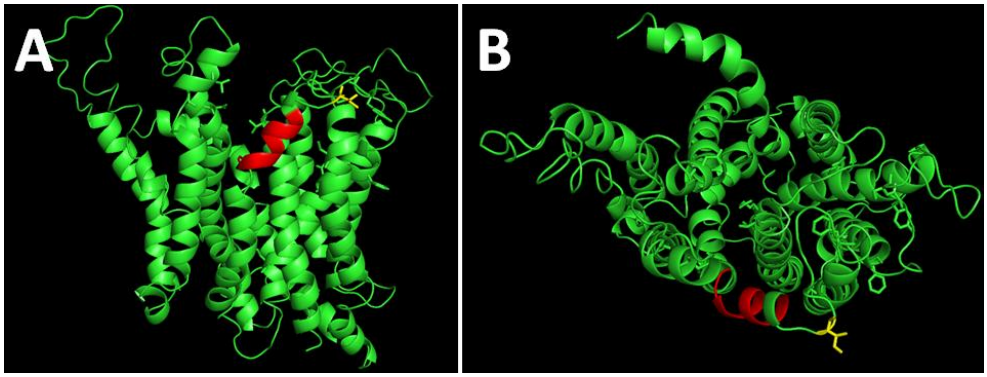

Figure 6: Model of the tertiary structure of wild-type *Pv*Suf1. Extracellular loops are shown at the top and intracellular loops at the bottom of the figure **(A)**. View from the extracellular space is shown in **(B)**. The 8 amino-acid region in loop 5/6 that was duplicated in strains IMS644 and IMS647 is shown in red. In the same loop, isoleucine 209, which was mutated to phenylalanine (I209F) in strain IMS649 is highlighted in yellow. Images were prepared with PyMOLTM (version 1.7.4.5 Educational Product, Schrodinger, LLC).

IMS646 and IMS649. A central region of chromosome 13 (position ~300,000 to ~350,000) was triplicated in strains IMS644 and IMS647. Strain IMS644 showed a complete duplication of chromosome 1, as well as of the right arm of chromosome 2 (after position ~600,000; (**Table 5, Figure S2**). To investigate whether these mutations contribute to the phenotype, reverse engineering of the mutated *PvSUF1* alleles was carried out both in an non-evolved and in an evolved strain background, as detailed in the next section.

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

To investigate whether the faster growth on sucrose observed after evolution of *PvSUF1*-expressing strains could be exclusively attributed to the mutations found in *PvSUF1*, *PvSUF1* expression cassettes (*TEF1*p-*PvSUF1*-*CYC1*t) were first isolated from each of the evolved strains (IMS646, IMS647 and IMS649) and cloned into a 2-µm plasmid. Two versions were constructed,

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

one carrying an additional *PGM2*-expressing cassette and another one without it. The resulting plasmids were transformed into an unevolved background strain, IMZ630 (*malΔ mphΔ suc2Δ imaΔ SPase*), resulting in strains IMZ712-715 and IMZ729 (**Table 6**). Strain IMZ712 (expressing *PvSUF1*^{G326C}) grew anaerobically on sucrose at 0.05 h^{-1} (**Table 6**), which corresponds to the specific growth rate displayed by the unevolved parental strain (IMZ636, **Table 3**). Co-expression of *PGM2* did not lead to a higher growth rate (IMZ714, μ = 0.05 h^{-1}, **Table 6**). Overexpression of a *PvSUF1*$^{YAAGSFSG\text{-duplication}}$ variant that encoded the 8 amino-acid duplication described above (from strain IMS647) in the unevolved background also, by itself, did not result in an increased specific growth rate (IMZ713 μ = 0.05 h^{-1}) (**Table 6**). However, when this mutation was combined with the overexpression of *PGM2* (IMZ715), a specific growth rate of 0.10 h^{-1} was observed (**Table 6**). The *PvSUF1* allele derived from strain IMS649, the evolved strain that grew at 0.20 h^{-1} (**Table 3**), supported high specific growth rates upon introduction in a non-evolved background when combined with *PGM2* overexpression (strain IMZ729, $\mu = 0.17 \pm 0.02$ h^{-1}) (**Table 6**).

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

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

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

DISCUSSION

Optimal expression of heterologous transporters in yeast can be hampered by protein misfolding, incorrect sorting, cell toxicity due to protein accumulation in intracellular compartments (unfolded protein response - UPR), rapid endocytosis and turnover, among other phenomena (Bassham and Raikhel 2000; Hernández 2005; Froissard *et al.* 2006; Nielsen 2013). In this

study, genetic factors involved in the efficient expression of a putative plant sucrose uniporter (*Pv*Suf1) in *S. cerevisiae* were identified by a combination of laboratory evolution, whole genome sequencing, reverse engineering of mutations observed in evolved strains and physiological analysis of evolved and reverse engineered strains.

Laboratory evolution of yeast dependent on sucrose uptake via *Pv*Suf1 resulted in faster growing strains – when compared to the parental ones – which contained mutations in the *PvSUF1* allele (**Figure 5**). Besides faster growth, the mutations may also be responsible for lowering the residual sucrose concentration in the accelerostat reactor (from 8 to 2.5 g/L, see Results section). In fact, the observed decrease in biomass yield occurred simultaneoulsy to the increase in proton uptake capacity. Mutations were also found in the genome of the evolved strains (**Table 5**, **Figure S2**) affecting genes that encode proteins involved in transporter sorting, ubiquitination and degradation: *COS10, SEC12 and SIS1*, which are present on chromosome 14 (Nakano, Brada and Schekman 1988; Luke, Sutton and Arndt 1991; Macdonald *et al.* 2015); *CUR1* and *SEC23* from chromosome 16 (Alberti 2012) and *UBC7* found on chromosome 13 (Hiller *et al.* 1996). However, these genomic alterations are unlikely to be the main cause of the improved specific growth rate of the evolved strains since reverse engineering of a mutated version of *PvSUF1* in unevolved *S. cerevisiae* sufficed to generate a strain that grew as fast as the fastest-growing evolved strain (**Table 6**). Among the mutations found in *PvSUF1*, those involving cysteine residues predominated (*PvSUF1*^{G326C}, *PvSUF1*$^{G457D\ Y128C\ C228G}$, *PvSUF1*$^{I209F\ C265F\ G326C}$; **Figure 5**). Correct formation of disulfide bonds has previously been reported to be important for inter- and intramolecular interactions of SUT (sucrose transporter) proteins and sucrose uptake activity (Krügel *et al.* 2008, 2012). *Pv*Suf1 contains only 3 out of the 4 cysteine residues conserved in all other plant sucrose transporters (**Figure 5**) (Lemoine 2000). Since the *PvSUF1*^{G326C} mutation was found in two independently evolved strains (IMS649 and IMS646), presence of a cysteine in position 326 of *Pv*Suf1 may be important for protein folding and oligomerization in yeast, but further research is clearly needed to test this

hypothesis. Study of the mutations identified in this work in different transporters, preferably combined with structural information, are required to identify underlying mechanisms and increase the predictability of functional expression of heterologous transporters in yeast.

Proton coupled studies showed that all *PvSUF1*-expressing *S. cerevisiae* strains tested (including those that were not subjected to laboratory evolution) displayed at least some sucrose/H^+ symport activity, which was high enough to explain their observed rates of sucrose uptake (**Figure 4**). This study unequivocally shows that *Pv*Suf1, which in a plant context was described to be a sucrose uniporter (Zhou *et al.* 2007) is not an ideal candidate transporter for improving the ATP stoichiometry of sucrose metabolism in *S. cerevisiae*. While, based on heterologous expression, we cannot draw definitive conclusions on energy coupling of wild type *Pv*Suf1-mediated sucrose transport, our results warrant reinvestigation of *Pv*Suf1's mechanism in a plant context.

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SUPPLEMENTARY MATERIAL

Table S1: Primers used in this study

Primer	Sequence (5' → 3')
Primers for plasmid construction	
2889	AAGGGGGATGTGCTGCAAG
5975	AACGAGCTACTAAAATATTGCGAA
6018	GTGGAAGAACGATTACAACAGG
7822	AGTTTATCATTATCAATACTCGCCATTTC
9041	GAGCTCCAGCTTTTGTTCCCTTTAGTG
10307	AACAGCTATGACCATGATTA
Primers for *PvSUF1* Sanger sequencing	
2889	AAGGGGGATGTGCTGCAAG
5543	ATCCCCAACAAAGCCTATC
6874	TGCCGCTGATATTGGTTACTC
6875	CATACGCCGAAGGTGTTAGAG
10443	CTTTACACTTTATGCTTCCGG
5544	TTAATGAAAACCACCTGCTAC
6876	CTGTCATACCGAAACCGATAGCC
6877	CGTTACCAACGGCCATAAAG
7999	GGATCCACTAGTTCTAGAAAACTTAGATTAG
Primers for *PGM2* Sanger sequencing	
2754	TGTACAAACGCGTGTACGCATG
5324	TAGTGTGAGCGGGATTTAAACTGTG
11098	TGCGTAAGAAGACAAAGG
11099	AGTATGAACGGTGTAACTG
11100	AGAATGAATTCTGGGCAAAG
1768	GCTTCCGGCTCCTATGTTG
11102	TACGCGATAATTTGACCCAG
11103	GAACGAATCTTGCACCATTG
11104	ACCTGGAGAAACGAAAGATG
Primers for *SPase* Sanger sequencing	
4226	ACTCGTACAAGGTGCTTTTAACTTG
2672	ACCTGACCTACAGGAAAGAG
6872	CCAGCCAACAAACCAACGTAG
6873	CTTCACCGAAAGTGTTCCACAAG
4175	GCGCCGCGGATCCACTAGTTCTAGAATCCG
4369	TGGGCATGTACGGGTTACAG
4224	TTGATGTAAATATCTAGGAAATACACTTG
7214	CCTGAAATTATTCCCCTACTTG
5545	CTACAAGAGAAAGGACAAGG
6871	GACGAAATCGACTACGCTTCTG
3903	GCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAG

Table S2: Extension of Table 4. Growth characteristics of evolved strains *S. cerevisiae* IMS646 (*PvSUF1, SPase*) and IMS649 (*PvSUF1, SPase, PGM2*) and the controls IMZ665 (*MAL11, SPase*) and IMZ709 (*MAL11, SPase, PGM2*) in sucrose-limited anaerobic chemostat cultures. The cultures of IMZ665 and IMS646 were grown at a dilution rate of 0.07 h^{-1} and the cultures of IMZ709 and IMS649 at a dilution rate of 0.15 h^{-1}. Biomass specific production or consumption rates are shown with the denotation $q_{metabolite}$. Averages and mean deviations were obtained from duplicate experiments.

Strain	IMZ665 (control)	IMS646 (evolved)	IMZ709 (control)	IMS649 (evolved)
Relevant genotype	*MAL11 SPase*	*PvSUF1 SPase*	*MAL11 SPase PGM2*	*PvSUF1 SPase PGM2*
Biomass yield (g/g glucose equivalent)	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
$q_{ethanol}$ (mmol/g biomass/h)	7.32 ± 0.17	7.43 ± 0.17	14.78 ± 0.02	15.6 ± 0.4
q_{CO2} (mmol/g biomass/h)	7.69 ± 0.17	7.59 ± 0.19	16.0 ± 0.2	16.7 ± 0.2
$q_{glycerol}$ (mmol/g biomass/h)	0.59 ± 0.03	0.55 ± 0.01	1.29 ± 0.05	1.37 ± 0.05
$q_{lactate}$ (mmol/g biomass/h)	0.06 ± 0.00	0.06 ± 0.00	0.16 ± 0.00	0.16 ± 0.00
$q_{pyruvate}$ (mmol/g biomass/h)	0.01 ± 0.00	0.02 ± 0.02	0.033 ± 0.001	0.04 ± 0.00
$q_{acetate}$ (mmol/g biomass/h)	0.02 ± 0.01	0.02 ± 0.00	0.06 ± 0.01	0.07 ± 0.00
Residual sucrose (g/L)	0.08 ± 0.02	1.90 ± 1.18	2.03 ± 0.15	4.33 ± 0.80
Carbon recovery (%)[a]	101 ± 1	95 ± 4	105 ± 7	102 ± 1
Actual dilution rate (h^{-1})	0.070 ± 0.000	0.071 ± 0.001	0.147 ± 0.001	0.152 ± 0.001

[a] Calculations of the carbon recovery were based on a carbon content of biomass of 48% (w/w).

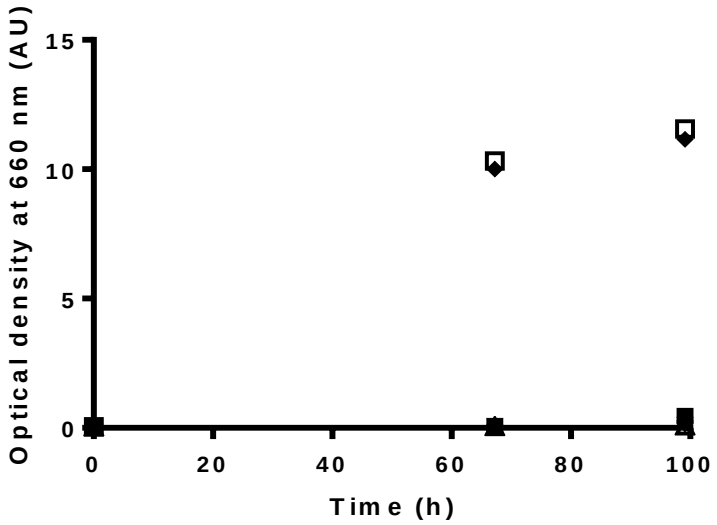

Figure S1: Growth of the evolved *S. cerevisiae* strains after plasmid removal on sucrose-based medium under anaerobiosis. Only IMS655 (IMS648 after plasmid removal, closed diamond) and the positive control (evolved strain IMS648, open square) grew on sucrose-based medium. All the other strains did not grow after 100 h incubation time: IMS652 (IMS644 plasmid cured, closed square), IMS653 (IMS646 plasmid cured, closed triangle), IMS654 (IMS647 plasmid cured, inverted closed triangle), IMS656 (IMS649 plasmid cured, closed circle) and IMZ730 (*malΔ mphΔ suc2Δ imaΔ SPase PGM2*). Apart from IMS648 and IMS655, the datapoints corresponding to all other strains are overlapped on the horizontal axis. The inoculum was grown in SMD, washed with distilled water and transferred to SMS. Incubation parameters: 30 ºC, 250 rpm, initial pH 6.

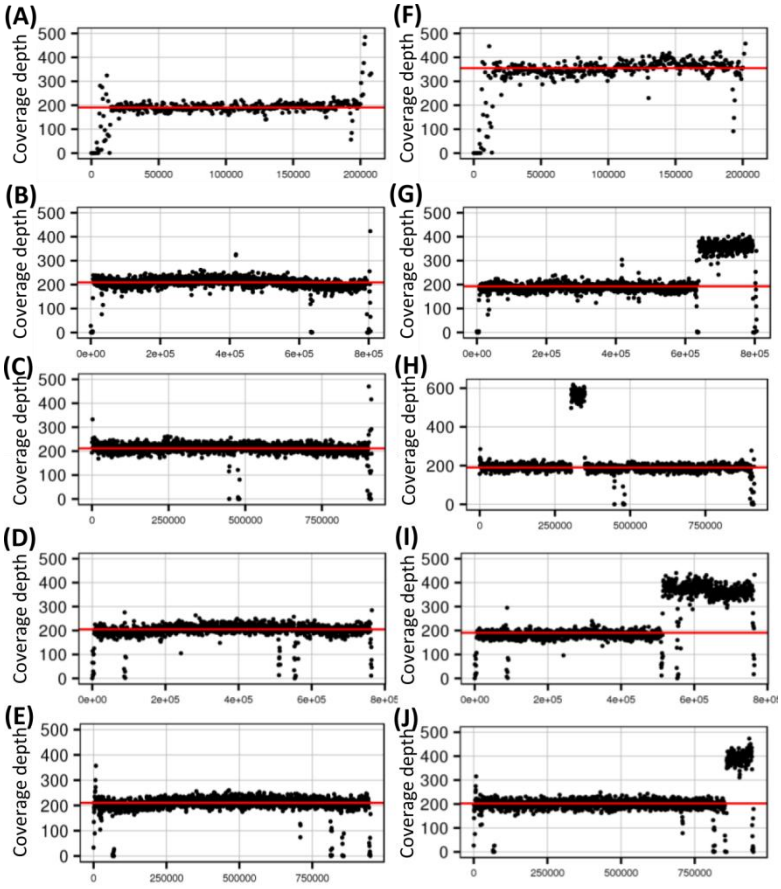

Figure S2: Depth of coverage analysis of chromosomes that were mutated after evolution of *PvSUF1*-expressing strains on sucrose-based medium. Panels on the left (**A** to **E**) displays chromosomes from the reference unevolved strain IMZ630 and panels in the right (**F** to **J**) display those of the evolved strains: IMS644 (**F** to **I**) and IMS649 (**J**). Chromosome 1 (**A** and **F**); chromosome 2 (**B** and **G**); chromosome 13, (**C** and **H**), chromosome 14 (**D** and **I**) and chromosome 16 (**E** and **J**). Panel **I** is similar to chromosome 14 from IMS646 and IMS649. Panel **J** is also similar to chromosome 16 from IMS649. Panel **H** is similar to chromosome 13 from IMS647. Sequencing reads from the strains shown have been mapped onto the CEN.PK113-7D genome sequence (Salazar *et al*., 2017). Read count data represent the average coverage of non-overlapping 500 bp windows.

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Outlook

The beginning of the 21st century saw a drastic increase in oil prices (Albers *et al*., 2016). As a response to this and due to environmental policy incentives, important investments were made in biotechnology (Albers *et al*., 2016; Werpy and Petersen, 2004). These investments yielded significant outcomes: almost twenty years later, several oil-based chemicals can now be obtained through microbial fermentation (Biddy *et al*., 2016). Sugars, cellulosic feedstocks and waste streams have been used as substrates for such fermentation processes. In the first chapter of this thesis – which was published in January 2016 – it is stated that "sucrose has been a neglected sugar or carbon source by the research community" (Marques *et al*., 2016). At that time, investments were mainly targeted at the use of lignocellulosic biomass as a substrate for microbial fermentation. As a result of that research, microbial performance ceased to be a bottleneck in second-generation (2G) ethanol processes. Currently, other issues prevail, mainly around handling and pre-treatment of biomass (Jansen *et al*., 2018). After a recent decrease in oil prices (2013), 2G biofuels face a tough challenge to compete in the market with oil and research has turned its focus into higher-value chemicals (fragrances, pharmaceuticals, polymers, etc) (Albers *et al*., 2016). In this context, 'clean' and cheap substrates such as sucrose are attracting increased interest in industry (Amyris, 2018; Braskem, 2018; DSM, 2017; Karp *et al*., 2017; Lallemand, 2016; Li *et al*., 2018; Voelker *et al*., 2017).

The research described in this thesis focused on sucrose metabolism in one of the most important industrial microbes: *Saccharomyces cerevisiae*. **Chapter 2** represents the first report on the complete abolishment of sucrose transport and hydrolysis in yeast (Marques *et al*., 2017). Availability of a strain that completely lacks the ability to consume sucrose is of paramount importance for academy and industry. Using the yeast chassis constructed in this thesis, alternative disaccharide consumption routes can be easily tested. The impact of this platform strain extends beyond the yeast research

community, as it can also be used, for instance, by plant researchers interested in studying plant sugar transporters and enzymes (Zhou *et al*., 2014).

Besides the chassis strain itself, the way in which CRISPR/Cas9 was used for gene deletion was innovative. Previous studies on CRISPR/Cas9-mediated gene editing showed up to six simultaneous gene editing events (Mans *et al*., 2015), with each deletion requiring the expression of a specific Cas9-gRNA. Our report was the first to apply the concept of a shared Cas9 targeting-sequence for multiplex gene deletion in yeast. Five isomaltase encoding genes were deleted using just one Cas9 targeting-sequence (Marques *et al*., 2017). There is a tremendous potential to further explore this approach, since the current *S. cerevisiae* genome is the result of a whole genome duplication event, hence it contains several gene families (e.g. *HXT*, *MAL*, etc). In **Chapter 3**, the deletion strategy mentioned was repeated in a different strain and combined with the expression of a putative sucrose facilitator (*Pv*Suf1) and a sucrose phosphorylase (SPase). The resulting engineered yeast strain conserved more energy (8% more ATP/sucrose) than the reference strain (Marques *et al*., 2018). Under anaerobic conditions, ATP supply can limit synthesis and/or export of a molecule of interest (e.g. lactic acid) (Van Maris *et al*., 2004). Thus, by increasing the ATP yield on sucrose, it is possible to convert aerobic bioprocesses into less oxygen demanding or even fully anaerobic bioprocesses, which would result in considerable reduction of industrial fermentation costs (Cueto-Rojas *et al*., 2015; de Kok *et al*., 2012; Feng *et al*., 2017; Ha *et al*., 2013; Sadie *et al*., 2011).

In **Chapter 4**, the power of laboratory evolution combined with DNA-sequencing technologies was used to identify mutations that could contribute to optimal expression and activity of plant sugar transporters in yeast. *Pv*Suf1 – a putative sucrose facilitator from beans (*Phaseolus vulgaris*) that was a crucial element in the strategy used to increase energy conservation in yeast (**Chapter 3**) – was mutated in diverse amino acid residues during evolution of *S. cerevisiae* in a sucrose-based medium. Reverse engineering of the mutations restored fast growth on sucrose and led to the identification of some amino acids (mainly cysteines) related to efficient transporter expression in yeast.

Further investigations on the mutations identified in this study (e.g. through the characterization of protein structure) will shed light on the yeast requirements for the efficient expression of (heterologous) plant transporters. Further research in this field is highly relevant because functional expression of heterologous membrane transporters in yeasts, while essential in many metabolic engineering strategies, still presents significant technical challenges.

Besides the contributions given by this thesis to the understanding and engineering of disaccharide metabolism in yeast, additional important questions could be formulated, such as: why have sucrose uniporters not been found in fungi? A few plant sucrose facilitators have been identified in plants (the SUFs and the SWEETs mentioned extensively in this thesis) (Chen *et al*., 2012, 2010; Lin *et al*., 2014; Zhou *et al*., 2014, 2007), but characterization of these transporters in yeast is still immature. Also in bacteria, sucrose uniporters have not been described so far (Reid and Abratt, 2005; Vadyvaloo *et al*., 2006) and, even if they had been, expressing such proteins in yeast would not be a simple task, since prokaryotic transporters generally do not express well in eukaryotic cells (Hildebrandt *et al*., 1993; Opekarová and Tanner, 2003). Besides this, there are only few publications that report decoupling of disaccharide and proton transport (Henderson and Poolman, 2017; Trichez, 2012). Finally, in none of these works the mutated transporter allowed for a high enough sugar uptake rate that would enable accurate physiological assessments or support anaerobic growth of *S. cerevisiae*. In **Chapter 4**, we present several mutants of *Pv*Suf1, which all behaved as sucrose/H^+ symporters. Since some of the mutations did not affect acid amino acids it is unlikely that these were responsible for changing the mechanism of transport from passive to active.

Other important questions are: why is sucrose mainly hydrolyzed extracellularly in yeast? Why does not this fungus contain a sucrose facilitator to be employed when sucrose is abundant in the environment? Is it simply because higher rates of substrate uptake are favored over higher yields of substrate conversion (Frank, 2010), so that active transporters would be

ecologically more advantageous over facilitators? If the answer is yes, then why are monosaccharides transported passively? Is it an example of cooperation between individuals where natural (or human) selection has favored the population rather than individual cells, in such a way that cells that make monosaccharides available extracellularly – for the other "cheater" cells – were favored? (Gore *et al*., 2009; Koschwanez *et al*., 2011; Lindsay *et al*., 2018). These questions highlight that metabolic engineering and microbial physiology are better explored and understood when ecological information is available.

This thesis exposes the need for research on metabolic engineering of trans-membrane transport, specifically because of the tremendous impact that it exerts on cell energy conservation and also on fermentation rates (Hara *et al*., 2017; Kell *et al*., 2015). In the long term, scientists will be able to design "synthetic transporters" according to their needs: substrate affinity and preference, transport mechanism (active or passive), regulation, cell sorting, etc (Davis *et al*., 2007; McNally *et al*., 2007). In this line, natural or genetically modified microorganisms may be replaced by "synthetic cells" (specifically designed according to the bioprocess characteristics) (Spoelstra *et al*., 2018), or simply by "cell-free" fermentations (Hodgman and Jewett, 2012). On the way to these projections, industrial microbiology should make the most of using and recombining diversity in expression host range and product pathways as well as in choosing optimal bioprocess setups and substrates. A major goal in this respect is the decentralization of fermentation technology, thereby allowing for local production of "biologics" at low cost (Jacquemart *et al*., 2016; Klutz *et al*., 2015).

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Curriculum vitae

Wesley Marques was born on May 4, 1990 in Campinas, São Paulo, Brazil. Before university (2005 to 2007), Wesley took secondary education classes together with a Mechanics (automation) course at the Technical High School of Campinas (Cotuca). In 2008, Wesley entered the University of Campinas (Unicamp) to study Molecular Biology. From 2009 to 2011, Wesley conducted research on plant cell wall formation under the supervision of Prof. dr. ir. Gonçalo Amarante Guimarães Pereira and dr. Marcela Salazar. In the last semester of university (2011), Wesley did an internship in plant biotechnology research at International Paper do Brazil LTDA. In 2012, Wesley started his masters course in Biotechnology at the University of São Paulo (USP) under the supervision of Prof. dr. ir. Andreas Karoly Gombert (Chemical Engineering Department, POLI, USP) and co-supervision of Prof. dr. ir. A.J.A. (Ton) van Maris and Prof. dr. J. T. Pronk (TuDelft, Biotechnology Department, Industrial Microbiology Group, IMB). From April to September 2013, Wesley carried out experiments in Delft. The topic of his masters research was the same as that of this thesis. In 2014, Wesley started his PhD in the context of an agreement on joint doctoral supervision between University of Campinas, Brazil and Delft University of Technology, the Netherlands. In parallel to his PhD activities, Wesley acted as students representative at the Dual Degree PhD program (2017- 2018) and at the Bioenergy PhD program (2017-2018). In November 2018, Wesley will join the group of Prof. Kristala Prather at MIT as a postdoctoral associate. He will conduct research on glucaric acid production by yeast.

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