TU Delft

Delft University of Technology

Strategies and genetic tools for engineering free-energy conservation in yeast

Mans, Robert

DOI
10.4233/uuid:d99fcde3-4b01-4077-9a1f-a8b6eda6a5e7

Publication date
2017

Document Version
Final published version

Citation (APA)
Mans, R. (2017). *Strategies and genetic tools for engineering free-energy conservation in yeast*. [Dissertation (TU Delft), Delft University of Technology]. https://doi.org/10.4233/uuid:d99fcde3-4b01-4077-9a1f-a8b6eda6a5e7

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

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

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

This work is downloaded from Delft University of Technology.
For technical reasons the number of authors shown on this cover page is limited to a maximum of 10.

Strategies and genetic tools for engineering free-energy conservation in yeast

Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof. ir. K.Ch.A.M. Luyben,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen op
maandag 16 oktober 2017 om 15:00 uur

door

Robert MANS

Ingenieur in Life Science and Technology
Technische Universiteit Delft, Nederland,

geboren te Amsterdam, Nederland

Dit proefschrift is goedgekeurd door de promotoren:

Prof. dr. ir. A.J.A. van Maris
Prof. dr. J.T. Pronk

Samenstelling promotiecommissie:

Rector magnificus,	voorzitter
Prof. dr. ir. A.J.A. van Maris	promotor
Prof. dr. J.T. Pronk	promotor

Onafhankelijke leden:

Dr. K. Benjamin	Amyris Inc, USA
Dr ir. S.J.J. Brouns	Technische Universiteit Delft
Prof. dr. B. Poolman	Rijksuniversiteit Groningen
Prof. dr. ir. S.J. Tans	Technische Universiteit Delft
Prof. dr. ing. L.M. Blank	RWTH Aachen University

Reservelid:

Prof. dr. I.W.C.E. Arends	Technische Universiteit Delft

The research presented in this thesis was performed at the Industrial Microbiology Section, Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, The Netherlands.

The project was financed by the BE-Basic R&D Program, which was granted a FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I).

The cover photo of sugar crystals under polarized light was made by Alexander Klepnev (www.facebook.com/alexandr.klepnev, own work) and licenced under CC BY-SA 4.0.

This thesis was printed with the help of www.proefschriftmaken.nl.

Table of Contents

Summary 1
Samenvatting 7
Chapter 1 15
General Introduction
Chapter 2 33
CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*
Chapter 3 65
Elimination of sucrose transport and hydrolysis in *Saccharomyces cerevisiae*: a platform strain for engineering sucrose metabolism
Chapter 4 91
Combined engineering of disaccharide transport and phosphorolysis for enhanced ATP yield from sucrose fermentation in *Saccharomyces cerevisiae*
Chapter 5 117
A CRISPR/Cas9-based exploration into the elusive mechanism for lactate export in *Saccharomyces cerevisiae*
Outlook 141
Vooruitblik 145
Bibliography 149
Acknowledgements 175
Curriculum vitae 181
List of publications 183

Summary

Microbial production of fuels and chemicals provides opportunities for replacing conventional production processes, which are based on chemical synthesis from non-renewable raw materials or on labour- and capital-intensive extraction from animal or plant tissues. Aeons of evolution, in which astronomical numbers of microorganisms competed for scarce resources, have optimized and streamlined the thousands of biochemical conversions in their cells for growth in specific natural environments. The resulting metabolic diversity, represented by many millions of microbial species, offers a great potential for developing novel microbial conversions of renewable substrates to products. Major advances in (recombinant) DNA technology have enabled the engineering of several microorganisms into efficient production platforms, which can be further modified for the production of a wide range of fuels and chemicals. For high-volume products based on microbial fermentation, such as transport fuels and commodity chemicals, the use of substrate can comprise up to 70% of the total product costs. These high substrate costs make a high product yield on the substrate essential for economic viability.

Saccharomyces cerevisiae is a highly popular industrial microorganism. This yeast has been engineered for production of chemicals ranging from simple molecules such as ethanol, butanol, lactic acid and succinic acid to more complex molecules such as the anti-malarial drug artemisinin or the antioxidant resveratrol. Optimizing productivity and product yields can be achieved via modulation of (heterologous) gene expression or via modifications to the native metabolism, such as the disruption of competing metabolic pathways, the introduction of alternative metabolic routes to prevent carbon loss (e.g. via decarboxylation reactions) and/or expansion of the flexibility in balancing the oxidation and reduction of redox cofactors.

Free-energy (ATP) conservation is an important metabolic engineering target for optimisation of product yields. Compounds whose redox-neutral synthesis from a substrate leads to the formation of ATP can be the sole product under anaerobic conditions. Such a scenario is favoured over aerobic production processes due to lower costs associated with reactor design, air compression, stirring and cooling. In such anaerobic processes, cellular energy requirements for growth and maintenance are supplied directly from substrate-level phosphorylation in the product pathway. Consequently, biomass formation is linked to product formation by the ATP stoichiometry of the product pathway. Loss of substrate carbon in the form of biomass can therefore be minimized by engineering product pathways such that they have a low but positive ATP yield.

In microbial biotechnology, products whose synthesis from a substrate requires ATP are typically made under aerobic conditions. In such processes, part of the substrate

is respired to CO_2 and water to generate the ATP needed for product formation, cell growth and/or maintenance. Increasing free-energy conservation in ATP-requiring product pathways, i.e. by reducing their net ATP requirement, reduces the amount of substrate that has to be respired and, therefore, enables higher product yields. Especially interesting targets for engineering free-energy conservation are pathways that catalyse an ATP-neutral conversion of substrate to product. Any change to such a product pathway that improves free-energy conservation has the potential to eliminate the need for substrate respiration to provide ATP and to enable anaerobic production with the aforementioned economic benefits.

Two strategies for increasing free-energy conservation in *S. cerevisiae* were investigated in the research described in this thesis. The first strategy focussed on the uptake and cleavage of the disaccharide sucrose, an important sugar substrate in large-scale industrial fermentation processes. In the second strategy, the focus was on the identification of genes involved in energy-dependent export of lactic acid, an important platform molecule, by engineered yeast strains. In order to facilitate the genetic modifications required for engineering sucrose utilization and lactic acid export, efficient genetic engineering tools are essential. Recently, the discovery of the CRISPR/Cas9 system provided a revolutionary new tool for genetic engineering. Originally identified as a bacterial defence mechanism against viral and other foreign DNA, the CRISPR/Cas9 system essentially consists of two parts, a Cas9 nuclease and a short "guide RNA" (gRNA). As the name already indicates, the gRNA guides Cas9 to a highly specific complementary sequence of DNA, which is then cut by Cas9, resulting in a double strand break. By guiding Cas9 to a genetic locus in yeast and supplying a DNA repair fragment with homology to the up- and downstream flanking regions of the resulting double strand break, (heterologous) DNA can then be introduced and/or parts of the yeast DNA can be removed. The flexible and precise RNA-based targeting of Cas9 has the potential to facilitate introduction of genetic modifications almost anywhere in the genome. In **Chapter 2**, the CRISPR/Cas9 system was optimized for use in *S. cerevisiae*. Two methods for Cas9-mediated genetic modifications were developed and evaluated. The first method exploited the exceptional efficiency of *in vivo* recombination by *S. cerevisiae* by co-transforming a plasmid backbone and gRNA-coding sequence fragment. The two fragments assembled *in vivo* into a functional gRNA-expression plasmid. This approach facilitated the genetic modification of a single genetic locus by obviating the need for prior plasmid construction. For the second method, plasmids were developed that enabled the simultaneous expression of two gRNAs and, thereby, the simultaneous guiding of Cas9 to two different genomic target sequences in a single transformation step. Simultaneous transformation of multiple of these double-gRNA plasmids allowed the concurrent modification of up to 6 genomic loci in a single transformation step. The versatility and specificity of CRISPR/Cas9-based genome editing was further demonstrated by the simultaneous introduction of two single-nucleotide changes and by combining the introduction of a multi-gene construct with two gene deletions. To

aid future genetic engineering studies, a set of Cas9-expressing strains with various auxotrophies was constructed via stable genomic integration of a constitutive copy of Cas9. Furthermore, for each method, a set of plasmids with various auxotrophic and dominant markers was constructed and made available to the scientific community. Finally, a web-based tool was developed for selection of suitable gRNA targets and the automated design of the oligonucleotide primers required for construction of gRNA expression plasmids.

The availability of the efficient CRISPR/Cas9-based genetic engineering toolbox described in **Chapter 2** enabled extensive modification of sucrose metabolism in yeast. In wild-type *S. cerevisiae*, cleavage of sucrose is catalysed by invertase (Suc2). This enzyme catalyses the, predominantly extracellular, hydrolysis of sucrose into glucose and fructose. These monosaccharides are imported via facilitated diffusion and subsequently converted via the Embden-Meyerhof-Parnas (EMP) glycolytic pathway. This conversion results in the formation of 4 ATP per sucrose by substrate-level phosphorylation. While the free-energy of sucrose hydrolysis ($\Delta G_0'$ = -29 kJ/mol) is not conserved by *S. cerevisiae*, some other organisms cleave sucrose by phosphorolysis. In this process, sucrose and inorganic phosphate are intracellularly converted into glucose-1-phosphate and fructose. Phosphoglucomutase can then convert glucose-1-phosphate into the glycolytic intermediate glucose-6-phosphate, thereby circumventing the ATP-requiring hexokinase reaction. By bypassing the ATP-dependent phosphorylation of glucose by hexokinase, this phosphorolytic pathway achieves a higher free-energy conservation than the invertase pathway. However, before the potential of this reaction could be explored, the native sucrose metabolism of *S. cerevisiae* had to be severely modified.

In *S. cerevisiae* uptake of sucrose involves symport with a single proton. Subsequent proton extrusion is catalysed by the plasma-membrane ATPase, which, in *S. cerevisiae*, has a stoichiometry of 1 ATP per exported proton (Van Leeuwen *et al.* 1992; Weusthuis *et al.* 1993). The resulting cost of 1 ATP per sucrose from proton-coupled uptake dictates that replacement of the proton-coupled sucrose symporters by a sucrose uniporter is necessary to gain an energetic benefit from sucrose phosphorolysis. In **Chapter 3**, all known sucrose-transporter genes (*MAL11*, *MAL21*, *MAL31*, *MPH2* and *MPH3*) and genes encoding sucrose hydrolysing enzymes (*SUC2*, *MAL12*, *MAL22* and *MAL32*) in *S. cerevisiae* were deleted in a single strain. In order to identify suppressor mutations that could alleviate the sucrose-negative phenotype and thereby complicate further strain engineering, the platform strain was first transformed with either a sucrose transporter gene (*MAL11*) or a gene encoding an intracellular sucrose hydrolase (*MAL12*). Subsequently, the resulting strains were subjected to laboratory evolution in sucrose-based synthetic medium. While the strain in which all sucrose-transporter genes were deleted did not grow after two months of incubation, the strain with deletions of the known sucrose-hydrolase genes was able to grow on sucrose with a specific growth rate of 0.08 h^{-1}. After laboratory evolution in sequential batch cultures, its specific growth

rate even increased to 0.25 h^{-1}. This increase in specific growth rate was accompanied by a 3-fold increase of the *in vitro* sucrose-hydrolysis activity. The increase in sucrose hydrolytic activity coincided with a 3- to 5-fold increase in expression of isomaltase (*IMA*) genes. Subsequent CRISPR/Cas9-based deletion of all isomaltase-encoding genes (*IMA1-5*) completely abolished sucrose hydrolysis. The resulting sucrose-negative strain was then used as a platform for further engineering of the sucrose metabolism.

In theory, replacement of extracellular sucrose hydrolysis by facilitated sucrose uptake (uniport) and intracellular phosphorolysis should increase the ATP yield from sucrose fermentation from 4 to 5 ATP per sucrose. In **Chapter 4**, the platform strain (constructed in **Chapter 3**) was first transformed with a known sucrose-proton symporter gene (*MAL11*) and then used for the expression of sucrose phosphorylase from *Leuconostoc mesenteroides* (LmSPase). In order to quantify the energetic benefit of LmSPase expression, the resulting strain was grown in anaerobic sucrose-limited chemostat cultures and compared to an isogenic reference strain expressing the intracellular sucrose hydrolase Mal12. Under anaerobic conditions, the formation of ATP from substrate utilization is directly linked to biomass formation. Combined proton-coupled uptake and intracellular phosphorolysis should, theoretically, increase the yield of sucrose fermentation from 3 to 4 ATP compared to the Mal12 expressing reference strain. In line with the expected energetic benefit of LmSPase expression, the biomass yield of the LmSPase expressing strain was 31% higher than that of the isogenic reference, which was very close to the theoretical prediction of 33%. The relatively low specific growth rate of the LmSPase expressing strain in anaerobic, sucrose-grown cultures (0.09 ± 0.02 h^{-1}) compared to that of the isogenic reference strain (0.19 ± 0.01 h^{-1}) was found to be caused by a metabolic bottleneck in the conversion of glucose-1-phosphate to glucose-6-phosphate. Overexpression of the native yeast phosphoglucomutase *PGM2* restored the anaerobic growth rate of the LmSPase expressing strain to 0.23 ± 0.01 h^{-1}. To further increase the ATP yield of sucrose fermentation, the LmSPase expressing strain was first used to screen for functional expression of the plant sucrose facilitators *PvSUF1*, *PsSUF1*, *PsSUF4*, *OsSWEET11* and *AtSWEET12*. After 5 days of incubation, the strain expressing a sucrose facilitator from *Phaseolus vulgaris* (PvSUF1) grew on synthetic medium with sucrose as the sole carbon source and was selected for further characterization. Expression of PvSUF1 together with LmSPase further increased the biomass yield on sucrose by 8% in anaerobic sucrose-limited chemostats, compared to a strain expressing Mal11. While this yield increase was smaller than theoretically predicted, it provides the first experimental demonstration that co-expression of a phosphorylase and a uniporter can improve energy coupling in *S. cerevisiae*.

Lactic acid plays an important role in the food industry (e.g. for its mildly acidic taste, as an emulsifier or to prevent spoilage), but is also an important platform molecule for synthesis of chemicals such as the biodegradable thermoplastic polyester polylactic acid (PLA). The intracellular conversion of lactic acid was expected to be equivalent to alcoholic fermentation in terms of redox balance and ATP yield. However, earlier

research showed that engineered homolactic strains of *S. cerevisiae* are unable to grow under anaerobic conditions and have a strict requirement for oxygen to provide the ATP for cell growth and maintenance. This unexpected phenotype is likely to reflect an energy cost associated with lactic acid export, which makes the overall conversion from glucose to extracellular lactic acid ATP neutral. Despite several attempts, the *S. cerevisiae* genes involved in lactic acid export have not been identified. It is very likely that multiple genes are involved in the export of this molecule, since single gene deletion mutants of the *S. cerevisiae* knock-out collection did not exhibit complete abolishment of lactic acid transport in previous research. The development of CRISPR/Cas9 based genetic engineering tools (**Chapter 2**) facilitates the use of a brute-force gene-deletion approach to identify genes involved in specific metabolic functions. Such an approach was applied in **Chapter 5** with the goal to identify the genes involved in lactic acid export. First, a subset of candidate lactic acid exporter genes was composed, based on their proposed role in carboxylic acid transport and/or resistance. This set of candidate genes, comprising 25 (putative) organic acid transporters, was then systematically knocked out in a single *S. cerevisiae* strain, using the CRISPR/Cas9 based approach described in **Chapter 2**. To investigate the effect of the gene deletions on lactic acid export, the final strain was transformed with a lactate dehydrogenase gene from *Lactobacillus casei*. To rule out growth-rate dependent effects, lactic acid production by the engineered strain with 25 gene deletions and an isogenic reference strain was compared in anaerobic, glucose-limited chemostat cultures. Under these conditions, no difference in lactic acid production could be observed between the two strains, which indicated that, most probably, the 25 gene deletions did not target (all of) the lactic acid transporters in *S. cerevisiae*.

Samenvatting

Het produceren van brandstoffen en chemicaliën met micro-organismen zou conventionele productieprocessen kunnen vervangen die gebaseerd zijn op chemische synthese vanuit fossiele reserves of op arbeids- en kapitaalintensieve extractie vanuit dierlijke of plantaardige weefsels. Miljoenen jaren van evolutie, waarbij astronomische aantallen micro-organismen verwikkeld zijn geweest in een competitie om schaarse grondstoffen, hebben geleid tot duizenden gestroomlijnde en geoptimaliseerde biochemische omzettingen in cellen, die nodig zijn om te groeien in specifieke natuurlijke milieus. De resulterende diversiteit van de stofwisseling, weerspiegeld in miljoenen microbiële soorten, biedt een enorm potentieel voor het ontwikkelen van nieuwe microbiële omzettingen van hernieuwbare grondstoffen in producten. De snelle ontwikkelingen in (recombinant-) DNA-technieken hebben het mogelijk gemaakt om verscheidene micro-organismen om te bouwen tot efficiënte productieplatformen, die verder kunnen worden aangepast voor de productie van een breed scala aan brandstoffen en chemicaliën. Voor bulkproducten, die gemaakt worden met behulp van microbiële fermentatie, kan het substraatgebruik tot wel 70% van de totale kosten vormen. Deze hoge kosten van het substraat maken een hoge productopbrengst noodzakelijk om processen economisch haalbaar te maken.

Saccharomyces cerevisiae (bakkersgist) is een erg populair micro-organisme, dat is geoptimaliseerd voor productie van simpele chemicaliën als ethanol, butanol, melkzuur en succinaat en voor het maken van complexe moleculen zoals het anti-malariamedicijn artimisinine en het antioxidant resveratrol. De productiviteit kan worden geoptimaliseerd door aanpassingen in de mate van (heterologe) genexpressie of via aanpassingen van de natuurlijke stofwisselingsroutes in de cel, bijvoorbeeld door het uitschakelen van concurrerende routes, het toevoegen van routes die koolstofverlies (bijvoorbeeld door decarboxyleringsreacties) voorkomen en/of door uitbreiding van de metabole flexibiliteit door het balanceren van de oxidatie en reductie van redoxcofactoren.

Het conserveren van vrije energie (ATP) is een belangrijk doelwit voor aanpassingen van de stofwisseling die als doel hebben om de productopbrengst te optimaliseren. Moleculen waarvan de redox-neutrale synthese vanuit het substraat leidt tot de vorming van ATP kunnen als enig product van de stofwisseling gemaakt worden onder anaërobe condities. Een dergelijk scenario heeft de voorkeur boven aërobe productieprocessen, omdat er bij anaërobe processen minder kosten verbonden zijn aan het ontwerp van de reactor, luchtcompressie en het mengen en koelen van de cultuur. In deze anaërobe processen kan de energie die nodig is voor groei en onderhoud van de cellen volledig gehaald worden uit substraat-niveau-fosforylering in de productroute. Als gevolg hiervan is de vorming van biomassa direct gekoppeld aan de ATP-stoichiometrie

van de productroute. Verlies van het koolstofsubstraat in de vorm van biomassa kan daarom worden geminimaliseerd door het inbouwen van een productieroute met een lage, maar positieve, ATP-opbrengst.

In de microbiële biotechnologie worden producten waarvan de synthese vanuit het substraat ATP nodig heeft, vaak juist gemaakt onder aërobe condities. In zulke processen wordt een gedeelte van het substraat verademd (verbrand) naar CO_2 en water om de ATP te genereren die nodig is voor productvorming, celgroei en/of onderhoud van de cellen. Het verhogen van de conservering van vrije energie in zulke ATP-behoevende productroutes, bijvoorbeeld door hun ATP-gebruik te verlagen, vermindert de hoeveelheid substraat die moet worden verbrand en maakt daarmee een verhoogde productopbrengst mogelijk. Routes die een energie-neutrale omzetting van het substraat naar product katalyseren zijn extra interessant voor het aanpassen van de conservering van vrije energie. Elke verandering aan zo'n productroute die de conservering van vrije energie verhoogt, heeft namelijk de potentie om het verbranden van substraat voor vorming van ATP overbodig te maken. Hiermee wordt anaërobe productie mogelijk, met de eerder genoemde economische voordelen.

Twee strategieën voor het verhogen van de conservering van vrije energie zijn bestudeerd in het onderzoek beschreven in dit proefschrift. De eerste strategie richtte zich op de opname en splitsing van de disacharide sacharose, een belangrijk substraat voor grootschalige industriële fermentatieprocessen. De tweede strategie richtte zich op het identificeren van gistgenen die betrokken zijn bij de energie-afhankelijke export van melkzuur. Melkzuur is een belangrijk platform-molecuul dat door gemodificeerde giststammen kan worden gemaakt.

Om de genetische modificaties die nodig zijn voor het aanpassen van de sacharosestofwisseling en melkzuurexport te vereenvoudigen, is een efficiënte methode voor genetische modificatie essentieel. De recente ontdekking van het CRISPR/Cas9 systeem bood een revolutionair nieuw gereedschap voor het introduceren van genetische modificaties. CRISPR/Cas9, oorspronkelijk ontdekt als een bacterieel afweermechanisme, bestaat in essentie uit twee onderdelen, het Cas9 endonuclease en een kort "gids-RNA" (gRNA). Zoals de naam al suggereert, leidt het gRNA Cas9 naar een uiterst specifieke complementaire sequentie van DNA. Cas9 brengt vervolgens op deze plaats in het genoom een dubbelstrengs DNA-breuk aan. Door Cas9 naar een specifieke plaats in het gistgenoom te sturen en tegelijkertijd een DNA-fragment aan te bieden dat homoloog is met de omliggende stukken DNA, en daardoor de dubbelstrengs breuk via homologe recombinatie kan repareren, kan (heteroloog) DNA worden toegevoegd en/of een deel van het oorspronkelijke gist-DNA worden verwijderd. De flexibele en precieze, gRNA-gebaseerde specificiteit van Cas9 heeft de potentie om introductie van genetische modificaties bijna overal in een genoom eenvoudiger te maken. In het onderzoek beschreven in **Hoofdstuk 2** is het CRISPR/Cas9 systeem geoptimaliseerd voor gebruik in *S. cerevisiae*. Hierbij zijn twee methoden voor het maken van genetische modificaties met Cas9 ontwikkeld en geëvalueerd. De eerste methode maakte gebruik

van de uitzonderlijke efficiëntie van *in vivo*-recombinatie in *S. cerevisiae*. Hierbij werden een plasmide "backbone" en een gRNA-coderend DNA-fragment tegelijkertijd in gistcellen ingebracht. De twee fragmenten werden vervolgens in de cel in elkaar gezet om een functioneel gRNA-expressieplasmide te vormen. Deze aanpak vergemakkelijkte de genetische modificatie van een enkele locatie in het gistgenoom, zonder de noodzaak om het plasmide vooraf in elkaar te zetten. Voor de tweede methode werden plasmiden ontwikkeld die gelijktijdige expressie van twee verschillende gRNAs mogelijk maakten. Met deze plasmiden kon Cas9, in een enkele transformatie-stap, naar twee verschillende plekken in het genoom worden geleid. Vervolgens werd de flexibiliteit en specificiteit van CRISPR/Cas9 gedemonstreerd door de simultane aanpassing van twee enkele nucleotiden op twee verschillende plekken in het chromosoom en door de introductie van meerdere DNA-fragmenten te combineren met het verwijderen van twee genen. Om toekomstige genetische modificaties te vereenvoudigen, werd een set van giststammen met Cas9 gemaakt met verschillende auxotrofe benodigdheden. Hierbij werd een constitutief tot expressie komend *cas9*-gen stabiel in het gistgenoom geïntegreerd. Verder werd voor beide methoden een set van plasmiden gemaakt met een aantal auxotrofe en dominante selectiemarkers. Deze plasmiden werden beschikbaar gemaakt voor de wetenschappelijke gemeenschap. Tot slot werd een online applicatie gemaakt voor de selectie van bruikbare gRNA 'targets' en voor geautomatiseerd ontwerp van de primers die nodig zijn voor de constructie van de gRNA-plasmiden.

De beschikbaarheid van de in **Hoofdstuk 2** beschreven efficiënte CRISPR/Cas9-gebaseerde methode maakte het mogelijk om ingrijpende veranderingen aan te brengen in de sacharosestofwisseling van gist. Natuurlijke stammen van *S. cerevisiae* splitsen sacharose met behulp van invertase. Dit enzym katalyseert de, voornamelijk extracellulaire, hydrolyse van sacharose naar glucose en fructose. Deze monosachariden worden geïmporteerd via gefaciliteerde diffusie en vervolgens omgezet via de Embden-Meyerhof-Parnas (EMP) variant van de glycolyse. Deze omzetting leidt tot de vorming van 4 ATP per sacharose door substraat-niveau-fosforylering. De vrije energie van sacharosehydrolyse ($\Delta G_o'$ = -29 kJ/mol) wordt door *S. cerevisiae* niet geconserveerd. Een aantal andere micro-organismen zijn echter in staat om sacharose te splitsen door fosforolyse. Bij dit proces worden sacharose en anorganisch fosfaat intracellulair omgezet naar glucose-1-fosfaat en fructose. Vervolgens kan fosfoglucomutase het gevormde glucose-1-fosfaat omzetten naar het glycolytische intermediair glucose-6-fosfaat. Door de ATP-afhankelijke fosforylering van glucose door hexokinase te omzeilen, maakt de fosforolytische route een hogere conservering van vrije energie mogelijk dan de invertase route. Echter, voordat de potentie van deze reactie onderzocht kon worden, waren ingrijpende aanpassingen aan de natuurlijke stofwisseling van sacharose in *S. cerevisiae* nodig.

In *S. cerevisiae* verloopt de opname van sacharose via een symport-mechanisme, waarbij een enkel proton met sacharose mee de cel in wordt getransporteerd. Vervolgens wordt dit proton de cel weer uitgepompt door het plasmamembraan-ATPase. Dit

ATPase heeft in *S. cerevisiae* een stoichiometrie van 1 ATP per geëxporteerd proton. De hieruit voortkomende kosten van 1 ATP voor de proton-gekoppelde opname van een sacharosemolecuul, maken het noodzakelijk om de proton-sacharose symporters te vervangen door een sacharoseuniporter als we een energetisch voordeel willen halen uit de intracellulaire fosforolyse van sacharose. In het onderzoek beschreven in **Hoofdstuk 3** werden alle bekende sacharosetransportergenen (*MAL11*, *MAL21*, *MAL31*, *MPH2* en *MPH3*) en alle genen die coderen voor sacharose hydrolyserende enzymen (*SUC2*, *MAL12*, *MAL22* en *MAL32*) in een enkele stam van *S. cerevisiae* verwijderd. Om mutaties te identificeren die groei op sacharose weer mogelijk maken, en daarmee toekomstige toepassingen kunnen bemoeilijken, werd deze platformstam vervolgens getransformeerd met ofwel een sacharosetransportergen (*MAL11*) óf een gen coderend voor een intracellulair sacharosehydrolase (*MAL12*). Vervolgens werden beide stammen onderworpen aan laboratoriumevolutie-experimenten in een synthetisch medium met sacharose als enige koolstof- en energiebron. Terwijl voor de stam waarin alle sacharosetransportergenen waren uitgeschakeld zelfs na twee maanden incubatie geen groei te zien was, groeide de stam met deleties in alle bekende sacharosehydrolasegenen op sacharose met een specifieke groeisnelheid van 0,08 uur^{-1}. Na de laboratoriumevolutie, via opeenvolgende batchcultures, nam deze groeisnelheid zelfs toe tot 0,25 uur^{-1}. Deze toename van de specifieke groeisnelheid ging gepaard met een drievoudige verhoging van de *in vitro* sacharosehydrolyseactiviteit evenals een drie- tot vijfvoudige verhoging van de expressie van de isomaltasegenen (*IMA*). Een met CRISPR/Cas9 gerealiseerde deletie van alle isomaltasegenen (*IMA1-5*) leidde vervolgens tot complete afwezigheid van alle sacharosehydrolyseactiviteit. De resulterende sacharose-negatieve stam werd daarna gebruikt als platform voor het verder aanpassen van de sacharosestofwisseling.

In theorie zou het vervangen van de extracellulaire hydrolyse van sacharose door de gefaciliteerde opname van sacharose (uniport) en een intracellulair fosforylase moeten leiden tot een verhoging van de ATP-opbrengst van sacharosefermentatie van 4 naar 5 ATP per sacharose. In het onderzoek beschreven in **Hoofdstuk 4** werd de platformstam (beschreven in **Hoofdstuk 3**) eerst getransformeerd met een bekend sacharose-proton symportergen (*MAL11*) en werd daarna gebruikt voor de expressie van sacharosefosforylase uit *Leuconostoc mesenteroides* (LmSPase). Om de verhoogde energetische efficiëntie van een LmSPase-expresserende stam te kunnen kwantificeren, werd de uiteindelijke stam gekweekt in anaërobe sacharose-gelimiteerde chemostaatculturen en vergeleken met een genetisch identieke referentiestam die het intracellulaire sacharosehydrolase Mal12 tot expressie bracht. Onder anaërobe, sacharose-gelimiteerde condities is vorming van ATP, door omzetting van het substraat, direct gerelateerd aan vorming van nieuwe biomassa. De combinatie van de proton-gekoppelde opname en de intracellulaire fosforylering zou, in theorie, de opbrengst van sacharosefermentatie moeten verhogen van 3 naar 4 ATP vergeleken met de Mal12-expresserende referentiestam. In overeenstemming met dit verwachte energetische voordeel was de biomassaopbrengst van een stam, waarin LmSPase tot expressie was

gebracht, 31% hoger dan dat van de genetisch identieke referentiestam. Deze toename lag erg dicht bij de verwachte verhoging van 33%. De relatief lage groeisnelheid van de stam met LmSPase-expressie tijdens anaërobe groei op sacharose (0,09 ± 0,02 uur^{-1}), vergeleken met die van de referentiestam (0,19 ± 0,01 uur^{-1}), bleek het resultaat van een limitatie bij de omzetting van glucose-1-fosfaat naar glucose-6-fosfaat. Overexpressie van het natuurlijke fosfoglucomutase van gist, *PGM2*, herstelde de anaërobe groeisnelheid naar 0,23 ± 0,01 uur^{-1}. Om vervolgens de ATP-opbrengst van sacharosefermentatie nog verder te verhogen, werd de stam met LmSPase-expressie eerst gebruikt om functionele expressie van plantaardige genen voor sacharosefacilitators (*PvSUF1*, *PsSUF1*, *PsSUF4*, *OsSWEET11* en *AtSWEET12*) te onderzoeken. Na 5 dagen incubatie groeide alleen de stam met een sacharosefacilitator uit *Phaseolus vulgaris* (PvSUF1) op synthetisch medium met sacharose als enige koolstofbron. Deze stam werd daarom uitgekozen voor een nadere karakterisering. De gecombineerde expressie van *PvSUF1* en *Lm*SPase verhoogde de biomassaopbrengst op sacharose met nog eens 8% in anaërobe sacharose-gelimiteerde chemostaatculturen ten opzichte van een stam met Mal11-expressie. Hoewel deze verhoging van de opbrengst lager was dan verwacht, werd door dit experiment wel voor het eerst aangetoond dat de combinatie van expressie van een fosforylase en een uniporter de energetische efficiëntie van *S. cerevisiae* kan verhogen.

Melkzuur speelt een belangrijke rol in de levensmiddelenindustrie, bijvoorbeeld als mild-zure smaakstof, voor het maken van emulsies en/of om bederf te voorkomen. Bovendien is melkzuur een belangrijk platformmolecuul voor de synthese van chemicaliën, zoals de thermoplastische biologisch-afbreekbare polyester polymelkzuur (polylactic acid, (PLA)). Van de intracellulaire omzetting van glucose naar melkzuur werd verwacht dat deze qua redoxbalans en ATP-opbrengst equivalent zou zijn aan alcoholische fermentatie. Echter, eerder onderzoek heeft al aangetoond dat genetisch gemodificeerde, homofermentatieve melkzuur-producerende stammen van *S. cerevisiae* niet in staat zijn om anaëroob te groeien. Kennelijk hebben zulke stammen zuurstof nodig om de ATP te leveren voor de groei en onderhoud van cellen. Hoogstwaarschijnlijk wordt dit onverwachte fenotype veroorzaakt door het gebruik van energie voor de export van melkzuur, waardoor de totale omzetting van glucose naar extracellulair melkzuur ATP-neutraal is. Ondanks verscheidene pogingen zijn de genen die betrokken zijn bij melkzuurexport in *S. cerevisiae* nog steeds niet geïdentificeerd. Omdat in geen van de enkele-deletie-mutanten uit de *S. cerevisiae* 'knock-out collectie' de capaciteit om melkzuur te exporteren ontbrak, is het waarschijnlijk dat verschillende transporteiwitten betrokken zijn bij export van dit molecuul. De ontwikkeling van CRISPR/Cas9-gebaseerde technieken voor genetische modificatie (**Hoofdstuk 2**) maakt het makkelijker om via 'brute kracht' gen-deletie methoden de genen te ontdekken die betrokken zijn bij specifieke metabole functies. Een dergelijke aanpak werd toegepast in het onderzoek beschreven in **Hoofdstuk 5** met het doel om de genen die betrokken zijn bij melkzuurtransport te identificeren. Eerst werd er een selectie gemaakt van de genen die betrokken zouden kunnen zijn bij melkzuurtransport, gebaseerd op een veronderstelde

rol in het transport van, of resistentie tegen, organische zuren. De geselecteerde set van genen, coderend voor 25 (veronderstelde) transporters van organische zuren, werd vervolgens uitgeschakeld in een enkele *S. cerevisiae* stam met behulp van de CRISPR/Cas9-methode die beschreven is in **Hoofdstuk 2**. Om vervolgens het effect van de gen-deleties op melkzuurexport te kunnen bestuderen, werd de uiteindelijke stam getransformeerd met een lactaatdehydrogenasegen uit *Lactobacillus casei*. Om groeisnelheid-gerelateerde effecten te voorkomen, werd productie van melkzuur door de stam met 25 deleties en een genetisch identieke referentiestam vergeleken in anaërobe, glucose-gelimiteerde chemostaatculturen. Onder deze kweekomstandigheden werden er geen verschillen gevonden tussen beide stammen. Dit wijst er hoogstwaarschijnlijk op dat de set van 25 gen-deleties geen, of niet alle, melkzuurtransporters in *S. cerevisiae* omvat.

Chapter 1

General introduction



1.1 Biotechnology

Throughout human history, use of living organisms for human benefit has played a central role in society. The original definition of the word "biotechnology", first coined in 1919, covers this very broad field and encompasses any process that makes use of (parts of) living organisms to manufacture products or to upgrade their value (Ereky 1919; Kennedy 1991). The definition given by the European Federation of Biotechnology (EFB) states that biotechnology is "the integration of natural science and organisms, cells, parts thereof and molecular analogues for products and services" (Wood 1997). The EFB definition includes the cultivation of soil to grow crops, the rearing of animals in agriculture and the use of microorganisms for various production processes. For the industrial production of fuels and chemicals, processes based on the use of microorganisms offer several advantages over traditional production methods. Advantages of these processes include the very short generation time of some microorganisms (under ideal conditions the popular bacterium *E. coli* can divide every 22 minutes (Helmstetter and Cooper 1968)), high volumetric productivity and the option to carefully control the production environment. Natural evolution, involving a constant competition for shared resources between astronomical numbers of microorganisms and microbial species, has optimized and streamlined the hundreds to thousands of biochemical conversions confined within cells of each microbial species (Wittmann *et al.* 2017). The resulting natural metabolic diversity and the innumerable combinations thereof provide opportunities for developing novel conversions from renewable resources to useful products, with the potential to replace costly and labour intensive extraction of active compounds from animal or plant tissues and to provide an alternative for their chemical synthesis (**Figure 1.1**) (Van Berkel 2000; Gavrilescu and Chisti 2005; Van Maris *et al.* 2006; Steen *et al.* 2010; Lee, Kim and Cheon 2015).

1.2 History of industrial microbiology

Without understanding of their biology or, indeed, awareness of their existence, microorganisms were already used for human benefit many thousands of years ago and, throughout human history, have been of great social and economic importance (Bud 1994). Historically, microorganisms were used for the production of bread, beer, wine, cheese and yoghurt, later followed by alcohol, acetic acid and lactic acid (Buchholz and Collins 2013). In 1857, Pasteur demonstrated that living organisms are responsible for fermentation and this discovery led to the development of concepts that have been crucial for the robust application of microbial processes on an industrial scale. For example, he postulated that each fermentation profile could be traced to a specific microbe. This notion was rapidly followed by the concept of using inoculation with pure cultures for reliable fermentation and the invention of a closed vessel to protect a fermentation process from air-borne contaminations (Pasteur 1857a, 1857b, 1859, 1873, 1876; Barnett 2000).

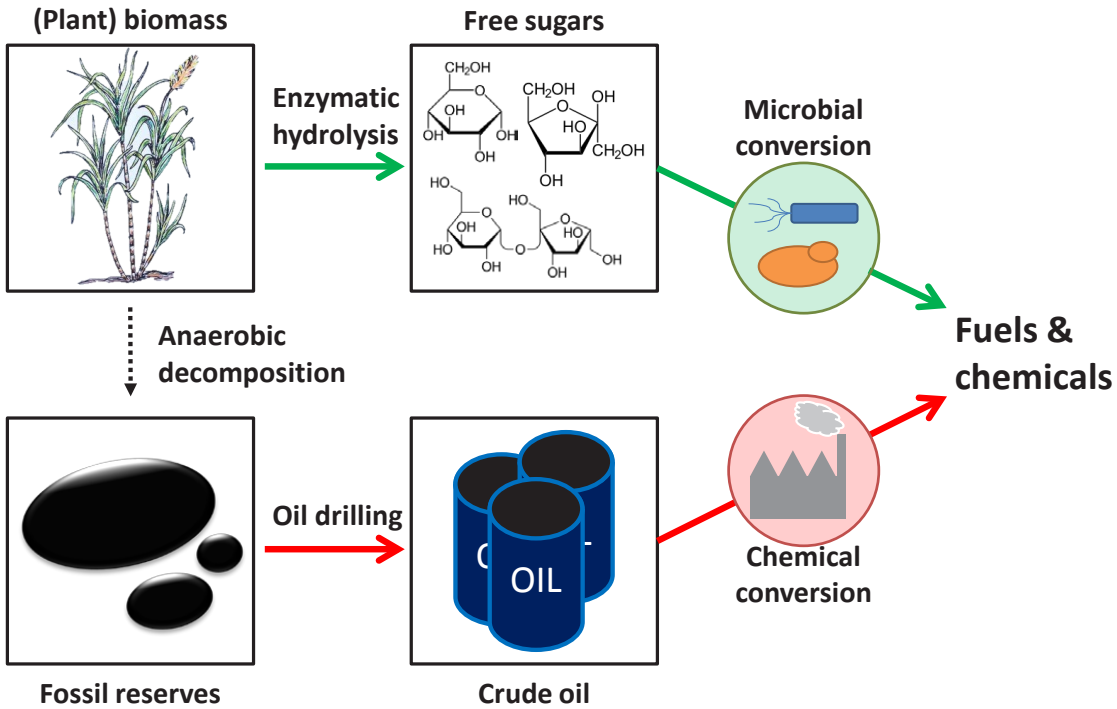
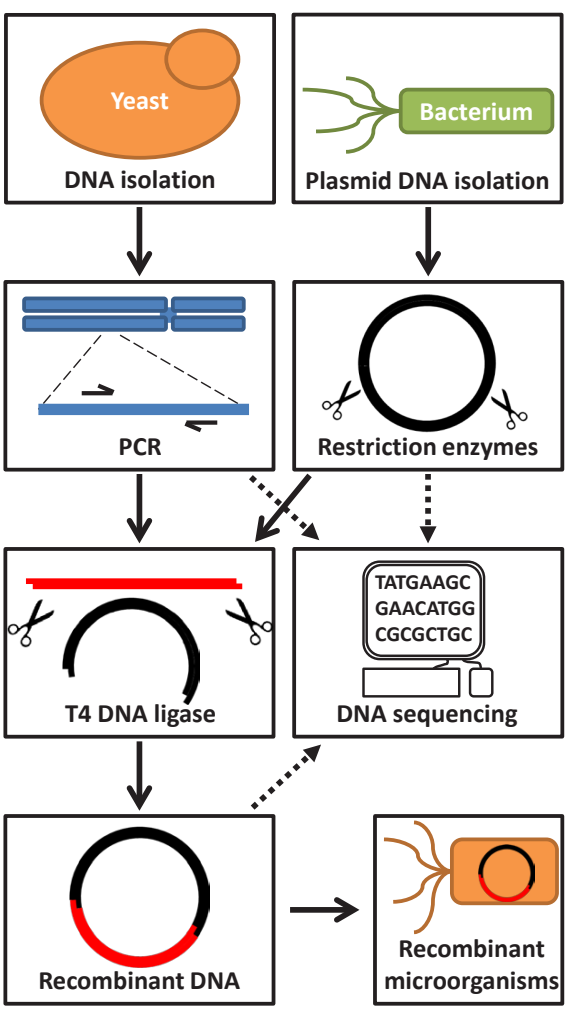


Figure 1.1: Microbial conversions (green) can provide a sustainable alternative for some chemical conversions (red). Prior to the microbial conversion of biomass, the microbial availability of the biomass-contained sugars is usually increased via a pre-treatment step, for example via enzymatic hydrolysis.

By the end of the nineteenth century, the fermentation industry was growing fast and focussed on applications in food production, medical care and hygiene (Bud 1994). Industrial fermentation processes for other chemicals rapidly developed in the years leading up to and during the First World War. The war caused a shortage of acetone as a solvent for the production of explosives in England, which led to the development of the Weizmann process for the mixed fermentation of acetone and butanol from molasses (the latter can be used to make synthetic rubber) (Nathan 1919; Weizmann and Anthony 1920; Bud 1994). At the same time, a shortage of glycerol for the production of the explosive nitroglycerin led to the rapid development of industrial-scale glycerol fermentation in Germany (Connstein and Lüdecke 1919). After the First World War, many other industrial fermentation processes for the manufacturing of product such as organic acids, pharmaceuticals and enzymes were developed (Buchholz and Collins 2013).

In 1928, Fleming observed that bacterial growth can be inhibited by cultures of *Penicillium notatum* and named the then still unidentified antibacterial substance in the culture broth "penicillin" (Bud 2007). When the therapeutic value of penicillin became clear, efforts by the Northern Regional Research Laboratory (NRRL) and the US Department of Agriculture (USDA) resulted in an interdisciplinary cooperation between various universities and companies with the goal to increase penicillin production (Coghill 1970; Buchholz and Collins 2013). The urgent need for large quantities of

penicillin during the Second World War rapidly expanded our knowledge regarding microbial fermentation on industrial scale. In as little as 2 years, early strain development combined with fermentation process optimization facilitated the increase in the level of output of penicillin by 140,000-fold (Podbielniak, Kaiser and Ziegenhorn 1970). Methods were developed for strain improvement via repeated cycles of mutagenesis (the introduction of random genetic changes) and selection of mutants with better properties (e.g. robustness, production rate, and titre), a technique now referred to as "classical strain improvement" that is still intensively applied today. The success story of penicillin and the novel concepts developed in strain engineering and fermentation sparked the emergence of many new high-value biotechnological production processes. By the 1950s large-scale biotechnological production of antibiotics, steroids, amino- and organic acids and carbohydrates was well established. As a result, industrial microbiology gained widespread attention due to its innovative and economic growth potential, and again in the 1970s when the first oil crisis brought a realization that production from renewable resources could alleviate the technological dependency on the petrochemical industry. Replacing existing chemical production processes by more sustainable microbial conversions from renewable substrates remains a major incentive for biotechnological research to this day (Van Maris *et al.* 2006).



In the field of biotechnology, the last 50 years were marked by the emergence and integration of molecular biology (**Figure 1.2**). Key discoveries included the elucidation of the structure of DNA (Watson and Crick 1953), the discovery of restriction enzymes (Linn and Arber 1968) and T4 DNA ligase (Weiss and Richardson 1967; Zimmerman *et al.* 1967), development of recombinant-DNA technology (Mertz and Davis 1972) and recombinant microorganisms (Cohen, Chang and Boyer 1973), DNA sequencing

Figure 1.2: Development of 'classical' molecular biology. DNA is first extracted from the organism of interest. The DNA-fragments of interest can then be obtained by the use of restriction enzymes or via PCR. PCR fragments can subsequently be cleaved by restriction enzymes to obtain compatible cohesive ends. By the use of T4 DNA ligase the fragments are then assembled into a final recombinant plasmid, which can be transformed to engineer recombinant microorganisms. Sequencing of DNA facilitates the design of genetic engineering strategies and can be used as quality control for cloning procedures.

(Sanger and Nicklen 1977; Maxam and Gilbert 1980) and development of PCR (Mullis *et al.* 1986; Saiki *et al.* 1988). These discoveries resulted in a tremendous expansion of the range of products from microbial fermentations, including biopolymers, heterologous proteins and pharmaceuticals. The development of recombinant human insulin and its subsequent approval for medical use by the US Food and Drug Administration in 1982 marked an important milestone, as it paved the way for biotechnological production of other recombinant pharmaceuticals (Bud 1994; Primrose and Twyman 2013). Additionally, the ability to inactivate and overexpress genes in a targeted manner enabled improvements in the product yields of existing processes and mutagenesis within coding regions of genes allowed production of enzymes with improved performance. For a more extensive review on the history of industrial microbiology the reader is referred to Buchholz & Collins, 2013.

1.3 *Saccharomyces cerevisiae* as a production host

Major advances in genetic engineering over the last five decades enabled researchers to design and engineer novel microbial strains by combining genetic information of various organisms – and, in an increasing number of cases, synthetic genetic information (Kosuri and Church 2014) - into a single production host. Nowadays, a limited number of organisms that can be easily engineered and grown in large-scale industrial processes are often applied in industry. While fundamental research on a wide variety of microbial strains expands the perspective of the potential of biotechnological processes, for industrial production of chemicals, the use of a few platform organisms for the production of a wide range of molecules is preferred. The use of a limited number of platform microorganisms allows for flexible use of production facilities and in some cases direct transfer of new knowledge from one production process to another (Borodina and Nielsen 2014).

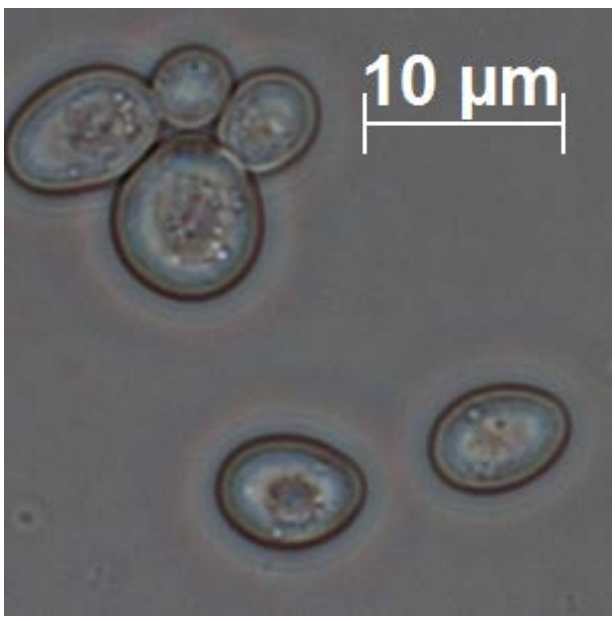


Figure 1.3: Phase-contrast microscopy image of *Saccharomyces cerevisiae* cells in a growing culture.

The budding yeast *Saccharomyces cerevisiae* (**Figure 1.3**) is one of the important platform organisms in modern industrial biotechnology. *S. cerevisiae* exhibits various properties advantageous for industry, including: i) high robustness under industrial conditions, including resistance to inhibitors in industrially relevant feedstocks and low pH tolerance, which reduces the risk of bacterial

contamination and lowers or eliminates the need for neutralization agents when producing acids; ii) a generally-recognized-as-safe (GRAS) status, based on its extensive historical use for the production of beer, bread and wine, which allows for convenient handling and disposal of cell matter; iii) a capacity to rapidly consume several cheap and abundant carbon substrates, such as sucrose and its monomers glucose and fructose; iv) excellent genetic accessibility due to a preference of homologous recombination (HR) over non-homologous end joining (NHEJ) as a DNA repair mechanism, combined with the availability of well-established tools for classical genetics and genetic engineering, including a high quality whole-genome sequence (Goffeau *et al.* 1996; Van Maris *et al.* 2006; Nijkamp *et al.* 2012; Kuijpers *et al.* 2013b; Borodina and Nielsen 2014).

Its advantageous traits for application-inspired research have stimulated the development of a wide toolkit for genetic modification techniques for *S. cerevisiae*. Nowadays, fast and reliable, high-efficiency transformation protocols are available (Gietz and Woods 2002) and strain platforms are available with various auxotrophies for use with standardized sets of expression vectors (Sikorski and Hieter 1989; Christianson *et al.* 1992; Mumberg, Müller and Funk 1995; Brachmann *et al.* 1998). Additionally, multiple counter-selectable genetic markers for gene integration and deletion have been developed (Boeke, La Croute and Fink 1984; Gueldener *et al.* 2002; Solis-Escalante *et al.* 2013, 2014). Based on its strong preference for homologous-recombination-based DNA repair, methods have been developed for *in vivo* assembly of large genetic constructs (Gibson *et al.* 2008; Kuijpers *et al.* 2013a, 2013b). Recently, the first CRISPR/Cas9-mediated genetic manipulations have been demonstrated in *S. cerevisiae* (DiCarlo *et al.* 2013b). Being part of a bacterial immune mechanism in *Streptococcus pyogenes*, the CRISPR/Cas9 machinery has a tremendous specificity for targeted genetic modifications. A unique feature of Cas9 is the use of a small RNA molecule to guide it to a target sequence where it introduces a double strand DNA break. The RNA-based targeting of Cas9 provides the potential to engineer the system for genetic modifications almost anywhere in the genome very fast and at low cost (Barrangou *et al.* 2007; Brouns *et al.* 2008; Marraffini and Sontheimer 2008; Deltcheva *et al.* 2011).

The ease of genetic engineering of *S. cerevisiae* has led to the exciting development of a wide range of products derived from novel yeast-based fermentations. An example demonstrating the potential of genetic modification in yeast is the industrial production of farnesene by the USA-based company Amyris. Farnesene can be derived in one step from farnesyl diphosphate, an intermediate in the ergosterol pathway, by farnesene synthase. Their engineered yeast strain achieved product titres in excess of 130 g L^{-1} farnesene, thus demonstrating the power of genetic engineering for the production of novel products in *S. cerevisiae* (Westfall *et al.* 2012; Sandoval *et al.* 2014; Meadows *et al.* 2016). However, high level production of farnesene required a substantial number of genetic interventions: i) overexpression of 8 genes involved in the ergosterol pathway, ii) introduction of heterologous phosphoketolase and phosphotransacetylase which, together, provide a carbon-efficient alternative for natural precursor-supply pathways,

iii) replacement of acetaldehyde dehydrogenase and acetyl-CoA synthase activity by acetylating acetaldehyde dehydrogenase, which lowers the energetic cost of producing a molecule of farnesene by 18 ATP, iv) replacement of the native NADPH-dependent HMG-CoA reductase by an NADH-dependent version to match the change in cofactor usage by acetylating acetaldehyde dehydrogenase and v) deletion of *HOR2*, which competes with phosphotransacetylase for acetyl phosphate. In addition to the aforementioned genetic modifications, various rounds of classical strain improvement via random mutagenesis and selection were employed. Another example of a recently developed process in *S. cerevisiae* is the production of vanillin, which required overexpression of heterologous 3-dehydroshikimate dehydratase, *O*-methyltransferase and aromatic carboxylic acid reductase. To limit the conversion of vanillin to vanillyl alcohol, *ADH6* was deleted and an additional deletion of *BGL1* reduced vanillin hydrolysis (Hansen *et al.* 2009). A final example is the production of the antioxidant resveratrol, which was enabled in yeast via expression of 4-coumarate-CoA ligase and stilbene synthase (Becker *et al.* 2003). Further optimization and realization of *de novo* production of resveratrol in *S. cerevisiae* was later achieved via the use of a codon-optimized version of tyrosine ammonia lyase and co-expression of a bacterial arabinose transporter (Wang *et al.* 2011). For other examples of novel production processes with *S. cerevisiae* see Borodina and Nielsen, 2014 and Dai *et al.*, 2014.

1.4 Energy coupling in *S. cerevisiae*

Although industrial production of chemicals with *S. cerevisiae* is already well established, engineering of its metabolism continues to improve its performance in existing processes and enable the development of novel processes. Improvement of existing processes can be achieved for example via i) elimination of competing metabolic pathways leading to by-product formation, such as the disruption of glycerol formation in ethanol production (Guadalupe-Medina *et al.* 2010), ii) overexpression of genes to overcome bottlenecks in metabolic pathways, such as overexpression of genes in valine metabolism to improve isobutanol production (Chen *et al.* 2011) and iii) engineering of the yeast to access a wider range of industrially relevant substrates, such as glycerol and pentose sugars (Van Maris *et al.* 2006; Swinnen *et al.* 2013).

In addition to targeted genetic modification, laboratory evolution can be used to select for mutants with industrially relevant phenotypes (**Figure 1.4**). This approach, often referred to as evolutionary engineering (Sauer 2001; Winkler and Kao 2014; Bachmann *et al.* 2015), can be extremely effective when an industrially relevant trait can be directly linked to a specific selective advantage, such as an increased growth rate or cell survival. Once mutants with improved characteristics have been obtained, whole-genome sequencing of the evolved and parental strains can rapidly provide insight into the underlying mutations (Oud *et al.* 2012). The genetic mutations can then be introduced in the unevolved parental strain to elucidate their biological function (reverse

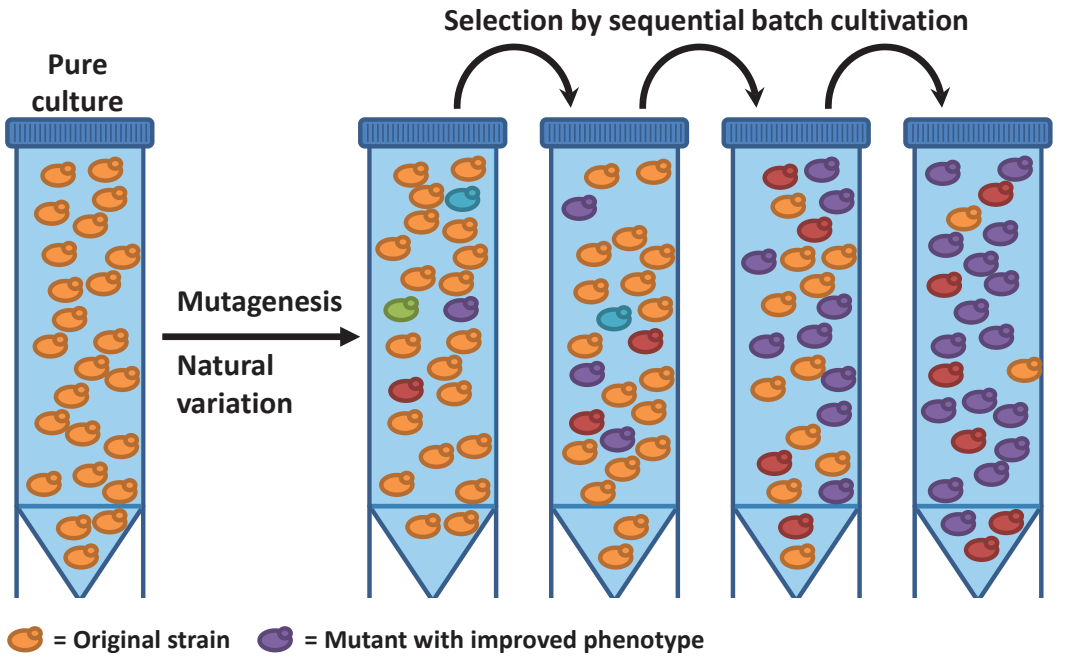


Figure 1.4: Laboratory evolution via repeated batch cultures. When a desired phenotypical trait can be coupled to growth- or survival-rate, evolutionary engineering can be applied to obtain improved microbial strains. First, mutations are introduced into a pure culture, either by chemical- or UV-mediated mutagenesis or by imperfect DNA-duplication in growing cultures. Subsequently, cycles of batch-cultivation can be applied to select and enrich for mutants that grow faster than the original strain.

engineering). The power of the integration of evolutionary- and reverse-engineering is illustrated by studies on high temperature tolerance, fast sedimentation and butanol tolerance of *S. cerevisiae* (González-Ramos *et al.* 2013; Oud *et al.* 2013; Caspeta *et al.* 2014).

This thesis focusses on understanding and addressing an important engineering target for increasing the performance of industrial microorganisms in industrial processes: the conservation of free-energy (ATP) in product formation pathways. Many cellular processes, including product pathways, which require a net input of free energy derive this free energy from hydrolysis of a phosphodiester bond in ATP. Similarly, free-energy can be conserved by the phosphorylation of ADP to ATP. In *S. cerevisiae*, ATP and the energetic equivalent GTP can be formed directly by substrate-level phosphorylation in glycolysis and the TCA-cycle, respectively. The $\Delta G^{\circ\prime}$ for synthesis of ATP from ADP and inorganic phosphate is 26 - 32 kJ mol^{-1} (Alberty 2003). Alternatively, in the presence of oxygen, yeast cells can form ATP via mitochondrial respiration. Complete dissimilation of glucose to CO_2 via glycolysis and the TCA cycle results in the formation of 4 ATP/GTP via substrate-level phosphorylation as well as 10 NADH and 2 $FADH_2$ (**Figure 1.5**). The transfer of electrons from NADH or $FADH_2$ to oxygen via the electron transport chain of the mitochondria can then be used to translocate protons, resulting in a proton gradient over the mitochondrial membrane. This gradient results in a driving force for proton translocation (the proton motive force) which is used for the formation of ATP

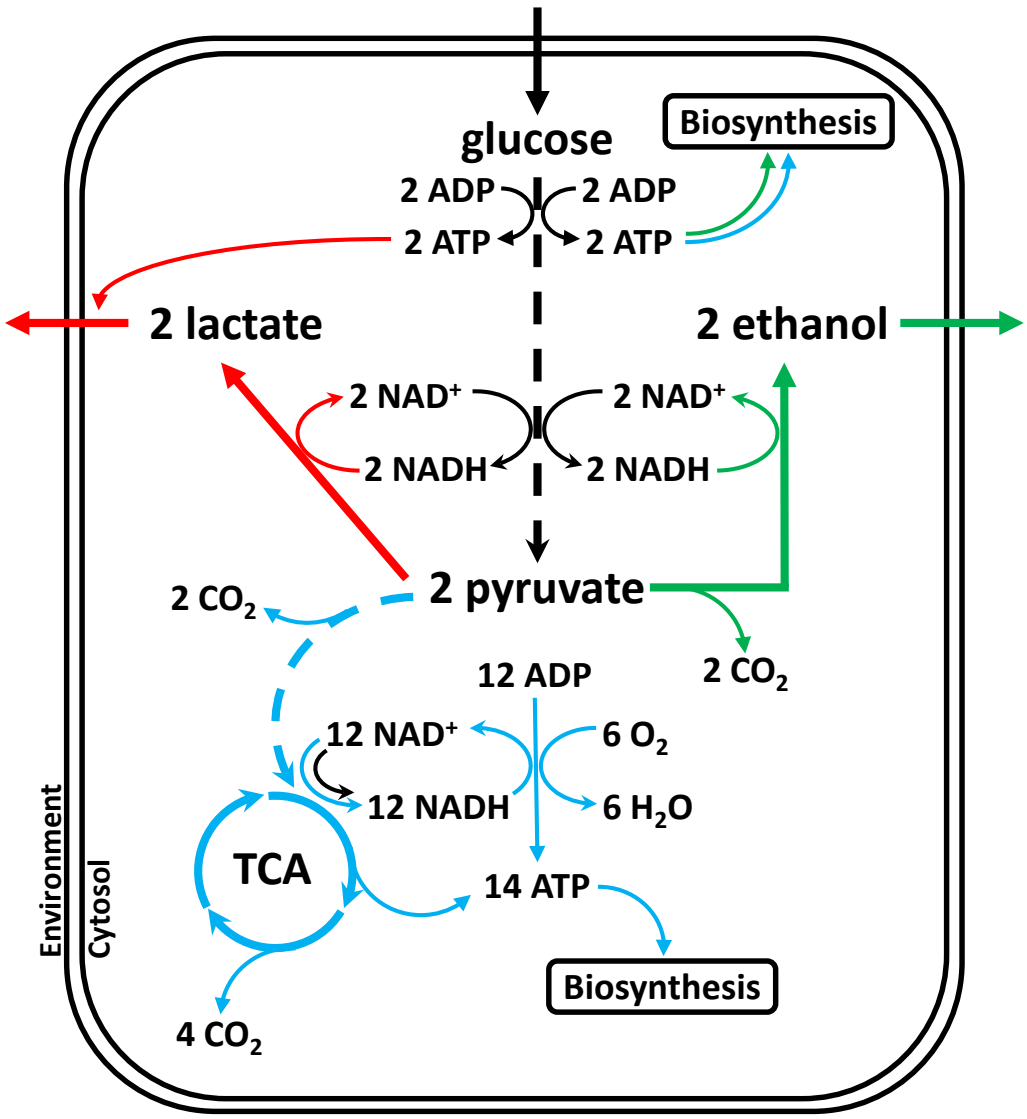


Figure 1.5: Schematic overview of examples of energy coupling in *S. cerevisiae*. The Embden-Meyerhof-Parnas (EMP) glycolytic pathway is depicted in black. An ATP-yielding product pathway (alcoholic fermentation, green) can directly provide the energy for anabolic reactions. A 'zero-ATP' product pathway (engineered pathway for lactate fermentation, red) catalyses the energy-neutral conversion from substrate to products and requires alternative metabolic conversions to provide the energy for anabolic reactions. ATP-generating aerobic respiration (blue) is often used to provide ATP for anabolic reactions in processes based on 'zero-ATP' or energy-requiring product pathways. 'Biosynthesis' refers to the reactions required for cellular- growth and maintenance and/or reactions in energy-requiring product pathways. For visualization purposes, $FADH_2$ is depicted as NADH, GTP is depicted as ATP. Furthermore, in the representation of aerobic respiration, the reduction of 2 NAD^+ to 2 NADH in the EMP-pathway has been pooled with the reduction of the additional 10 NAD^+ from the reactions leading to and in the TCA cycle (double arrow, black and blue).

via ATP synthase, where translocation of ca. 3 protons results in the formation of one ATP molecule (Nicholls and Ferguson 2004; Walker 2013). The energy stored in the form of ATP can be used by the cell for cellular maintenance and the formation of new macromolecules such as DNA, RNA and proteins, for the transport of molecules across membranes and, if required, for product formation (**Figure 1.5**).

When it comes to ATP and product formation, a distinction can be made between three types of products: i) products that require a net input of ATP for their synthesis; ii) products whose formation from the substrate is energy neutral and; iii) products whose formation leads to the release of energy which can be conserved in the form of ATP (De Kok *et al.* 2012a).

Products whose synthesis is coupled to a net formation of ATP can, in principle, serve as sole catabolic products in *S. cerevisiae*. All of the energy required for cell growth and maintenance can then be conserved in the form of ATP in the product formation pathway by substrate-level phosphorylation. Such a situation implies that no oxygen should be required to supply additional ATP via respiration and therefore production can be performed under anaerobic conditions, which is preferred in view of the lower operating costs of anaerobic industrial fermentation processes (Weusthuis *et al.* 2011; Cueto-Rojas *et al.* 2015). An example is anaerobic ethanol formation by *S. cerevisiae*, whose production from glucose results in the formation of 2 ATP molecules per glucose molecule consumed (**Figure 5**). In anaerobic, fermentative production systems, product formation and cell growth are directly linked via the ATP stoichiometry of the product pathway. As a consequence, a product pathway with a high ATP yield can lead to the formation of excess biomass as a by-product, lowering the yield of the intended product. Additionally, biomass formation from substrate leads to the reduction of NAD^+ to NADH, which, in anaerobic cultures of *S. cerevisiae*, is reoxidized via formation of an additional by-product, glycerol (Dijken and Scheffers 1986; Bakker *et al.* 2001; Van Maris *et al.* 2006). The impact of the ATP stoichiometry of catabolic pathways on product yield is well demonstrated by comparing ethanol formation by *S. cerevisiae* and the bacterium *Zymomonas mobilis*. Alcoholic fermentation in *S. cerevisiae* proceeds via the Embden-Meyerhof-Parnas (EMP) pathway, resulting in the aforementioned formation of 2 ATP molecules and 2 molecules of ethanol per glucose. *Z. mobilis* uses the Entner-Doudoroff (ED) pathway. This pathway also generates 2 ethanol per glucose, but yields only a single ATP molecule for each glucose molecule (Sprenger 1996). As a result of the its lower ATP yield, the anaerobic biomass yield of *Z. mobilis* on glucose is more than 2 fold lower and its ethanol yield on glucose is 20% higher than in *S. cerevisiae* (Rogers, Lee and Tribe 1979; Lee *et al.* 1980; Kuyper *et al.* 2005). This example demonstrates how higher product yields are achieved when product formation from substrate leads to a low but positive ATP yield (De Kok *et al.* 2012a).

For some products, the formation from substrate is either energy neutral ('zero-ATP pathway') or requires a net input of energy for their synthesis. In such cases, *S. cerevisiae* can meet the ATP requirements for cell growth, maintenance and/or product formation in two ways (**Figure 1.5**). Firstly, part of the sugar substrate can be co-fermented to ethanol, which, for conversion of one glucose molecule, leads to the formation of 2 ATP. However, part of the available carbon is directed towards ethanol instead of the intended product, furthermore the recovery of both products from such a mixed fermentation requires extensive downstream processing. Alternatively, in aerobic

processes, part of the substrate can be respired to CO_2, which is subsequently released from the culture broth. Compared to alcoholic fermentation, the ATP yield of respiration is much higher, resulting in the formation of an additional 14 ATP per glucose by the respiratory chain for a total of 16 ATP in *S. cerevisiae* (Verduyn *et al.* 1991; Pronk *et al.* 1994). While the higher ATP yield of respiration compared to fermentation results in a reduced loss of carbon for energy generation, aerobic conditions are not preferred for the industrial production of chemicals. On an industrial scale, maintaining aerobic conditions adds to production costs due to a more complex reactor design to enable high oxygen transfer rates and, as more heat is dissipated for each ATP that is formed via respiration, intensive cooling (Weusthuis *et al.* 2011; Cueto-Rojas *et al.* 2015). Therefore, for products with very small profit margins, such as bulk chemicals, avoiding the need for aeration in the production process is of great importance to be cost-competitive with their chemically synthesized counterparts (Weusthuis *et al.* 2011; De Kok *et al.* 2012a).

1.5 Engineering free-energy conservation in *S. cerevisiae*

As discussed above, synthesis of catabolic products from substrate leads to the net formation of ATP. This ATP excess is then used by the cell for the formation of biomass and, in anaerobic processes, glycerol. Biomass can be considered an undesirable by-product in industrial processes if its production levels exceed those required for fulfilling its catalytic role. In such cases, anaerobic biomass formation can be reduced by lowering the free-energy conservation for ATP-yielding catabolic product pathways. An example of such an approach in *S. cerevisiae* is the engineering of sucrose metabolism for improved ethanol yields (Basso *et al.* 2011). Wild-type *S. cerevisiae* predominantly hydrolyses sucrose with an extracellular invertase (Suc2) and takes up the resulting monomers glucose and fructose via facilitated diffusion. Subsequent fermentation of glucose and fructose to 4 ethanol molecules results in the net formation of 4 ATP per sucrose (**Figure 1.6A**). Via removal of the signal peptide of Suc2, which is required for extracellular targeting, Basso et al., (2011) relocated sucrose hydrolysis to the yeast cytosol. This modification was combined with evolutionary engineering to increase the capacity of proton-coupled uptake of sucrose prior to hydrolysis, at the net expense of 1 ATP per sucrose. This ATP requirement for the sucrose transport reaction lowered the overall ATP stoichiometry of sucrose fermentation from 4 to 3 moles of ATP per mole sucrose (**Figure 1.6B**). Accordingly the anaerobic biomass yield on sucrose was 30% lower and the ethanol yield was 11% higher (for other examples see Gombert and van Maris, 2015).

For products whose synthesis requires a net input of ATP, increasing free-energy conservation will lower the requirement of substrate necessary to provide ATP and therefore results in a higher product yield. In a recent study, the ATP requirement for production of the isoprenoid farnesene was reduced via modification of the cytosolic acetyl-CoA pathway (Meadows *et al.* 2016). By replacing acetaldehyde dehydrogenase

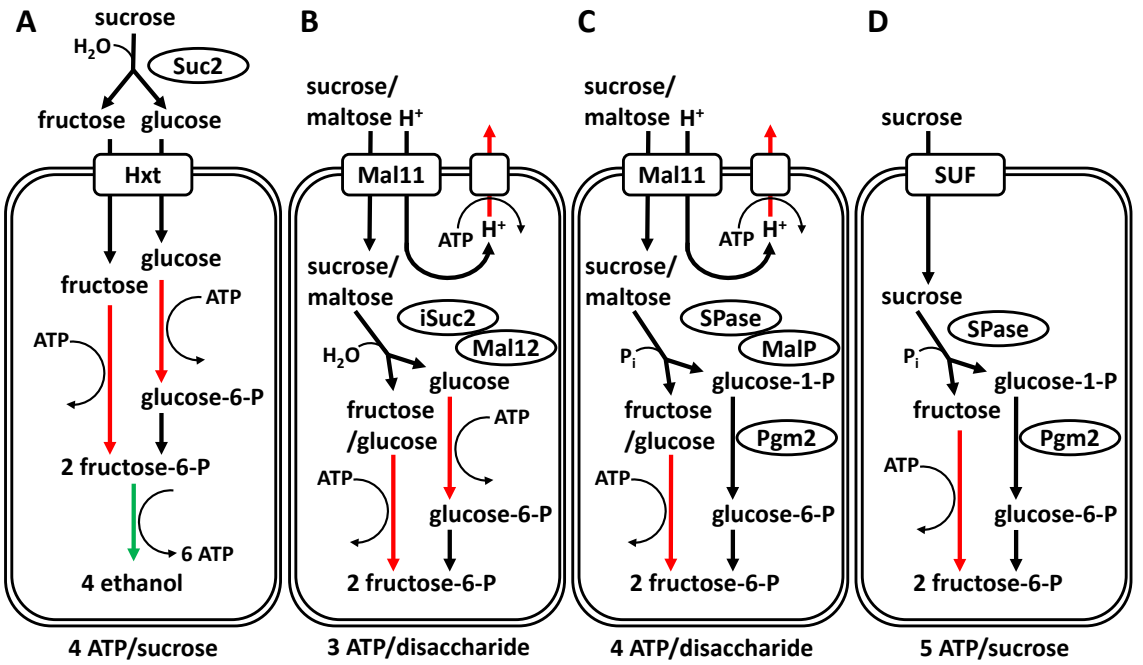


Figure 1.6: Different conceivable configurations of disaccharide metabolism in (engineered) yeast strains. Extracellular hydrolysis of sucrose, followed by uptake of the released monosaccharides and subsequent conversion by the EMP-pathway to ethanol result in the net formation of 4 ATP per sucrose (**A**). Proton-coupled uptake (catalysed by Mal12) and intracellular hydrolysis (catalysed by iSUC2 (sucrose) or Mal12) of disaccharides leads to the formation of 3 ATP per disaccharide due to the need of proton extrusion by the plasma membrane proton ATPase at a net cost of 1 ATP per transported disaccharide (**B**). Proton-coupled uptake (catalysed by Mal12) and intracellular phosphorolysis (catalysed by SPase and MalP for sucrose and maltose, respectively) result in the formation of 4 ATP per disaccharide by circumventing the ATP-requiring hexokinase step (which catalyses the conversion of glucose to glucose-6-phosphate) (**C**). Facilitated diffusion and intracellular phosphorolysis of sucrose leads to the formation of 5 ATP per sucrose as, in contrast to proton-coupled uptake, the use of a sucrose facilitator does not result in a net energetic cost for sucrose uptake (**D**).

and acetyl-CoA synthetase activity with acetylating acetaldehyde dehydrogenase (Kozak *et al.* 2014a) or by the combination of phosphoketolase and phosphotransacetylase, two ATP were saved for the synthesis of each cytosolic acetyl-CoA molecule. Since 9 acetyl-CoA molecules are required for the synthesis of one molecule of farnesene, this modification lowered the overall ATP requirements of farnesene synthesis from glucose by 18 ATP, and increased the product yield past the theoretical limits of synthesis via the native yeast metabolic pathway (Meadows *et al.* 2016; Van Rossum *et al.* 2016b).

Pathways that catalyse an ATP-neutral conversion of substrate to product represent an interesting case for metabolic engineering research. Any change to such a catabolic pathway that improves free-energy conservation can eliminate the need of substrate for ATP synthesis and, additionally, has the potential to enable anaerobic production at high yields. Furthermore, coupling of product- and biomass formation under these conditions should, at least in theory, allow for simple, growth-coupled evolutionary engineering strategies to achieve higher conversion rates. In this thesis,

production of lactic acid from glucose by engineered 'homolactic' *S. cerevisiae* strains will be used as a model for such a 'zero-ATP process' (**Figure 1.5**).

As discussed above, conversion of glucose to 2 pyruvate, via the Embden-Meyerhof-Parnas (EMP) pathway, yields 2 ATP from substrate-level phosphorylation and results in the reduction of 2 NAD^+ to 2 NADH. Subsequent decarboxylation of pyruvate to acetaldehyde (by pyruvate decarboxylase) and reduction of acetaldehyde to ethanol (by alcohol dehydrogenase) results in the oxidation of NADH to NAD^+ and does not affect ATP stoichiometry. Export of ethanol from the cell proceeds via passive diffusion. Therefore the overall conversion of glucose to 2 ethanol and 2 CO_2 is redox-neutral and results in the overall formation of 2 ATP. Inactivation of all three *S. cerevisiae* pyruvate decarboxylase genes (*pdc1Δ pdc5Δ pdc6Δ*) and expression of a heterologous NAD^+-linked lactate dehydrogenase (*LDH*) gene completely abolishes the formation of ethanol from pyruvate and allows the conversion of pyruvate to lactic acid as sole fermentation product (Porro *et al.* 1995). While this conversion is also redox-neutral, the requirement of 1 ATP per lactic acid for product export, via an as yet unidentified lactic acid transport mechanism, cancels out the ATP formed by substrate level phosphorylation (Van Maris *et al.* 2004b; Abbott *et al.* 2009a) (**Figure 1.5**). Therefore, enabling anaerobic production of lactic acid by *S. cerevisiae* requires a further genetic intervention, either resulting in increased free-energy (ATP) conservation or a reduction of the ATP requirement for product export.

1.6 Disaccharide uptake and cleavage and product export as targets for engineering free-energy conservation.

In addition to glucose and other six-carbon monosaccharides, most wild-type strains of *S. cerevisiae* can also use disaccharides such as sucrose and maltose as carbon sources. Maltose is an important sugar in beer brewing while sucrose, derived from sugar cane, sugar beets or from molasses, generated during refinery of sugar from these crops, is a tremendously important carbon source for large-scale industrial fermentation processes (Lodolo *et al.* 2008; Marques *et al.* 2015). Disaccharides provide an additional opportunity for engineering free-energy conservation because of the energy-rich bond between the associated monosaccharides. For example, the conversion of maltose to ethanol by *S. cerevisiae* leads to the formation of 3 ATP and proceeds via proton-coupled uptake followed by intracellular hydrolysis of maltose (**Figure 1.6B**), a process in which the free-energy of maltose hydrolysis ($\Delta G_0'$ = -15.5 kJ mol^{-1}) is not conserved (Van Leeuwen *et al.* 1992; Weusthuis *et al.* 1993; De Kok *et al.* 2011). Some organisms do not cleave maltose by hydrolysis but, instead, use a reaction catalysed by a maltose phosphorylase (MalP), in which inorganic phosphate and maltose are converted into glucose and glucose-1-phosphate (Doudoroff 1955). Glucose-1-phosphate can subsequently be converted into glucose-6-phosphate (an intermediate of the EMP pathway) by phosphoglucomutase, thus circumventing ATP-dependent hexokinase reaction and thereby increasing the ATP

yield of maltose catabolism by 1 ATP. It has been previously demonstrated that replacing maltose hydrolysis by this phosphorolytic pathway in *S. cerevisiae* increases free-energy conservation from maltose fermentation (De Kok *et al.* 2011) (**Figure 1.6C**). However, proton-coupled uptake of maltose by *S. cerevisiae* requires subsequent proton extrusion via the plasma membrane H^+-ATPase at the expense of 1 ATP per proton. Therefore, to fully benefit from disaccharide phosphorolysis, the proton-coupled transport system should be replaced by facilitated diffusion.

In addition to uptake and conversion of the substrate, also energy-requiring product export can be considered as a target for engineering free-energy conservation. For the model case of lactic acid production by engineered *S. cerevisiae*, export of the product comes with an energetic cost of 1 ATP per lactic acid (Van Maris *et al.* 2004b; Abbott *et al.* 2009a). ATP-driven export of lactic acid has the advantage of enabling high extracellular lactic acid concentrations while keeping the intracellular acid concentration low (Van Maris *et al.* 2004a). Replacement of the native lactic acid export system by energy-independent transport would not allow the build-up of such a steep concentration gradient over the plasma membrane and therefore necessitate *in situ* product removal for industrial production. Alternatively, an energy-dependent transport mechanism with a stoichiometry of >1 lactic acid per ATP has the potential to allow homofermentative lactic acid production at low pH to high final concentrations. Although thermodynamically feasible, examples of mechanisms with such an atypical fixed stoichiometry are not known. In order to replace the native lactic acid transport system, the responsible transporter genes will have to be removed. However, despite several attempts (see below), the genes involved in lactic acid export by *S. cerevisiae* have not yet been identified. Initial research efforts on engineering lactic acid export should therefore focus on the identification of the complete set of lactic acid transport genes in this yeast.

Various studies have found the proton-symporters Jen1 and (mutated) Ady2 to be responsible for uptake of lactic acid in *S. cerevisiae*. However, while the combined deletion of *JEN1* and *ADY2* was sufficient to completely abolish lactic acid uptake, excretion of lactic acid remained unaffected (De Kok *et al.* 2012b; Pacheco *et al.* 2012). In another study, a knock-out library of 4802 single gene deletion *S. cerevisiae* strains was transformed with a lactate dehydrogenase (*LDH*) gene and screened for the ability to produce lactic acid. Only the knock-out of one transporter lowered the final lactic acid titre by >90%, but the effect of this gene deletion (*PDR12*) was most likely an artefact created by the auxotrophic strain background (Bauer *et al.* 2003; Hirasawa *et al.* 2013). Since neither the combined knock out of *JEN1* and *ADY2* nor any of the single gene deletions eliminated or strongly reduced lactic acid export, it is likely that multiple genes are involved in this transport function. Identification of all lactic acid transport genes requires a careful evaluation of known yeast transporter genes for a potential role in lactic acid export followed by systematic gene deletions in a single strain.

1.7 Scope of this thesis

The objectives of the research described in this thesis were to implement methods and explore strategies for increasing free-energy conservation in the model organism *Saccharomyces cerevisiae*, with the ultimate goal to enable anaerobic, fermentative production of products whose current formation from substrate does not result in a net formation of ATP.

Replacement of sucrose hydrolysis by sucrose phosphorylase (SPase) has the potential to increase free-energy conservation of sucrose fermentation by 1 ATP (**Figure 6B and 6C**). Phosphorolysis of sucrose proceeds intracellularly and therefore competition by intracellular hydrolysis activity needs to be completely eliminated to achieve the highest energetic benefit. Additionally, intracellular phosphorolysis necessitates sucrose uptake, which proceeds via proton-coupled uptake in *S. cerevisiae*. Subsequent proton extrusion via the plasma membrane H^+-ATPase requires 1 ATP, completely negating the energetic benefit of sucrose phosphorylase. Therefore, to benefit from sucrose phosphorylase activity, the native *S. cerevisiae* proton-coupled transport system for sucrose also needs to be replaced by facilitated diffusion. A strain combining facilitated uptake and phosphorolytic cleavage of sucrose has a theoretical ATP yield of 5 ATP per sucrose (**Figure 6D**), or 2.5 ATP per hexose-equivalent, exceeding the efficiency of a wild type strain on both mono- and disaccharides. In *S. cerevisiae*, 5 native disaccharide transporters have been described and, next to invertase, at least 3 intracellular sucrose hydrolases are present. Therefore, replacement of the native sucrose transport and cleavage mechanism requires a minimum of 9 genetic interventions, which makes an efficient genetic modification tool essential.

The use of CRISPR/Cas9 for genetic modification was recently demonstrated in *S. cerevisiae*. A unique feature of the endonuclease Cas9 is that it can be directed to highly specific sequences in the genome by a "guide RNA" (gRNA) for the introduction of a double-strand DNA break. This characteristic provides the potential for simultaneous introduction of multiple gRNAs, facilitating multiple genetic interventions in a single transformation step. The goal of the research described in **Chapter 2** was to further develop the CRISPR/Cas9 system by providing a set of plasmids that can be easily modified for flexible gRNA expression in *S. cerevisiae*. Subsequently, the potential of this set of plasmids for making simultaneous genetic interventions was evaluated experimentally.

The availability of CRISPR/Cas9-based genome editing allowed for a thorough investigation of the native sucrose transport and cleavage genes. Identification and subsequent deletion of all genes involved in proton-coupled sucrose uptake and hydrolysis is crucial for engineering facilitated diffusion and phosphorolysis of sucrose. Therefore, the goal of **Chapter 3** was to determine if deletion of all known sucrose transporters and hydrolysis genes is sufficient to completely abolish both functions. To this end, a strain was constructed in which all genes described to be involved in sucrose

uptake and cleavage were deleted. This strain was then subjected to laboratory evolution for the identification of suppressor mutations and genes previously unknown to catalyse sucrose transport or hydrolysis.

The construction of a sucrose negative strain allowed for the implementation of facilitated diffusion of sucrose followed by phosphorolytic cleavage. In **Chapter 4**, a sucrose phosphorylase from *Leuconostoc mesenteroides* (*LmSPase*) was expressed in this platform strain. Then, to further increase the energetic efficiency and to exceed the efficiency of wild type *S. cerevisiae*, various sucrose facilitators from plants were screened for functional sucrose transport. Finally, the energetic efficiency of a strain expressing both *LmSPase* and a sucrose facilitator from *Phaseolus vulgaris* was analysed in anaerobic, sucrose-limited chemostat cultivation.

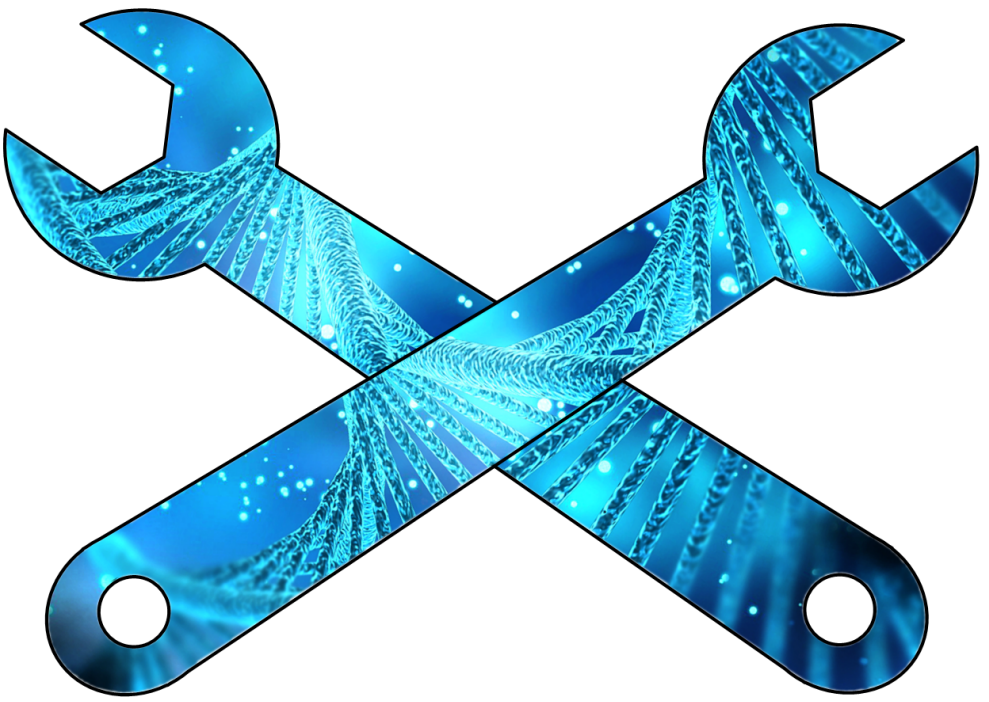
Engineering energy-dependent product export also has the potential to increase free-energy conservation. However, for some products produced by (engineered) *S. cerevisiae*, such as lactic acid, the exact mechanism and the genes involved in product export have not yet been identified. Therefore, the goal of **Chapter 5** was to investigate which genes are involved in lactic acid export in *S. cerevisiae*. First, based on available literature data, a set of 25 candidate transporter genes was defined, consisting of, for example, the mammalian monocarboxylate transporter homologues *MCH1-5*, the aqua(glycerol)prions *AQY1-3* and *YLL053c* and the known transporters involved in lactic acid uptake *JEN1* and *ADY2*. Then, the availability of a CRISPR/Cas9 based gene deletion approach (described in Chapter 2) allowed the deletion of each candidate gene in a single strain. Finally, the lactic acid export capacity of this strain was evaluated via expression of a lactate dehydrogenase (*LDH*) gene, which competes for pyruvate with alcoholic fermentation.

1.8 Acknowledgements

The image “Red Cane” which is featured on the title page of this chapter (page 15) was kindly supplied by David Gil and is licensed under CC BY 2.0.

Chapter 2

CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*



Robert Mans*, Harmen M. van Rossum*, Melanie Wijsman, Antoon Backx, Niels G.A. Kuijpers, Marcel van den Broek, Pascale Daran-Lapujade, Jack T. Pronk, Antonius J.A. van Maris and Jean-Marc G. Daran

*Joint first authorship

Abstract

A variety of techniques for strain engineering in *Saccharomyces cerevisiae* have recently been developed. However, especially when multiple genetic manipulations are required, strain construction is still a time-consuming process. This study describes new CRISPR/Cas9-based approaches for easy, fast strain construction in yeast and explores their potential for simultaneous introduction of multiple genetic modifications. An open-source tool (http://yeastriction.tnw.tudelft.nl) is presented for identification of suitable Cas9 target sites in *S. cerevisiae* strains. A transformation strategy, using *in vivo* assembly of a guideRNA plasmid and subsequent genetic modification, was successfully implemented with high accuracies. An alternative strategy, using *in vitro* assembled plasmids containing two gRNAs, was used to simultaneously introduce up to six genetic modifications in a single transformation step with high efficiencies. Where previous studies mainly focused on the use of CRISPR/Cas9 for gene inactivation, we demonstrate the versatility of CRISPR/Cas9-based engineering of yeast by achieving simultaneous integration of a multigene construct combined with gene deletion and the simultaneous introduction of two single-nucleotide mutations at different loci. Sets of standardized plasmids, as well as the web-based Yeastriction target-sequence identifier and primer-design tool, are made available to the yeast research community to facilitate fast, standardized and efficient application of the CRISPR/Cas9 system.

2.1 Introduction

For decades, *Saccharomyces cerevisiae* has been successfully used as a model organism to decipher biological processes in higher eukaryotes (Botstein and Fink 2011) and as a popular metabolic engineering platform (Nielsen *et al.* 2013). Expression and optimization of heterologous product pathways in *S. cerevisiae* (see e.g. Paddon *et al.*, 2013; Beekwilder *et al.*, 2014), requires introduction of multiple (successive) genetic modifications, including integration of product pathway genes at multiple genetic loci and rewiring central metabolism by modifying properties of specific metabolic reactions (e.g. via gene deletion, changing regulatory properties or replacement of native genes by heterologous counterparts) (Wieczorke *et al.* 1999; Ro *et al.* 2006). Introduction of the required genetic modifications has so far remained a time-consuming and labour-intensive process, as each individual alteration requires a cycle of transformation, selection and confirmation. Furthermore, since each modification is accompanied by the integration of a selection marker gene, the maximum number of sequential modifications may be limited by selection marker availability. This limitation stimulated extensive research into the identification of novel genetic markers for *S. cerevisiae* (Chee and Haase 2012; Solis-Escalante *et al.* 2013; Siewers 2014). Additionally, multiple strategies for the recycling of genetic markers have been developed, such as homologous- recombination-mediated counter selection (gene "loop-out") and use of recombinases such as the Cre/*loxP* or 2µm-plasmid-based Flp/FRT methods (Güldener *et al.* 1996; Storici, Coglievina and Bruschi 1999; Hegemann and Heick 2011). These recombinase-based methods leave a copy of a repeat sequence (e.g. *loxP* or FRT site) in the genome, which leads to genome instability after multiple, repeated rounds of marker recovery (Delneri *et al.* 2000; Solis-Escalante *et al.* 2015). 'Scarless' removal of counter-selectable markers has been made possible via the *delitto perfetto* method (Storici *et al.*, 2001), while a recently reported marker-recovery method based on generation of I-SceI-induced double-stranded breaks even allows simultaneous, seamless removal of multiple markers (Solis-Escalante *et al.*, 2014). While these methods largely eliminate limitations by marker-gene availability, substitution of target genes by marker cassettes remained a time-consuming process, due to the absence of robust methods for simultaneous introduction of multiple genetic modifications in a single transformation step. Alternative methods such as meganucleases, zinc finger nucleases (ZFNs) (Urnov *et al.* 2010; Carroll 2011) and transcription activator-like effector nucleases (TALENs) (Christian *et al.* 2010; Miller *et al.* 2011; Mussolino *et al.* 2011) utilize double-stranded DNA breaks (DSBs) for site-directed genome editing. Due to the lethal nature of DSBs in yeast, these methods could theoretically be used for marker-free modifications. However, for each genetic modification a new ZFN or TALEN protein has to be designed and generated.

Bacteria have developed several systems to degrade foreign DNA. Very quickly after their discovery, restriction enzymes became the "workhorses of molecular biology"

(reviewed by Roberts, 2005). Another prokaryotic immune mechanism, consisting of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) systems was discovered in 2007 (Barrangou *et al.* 2007; Brouns *et al.* 2008; Marraffini and Sontheimer 2008). To function *in vivo*, the type-II bacterial CRISPR system of *Streptococcus pyogenes* requires the Cas9 nuclease and the RNA complex that guides it to a specific sequence of the (foreign) DNA. This RNA complex generally consists of two RNA molecules; the CRISPR RNA (crRNA) and the trans-activating CRISPR RNA (tracrRNA). The crRNA contains the 20-30 bp target sequence and a sequence that binds to the tracrRNA, resulting in a duplex RNA complex, recognized by the Cas9 nuclease. When directly after the target sequence, a proper protospacer adjacent motif (PAM) is present (in case of the *S. pyogenes* Cas9 this sequence is NGG), Cas9 will bind to and restrict the target sequence of the (invading) DNA (Deltcheva *et al.* 2011). A unique feature of this system is the RNA-dependence for targeting of the nuclease Cas9, which makes selective targeting of any locus for the introduction of DSBs possible. Since its discovery, Cas9 based systems have been used for the construction of (multiplexed) genetic modifications in a variety of organisms, including human pluripotent stem cells (González *et al.* 2014), zebrafish (Hwang *et al.* 2013), plants (Feng *et al.* 2013), fly (Gratz *et al.* 2013) and mice (Wang *et al.* 2013) (for a more extensive list see (Hsu, Lander and Zhang 2014)).

In 2013, DiCarlo and coworkers employed the CRISPR/Cas9 system for the introduction of DSBs in *S. cerevisiae* (DiCarlo *et al.* 2013b). In a strain expressing a plasmid-borne *cas9* gene from *S. pyogenes*, a second plasmid was introduced, containing the *SNR52* promoter followed by a sequence encoding for a chimeric crRNA-tracrRNA, or guide-RNA (gRNA) with a 20 bp targeting sequence for *CAN1*. The gRNA was recognized by the Cas9 protein, resulting in a double-strand break at the *CAN1* locus. Subsequently, this otherwise lethal break was repaired by the yeast homologous-recombination machinery, using a co-transformed repair fragment that bridged the flanking regions of the break. Since the repair fragment was designed to introduce a premature stop-codon, introduction and repair of a DSB resulted in colonies that were resistant to canavanine. It has recently been shown that the CRISPR/Cas9 system can be used for making up to three simultaneous gene deletions in yeast (Bao *et al.* 2015).

The goal of the present study is to explore the use of CRISPR/Cas9 for, standardized (multiplexed) construction of gene deletions, multi-pathway integrations and site-directed single-nucleotide mutagenesis. To this end, we present a web-based CRISPR tool to facilitate selection of suitable targets and to design the primers necessary for construction of plasmids that express specific gRNAs. Furthermore, we report on the construction of standardized plasmids for expression of one or two gRNAs and explore their use for multiplexed gene deletions, both alone and in combination with multigene chromosomal integrations and/or with the introduction of single-nucleotide changes.

2.2 Methods

Strains, growth conditions and storage

The *S. cerevisiae* strains used in this study (**Table 3.1**) share the CEN.PK genetic background (Entian and Kötter 2007; Nijkamp *et al.* 2012). Shake flask cultures were grown at 30°C in 500 mL flasks containing 100 mL synthetic medium (SM) (Verduyn *et al.* 1992) with 20 $g{\cdot}L^{-1}$ glucose in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ) set at 200 rpm. When required, auxotrophic requirements were complemented via addition of 150 $mg{\cdot}L^{-1}$ uracil, 100 $mg{\cdot}L^{-1}$ histidine, 500 $mg{\cdot}L^{-1}$ leucine, 75 $mg{\cdot}L^{-1}$ tryptophan (Pronk 2002) or by growth in YP medium (demineralized water, 10 $g{\cdot}L^{-1}$ Bacto yeast extract, 20 $g{\cdot}L^{-1}$ Bacto peptone). As a carbon source, 20 $g{\cdot}L^{-1}$ glucose was used. Frozen stocks were prepared by addition of glycerol (30% v/v) to exponentially growing shake-flask cultures of *S. cerevisiae* and overnight cultures of *E. coli* and stored aseptically in 1 mL aliquots at -80 °C.

Plasmid construction

Construction of the single gRNA plasmid series (pMEL10 – pMEL17)

The single gRNA plasmids (pMEL10 – pMEL17) were constructed via Gibson assembly (New England Biolabs, Beverly, MA) of a marker cassette with one fragment containing both the gRNA *CAN1*.Y (DiCarlo *et al.* 2013b) and the 2µm replication sequence. This fragment was obtained by PCR from plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t, using primers 6845 & 6846 (Table S1). The various marker cassettes were PCR amplified from plasmid templates pUG72, pUG-amdSYM, pUG-hphNT1, pUG6, pUG73 and pUG-natNT2 with primers 3093 & 3096 resulting in the *KlURA3, amdSYM, hphNT1, kanMX, KlLEU2* and *natNT2* cassettes, respectively. The *HIS3* and *TRP1* cassettes were obtained by PCR with primers 6847 & 6848 on plasmid templates pRS423 and pRS424. Assembly of the single gRNA plasmids was done by combining the appropriate marker cassette with the backbone containing the gRNA *CAN1*.Y and 2µm sequences in a Gibson assembly reaction, following the manufacturer's recommendations. For each single gRNA plasmid (pMEL10 – pMEL17), an *E. coli* clone containing the correctly assembled plasmid (confirmed by restriction analysis) was selected, stocked and deposited at EUROSCARF.

Construction of the double gRNA plasmid series (pROS10 – pROS17)

To construct the double gRNA plasmids (pROS10 – pROS17), an intermediate plasmid was first constructed, carrying two gRNA cassettes that both targeted *CAN1*.Y (DiCarlo *et al.* 2013b). This intermediate plasmid was assembled out of four different overlapping fragments: the two gRNA cassettes overlapping with each other in the 2µm replicon, one *ScURA3* marker cassette and a cassette containing all sequences for amplification in *E. coli*. The gRNA cassettes were obtained in a two-step PCR approach. First a 2µm fragment was obtained from pUD194 (**Table 2.2**) with primers 3289 & 4692 and two

Table 2.1: *Saccharomyces cerevisiae* strains used in this study.

Name (Accession no.)	Relevant genotype	Parental strain	Origin
CEN.PK113-7D	*MAT*a *URA3 TRP1 LEU2 HIS3*		P. Kötter
CEN.PK113-5D	*MAT*a *ura3-52 TRP1 LEU2 HIS3*		P. Kötter
CEN.PK122	*MAT*a/*MAT*α *URA3/URA3 TRP1/TRP1 LEU2/LEU2 HIS3/HIS3*		P. Kötter
CEN.PK2-1C	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ*		P. Kötter
CEN.PK115	*MAT*a/*MAT*α *ura3-52/ura3-52 TRP1/TRP1 LEU2/LEU2 HIS3/HIS3*		P. Kötter
IMX585 (Y40592)*	*MAT*a *can1Δ::cas9-natNT2 URA3 TRP1 LEU2 HIS3*	CEN.PK113-7D	This study
IMX581 (Y40593)*	*MAT*a *ura3-52 can1Δ::cas9-natNT2 TRP1 LEU2 HIS3*	CEN.PK113-5D	This study
IMX664 (Y40594)*	*MAT*a/*MAT*α *CAN1/can1Δ::cas9-natNT2 URA3/URA3 TRP1/TRP1 LEU2/LEU2 HIS3/HIS3*	CEN.PK122	This study
IMX672 (Y40595)*	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2*	CEN.PK2-1C	This study
IMX673 (Y40596)*	*MAT*a/*MAT*α *ura3-52/ ura3-52 CAN1/can1Δ::cas9-natNT2 TRP1/ TRP1 LEU2/LEU2 HIS3/HIS3*	CEN.PK115	This study
IMX711	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ* pMEL10-gRNA-*MCH1*	IMX672	This study
IMX712	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch2Δ* pMEL10-gRNA-*MCH2*	IMX672	This study
IMX713	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch5Δ* pMEL10-gRNA-*MCH5*	IMX672	This study
IMX714	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch5Δ pMEL10*-gRNA-*MCH1* pMEL10-gRNA-*MCH5*	IMX672	This study
IMX715	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 itr1Δ pdr12Δ* pUDR005	IMX672	This study
IMX716	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ itr1Δ pdr12Δ* pUDR002 pUDR005	IMX672	This study
IMX717	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ* pUDR002 pUDR004 pUDR005	IMX672	This study
IMX718	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2* $GET4^{G315C}$ $NAT1^{C1139G}$ pUDR020	IMX672	This study
IMX719	*MAT*a *can1Δ::cas9-natNT2 URA3 TRP1 LEU2 HIS3 acs1Δ acs2Δ::(pADH1-aceF-tPGI1 pPGI1-lplA2-tPYK1 pPGK1-lplA-tPMA1 pTDH3-pdhB-tCYC1 pTEF1-lpd-tADH1 pTPI1-pdhA-tTEF1)*	IMX585	This study

*These strains have been deposited at euroscarf

different gRNA cassettes were PCR amplified from p426-SNR52p-gRNA.CAN1.Y-SUP4t with primers 5972 & 5976 for the first cassette and 5977 & 5973 for the second cassette. Each gRNA cassette was separately pooled with the 2µm fragment and in a second PCR reaction the gRNA cassettes were extended with either the 5' or 3' halve of the 2µm fragment, resulting in two different gRNA cassettes, overlapping in the 2µm sequence, by using primer pair 5975 & 4068 for the first and 5974 & 3841 for the second fragment. The marker fragment containing *ScURA3* was obtained from pUD192 with primers 3847 & 3276 (**Table 2.2**) and the fragment containing all sequences for amplification in *E.coli* was PCR amplified from pUD195 (**Table 2.2**) with primers 3274 & 3275. Using Gibson assembly, the four overlapping fragments were assembled into the intermediate plasmid pUDE330. To obtain pROS10, pUDE330 was linearized by PCR-amplification of the backbone, excluding the gRNA fragments, and co-transformed with two gRNA cassettes for *in vivo* assembly by homologous recombination in yeast (Kuijpers *et al.*

2013b). For linearizing the backbone, a single primer was used (5793) and the gRNA fragments were obtained by PCR from pUDE330 with primers 6008 & 5975 and 6007 & 5974. The plasmid was extracted from yeast and transformed into *E. coli* for storage and plasmid propagation. The other double gRNA plasmids were assembled by the Gibson assembly method with a marker cassette and the pROS10 plasmid backbone fragment. This backbone fragment was obtained by linearization of pROS10 with restriction enzymes PvuII and NotI. The various marker cassettes were PCR amplified from plasmid templates pUG-amdSYM, pUG-hphNT1, pUG6, pUG73 and pUG-natNT2 with primers 3093 & 3096 resulting in the *amdSYM, hphNT1, kanMX, KlLEU2* and *natNT2* cassettes, respectively. The *HIS3* and *TRP1* cassettes were obtained by PCR with primers 6847 & 6848 on plasmid templates pRS423 and pRS424. Combining the appropriate marker cassette with the pROS10 backbone fragment in a Gibson assembly reaction, following the manufacturer's recommendations, resulted in pROS11 – pROS17. For each of these double gRNA plasmids, an *E. coli* clone containing the correctly assembled plasmid was selected, stocked and deposited at EUROSCARF.

Strain construction

S. cerevisiae strains were transformed according to Gietz *et al.* (Gietz and Woods 2002). Mutants were selected on solid YP medium (demineralized water, 10 g·L^{-1} Bacto yeast extract, 20 g·L^{-1} Bacto peptone, 2% (w/v) agar), supplemented with 200 mg·L^{-1} G418, 200 mg·L^{-1} hygromycin B or 100 mg·L^{-1} nourseothricin (for dominant markers) or on synthetic medium supplemented with appropriate auxotrophic requirements (Verduyn *et al.* 1992). In all cases, gene deletions and integrations were confirmed by colony PCR on randomly picked colonies, using the diagnostic primers listed in Table S1. Integration of *cas9* into the genome was achieved via assembly and integration of two cassettes containing *cas9* and the *natNT2* marker into the *CAN1* locus. The *cas9* cassette was obtained by PCR from p414-TEF1p-cas9-CYC1t (DiCarlo *et al.* 2013b), using primers 2873 & 4653. The *natNT2* cassette was PCR amplified from pUG-natNT2 with primers 3093 & 5542. 2.5 µg *cas9* and 800 ng *natNT2* cassette were pooled and used for each transformation. Correct integration was verified by colony PCR (Supplemental information) using the primers given in Table S1, the resulting strains have been deposited at EUROSCARF. IMX719 was constructed by co-transformation of pUDR022 (see below) with genes required for functional *Enterococcus faecalis* PDH expression (Kozak *et al.* 2014b). The gene cassettes were obtained by PCR using plasmids pUD301 — pUD306 as template (**Table 3.2**) with the primers indicated in Table S1 and the *ACS1* dsDNA repair fragment, obtained by annealing two complementary single stranded oligos (6422 & 6423). After confirmation of the relevant genotype (**Figure 2.4B**), the pUDR022 plasmid was removed as explained in Supplemental information.

Single gRNA method (cloning free)

The yeast homologous recombination (HR) machinery was used to assemble plasmids

Table 2.2: Plasmids used in this study.

Name (Accession no.)	Relevant characteristics	Origin
pUG6	Template for A-*kanMX*-B† cassette	(Gueldener *et al.* 2002)
pUG72	Template for A-*KlURA3*-B cassette	(Gueldener *et al.* 2002)
pUG73	Template for A-*KlLEU2*-B cassette	(Gueldener *et al.* 2002)
pUG-hphNT1	Template for A-*hphNT1*-B cassette	(De Kok *et al.* 2011)
pUG-natNT2	Template for A-*natNT2*-B cassette	(De Kok *et al.* 2012b)
pUG-amdSYM	Template for A-*amdSYM*-B cassette	(Solis-Escalante *et al.* 2013)
pRS423	Template for A-*ScHIS3*-B cassette	(Christianson *et al.* 1992)
pRS424	Template for A-*ScTRP1*-B cassette	(Christianson *et al.* 1992)
p414-TEF1p-Cas9-CYC1t	*CEN6/ARS4* ampR *TRP1 pTEF1-cas9-tCYC1*	(DiCarlo *et al.* 2013b)
p426-SNR52p-gRNA.CAN1.Y-SUP4t	2µm ampR *URA3* gRNA-*CAN1*.Y	(DiCarlo *et al.* 2013b)
pUD192	pUC57 + *ScURA3*	(Kozak et al. 2014a)
pUD194	pUC57 + 2µm	(Kozak et al. 2014a)
pUD195	pUC57 + pMB1 + ampR	(Kozak et al. 2014a)
pUD301	pUC57 + *pTPI1-pdhA E. faecalis-tTEF1*	(Kozak et al. 2014a)
pUD302	pUC57 + *pTDH3-pdhB E. faecalis -tCYC1*	(Kozak et al. 2014a)
pUD303	pUC57 + *pADH1-aceF E. faecalis -tPGI1*	(Kozak et al. 2014a)
pUD304	pUC57 + *pTEF1-lpd E. faecalis -tADH1*	(Kozak et al. 2014a)
pUD305	pUC57 + *pPGK1-lplA E. faecalis -tPMA1*	(Kozak et al. 2014a)
pUD306	pUC57 + *pPGI1-lplA2 E. faecalis -tPYK1*	(Kozak et al. 2014a)
pUDE330	2µm ampR *ScURA3* gRNA-*CAN1*.Y [2x]	This study
pMEL10 (P30779)*	2µm ampR *KURA3* gRNA-*CAN1*.Y	This study
pMEL11 (P30780)*	2µm ampR *amdSYM* gRNA-*CAN1*.Y	This study
pMEL12 (P30781)*	2µm ampR *hphNT1* gRNA-*CAN1*.Y	This study
pMEL13 (P30782)*	2µm ampR *kanMX* gRNA-*CAN1*.Y	This study
pMEL14 (P30783)*	2µm ampR *KlLEU2* gRNA-*CAN1*.Y	This study
pMEL15 (P30784)*	2µm ampR *natNT2* gRNA-*CAN1*.Y	This study
pMEL16 (P30785)*	2µm ampR *HIS3* gRNA-*CAN1*.Y	This study
pMEL17 (P30786)*	2µm ampR *TRP1* gRNA-*CAN1*.Y	This study
pROS10 (P30787)*	2µm ampR *ScURA3* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	This study
pROS11 (P30788)*	2µm ampR *amdSYM* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	This study
pROS12 (P30789)*	2µm ampR *hphNT1* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	This study
pROS13 (P30790)*	2µm ampR *kanMX* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	This study
pROS14 (P30791)*	2µm ampR *KlLEU2* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	This study
pROS15 (P30792)*	2µm ampR *natNT2* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	This study
pROS16 (P30793)*	2µm ampR *HIS3* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	This study
pROS17 (P30794)*	2µm ampR *TRP1* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	This study
pUDR002	2µm ampR *TRP1* gRNA-*MCH1* gRNA-*MCH2*	This study
pUDR004	2µm ampR *HIS3* gRNA-*MCH5* gRNA-*AQY1*	This study
pUDR005	2µm ampR *ScURA3* gRNA-*ITR1* gRNA-*PDR12*	This study
pUDR020	2µm ampR *ScURA3* gRNA-*NAT1* gRNA-*GET4*	This study
pUDR022	2µm ampR *kanMX* gRNA-*ACS1* gRNA-*ACS2*	This study

† A and B refer to 60 bp tags that are incorporated via PCR, enabling homologous recombination.
* These plasmids have been deposited at EUROSCARF

with specific gRNA sequences out of two different fragments: a plasmid backbone and a gRNA target sequence. Depending on the preferred selectable marker, the linearized plasmid backbone was obtained via PCR using the appropriate single gRNA plasmid (pMEL10 – pMEL17) as a template, with primers 6005 & 6006. To obtain the double stranded gRNA cassettes (the target sequences are listed in **Table 2.3**), two complementary single stranded oligos (**Table S2.1**) were mixed in a 1:1 molar ratio, heated to 95 °C and then cooled down to room temperature. The resulting gRNA fragments contained the 20 bp gRNA recognition sequences, flanked by 50 bp overlaps with the

Table 2.3: Target sequences used in this study

Locus	Sequence (including PAM)	Restriction site	AT score	RNA score
MCH1	TATTGGCAATAAACATCTCGAGG		0.65	0.50
MCH2	ATCTCGATCGAGGTGCCTGATGG	PvuI	0.45	0.30
MCH5	ACTCTTCCGTTTTAGATATCTGG	EcoRV	0.65	0.50
AQY1	ACCATCGCTTTAAAATCTCTAGG	DraI	0.65	0.50
ITR1	ATACATCAACGAATTCCAACCGG	EcoRI	0.65	0.60
PDR12	GCATTTTCGGTACCTAACTCCGG	KpnI	0.55	0.65
NAT1	AAAGGAATTGGATCCTGCGTAGG	BamHI	0.55	0.60
GET4	GGGCTCGCTAGGATCCAATTCGG	BamHI	0.45	0.50
ACS1	TTCTTCACAGCTGGAGACATTGG	PvuII	0.55	0.45
ACS2	TCCTTGCCGTTAAATCACCATGG		0.55	0.75

linearized plasmid backbone. The 120 bp repair fragments were obtained by following the same procedure and were identical to the up- and downstream regions of the DSB break, allowing for effective repair by the HR-machinery. For each transformation, a linearized plasmid backbone, a double stranded cassette containing the gRNA sequence of choice and the double stranded DNA cassette for repair of the DSB were pooled and co-transformed to the appropriate strain.

Double gRNA method

Plasmids with two gRNAs were assembled *in vitro*, using Gibson assembly of a 2µm fragment containing the two gRNA sequences and a double gRNA (pROS10 – pROS17) plasmid backbone. The 2µm fragment was obtained via PCR, using pROS10 as a template with two primers containing the 20 bp gRNA recognition sequences and a 50 bp sequence, homologous to the linearized plasmid backbone (Table S1). The linearized plasmid backbone was obtained via PCR using one of the double gRNA plasmids (pROS10 – pROS17, depending on the preferred selective marker) as a template with a single primer (6005), binding at each of the two *SNR52* promoters (Supplemental information). The two fragments were combined using Gibson assembly, followed by transformation to *E. coli* for storage and plasmid propagation. Since both of the gRNA containing primers could bind on either side of the 2µm fragment, it was important to check that the final plasmid contained one copy of each gRNA (theoretically this would be the case in 50% of the *E. coli* transformants). To simplify this confirmation step, the gRNA target sequences were selected for the presence of a restriction site. Alternatively, diagnostic primers specific for the introduced 20 bp recognition sequences were used for the identification of correctly assembled gRNA plasmids using PCR (see Protocol in Supplemental information).

To construct the plasmid pUDR002 (targeting *MCH1&MCH2*, *TRP1* marker), the 2µm fragment was amplified using DreamTaq (Fisher Scientific) from pROS10 using primers 6835 & 6837 (Supplemental information). The backbone of pROS17 was amplified using the Phusion polymerase (Fisher Scientific) with a single primer 6005 (Supplemental information). The two fragments were assembled using Gibson assembly and confirmed via restriction analysis. Similarly, the following plasmids were

constructed: pUDR004 (targeting *MCH5&AQY1, HIS3* marker), pUDR005 (targeting *ITR1&PDR12, KlURA3* marker), pUDR020 (targeting *NAT1*&*GET4, URA3* marker), pUDR022 (targeting *ACS1*&*ACS2*, *kanMX* marker). Transformations using the double gRNA method required co-transformation of 2 µg of (each) pUDR plasmid together with 1 µg of (each) corresponding double stranded DNA cassette for DSB repair.

Molecular biology techniques

PCR amplification with Phusion® Hot Start II High Fidelity Polymerase (Thermo Fisher Scientific) was performed 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 to confirm successful deletions by one of the two described methods can be found in Table S1. DNA fragments obtained by PCR were separated by gel electrophoresis on 1% (w/v) agarose gels (Thermo Fisher Scientific) in TAE buffer (Thermo Fisher Scientific) at 100V for 30 minutes. Fragments were excised from gel and purified by gel purification (Zymoclean™, D2004, Zymo Research, Irvine, CA). Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich) according to the supplier's manual. Yeast plasmids were isolated with Zymoprep Yeast Plamid Miniprep II Kit (Zymo Research). *E. coli* DH5α (18258-012, Life Technologies) was used for chemical transformation (T3001, Zymo Research) or for electroporation. Chemical transformation 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 DH5α cells were prepared according to BioRad's protocol, except for the use of LB medium without NaCl when pre-growing the cells.

Yeastriction webtool

The tool is written in Javascript and based on the MEAN.io stack (MongoDB, Express, AngularJS and Node.js). The source code is available at https://github.com/hillstub/Yeastriction. Genome and ORF sequences were downloaded from SGD (http://www.yeastgenome.org) in GFF and FASTA file format, respectively. ORFs, including their 1kb up- and downstream sequences were extracted and imported into Yeastriction, with the aid of an in-house script.

Yeastriction extracts all possible Cas9 target sequences (20 basepairs followed by NGG) from a specified ORF and from its complementary strand. Subsequently, sequences containing 6 or more Ts are discarded as this can terminate transcription (Braglia, Percudani and Dieci 2005; Wang and Wang 2008). Target sequences are then tested for off-targets (an off-target is defined as a sequence with either the NGG or NAG PAM sequence and 17 or more nucleotides identical to the original 20 bp target sequence (Hsu *et al.* 2013)) by matching the sequences against the reference genome using Bowtie

(version 1) (Langmead *et al.* 2009). If any off-target is found the original target sequence is discarded. In a next step, the AT content is calculated for the target sequence. Using the RNAfold library (essentially with the parameters --MEA --noLP –temp=30.) (Lorenz *et al.* 2011) the maximum expected accuracy structure of each RNA molecule is calculated. The target sequence is also searched for the presence of restriction sites based on a default list or a user-defined list. The targets can be ranked based on presence of restriction sites (1 for containing and 0 for lacking a restriction site), AT content (1 having the highest AT-content and 0 for the lowest AT-content) and secondary structure (1 having the lowest amount of pairing nucleotides and 0 for the highest number of nucleotides involved in secondary structures (indicated by brackets)). The range for every parameter is determined per locus and used to normalize the values. Subsequently, the target sequences are ranked by summation of the score for each parameter. These ranking scores should only be used to order the targets from a single locus and not to compare targets for different loci. The application can be accessed at the following URL: http://yeastriction.tnw.tudelft.nl/.

2.3 Results and discussion

Yeastriction: a CRISPR design tool

To streamline design and construction of CRISPR/Cas9 gRNA plasmids for introduction of (multiple) genetic modifications, the Yeastriction webtool (http://yeastriction.tnw.tudelft.nl) was developed. This webtool was designed to be compatible with the single and double gRNA plasmid series (pMEL10 – pMEL17 and pROS10 – pROS17, respectively), as described below. Because the CRISPR/Cas9 system can be highly sequence specific, it is crucially important to select target sequences based on correct reference genome sequence information. A difference of a single nucleotide in the gRNA, as compared to the genomic target sequence, can already completely abolish Cas9 nuclease activity (Hsu *et al.* 2013). To make Yeastriction useful for the entire yeast community, a set of 33 *S. cerevisiae* genomes (www.yeastgenome.org) was implemented. In the first step, the user can select the desired reference genome and enter (multiple) systematic names (e.g. YDL054C, YKL221W) or gene names (e.g. *MCH1*, *MCH2*). For each entered gene, the tool matches every potential target sequence to the selected reference genome. If there are potential off-targets (other sequences present in the same genome with either NAG or NGG as PAM sequence and with a 0 – 3 nucleotide difference in the 20 bp target sequence (Hsu *et al.* 2013)) the target sequence is discarded. Sequences that contain 6 or more consecutive Ts are also discarded as they may cause transcript termination (Braglia, Percudani and Dieci 2005; Wang and Wang 2008). A recent report indicates that the AT content of Cas9 target sequences should preferably be above 65% (Lin *et al.* 2014b). Furthermore, there are indications that target sequences without obvious nucleotide interactions in secondary structures are more efficient (Zhang 2014). The presence of unique restriction sites within the target sequence simplifies verification of

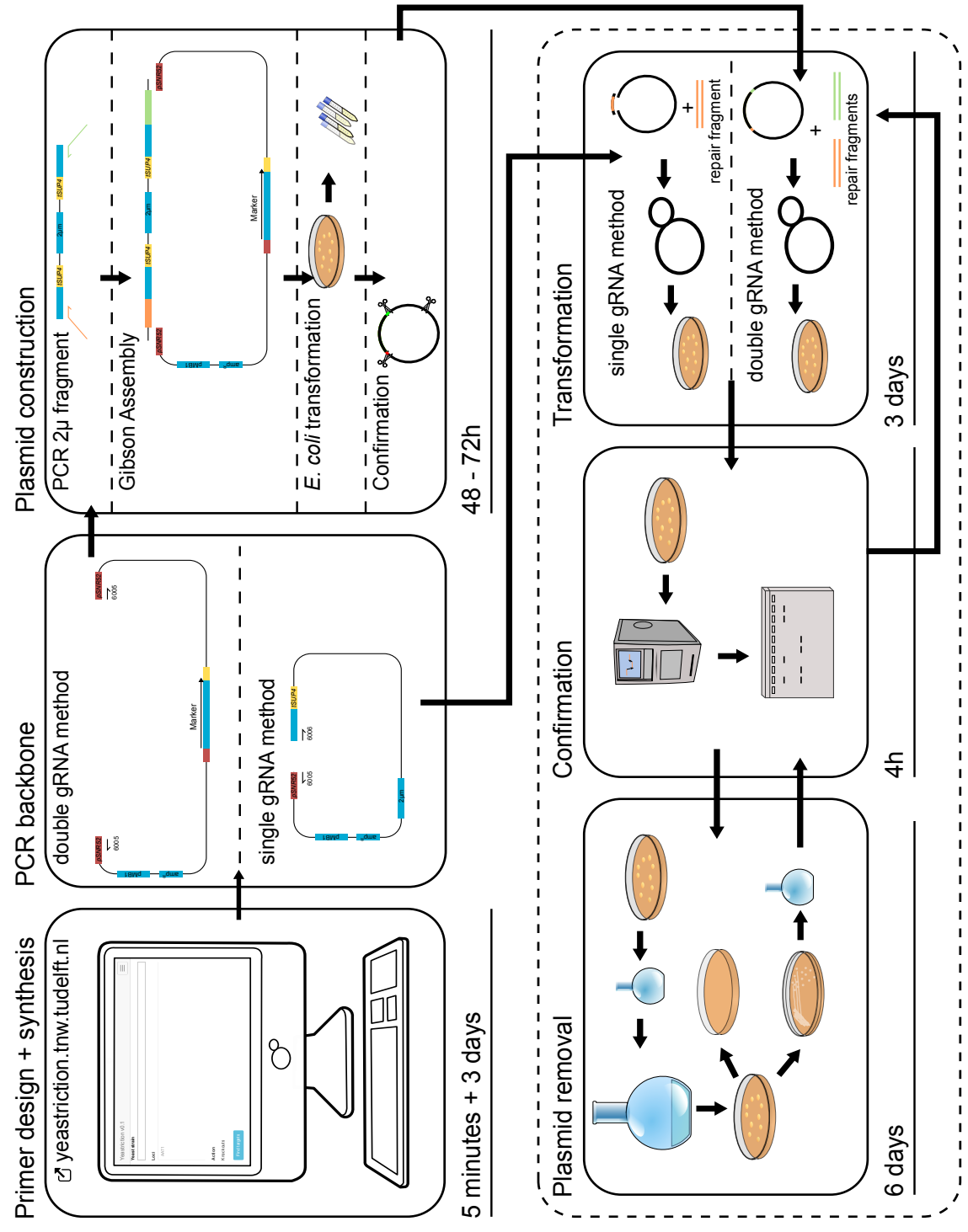
correct plasmid assembly. Yeastriction therefore ranks potential Cas9 target sequences according to AT-content, secondary structures and the presence of restriction sites. To increase flexibility, the user can also choose to leave out one of the parameters in the final ranking. For the top-ranked target sequence, the tool automatically designs the oligonucleotide primers required for plasmid construction and the oligonucleotides (which can be ordered as primers) needed to form the repair fragment when a gene deletion is desired. To increase flexibility, the user can also choose another target site than the top-ranked sequence (Supplemental information). In comparison with existing tools such as ChopChop (Montague *et al.* 2014), Yeastriction combines several features to improve gRNA sequence selection for *S. cerevisiae*: i) yeast strain specificity, ii) elimination of spacer sequences with potential off-targets, iii) ranking of the remaining gRNA sequences based on RNA structure and AT content and iv) direct generation of primer sequences, compatible with the transformation methods described below.

Construction of a set of plasmids for transformation with one or two gRNAs

DiCarlo and co-workers (DiCarlo *et al.* 2013b) described construction of plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t (Addgene www.addgene.org/43803/) for expression of a single gRNA in yeast, using the *SNR52* promoter and *SUP4* terminator. We hypothesized that the gRNA could be easily changed by *in vivo* homologous recombination via co-transformation of the linearized p426-SNR52p-gRNA.CAN1.Y-SUP4t backbone (from which the 20 bp recognition sequence "*CAN1*.Y" was omitted) together with a new 20 bp gRNA fragment, flanked by 50 bp overlaps with the plasmid backbone. In order to further increase the flexibility of this system, and to allow its use with genetic markers other than *URA3*, a standardized set of plasmids was constructed (**Figure 2.1B**). To this end, a linearized plasmid backbone of the p426-SNR52p-gRNA.CAN1.Y-SUP4t plasmid that excluded the *URA3* marker gene, was obtained by PCR. Subsequently, eight different genetic markers (*KlURA3, amdSYM, hphNT1, kanMX, KlLEU2, natNT2, HIS3* and *TRP1*) were PCR amplified and (re-)introduced in this backbone using Gibson assembly. The resulting plasmids were named pMEL10 to pMEL17 (**Figure 2.1B**, **Table 2.2**).

Use of *in vivo* recombination enabled the introduction of one gRNA sequence per single gRNA plasmid backbone (pMEL10 – pMEL17), without the need of prior cloning. When multiple genetic modifications are required, marker availability might become a limitation. In this case, plasmids containing multiple gRNA sequences would be preferred as this allows introduction of more genetic modifications before plasmid removal is required. For this purpose, a second set of plasmids was constructed that contained 2 gRNA sequences, between separate promoters and terminators.

First pROS10 was constructed (**Figure 2.1C**), containing the *ScURA3* marker, ampR for selection in *E. coli* and a 2µm fragment flanked by two p*SNR52*-gRNA-t*SUP4* cassettes in opposite directions, with one of these cassettes containing the target sequence *CAN1*.Y and the other *ADE2*.Y (DiCarlo *et al.* 2013b). This design enables construction of


A
Primer design + synthesis
yeastriction.tnw.tudelft.nl
5 minutes + 3 days
PCR backbone
double gRNA method
Marker
single gRNA method
48 - 72h
Plasmid construction
PCR 2µ fragment
Gibson Assembly
Marker
E. coli transformation
Confirmation
Plasmid removal
6 days
Confirmation
4h
Transformation
single gRNA method
repair fragment
double gRNA method
repair fragments
3 days

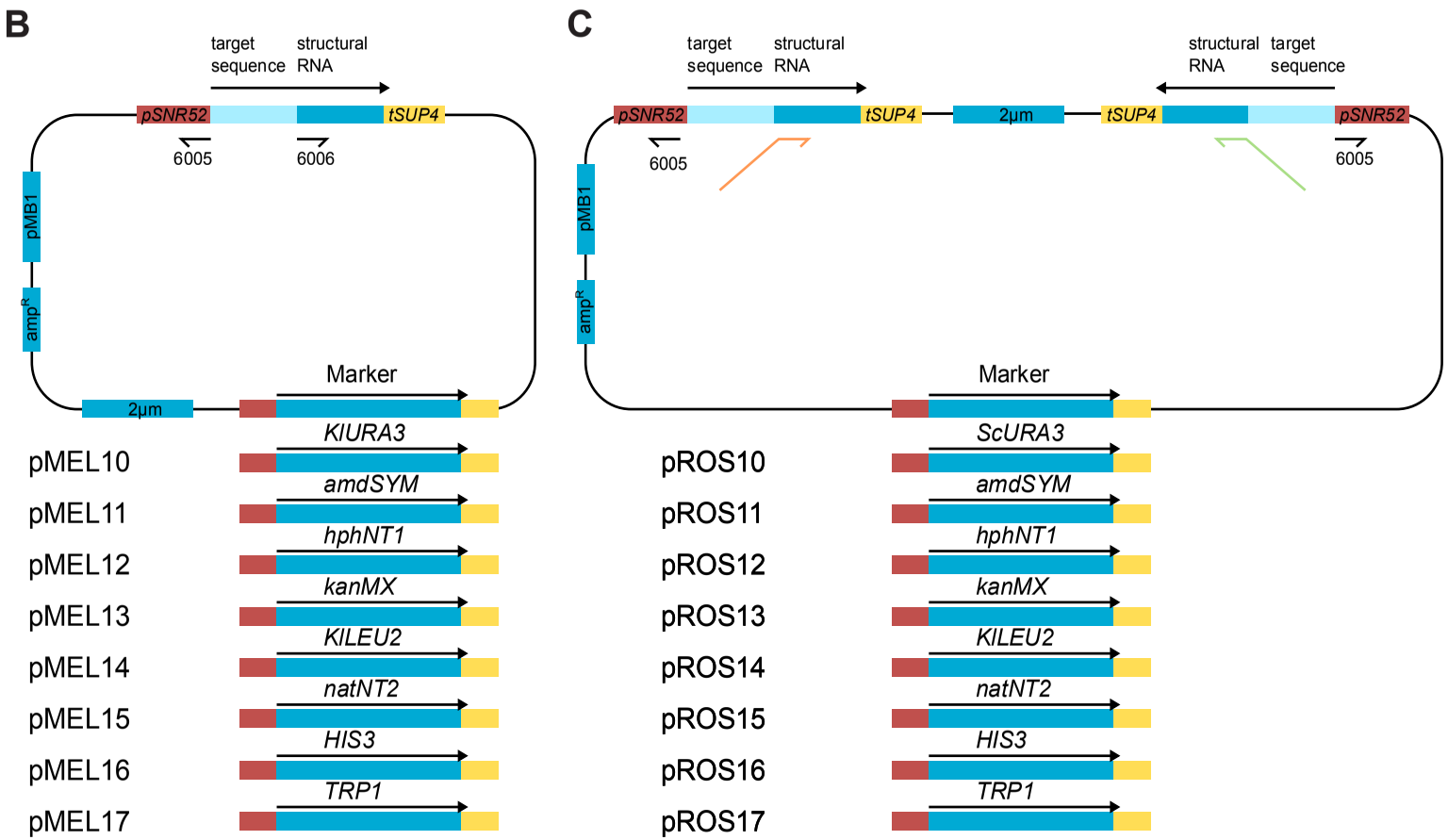


Figure 2.1: Workflow for CRISPR/Cas9 modification of *S. cerevisiae* genome using single and double gRNA plasmid series (pMEL10 - pMEL17 and pROS10 - pROS17, respectively). (**A**) All oligonucleotides, required for targeting the gene(s) of interest (GOI), can be automatically designed with the Yeastriction webtool (http://yeastriction.tnw.tudelft.nl/). For the single gRNA method, the tool designs complementary oligonucleotides that can be annealed to form (i) a double stranded repair fragment and (ii) a double stranded insert which contains the target sequence for the GOI. For expression of the gRNA, a plasmid backbone containing the genetic marker of choice is amplified from a single gRNA plasmid (pMEL10 – pMEL17). Gene deletion is achieved via co-transformation of the plasmid backbone, the dsDNA insert (containing the gRNA target sequence, flanked by sequences identical to both sides of the linearized plasmid backbone) and the repair fragment. *(see next page)*

Figure 2.1 *(continued)***:** For the double gRNA method, Yeastriction designs two sets of oligonucleotides, the first oligonucleotide binds to the 2µm fragment and has a tail containing the desired target sequence for the GOI and a sequence identical to both sides of a linearized double gRNA plasmid backbone (pROS10 – pROS17), the second set of oligonucleotides can be annealed to form the dsDNA repair fragment(s). To construct a gRNA plasmid with two target sequences, first a double gRNA plasmid backbone with the appropriate marker (pROS10 – pROS17) is amplified by PCR, excluding the 2µm fragment. Then, the 2µm fragment is PCR amplified using the primers harbouring the targets for the GOIs and sequences identical to the plasmid backbone. The final plasmid is then constructed *in vitro* using the plasmid backbone and the 2µm fragment, e.g. with the Gibson assembly method. After confirmation of correct plasmid assembly using restriction analysis or PCR, the resulting plasmid is transformed to yeast, together with the appropriate 120 bp dsDNA repair fragment(s). After transformation the desired genetic modification(s) are checked by PCR, Southern blot analysis or sequencing. Subsequently, the strain can be modified again in a new round of transformation (preferably using plasmids with other markers). Before physiological analysis, the gRNA plasmid(s) are preferably removed. This can be done by growing the strains in liquid media without selection pressure or, if possible, by counter-selection pressure (with 5-fluoroorotic acid, fluoroacetamide or 5-fluoroanthranilic acid for pMEL10+pROS10 plasmids, pMEL11+pROS11 plasmids and pMEL17+pROS17 plasmids, respectively). After confirming plasmid removal by restreaking the same colony on selective and non-selective medium and/or PCR analysis, the resulting strain is re-grown in liquid medium and stored at -80°C. (**B**) Architecture of the single gRNA plasmid series (pMEL10 – pMEL17). The primers used for PCR amplification of the plasmid backbone are indicated by black arrows. (**C**) Architecture of the double gRNA plasmid series (pROS10 – pROS17) with two gRNA cassettes. The plasmid backbone can be PCR amplified with a single primer (indicated with a black arrow). The 2µm fragment is amplified with primers designed using Yeastriction (indicated in orange and light green coloured arrows).

plasmids that target other loci, by first PCR-amplifying the 2µm fragment, using primers that incorporate the new target sites, followed by assembly into a linearized backbone that contains the desired marker gene via an *in vitro* method such as Gibson assembly. To generate plasmids with marker genes other than *URA3*, the backbone of pROS10 was first digested with restriction enzymes to remove the *ScURA3* marker. Subsequently, other markers were PCR amplified and inserted using Gibson assembly. This yielded plasmids pROS11 to pROS17 (**Figure 3.1C**, **Table 3.2**), which contained the marker genes *amdSYM, hphNT1, kanMX, KlLEU2, natNT2, HIS3 and TRP1*, respectively. Both plasmid series (pMEL10 - pMEL17 and pROS10 - pROS17) have been made available through EUROSCARF.

Construction of various *cas9* expressing CEN.PK strains

In the *cas9* bearing strains described by DiCarlo and co-workers (DiCarlo *et al.* 2013b), a centromeric plasmid was used to express *cas9*, either from a variant of the inducible *GAL1* promoter or from the constitutive *TEF1* promoter. When multiple rounds of transformation and gRNA plasmid removal are desired, a stable integrated copy of *cas9* is preferred, since this allows growth of strains on complex medium, which enables faster growth and efficient plasmid recycling. To this end, a *cas9* gene under the control of the *TEF1* promoter was integrated into the *CAN1* locus together with the *natNT2* marker. In order to increase the flexibility of the CRISPR/Cas9 system, *cas9* was integrated in

the haploid strains CEN.PK113-7D and CEN.PK113-5D (Ura$^-$) and the diploid strains CEN.PK122 and CEN.PK115 (Ura$^-$). Additionally, *cas9* was integrated in the quadruple auxotrophic strain CEN.PK2-1C (Ura$^-$, Leu$^-$, Trp$^-$, His$^-$), resulting in strain IMX672, which was used to test the efficiencies of the gRNA plasmids developed in this study. All these strains are deposited at EUROSCARF. Constitutive expression of *cas9* in CEN.PK113-7D (IMX585) did not significantly affect the maximum specific growth rate. IMX585 grew at 0.37 ± 0.003 h^{-1} (value and mean deviation are based on two independent experiments) on glucose synthetic medium in shake-flask cultures while the growth rate of the reference CEN.PK113-7D was of 0. 39 ± 0.01 h^{-1}.

Seamless and markerless gene deletion using *in vivo* assembled plasmids containing single gRNAs

The first plasmid series, pMEL10 – pMEL17, was designed to perform gene deletions from plasmids assembled *in vivo*, harnessing the high frequency and fidelity of homologous recombination in *S. cerevisiae* (Orr-Weaver *et al.*, 1983a; Orr-Weaver *et al.*, 1983b; Kunes *et al.*, 1985; Kuijpers *et al.*, 2013a) and obviating the need for prior cloning of the gRNA sequence. To test whether *in vivo* assembly of a plasmid carrying the gRNA sequence could be combined with CRISPR-mediated genetic modification, a plasmid backbone and insert containing the gRNA of choice were co-transformed with a 120 bp "repair fragment" for markerless and scarless gene deletion. The gRNA sequence targeting the gene *MCH1* (**Table 2.3**) was selected based on a high score (AT content 0.65, RNA score 0.50) in Yeastriction and contained 50 bp overlaps to each side of the linearized plasmid backbone with the *KlURA3* marker, facilitating homologous recombination. Transformation of IMX672 (*ura3-52 trp1-289 leu2-3,112 his3Δ*, *can1::cas9*) resulted in >5,000 colonies per plate and colony PCR was performed to confirm successful gene deletion. Out of 24 randomly tested colonies, 12 (50%) showed the intended single gene deletion of *MCH1* (**Figure 2.2**). The colonies that did not show the intended deletion could be caused by misassembly of the plasmid, as omitting either the insert or the repair fragment from the transformation mixture yielded ~2,000 colonies. To test whether these results could be reproduced when different loci were targeted, the same strategy was employed to target *MCH2* and *MCH5*. A gRNA recognition sequence was selected for each gene using Yeastriction, based on a low score for *MCH2* (AT content 0.45, RNA score 0.30) and a high score for *MCH5* (AT content 0.65, RNA score 0.50) targeting sequence respectively. Transformation with gRNA inserts targeting either of these loci and the corresponding repair fragments resulted in similar colony counts as observed for *MCH1*. Furthermore, colony PCR determined successful deletions for both loci at varying efficiencies, 25% and 75% for *MCH2* and *MCH5* respectively (Figure 2). This observed difference in deletion efficiency was in line with the targeted site quality scores predicted by Yeastriction for *MCH2* and *MCH5* selected gRNAs. This cloning-free approach might be extended to enable co-transformation of multiple gRNA inserts and their corresponding repair fragments. By assembly of multiple plasmids bearing different

gRNA sequences in a single cell, this might facilitate the simultaneous introduction of multiple gene deletions. To test this possibility, the same plasmid backbone, containing the *KlURA3* marker, was co-transformed with three different gRNA fragments (*MCH1*, *MCH2* & *MCH5*) and their corresponding repair fragments. Over 5,000 colonies were obtained after transformation and 24 randomly picked colonies were tested via colony PCR. Transformation with three inserts led to the identification of 4 mutants containing a single and 1 mutant containing a double deletion (24 colonies tested, **Figure 2.2A**) but none with three deletions. Only one of the 6 identified deletions was found in the *MCH1* locus (targeted by a gRNA with a low Yeastriction score, **Table 2.3**), while three and two deletions corresponded to ORFs of *MCH1* and *MCH5* respectively. These results indicated that mutants containing multiple gene deletions could be obtained via combined *in vivo* recombination and gene disruption, although at low (~4%) efficiencies. This low efficiency of multiple gene disruption might have several reasons (i) it can be the result of misassembly of the plasmid, as transformation of the backbone without insert or repair oligo already resulted in ~2,000 colonies when making single deletions (ii) a cell does not need to assemble all three plasmids, as one plasmid is enough to restore prototrophy and (iii) errors present in the gRNA inserts, as sequencing of *in vivo* assembled plasmids of three false positive colonies, showed insertions and deletions in the gRNA sequence, impairing *cas9* restriction in the target ORF (data not shown). All collectively these results showed that single gene deletions could be easily performed by this method, however when more than one locus has to be altered a more effective strategy is needed as is detailed in the next section.

Multiplexing seamless gene deletions using *in vitro* assembled plasmids containing two gRNAs

While in *vivo assembly* of the gRNA plasmid and CRISPR-assisted genetic modification can be combined in a single transformation, the relatively high incidence of false positive colonies limits use of this system for simultaneous introduction of multiple chromosomal modifications. Since some of the false positives could be the result of misassembly of the gRNA containing plasmid, it was expected that pre-assembly of the plasmid using *in vitro* Gibson assembly would result in a lower number of false positives. Therefore, a double gRNA plasmid was constructed, expressing guide RNAs designed to target *ITR1* and *PDR12*. First, the pROS10 backbone and the 2µm fragment flanked by the gRNAs containing the target sequences for *ITR1* and *PDR12* were amplified using PCR (**Figure 2.1**). The resulting DNA fragments were assembled and the plasmid (pUDR005) was verified by digestion with restriction enzymes specifically targeting the *ITR1* and *PDR12* target sequences (**Table 2.3**). Co-transformation of the resulting plasmid with the corresponding repair fragments into the *cas9* expressing strain IMX672 yielded 900 colonies per plate. In contrast, a single transformation in which repair fragments were omitted yielded only two colonies. 24 Colonies were randomly picked and PCR verification determined that all of these contained both gene disruptions (IMX715,

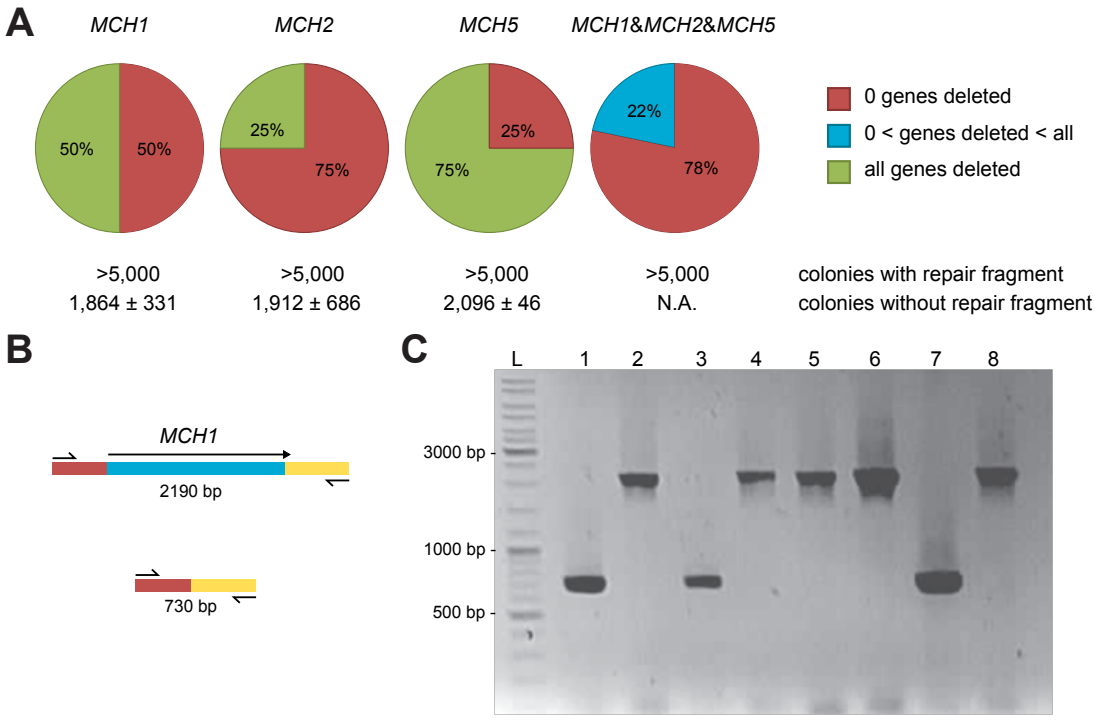


Figure 2.2: Efficiency of gene deletion obtained after transformation with a single gRNA plasmid. (**A**) Quantification of the number of colonies and corresponding gene deletion efficiencies, obtained after transformation of *S. cerevisiae* IMX672 (*ura3-52 trp1-289 leu2-3,112 his3Δ, can1Δ::cas9*) with 100 ng pMEL10 backbone, 300 ng of gRNA insert DNA and 2 µg of the corresponding 120 bp dsDNA repair fragment. The transformation targeting *MCH1*, *MCH2* and *MCH5* simultaneously was performed using 300 ng of each insert and 2 µg of each repair fragment. For the transformations with repair fragments, the exact number of transformants could not be determined, but exceeded 5,000 colonies per plate. The data represent average and standard deviation of transformants of three independent transformation experiments. The estimated total number of colonies carrying gene deletions was based on colony PCR results of 24 randomly picked colonies. In red: percentage of colonies without gene deletions, in blue: percentage of colonies containing one or two but not all deletions, green: percentage of colonies containing all desired gene deletions. No transformants with all three genes deleted were identified. (**B**) Diagnostic primers were designed outside of the target ORFs to differentiate between successful and non-successful colonies via PCR. In this colony PCR example, primers 6862 & 6863 were used to amplify the *MCH1* locus. (**C**) Example of a diagnostic gel from the transformation targeting the *MCH1* locus. The first lane (L) contains the GeneRuler DNA Ladder Mix. Lane 1 to 8 show the PCR results of 8 randomly picked colonies. Successful deletion of *MCH1* results in a PCR fragment with a length of 729 bp (lane 1, 3 and 7), when *MCH1* is still present a band is observed at 2190 bp (lane 2, 4, 5, 6 and 8).

Figure 3). The observed efficiency is very high compared to the method using *in vivo* assembled plasmids. Most likely, pre-assembly and confirmation of the plasmid containing two gRNAs greatly reduces the number of false positives. Additionally, transformation of an already assembled plasmid might enable immediate transcription of the gRNA, while the other method required assembly of a correct plasmid prior to transcription of the gRNA.

Encouraged by these extremely high efficiencies, transformations were performed using multiple plasmids that carried different genetic markers. To this end,

two new plasmids were constructed, each targeting two different genetic loci (*MCH1*/*MCH2* (pUDR002) and *MCH5*/*AQY1* (pUDR004)). Two and three plasmids were co-transformed, containing either the *KlURA3* and *TRP1* or the *KlURA3*, *TRP1* and *HIS3* selectable markers. In contrast to the transformations with a single plasmid, which yielded over 900 colonies, only 74 colonies and 14 colonies were obtained when transforming two and three plasmids, respectively. These lower numbers of colonies might reflect the decreasing probability that a single cell successfully takes up multiple plasmids and, subsequently, performs the corresponding gene deletions. The characterization of the colonies from the transformation with two plasmids revealed that out the 20 tested clones, 14 (70%) harboured all 4 deletions (IMX716). Similarly, out of 20 randomly tested colonies obtained from the transformation *with* 3 plasmids, 13 (65%) clones contained all 6 deletions (IMX717, **Figure 2.3**).

These results unequivocally demonstrate the efficiency of CRISPR/Cas9 mediated genetic modification of yeast in simultaneously generating multiple deletions in a single transformation step. Recently, CRISPR/Cas9 was successfully applied to simultaneously disrupt all homozygous alleles in the polyploid ATCC4124 strain. In four transformation iterations a quadruple *ura3 trp1 leu2 his3* auxotrophic strain was constructed (Zhang *et al.* 2014). A design similar to the native polycistronic CRISPR array consisting of a tracrRNA and crRNA instead of the chimeric gRNA was used to achieve three concurrent deletions using a single crRNA array (Bao *et al.* 2015). To the best of our knowledge, the present study is the first to demonstrate generation of a sextuple deletion strain of *S. cerevisiae* in a single transformation event.

Multiplexing deletion, multi-gene integration and introduction of single nucleotide mutations

Hitherto, reported applications of CRISPR/Cas9 in *S. cerevisiae* focused on gene inactivation. I-SceI-mediated introduction of double-stranded breaks has previously been shown to facilitate simultaneous integration of several gene expression cassettes at chromosomal loci (Kuijpers *et al.* 2013a). To explore the potential of CRISPR to combine gene deletion with the simultaneous *in vivo* assembly and chromosomal integration of multiple DNA fragments, we attempted to construct a *S. cerevisiae* strain with a double *ACS1 ACS2* deletion that also overexpresses the *Enterococcus faecalis* pyruvate dehydrogenase (PDH) complex (Kozak *et al.* 2014b) in a single transformation. To this end, IMX585 was transformed with a plasmid expressing the gRNAs targeting the *ACS* genes (pUDR022). A 120 bp repair fragment was co-transformed for the deletion of *ACS1* and the ORF of *ACS2* was replaced via integration of six gene cassettes expressing the genes of the E1α, E1β, E2 and E3 subunits of *Enterococcus faecalis* PDH encoded by *pdhA*, *pdhB*, *aceF*, and *lpd*, as well as the *lplA* and *lplA2* genes required for PDH lipoylation (Kozak *et al.* 2014b) (**Figure 2.4A**). Cytosolic acetyl-CoA is essential and a double *acs1*Δ *acs2*Δ mutant is not viable (Van den Berg *et al.* 1996) unless acetyl-CoA is provided by an alternative route (Kozak *et al.* 2014a, 2014b). The transformation targeting the two

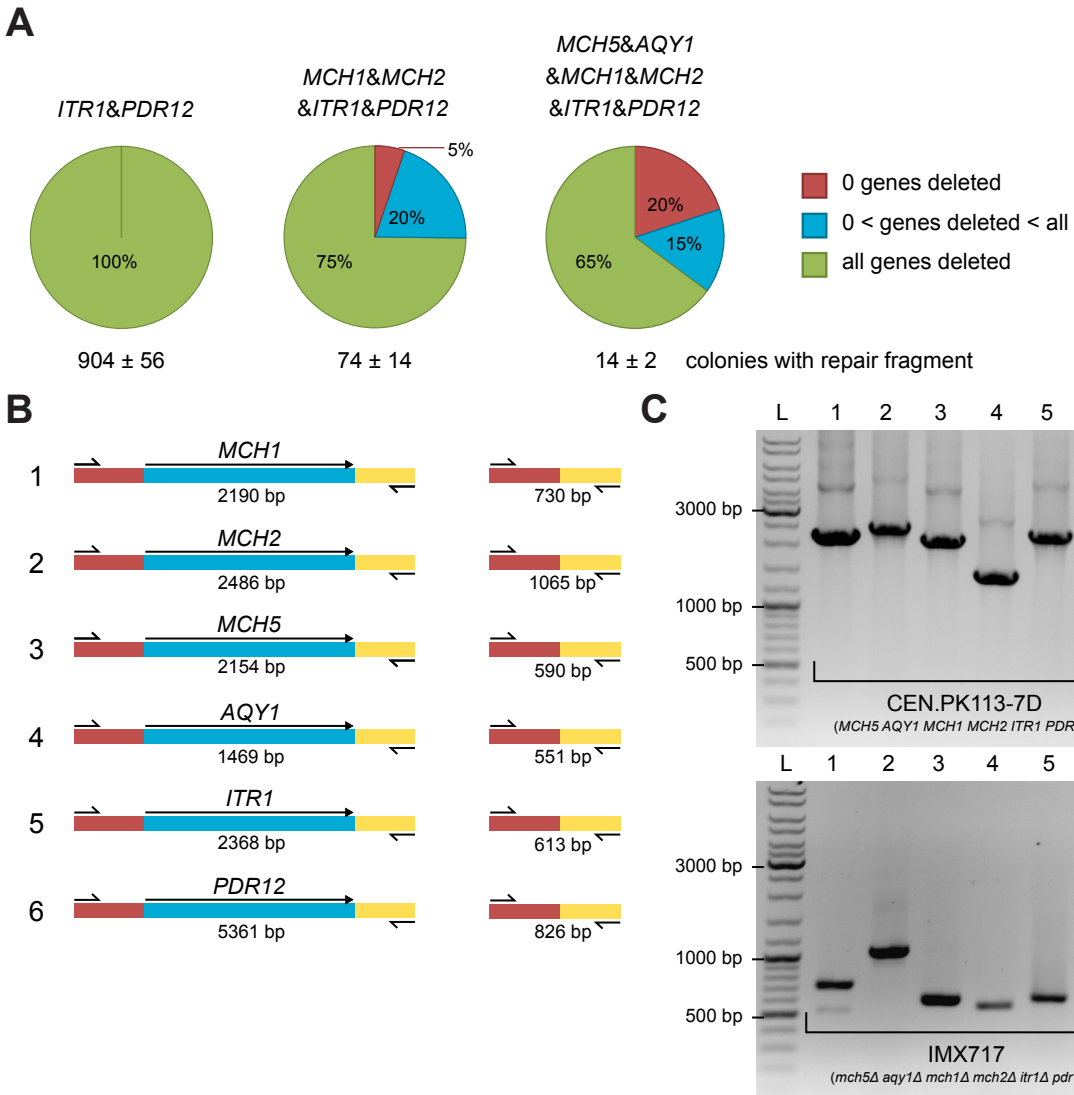
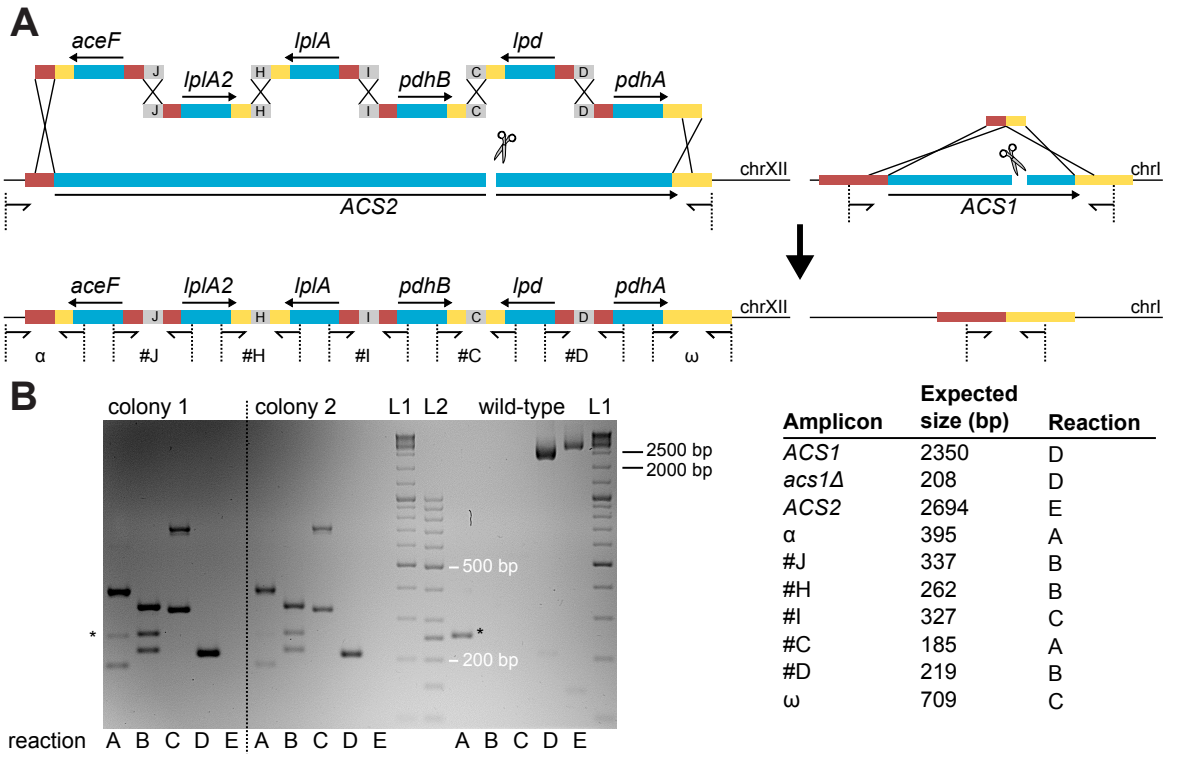


Figure 2.3: Efficiency of gene deletion obtained after transformation with double gRNA plasmid. (**A**) Quantification of the number of colonies and corresponding gene deletion efficiencies after transformation of *S. cerevisiae* IMX672 (*ura3-52 trp1-289 leu2-3,112 his3Δ, can1Δ::cas9*) with 2 µg of various double gRNA plasmids with 1 µg of the appropriate repair fragment(s). When multiple plasmids were transformed simultaneously, 2 µg of each plasmid was added. The data represent average and standard deviation of transformants of three independent transformation experiments. In red: percentage of colonies containing no gene deletions, in blue: percentage of colonies containing some but not all targeted gene deletions (1, 1-3 and 1-5 respectively), in green: percentage of colonies containing all targeted simultaneous gene deletions (2, 4 and 6 respectively). (**B**) Diagnostic primers were designed outside of the target ORFs to differentiate between successful and non-successful colonies via PCR. In this colony PCR example, primers 6862 & 6863, 6864 & 6865, 6866 & 6867, 6868 & 6870, 6869 & 6870 and 253 & 3998, were used to amplify the *MCH1, MCH2, MCH5, AQY1, ITR1* and *PDR12* loci respectively. The expected sizes of the PCR products obtained when the gene is present (left) or deleted (right) are indicated. (**C**) Example of a diagnostic gel from the transformation introducing 6 simultaneous gene deletions, resulting in IMX717. The first lane (L) contains the GeneRuler DNA Ladder Mix. Lane 1 to 6 show the PCR results of the reference strain CEN.PK113-7D (top) and a randomly picked colony of IMX717 (bottom) with primers 6862 & 6863 for *MCH1* (lane 1), 6864 & 6865 for *MCH2* (lane 2), 6866 & 6867 for *MCH5* (lane 3), 6868 & 5593 for *AQY1* (lane 4), 6869 & 6870 *ITR1* (lane 5) and 253 & 3998 for *PDR12* (lane 6).

ACS genes yielded 11 colonies, with 10 out of 10 picked colonies showing the desired genotype (**Figure 2.4B**). From one colony, the gRNA plasmid was removed by growing on non-selective YP medium, yielding strain IMX719. This strain showed only growth on synthetic medium with 2% glucose and lipoate and failed to grow on synthetic medium with 2% glucose and on YP with 2% ethanol. This phenotype is entirely consistent with the phenotype reported by (Kozak *et al.* 2014b) for an *acs1 acs2* strain of *S. cerevisiae* that expresses the *E. faecalis* PDH subunits and lipoylation genes, thereby further confirming the genotype of the strain IMX719.



Amplicon	Expected size (bp)	Reaction
ACS1	2350	D
acs1Δ	208	D
ACS2	2694	E
α	395	A
#J	337	B
#H	262	B
#I	327	C
#C	185	A
#D	219	B
ω	709	C

Figure 2.4: Multiplexing CRISPR/Cas9 in *S. cerevisiae.* (**A**) Chromosomal integration of the six genes required for expression of a functional *Enterococcus faecalis* pyruvate dehydrogenase complex in the yeast cytosol. All six genes are flanked by 60 bp sequences enabling homologous recombination (indicated with black crosses). The first and the last fragments are homologous to 60 bp just up- and downstream of the *ACS2* ORF, respectively, thus enabling repair of the Cas9-induced double strand break by homologous recombination (left panel). Deletion of *ACS1* using a 120 bp dsDNA repair fragment is shown in the right panel. (**B**) Multiplex colony PCR was performed on ten transformants to check their genotypes. Results are shown for two representative colonies, confirming the intended genotype. The PCR on the wild-type strain shows the predicted bands for the presence of the wild-type *ACS1* and *ACS2* alleles. Two DNA ladders were used; L1 refers to the GeneRuler DNA ladder (Thermo Scientific) and L2 to the GeneRuler 50 bp DNA ladder (Thermo Scientifc). (The bands indicated with an asterisk reflect aspecific PCR products)

To explore the potential of CRISPR/Cas9 to introduce specific single-nucleotide mutations in genomic DNA, a plasmid was constructed with gRNAs targeting the *GET4* and *NAT1* loci (pUDR020). Both of the target sequences contained a BamHI (G|GATCC) restriction site. The corresponding repair oligonucleotides were designed to introduce a

single-nucleotide change in the genomic target sequence, which at both sites resulted in the introduction of a restriction site for EcoRI (G|AATTC) and simultaneously disrupted the BamHI restriction site (**Figure 2.5A**). Transformation of the *cas9* bearing strain IMX672 with pUDR020 (*NAT1* and *GET4* gRNA, *KlURA3*) and the corresponding repair fragments resulted in ~1,500 colonies, while omitting both repair fragments did not result in any colonies. Eight colonies were randomly picked and a part of the ORF containing the target sequence was PCR amplified for both *GET4* and *NAT1*. Digestion of the amplified PCR product with BamHI and EcoRI, followed by gel electrophoresis showed that four of the eight colonies contained both mutations after transformation (**Figure 2.5B**). To confirm whether these four colonies indeed contained the desired mutations,

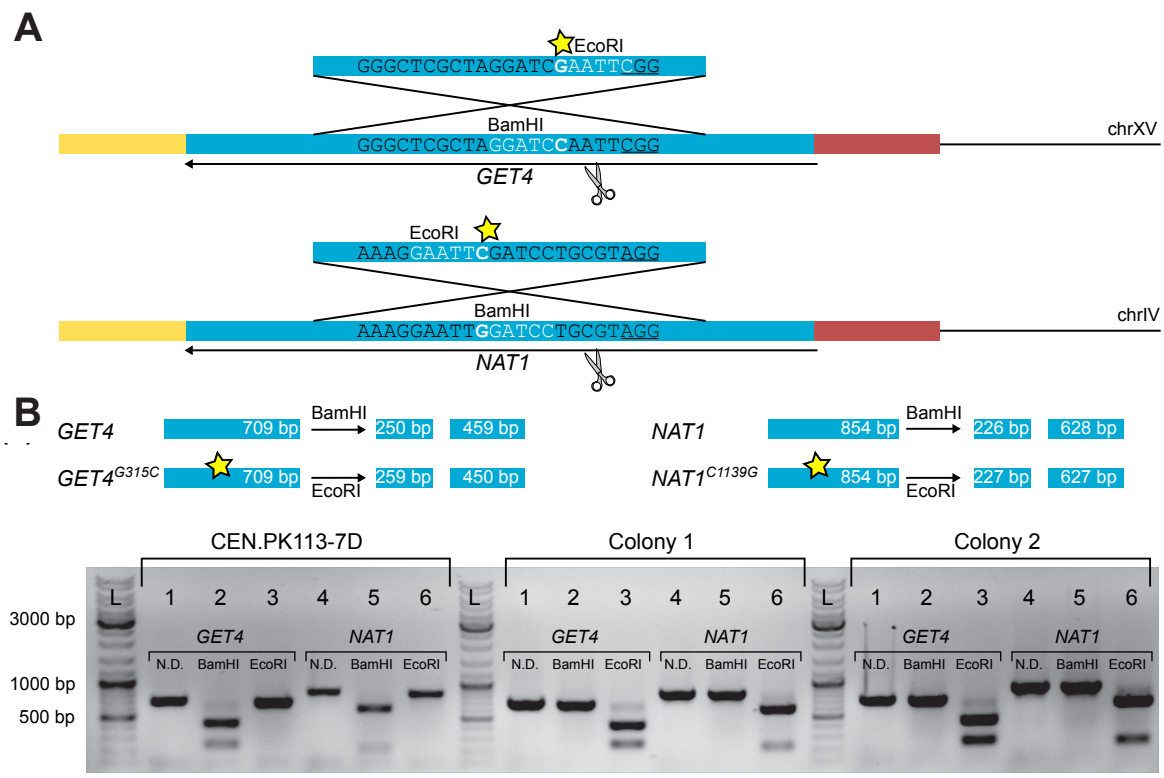


Figure 2.5: Simultaneous introduction of different single-nucleotide mutations in *S. cerevisiae*. (**A**) Transformation of IMX581 (ura3-52, can1Δ::CAS9) was performed with pUDR020, resulting in the introduction of two mutations in *NAT1* and *GET4*. Underlined: the protospacer adjacent motif (PAM) sequences associated with the gRNA targets. In white: restriction sites present in the original gRNA targeting sequence (BamHI) and in the repair fragment used to correct the double strand break (EcoRI). (**B**) Introduction of the double strand break and subsequent repair using the mutagenic repair fragment resulted in a change of restriction site from BamHI to EcoRI. In this colony PCR example, primers 7030 & 7031 and 7036 & 7037 were used to amplify a part of the *GET4* and *NAT1* locus (lane 1 and 4 respectively) from CEN.PK113-7D and two colonies of IMX581, transformed with pUDR020 and mutagenic repair fragments. In lanes labelled 2 and 5: digestion of the PCR fragments with BamHI, which only results in digestion fragments of sizes 250 bp + 459 bp and 226 bp and 628 bp when the original restriction sites in *GET4* and *NAT1* are still present (CEN.PK113-7D). In lanes labelled 3 and 6: digestion of the PCR fragments with EcoRI, which only results in digestion fragments of sizes 259 bp + 450 bp and 227 bp and 627 bp when this new restriction site has been introduced via the mutagenic repair fragment (Colony 1 and 2).

a fragment of 120 bp around the target site was sequenced. All colonies contained the desired mutations, although two of the four colonies also showed additional, undesired, mutations at the *GET4* locus (**Supplementary data S2.1**, colony 2 and 4). Although these results show that one mutation can already abolish restriction, Cas9 induced DSBs might still occur, as long as the gRNA cassette is expressed. We therefore advise to (re) sequence mutated sites after gRNA plasmid removal and/or use the two step strategy discussed below.

The ease with which specific single-nucleotide mutations can be simultaneously introduced, makes the CRISPR/Cas9 system a highly valuable tool for analysing the biological significance of mutations identified by whole genome resequencing of strains obtained by mutagenesis (e.g. UV, X-rays) (Jung *et al.* 2011) or evolutionary engineering (Hong *et al.* 2011; González-Ramos *et al.* 2013; Oud *et al.* 2013; Caspeta *et al.* 2014). Reverse engineering of such mutations essentially encompasses restoration of the reference nucleotide in the mutant strain and/or introduction of the mutated nucleotide position in a naïve (non-mutated or non-evolved) genetic background. Even laboratory evolution experiments performed in the absence of mutagenesis typically yield multiple mutations, not all of which contribute to the phenotype of interest (see e.g. (De Kok *et al.* 2012b; González-Ramos *et al.* 2013). Therefore availability of methods that enabled reintroduction of multiple point mutations at various genomic loci is invaluable for rapid identification of relevant mutations. Similar to the YOGE (Yeast Oligo-Mediated Genome Engineering) method (DiCarlo *et al.* 2013a), the CRISPR approach enables multiplexing and offers flexibility. In contrast to the YOGE method, which requires a specific background (*mlh1Δ msh2Δ RAD51*^{K342E}*↑ RAD54↑*) (DiCarlo *et al.* 2013a), the CRISPR approach should be applicable to any *S. cerevisiae* strain background that expresses a functional *cas9* gene. The two examples used in this study (*GET4* and *NAT1*) were designed for easy verification. If the desired point mutation is not present in a suitable target sequence, it may be possible to introduce (multiple) single Nucleotide Variation(s) (SNV) in two rounds of CRISPR/Cas9 mediated genome modification. In the first round, a larger part surrounding one or several SNVs could be deleted while repairing the DSBs with repair fragments that contain a generic synthetic target sequence. In a second round, these generic target sequences can then be cut by Cas9, combined with the repair of the DSBs with 120 bp sequences that contain the desired SNVs.

2.4 Outlook

The use of homologous recombination for the assembly of multi-gene constructs (Gibson *et al.* 2008; Shao, Zhao and Zhao 2009) had a tremendous impact on genetic engineering strategies (Annaluru *et al.* 2014; Casini *et al.* 2014). Even before the advent of CRISPR/Cas9 these developments have immensely decreased strain construction time in our group and enabled us to express complicated multi-step pathways and multi-

component enzymes (Koopman *et al.* 2012; Guadalupe-Medina *et al.* 2013; Kuijpers *et al.* 2013a; Beekwilder *et al.* 2014; Kozak *et al.* 2014b). Until recently the deletion of multiple genes and insertions at multiple loci remained a cumbersome and time-intensive process. Here, building on the pioneering conceptual proof of CRISPR functionality in lower eukaryotes (DiCarlo *et al.* 2013b), we show that CRISPR/Cas9 will further improve and accelerate yeast strain construction, not only by allowing multiplexing gene deletions (DiCarlo *et al.* 2013b; Zhang *et al.* 2014) but also by allowing simultaneous introduction of gene deletions, chromosomal integration of multi-gene constructs and the introduction of specific mutations. Although it is difficult to quantify the impact on the overall time requirements for complex pathway engineering, the examples presented here suggest that a three to four-fold acceleration is unlikely to be exaggerated.

This paper focuses on *S. cerevisiae*, a microbial species that is already known for its easy genetic accessibility. Therefore, it is logical to speculate that this methodology should have an even higher impact on species of yeasts and filamentous fungi that are notoriously more difficult to alter genetically. Indeed, a very recent example described the benefit of the introduction of CRISPR/Cas9 in *Schizosaccharomyces pombe* (Jacobs *et al.* 2014). While the methodology reported in this study streamlines the use of CRISPR in *S. cerevisiae*, the method can be further improved. The RNA polymerase III dependent promoter *SNR52* is not a broadly recognized promoter (Ryan *et al.* 2014). Recently, it has been shown that guideRNAs, flanked by the hammerhead and hepatitis delta virus ribozymes, can be expressed using polymerase II promoters (Gao and Zhao 2014). A promising promoter would then be the *TEF1* promoter from *Blastobotrys adeninivorans* (Terentiev *et al.* 2004) as this is a strong, constitutive promoter, recognized in different species, like *S. cerevisiae*, *Hansenula polymorpha* and *Pichia pastoris*.

We hope that by providing the easy-to-use Yeastriction design tool, two versatile plasmid series for gRNA expression, a set of *S. cerevisiae* CEN.PK strains harbouring the *cas9* expression cassette and standardized protocols (Supplemental information), this paper will help colleagues to facilitate and accelerate yeast strain engineering.

2.5 Acknowledgements

We thank James Dykstra for the construction of IMX719 and Arthur Dobbe for sharing the growth rates of IMX585. PDL and NK were financially supported by the Technology Foundation STW (Vidi Grant 10776). HMvR, RM, AJAvM, JTP and JMD were supported by the BE-Basic R&D Program, which was granted an FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I). The image on the title page (page 35) was modified from an image made by Nogas1974 (own work), which is licenced under CC BY-SA 4.0, via Wikimedia Commons.

Supplementary material

Table S2.1: Primers used in this study

Number	Name	Sequence 5' → 3'
***CAS9* integration**		
2873	CAN1DelcassFW	TCAGACTTCTTAACTCCTGTAAAAACAAAAAA AAAAAAAGGCATAGCAATAAGCTGGAGCTCAT AGCTTC
3093	tagA-pUG	ACTATATGTGAAGGCATGGCTATGGCACGGCA GACATTCCGCCAGATCATCAATAGGCACCTTCG TACGCTGCAGGTCGAC
4653	A-CYC1t-rv	GTGCCTATTGATGATCTGGCGGAATGTCTGCC GTGCCATAGCCATGCCTTCACATATAGTCCGC AAATTAAAGCCTTCGAG
5542	CAN1 KO rv	CTATGCTACAACATTCCAAAATTTGTCCCAAA AAGTCTTTGGTTCATGATCTTCCCATACGCAT AGGCCACTAGTGGATCTG
Cas9 integration confirmation		
5829	CAN1 cut rv	AGAAGAGTGGTTGCGAACAGAG
2673	m-PCR-HR4-RV	TGAAGTGGTACGGCGATGC
2668	m-PCR-HR2-FW	ACGCGTGTACGCATGTAAC
9	KanA	CGCACGTCAAGACTGTCAAG
2620	Nat Ctrl Fw	GCCGAGCAAATGCCTGCAAATC
2615	Can1RV	GAAATGGCGTGGGAATGTGA
Construction pMEL series		
3093	tagA-pUG	ACTATATGTGAAGGCATGGCTATGGCACGGCA GACATTCCGCCAGATCATCAATAGGCACCTTC GTACGCTGCAGGTCGAC
3096	tagB-pUG	GTTGAACATTCTTAGGCTGGTCGAATCATTTA GACACGGGCATCGTCCTCTCGAAAGGTGGCAT AGGCCACTAGTGGATCTG
6845	p426 cRNA-rv A	GTGCCTATTGATGATCTGGCGGAATGTCTGCC GTGCCATAGCCATGCCTTCACATATAGTACAGG CAACACGCAGATATAGG
6846	p426 cRNA-fw B	CACCTTTCGAGAGGACGATGCCCGTGTCTAAA TGATTCGACCAGCCTAAGAATGTTCAACGGCC CACTACGTGAACCATC
6847	pRS Marker fw A	ACTATATGTGAAGGCATGGCTATGGCACGGCA GACATTCCGCCAGATCATCAATAGGCACTGCG GCATCAGAGCAGATTG
6848	pRS Marker rv B	GTTGAACATTCTTAGGCTGGTCGAATCATTTA GACACGGGCATCGTCCTCTCGAAAGGTGCATC TGTGCGGTATTTCACACC
Construction pROS series		
5972	tSUP4 rv ol 2mu-5*	GTTCTACAAAATGAAGCACAGATGCTTCGTTG GAGGGCGTGAACGTAAG
5974	2mu inside fw	TACTTTTGAGCAATGTTTGTGGA
5975	2mu inside rv	AACGAGCTACTAAAATATTGCGAA
6007	struct-guideRNA ADE2.y (ol pSNR52)	GTGCGCATGTTTCGGCGTTCGAAACTTCTCCG CAGTGAAAGATAAATGATCACTTGAAGATTCT TTAGTGTGTTTTAGAGCTAGAAATAGCAAGTT AAAATAAG
6008	struct-guideRNA CAN1.y (ol pSNR52)	GTGCGCATGTTTCGGCGTTCGAAACTTCTCCG CAGTGAAAGATAAATGATCGATACGTTCTCTA TGGAGGAGTTTTAGAGCTAGAAATAGCAAGTT AAAATAAG
3289	Fus Tag F fw	CATACGTTGAAACTACGGCAAAGG
4692	Fus Tag G rv	AAGGGCCATGACCACCTG

5976	pSNR52 fw ol tag I-2	GCCTACGGTTCCCGAAGTATGCTGCTGATGTC TGGCTATACCTATCCGTCTACGTGAATACCCTC ACTAAAGGGAACAAAAG
5977	pSNR52 fw ol tag B	CACCTTTCGAGAGGACGATGCCCGTGTCTAAA TGATTCGACCAGCCTAAGAATGTTCAACCCCT CACTAAAGGGAACAAAAG
5973	tSUP4 rv ol 2mu 3*	GGCATAGTGCGTGTTTATGCTTAAATGCGTGG AGGGCGTGAACGTAAG
5975	2mu inside rv	AACGAGCTACTAAAATATTGCGAA
4068	Nic1 amp Fwd	GCCTACGGTTCCCGAAGTATGC
5974	2mu inside fw	TACTTTTGAGCAATGTTTGTGGA
3841	for f3h	CACCTTTCGAGAGGACGATG
3847	FUS Tag A fw	ACTATATGTGAAGGCATGGCTATGG
3276	Fus Tag B-rv	GTTGAACATTCTTAGGCTGGTCGAATC
3274	Fus Tag I-fw	TATTCACGTAGACGGATAGGTATAGC
3275	Fus Tag A-rv	GTGCCTATTGATGATCTGGCGGAATG
5793	pCAS9 rv	GATCATTTATCTTTCACTGCGGAG
Construction PDH cassettes		
5654	D_FW_E1a	GAATTCACGCATCTACGACTG
7426	tTEF1_yeast ol tACS2	GAAAATAGAAAACAGAAAAGGAGCGAAATTT TATCTCATTACGAAATTTTTCTCATTTAAGGAG GCACTATTACTGATGTGATTTC
3277	Fus Tag C rv	CTAGCGTGTCCTCGCATAGTTCTTAGATTG
7338	pTDH3 fw ol tag I	TATTCACGTAGACGGATAGGTATAGCCAGACA TCAGCAGCATACTTCGGGAACCGTAGGCATAA AAAACACGCTTTTTCAGTTCG
3284	Fus Tag J rv	CGACGAGATGCTCAGACTATGTGTTC
7356	tPGI1 ol pACS2	GGTTAGTGATTGTTATACACAAACAGAATACA GGAAAGTAAATCAATACAATAATAAAATTAAT TTTTAAAATTTTTACTTTTCGCGAC
5653	D_RV_E3	AAGCTTAATCACTCTCCATACAGGG
3283	Fus Tag C fw	ACGTCTCACGGATCGTATATGC
5661	I_RV_LL.LA1	TCTAGAGCCTACGGTTCCCGA
5663	H_FW_LA1	TCTAGAAGATTACTCTAACGCCTCAGC
2686	Tag H fusion reverse	GTCACGGGTTCTCAGCAATTCG
3285	Fus Tag J fw	GGCCGTCATATACGCGAAGATGTC
gRNA cassette construction		
6835	CrRNA insert MCH1 XhoI FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGC AGTGAAAGATAAATGATCTATTGGCAATAAAC ATCTCGGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAAC
6836	CrRNA insert MCH1 XhoI RV	GTTGATAACGGACTAGCCTTATTTTAACTTGCT ATTTCTAGCTCTAAAACCGAGATGTTTATTGCC AATAGATCATTTATCTTTCACTGCGGAGAAGT TTCGAACGCCGAAACATGCGCA
6837	CrRNA insert MCH2 PvuI FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGC AGTGAAAGATAAATGATCATCTCGATCGAGGT GCCTGAGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAAC
6838	CrRNA insert MCH2 PvuI RV	GTTGATAACGGACTAGCCTTATTTTAACTTGCT ATTTCTAGCTCTAAAACTCAGGCACCTCGATC GAGATGATCATTTATCTTTCACTGCGGAGAAG TTTCGAACGCCGAAACATGCGCA
6839	CrRNA insert MCH5 EcoRV FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGC AGTGAAAGATAAATGATCACTCTTCCGTTTTA GATATCGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAAC

6840	CrRNA insert MCH5 EcoRV RV	GTTGATAACGGACTAGCCTTATTTTAACTTGC TATTTCTAGCTCTAAAACGATATCTAAAACGG AAGAGTGATCATTTATCTTTCACTGCGGAGAA GTTTCGAACGCCGAAACATGCGCA
6841	CrRNA insert AQY1 DraI FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGC AGTGAAAGATAAATGATCACCATCGCTTTAAA ATCTCTGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAAC
6842	CrRNA insert AQY1 DraI RV	GTTGATAACGGACTAGCCTTATTTTAACTTGCT ATTTCTAGCTCTAAAACAGAGATTTTAAAGCG ATGGTGATCATTTATCTTTCACTGCGGAGAAG TTTCGAACGCCGAAACATGCGCA
6843	CrRNA insert ITRI EcoRI FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGC AGTGAAAGATAAATGATCATACATCAACGAAT TCCAACGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAAC
6844	CrRNA insert ITRI EcoRI RV	GTTGATAACGGACTAGCCTTATTTTAACTTGC TATTTCTAGCTCTAAAACGTTGGAATTCGTTGA TGTATGATCATTTATCTTTCACTGCGGAGAAG TTTCGAACGCCGAAACATGCGCA
7040	CrRNA insert PDR12 KpnI FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGC AGTGAAAGATAAATGATCGCATTTTCGGTACC TAACTCGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAAC
7041	CrRNA insert PDR12 KpnI RV	GTTGATAACGGACTAGCCTTATTTTAACTTGC TATTTCTAGCTCTAAAACGAGTTAGGTACCGA AAATGCGATCATTTATCTTTCACTGCGGAGAA GTTTCGAACGCCGAAACATGCGCA
7348	ACS2_targetRNA FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGC AGTGAAAGATAAATGATCTCCTTGCCGTTAAA TCACCAGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAAC
6414	ACS1 gRNA	GTGCGCATGTTTCGGCGTTCGAAACTTCTCCG CAGTGAAAGATAAATGATCTTCTTCACAGCTG GAGACATGTTTTAGAGCTAGAAATAGCAAGTT AAAATAAG
7026	GET4_targetRNA FW BamHI, DpnI	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGC AGTGAAAGATAAATGATCGGGCTCGCTAGGAT CCAATTGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAAC
7032	NAT1_targetRNA FW BamHI, DpnI	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGC AGTGAAAGATAAATGATCAAAGGAATTGGATC CTGCGTGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAAC
Repair oligos		
6849	MCH1 Repair Oligo FW	GGTGTCATTTATATAAGCTATGAATTTTAAAAA AAATAAATGTAGCAGTTTCTTTTTTGTGATTTG CACTTGGAAAATGGTTATTGCTATAAAATGAT ATGAAAGGAAACTAGTCTCGAT
6850	MCH1 Repair Oligo RV	ATCGAGACTAGTTTCCTTTCATATCATTTTATA GCAATAACCATTTTCCAAGTGCAAATCACAAA AAAGAAACTGCTACATTTATTTTTTTTAAAATT CATAGCTTATATAAATGACACC
6851	MCH2 Repair Oligo FW	TATAGAACTATATAACTGATACTAGAATATACT AATTCGTGCACTATTAACCGTTTGGCGAGGTC ACTTTTATTTCACACTGTAGATAAGAAGGGGA TAGAGTTGCCAGAAAATTTTTTG
6852	MCH2 Repair Oligo RV	CAAAAAATTTTCTGGCAACTCTATCCCCTTCT TATCTACAGTGTGAAATAAAAGTGACCTCGCC AAACGGTTAATAGTGCACGAATTAGTATATTC TAGTATCAGTTATATAGTTCTATA

6853	MCH5 Repair Oligo FW	TAAAAGAAAATATTATTGCATTACTTTTTTGAA GATCTATAAAGGGCACTGTCTTACTTTTATTTT TCTTTTTAATCTATAGTAAAATCAGAGCTTTTT AATCGATAGTATGCCCCCGTG
6854	MCH5 Repair Oligo RV	CACGGGGGCATACTATCGATTAAAAAGCTCTG ATTTTACTATAGATTAAAAAGAAAAATAAAAG TAAGACAGTGCCCTTTATAGATCTTCAAAAAA GTAATGCAATAATATTTTCTTTTA
6855	AQY1 Repair Oligo FW	CTTTGTATTTGGTGCTGTCTGTCAATACGGCA CATAAAGTAACATGTAATTAACTATAACTTTTT CCCTCCTTTTCTTTATTTCTCGCTCACTAGCAC TTAATGTTATAATACTCGGCAA
6856	AQY1 Repair Oligo RV	TTGCCGAGTATTATAACATTAAGTGCTAGTGA GCGAGAAATAAAGAAAAGGAGGGAAAAAGTT ATAGTTAATTACATGTTACTTTATGTGCCGTAT TGACAGACAGCACCAAATACAAAG
6857	ITR1 Repair Oligo FW	ATTTTCTACTATGTATTTGAATATTCAATTGCG TCTCCTTCCTTTTACCTCGTGAAAGGATTTAA CACCCACTGCAGAAACAAAGAAAATGAAAGA GATGTATACAGTAGGACGACCAAT
6858	ITR1 Repair Oligo RV	ATTGGTCGTCCTACTGTATACATCTCTTTCATT TTCTTTGTTTCTGCAGTGGGTGTTAAATCCTT TCACGAGGTAAAAGGAAGGAGACGCAATTGA ATATTCAAATACATAGTAGAAAAT
7042	PDR12 Repair Oligo FW	AAAATTGAAAATAAAAATTGTGTGTTAAACCA CGAAATACAAATATATTTGCTTGCTTGTTTTTT TATTAATAAGAACAATAACAATAAATCTGTAA ACCTTTTTTTTAAGTGAAAATTA
7043	PDR12 Repair Oligo RV	TAATTTTCACTTAAAAAAAAGGTTTACAGATT TATTGTTATTGTTCTTATTAATAAAAAAACAAG CAAGCAAATATATTTGTATTTCGTGGTTTAACA CACAATTTTTATTTTCAATTTT
6422	ACS1 repair fw	CTATCTATAAGCAAAACCAAACATATCAAAAC TACTAGAAAGACATTGCCCCACTGTGTTTGAT GATTTCTTTCCTTTTTATATTGACGACTTTTTT TTTCGTGTGTTTTTGTTCTCTTA
6423	ACS1 repair rv	TAAGAGAACAAAAACACACGAAAAAAAAAGT CGTCAATATAAAAAGGAAAGAAATCATCAAAC ACAGTGGGGCAATGTCTTTCTAGTAGTTTTGA TATGTTTGGTTTTGCTTATAGATAG
7028	GET4_repair oligo fw	GGAAGTTAAAGTTGATGACATCTCAGTTGCTA GATTGGTTAGATTAATAGCCGAATTCGATCCTA GCGAGCCCAATTTAAAGGACGTTATTACTGGT ATGAACAATTGGTCTATCAAATT
7029	GET4_repair oligo rv	AATTTGATAGACCAATTGTTCATACCAGTAATA ACGTCCTTTAAATTGGGCTCGCTAGGATCGAA TTCGGCTATTAATCTAACCAATCTAGCAACTG AGATGTCATCAACTTTAACTTCC
7034	NAT1_repair oligo fw	GTTTCACCACTATTGGAGAAAATTGTCCTTGA TTATTTGTCCGGATTAGATCCTACGCAGGATC GAATTCCTTTTATTTGGACCAATTATTACTTGT CTCAACATTTCCTTTTCCTTAAG
7035	NAT1_repair oligo rv	CTTAAGGAAAAGGAAATGTTGAGACAAGTAAT AATTGGTCCAAATAAAAGGAATTCGATCCTGC GTAGGATCTAATCCGGACAAATAATCAAGGAC AATTTTCTCCAATAGTGGTGAAAC
Diagnostic primers for confirming correct double gRNA plasmid assembly		
Primer A		CACCTTTCGAGAGGACGATG
Primer B		GCTGGCCTTTTGCTCACATG
7351	GET4 dg fw-2	AATTGGATCCTAGCGAGCCC
7352	NAT1 dg fw-2	ACGCAGGATCCAATTCCTTTG

Diagnostic primers for seamless deletion confirmation		
6862	MCH1 check FW	GTCCAGGATTCTCCGAAGAACTC
6863	MCH1 check RV	ACGGCTGTTTGTCCGATATTGC
6864	MCH2 check FW	GCACGACTTTGCAGGCTTTC
6865	MCH2 check RV	CACCGAACCAACATTAGGTAGC
6866	MCH5 check FW	GAAGACTGACGGGCACTTTG
6867	MCH5 check RV	GCCCTAGGCGGTATTGTATGAG
6868	AQY1 check FW	GATGTCCTTCCACCCTCTTACAC
5593	42 - CBS1483_Sc16_Scf39 _HPA2-Rv	CGTGTATTGGTGTACGGATGAGTC
6869	ITR1 check FW	AGGTCTTCAATGCCGGGTTAG
6870	ITR1 check RV	GTATCAGCCGTCAGTAGTATCCAG
253	PDR12 - CTRL FW	CGGTATCACATTTTCTCGACGG
3998	PDR12 KO rv	CGCGACAGACATTTGTTGG
Diagnostic primers for *GET4* and *NAT1* mutation confirmation		
7030	GET4_dg fw	TGTTCGTTTTGTCGCTCTTCG
7031	GET4_dg rv	AACTCAGCCACCGTGCTATC
7036	NAT1_dg fw	TATCCAAGATGCGAACCACCC
7037	NAT1_dg rv	AGATAGGCTCTTGGCGTACC
Diagnostic primers for confirming strain IMX719		
2619	acs2 KO Ctrl Rv	CCGATATTCGGTAGCCGATTCC
2430	Tag C-reverse short	GCGTCCAAGTAACTACATTATGTG
2668	m-PCR-HR2-FW	ACGCGTGTACGCATGTAAC
7330	NATDiagfw	CGAGCAAATGCCTGCAAATC
5044	ADH1 Flank right FW	CTAGCGGTTATGCGCGTCTCAC
2913	G_RV_2 (PDH construct ctrl)	AATAGCCGCCAGGAAATGCC
2914	J_FW (PDH construct ctrl)	GTCGTCATAACGATGAGGTGTTGC
2915	J_RV (PDH construct ctrl)	GGAGCCAACAAGAATAAGCCGC
2908	D_FW (PDH construct ctrl)	GGATTGGGTGTGATGTAAGGATTCGC
2909	D_RV (PDH construct ctrl)	CCCGCTCACACTAACGTAGG
2916	I_FW (PDH construct ctrl)	GCAGGTATGCGATAGTTCCTCAC
2905	B_RV (PDH construct ctrl)	TGGCAGTATTGATAATGATAAACTCG
5641	F_FW_EF	CCATTGCTGAAGCTGACAAG
2618	acs2 Ctrl Fw	TACCCTATCCCGGGCGAAGAAC
2927	ACS1 KO ctrl FW	AAACTGGGCGGCTATTCTAAGC
2928	ACS1 KO ctrl RV	AGCAGCTCGGTTATAAGAGAAC

Table S2.2: Alignment of 120 bp surrounding the target site at the mutated *GET4* locus of the four sequenced colonies (in bold the target site, in lowercase the intended mutation, in yellow additional, undesired mutations).

Reference	258	GGAAGTTAAAGTTGATGACATCTCAGTTGCTAGATTGGTTAGATTAATAG**CCGAATTGGA**
Colony #1		GGAAGTTAAAGTTGATGACATCTCAGTTGCTAGATTGGTTAGATTAATAGCCGAATTcGA
Colony #2		GGAAGTTAAAGTTGATGACATCTCAGTTGCTAGATTGGTTAGATTAATAGCCGAATTcGA
Colony #4		GGAAGTTAAAGTTGATGACATCTCAGTTGCTAGATTGGTTAGATTAATAGCCGAATTcGA
Colony #6		GGAAGTTAAAGTTGATGACATCTCAGTTGCTAGATTGGTTAGATTAATAGCCGAATTcGA

Reference	318	**TCCTAGCGAGCCC**AATTTAAAGGACGTTATTACTGGTATGAACAATTGGTCTATCAAATT
Colony #1		TCCTAGCGAGCCCAATTTAAAGGACGTTATTACTGGTATGAACAATTGGTCTATCAAATT
Colony #2		TCCTAGCGAGCCCAATTTAAAG - - - - - - - TTACTGGTATGAACAATTGGTCTATCAAATT
Colony #4		T - CTAGCGAGCCCAATTTAAAGGACGTTATTATTGGTATGAACAATTGGTCTATCAAATT
Colony #6		TCCTAGCGAGCCCAATTTAAAGGACGTTATTACTGGTATGAACAATTGGTCTATCAAATT

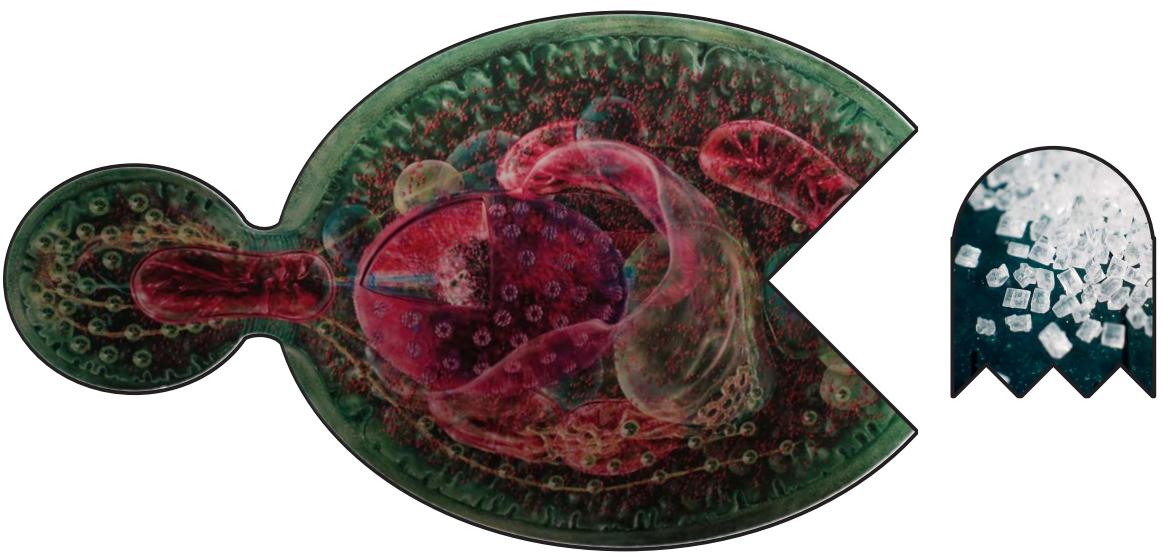
Table S2.3: Alignment of 120 bp surrounding the target site at the mutated *NAT1* locus of the four sequenced colonies (in bold the target site, in lowercase the intended mutation).

Reference	1075	GTTTCACCACTATTGGAGAAAATTGTCCTTGATTATTTGTCCGGATTAGAT**CCTACGCAG**
Colony #1		GTTTCACCACTATTGGAGAAAATTGTCCTTGATTATTTGTCCGGATTAGATCCTACGCAG
Colony #2		GTTTCACCACTATTGGAGAAAATTGTCCTTGATTATTTGTCCGGATTAGATCCTACGCAG
Colony #4		GTTTCACCACTATTGGAGAAAATTGTCCTTGATTATTTGTCCGGATTAGATCCTACGCAG
Colony #6		GTTTCACCACTATTGGAGAAAATTGTCCTTGATTATTTGTCCGGATTAGATCCTACGCAG

Reference	1135	**GATCCAATTCCTTT**TATTTGGACCAATTATTACTTGTCTCAACATTTCCTTTTCCTTAAG
Colony #1		GATCgAATTCCTTTTATTTGGACCAATTATTACTTGTCTCAACATTTCCTTTTCCTTAAG
Colony #2		GATCgAATTCCTTTTATTTGGACCAATTATTACTTGTCTCAACATTTCCTTTTCCTTAAG
Colony #4		GATCgAATTCCTTTTATTTGGACCAATTATTACTTGTCTCAACATTTCCTTTTCCTTAAG
Colony #6		GATCgAATTCCTTTTATTTGGACCAATTATTACTTGTCTCAACATTTCCTTTTCCTTAAG

Chapter 3

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



Wesley Leoricy Marques, Robert Mans, Eko Roy Marella, Rosa Lorizolla Cordeiro, Marcel van den Broek, Jean-Marc G. Daran, Jack T. Pronk, Andreas K. Gombert, Antonius J.A. van Maris

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 h^{-1} 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.

3.1 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.* 2015; 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.* 2015). 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.* 2015), 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.

3.2 Methods

Strains and maintenance

S. cerevisiae strains used in this work (**Table 3.1**) 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 °C.

Table 3.1: 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 3.2**) 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.* 2013b). 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.* 2013b) with primers 1768 & 7236 (**Table S3.1**). 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 3.1A; Table 3.2**) contains two gRNA cassettes: one for deletion of *IMA5* (*gRNA-IMA5*) and one targeting-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 S3.1**). 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 S3.1**). 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 3.2**). 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 3.2**). 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 S3.1**). 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

Table 3.2: Plasmids used in this study

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

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 3.2**) was linearized with *BstE*II (restriction site at *LEU2* marker) and transformed into IMK291, resulting in strain IMX469 (**Table 3.1**). Transformation of pUDE260 and pUDE044 into IMX469 resulted in IMU048 and IMU055, respectively. Linearization of pUDI084 (**Table 3.2**) 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 3.1**). Repair fragments were obtained by annealing two complementary PAGE purified single-stranded oligonucleotides according to Mans *et al.* (2015): 8592 & 8593 for *IMA1-4* and 8763 & 8764 for *IMA5* (**Table 3.2**).

In order to delete *IMA* genes from strains 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.

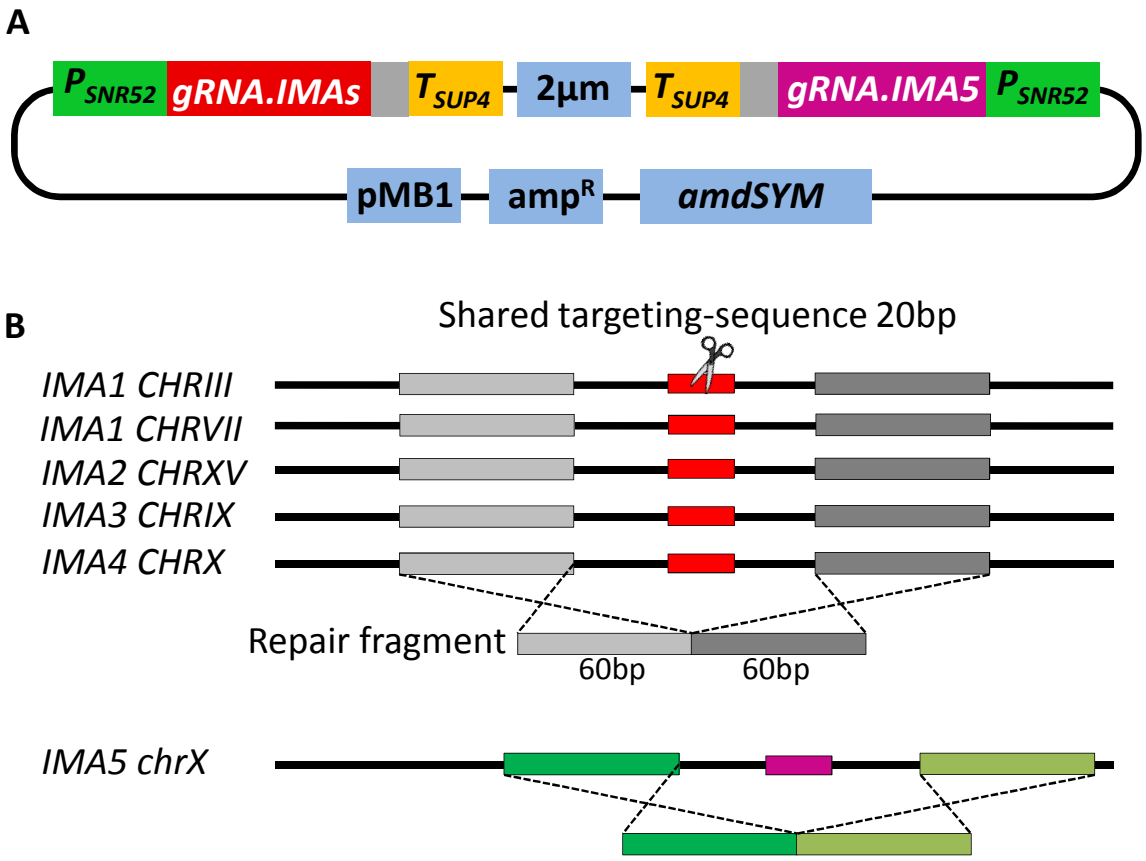


Figure 3.1: Strategy for deletion of the *IMA* genes using a shared Cas9 target site. (**A**) Plasmid pUDR127, that contains two gRNA-cassettes: '*gRNA-IMAs*' (in red) with a target sequence to cleave all *IMA genes* except *IMA5* and "*gRNA-IMA5*", in purple. The SNR52 promoter and SUP4 terminator are 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*.

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 3.2**) 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 °C 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 °C. 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 (OD_{660nm} = 3 – 4). For strains IMU054, IMU055, IMK700, IMK716, IMK717, IMK718, IMZ620, IMZ621, IMZ622 and IMZ623, ethanol (20 g L^{-1}) was used as carbon source instead of sucrose. 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 °C 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 S3.1**). A Rotor-Gene Q (Qiagen) was used with the following sequence: denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s and extension at 72 °C for 20 s. A melting curve up from 60 °C to 95 °C 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 S3.1**). *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.

3.3 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 3.2**). At pH values of 5-7, no growth was observed (**Figure 3.2A**). At pH 3, linear growth was observed (**Figure 3.2A**), which was consistent with the occurrence of sucrose hydrolysis in a sterile culture at this acidic pH (**Figure 3.2B**). Whilst sucrose hydrolysis also occurred at pH 2 (**Figure S3.2**, 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 3.3**). 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

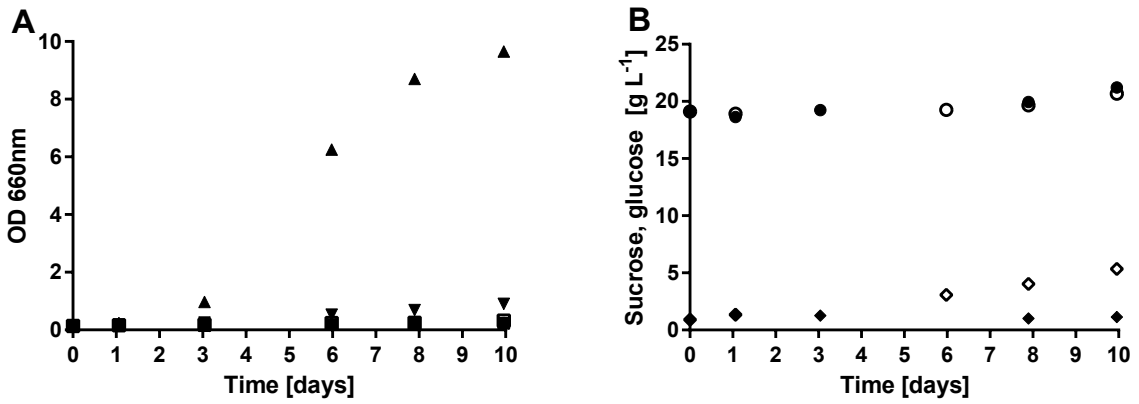


Figure 3.2: Non-biological hydrolysis of sucrose caused by medium acidity. (**A**) Growth of *Saccharomyces cerevisiae* strain IMU051 (*malΔ mphΔ suc2Δ*) in synthetic medium with 20 g L^{-1} sucrose as sole carbon source with initial pH set at different values: pH 2, ■; pH 3, ▲; pH 4, ▼; pH 5, □; pH 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 g L^{-1} were incubated at 30° C and at 200 rpm.

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 3.3**). 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 3.3**). 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 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 3.3**).

Laboratory evolution enables sucrose hydrolysis and increased *IMA* expression

To investigate which genes encode the enzymes responsible for residual sucrose

Table 3.3: 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

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 3.3**), 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 3.3**), 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 3.3**). 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 3.3**). In CEN.PK113-7D, isomaltase activity was only 0.13 µmol mg protein^{-1} min^{-1} (**Table 3.3**). 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 3.3**). 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 3.3A** and **B**). In all analysed strains, the relative expression level of *IMA5* was approximately 5-10 times higher than that of *IMA1* (**Figure 3.3A** and **B**).

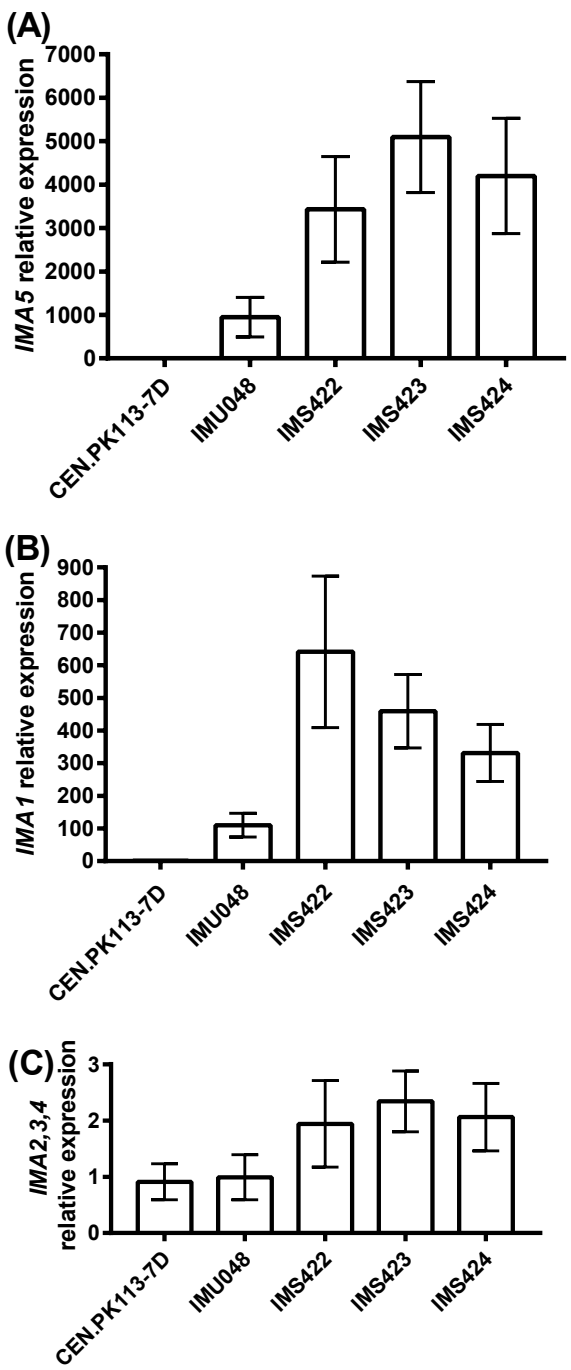


Figure 3.3: Relative expression of *IMA1-5* in *Saccharomyces cerevisiae* strains evolved for faster growth on synthetic medium with sucrose as the sole carbon source. Relative expression of *IMA5* (**A**); *IMA1* (**B**) and *IMA2*, *IMA3* and *IMA4* (**C**). Expression of *IMA2*, *IMA3* and *IMA4* 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 3.1**). All 6 copies of *IMA* genes present in strain IMZ571 and IMS422-424, all derived from strain CEN.PK102-3A (**Table 3.1**) (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 3.4**) or after a further 2-months incubation. Both the sucrose- and isomaltose hydrolysis activities in the resulting strains were below the detection limit (**Table 3.4**).

Table 3.4: 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}.

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 3.4**).

Relative to the 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 3.4**). 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 3.1**). 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 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 3.4**). 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 3.4**). No maltose hydrolysis was detected in any of the *IMA*-overexpressing strains.

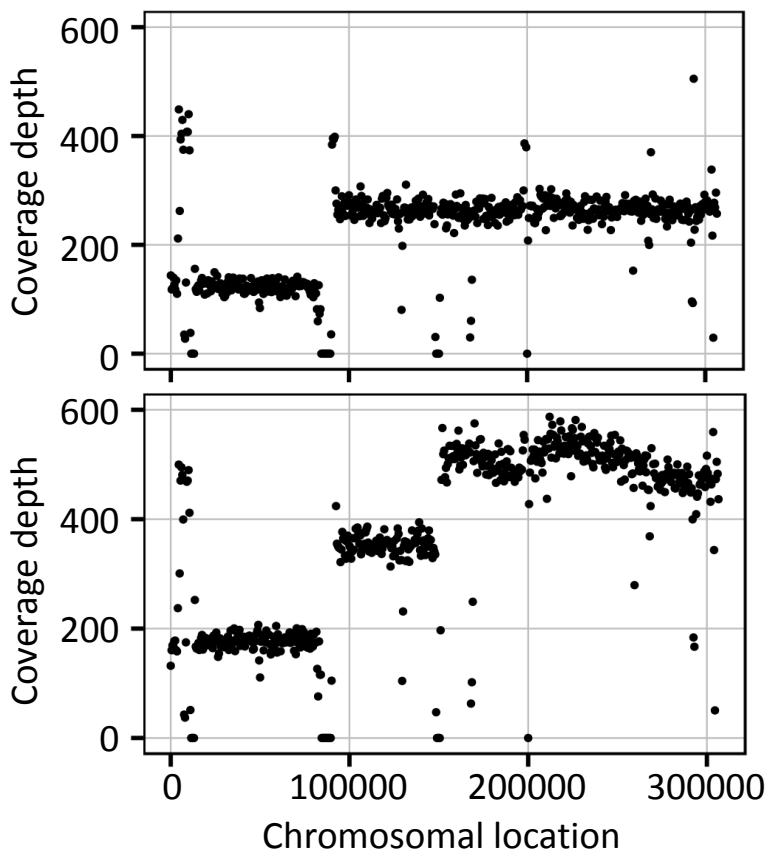


Figure 3.4: Depth of coverage analysis of chromosome III from unevolved strain IMU048 (**A**) and the evolved strain IMS422 (**B**). Sequencing reads from the IMU048 and IMS422 have been mapped onto the CEN.PK113-7D genome sequence (Nijkamp *et al.* 2012). Read count data represent the average coverage of non overlapping 500 bp window. These data are representative of the other two evolved strains (IMS423 and IMS424) .

3.4 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 3.3A** and **B**), but not of *IMA2,3,4* (**Figure 3.3C**), 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 3.4)**. 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.* 2012b). In all the three evolved strains obtained in this study, a third copy of the mentioned region was found (**Figure 3.4**). 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.

3.5 Acknowledgements

We thank Stefan de Kok for the construction of strain IMK291 and Harmen M. van Rossum for his advice on CRISPR/Cas9 genome editing. WLM was supported by the BE-Basic R&D Program, which was granted an FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I), and also by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo, Brazil), grant numbers 2012/05548-1 and 2012/16630-0. This work was carried out as part of a Dual Degree PhD project under the agreement between UNICAMP and Delft University of Technology. RLC was supported by FAPESP, grant number 2014/07962-5. The image on the title page (page 65) was modified from "Yeast Cell" by Col Ford and Natasha de Vere's photostream, which is licenced under CC BY 2.0 and an image made by Lauri Andler, which is licenced under CC BY-SA 3.0, via Wikimedia Commons

Supplementary material

Table S3.1: Primers used in this study.

Primer code	Name	Sequence (5'→ 3')
Primers for *SUC2* deletion		
1482	KO Fw SUC2	CCATTATGAGGGCTTCCATTATTCCCCGCATTTTTATTACTCTGAA CAGGCAGCTGAAGCTTCGTACGC
1483	KO Rv SUC2	CTTTAGAATGGCTTTTGAAAAAAATAAAAAAGACAATAAGTTTT ATAACCGCATAGGCCACTAGTGGATCTG
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	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAA ATGATC**TTCTTCTGGAGACCTCCTAA**GTTTTAGAGCTAGAAATA GCAAGTTAAAATAAG
8761	IMA5_taretRNA_F	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAA ATGATC**TGGCCAACCTGATTTCAATT**GTTTTAGAGCTAGAAATA GCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
6005	p426 CRISP rv	GATCATTTATCTTTCACTGCGGAGAAG
9302	pTDH3_IMA1start	CTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAGAACT TAGTTTCGACGGATATGACTATTTCTTCTGCACATCCAGAGACAG AAC
9303	pTDH3_IMA234strt	CTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAGAAC TTAGTTTCGACGGATATGACTATTTCTTCTGCACATC
9304	pTDH3_IA5star	CTACTTTTATAGTTAGTCTTTTTTTTAGTTTTAAAACACCAGAAC TTAGTTTCGACGGATATGACGATCATCCATAATCCTAAATGGTG GAAAG
9305	tCYC1_IMA1end	CGGTTAGAGCGGATGTGGGGGGAGGGCGTGAATGTAAGCGTGA CATAACTAATTACATGATCATTCGCTGATATATATTCTTCCTTC
9306	tCYC1_IMA234end	CGGTTAGAGCGGATGTGGGGGGAGGGCGTGAATGTAAGCGTGA CATAACTAATTACATGATCATTCAGATATGTAAATTCTGCCCTCC CATG
9307	tCYC1_IMA5end	CGGTTAGAGCGGATGTGGGGGGAGGGCGTGAATGTAAGCGTGA CATAACTAATTACATGATTACTTCAACAAGTAAAGTCTTCCTTCC CATGGCTGCAAGG
Repair fragments		
8592	IMA_repair oligo fw	CGACTCGCCACAAGATGATATGGGTTACGATATTGCCAACTACG AAAAGGTCTGGCCAACATGCTAGAACACCTATGCAATGGTCTC GTGAGGAGCCAAATGCTGGTTTTTCTGGTCCTA
8593	IMA_repair oligo rv	TAGGACCAGAAAAACCAGCATTTGGCTCCTCACGAGACCATTG CATAGGTGTTCTAGCATGTTGGCCAGACCTTTTCGTAGTTGGC AATATCGTAACCCATATCATCTTGTGGCGAGTCG
8763	IMA5_Repair_oligo_Fw	GCGAAAAACTACTGTTTCTTGATGACTATATAGAAAGTGCTCAAT ATTGAAAAGAAAAACCGAGCACATTTAACATAAACGTACTGATC TTTCGAAACAAATAGTTATGCCGATATTTAT
8764	IMA5_Repair_oligo_Rv	ATAAATATCGGCATAACTATTTGTTTCGAAAGATCAGTACGTTTA TGTTAAATGTGCTCGGTTTTTCTTTTCAATATTGAGCACTTTCTA TATAGTCATCAAGAAACAGTAGTTTTTCGC

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

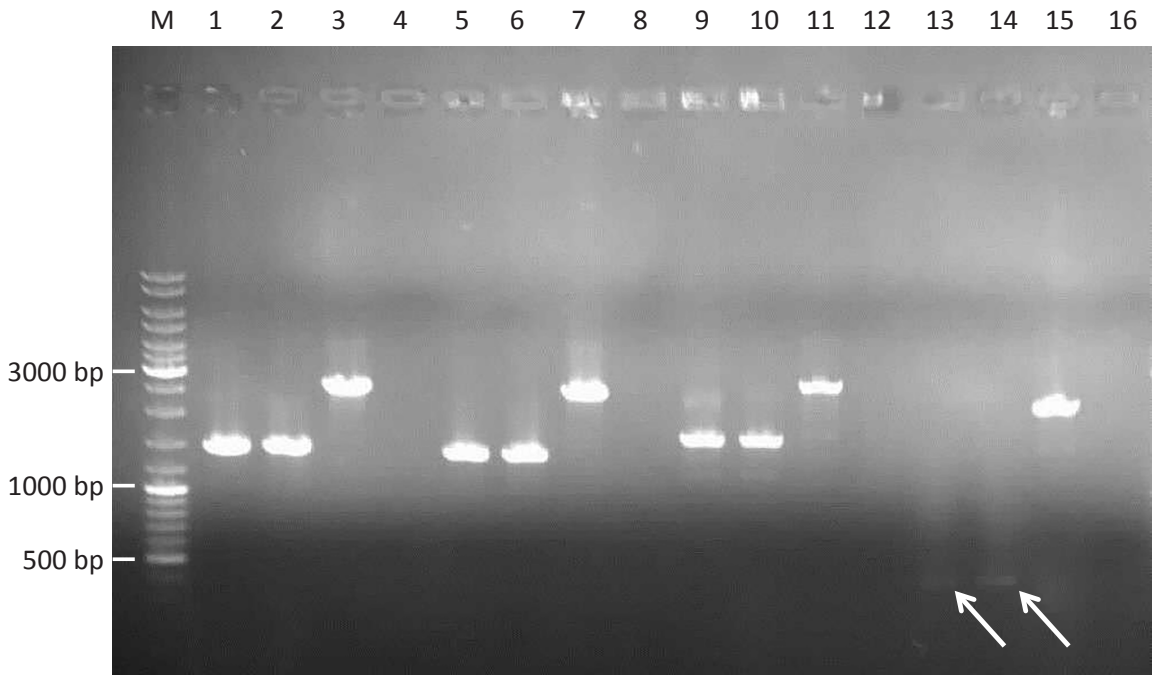


Figure S3.1: Diagnostic PCR of the deletion of the *IMA* genes. The deletion was carried out in strain IMK700 (lane 2, 6, 10 and 14). Lanes 1, 5, 9 and 13 correspond to a strain that is not under the scope of this paper. IMU048 was used for comparison since it carries all the *IMA* genes (lanes 3, 7, 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 S3.1**). 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.

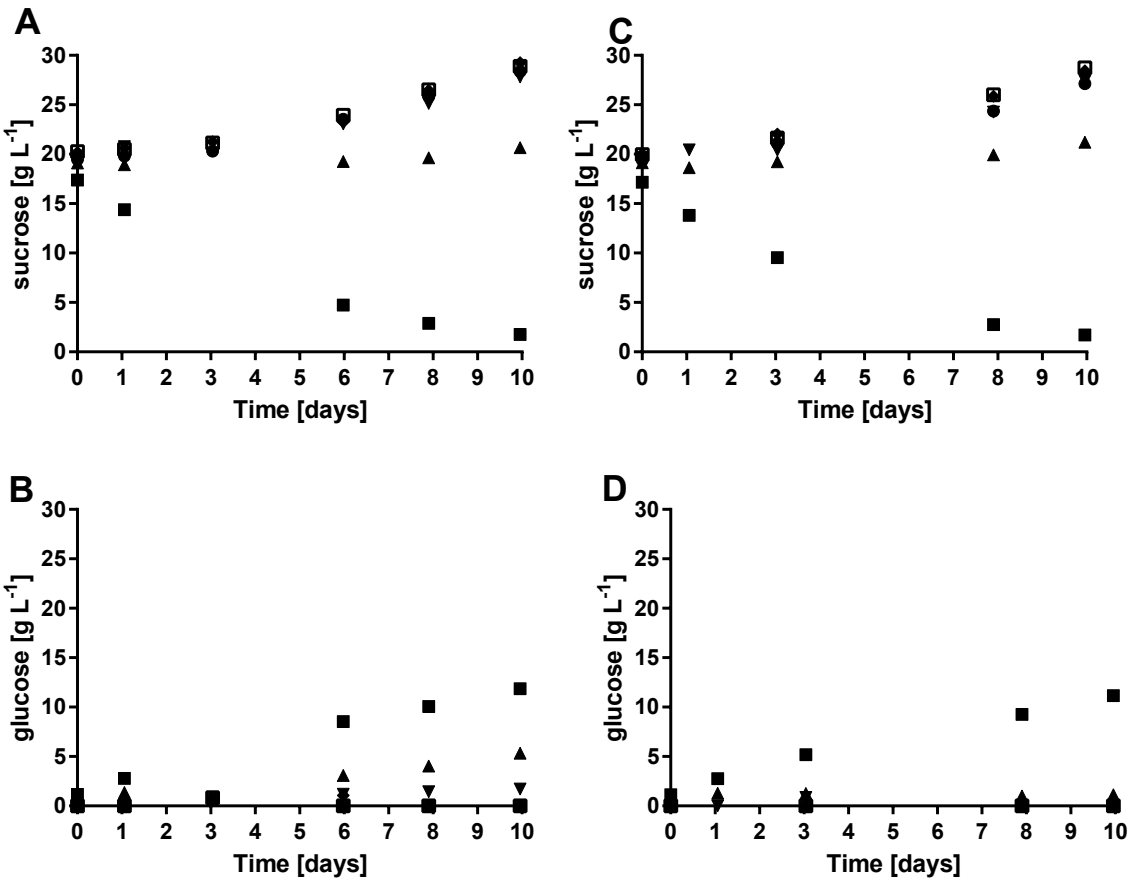
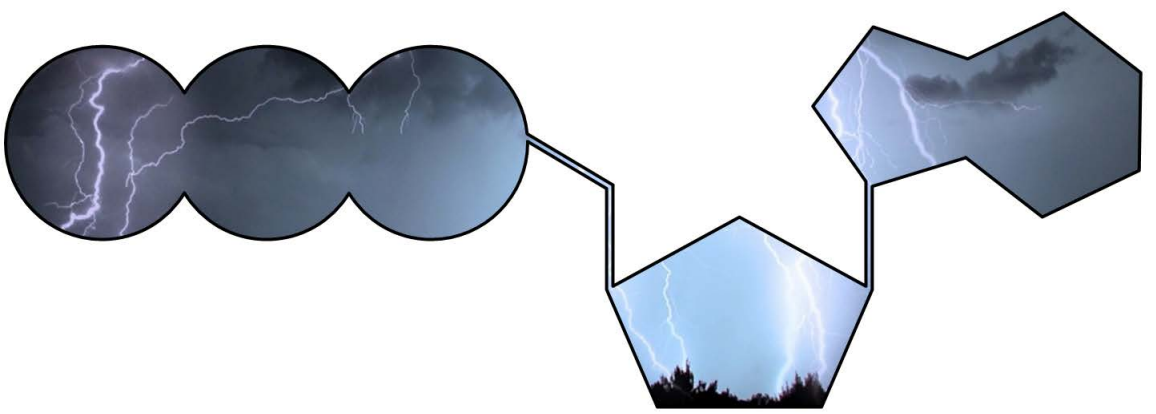


Figure S3.2: Non-biological hydrolysis of sucrose in synthetic medium set at different pH values. **A** and **B** show sucrose and glucose concentrations in the supernatant of shake-flasks without inoculum, respectively. **C** and **D** show sugar concentration in the presence of IMU051 (*malΔ mphΔ suc2Δ*), 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.

Chapter 4

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



Robert Mans*, Wesley Leoricy Marques*, Eko Roy Marella, Jolanda ter Horst, Erik de Hulster, Jean-Marc Daran, Jack T. Pronk, Andreas K. Gombert, Antonius J.A. van Maris

*Joint first authorship

Abstract

Anaerobic industrial fermentation processes do not require aeration and intensive mixing and are therefore economically attractive 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 sucrose facilitator from *Phaseolus vulgaris* (PvSUF1). The resulting strains grew anaerobically on sucrose at specific growth rates of 0.09 ± 0.02 h^{-1} (*LmSPase*) and 0.06 ± 0.01 h^{-1} (*PvSUF1*, *LmSPase*). Overexpression of the yeast *PGM2* gene, which encodes phosphoglucomutase, increased anaerobic growth rates on sucrose of these strains to 0.23 ± 0.01 h^{-1} and 0.08 ± 0.00 h^{-1}, respectively. Determination of the biomass yield in anaerobic sucrose-limited chemostat cultures was used to assess the free-energy conservation of the engineered strains. Replacement of intracellular hydrolase with a phosphorylase increased the biomass yield on sucrose by 31%. Additional replacement of the native proton-coupled sucrose uptake system by facilitated diffusion 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.

4.1 Introduction

Microbial conversion of sugars from renewable feedstocks into chemicals and fuels offers a sustainable alternative to conventional petroleum-based production processes (Nielsen *et al.* 2013). In microbial processes for production of commodity chemicals, the cost of the sugar substrate can be up to 70% of the variable cost price. This impact of substrate costs on process economics necessitates high yield of product on substrate (De Kok *et al.* 2012a; 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 implies 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.* 2012a; 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.* 2012a; 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 (Van Maris *et al.* 2004b; Abbott *et al.* 2009a). 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.* 2009b; 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 4.1A**) or uptake of sucrose by a proton-symport mechanism followed by intracellular hydrolysis (**Figure 4.1B**) (Santos *et al.* 1982; Stambuk, Batista and De Araujo 2000; Batista, Miletti and Stambuk 2005).

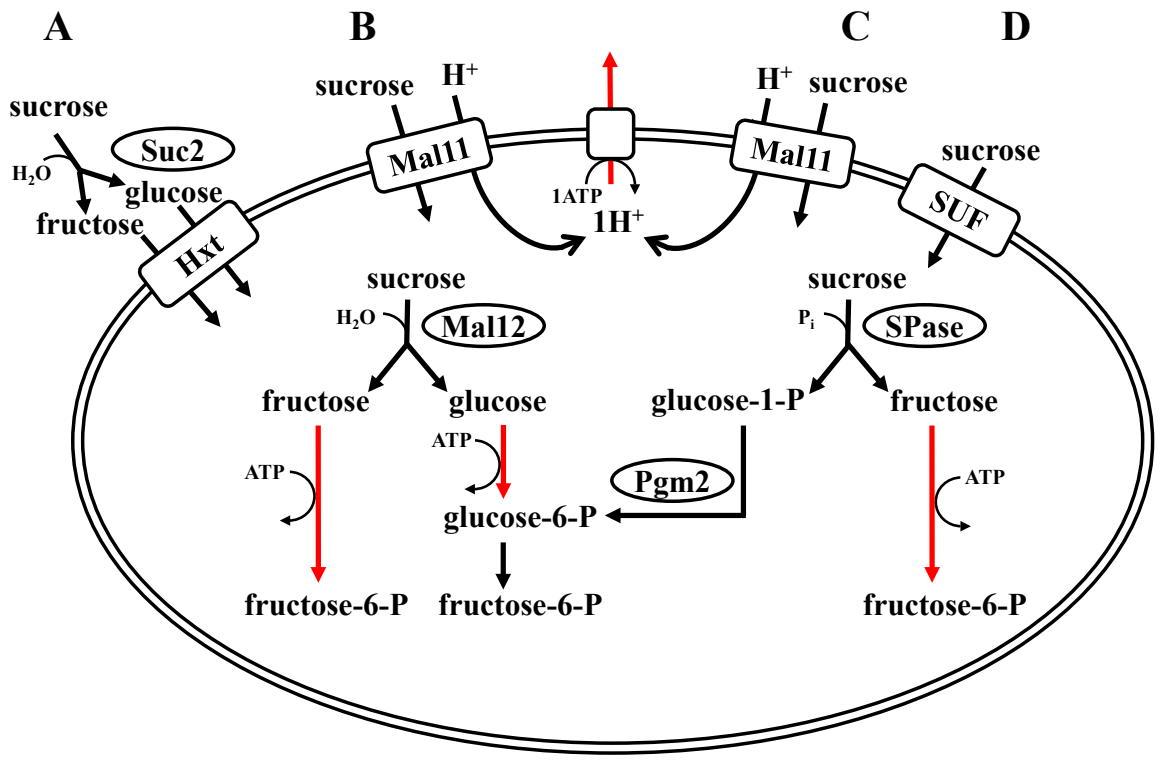


Figure 4.1: Schematic representation of different strategies for the uptake and cleavage of sucrose. (**A**) Extracellular hydrolysis of sucrose, facilitated by the external invertase Suc2, followed by facilitated diffusion of the monosaccharides. (**B**) Uptake of sucrose via the proton-symporter Mal11, followed by ATP-driven export of the proton and intracellular hydrolysis of sucrose catalysed by Mal12. (**C**) Uptake of sucrose via the proton-symporter Mal11, followed by ATP-driven export of the proton and intracellular phosphorolysis of sucrose catalysed by sucrose phosphorylase (SPase). (**D**) Uptake of sucrose via facilitated diffusion using a sucrose facilitator (SUF) followed by intracellular phosphorolysis of sucrose catalysed by SPase. In red: metabolic steps that require hydrolysis of ATP.

S. cerevisiae does not conserve the free energy of sucrose hydrolysis ($\Delta G_o'$ = -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 free-energy conservation than sucrose hydrolysis (**Figure 4.1C**). Genes encoding SPase are known from various bacterial species (Kawasaki *et al.* 1996). Other disaccharide phosphorylases, such as maltose- and cellobiose phosphorylase, have previously been functionally expressed in *S. cerevisiae* (De Kok *et al.* 2011; Sadie *et al.* 2011).

While intracellular phosphorolysis theoretically enables a higher free-energy conservation (gain of 1 ATP per sucrose molecule), it requires transport of extracellular sucrose to the cytosol. However, in wild-type *S. cerevisiae*, uptake of sucrose via a proton-symporter (e.g. Mal11 (Stambuk *et al.* 1999)) and subsequent export of the proton via the H^+-ATPase results in a net expense of 1 ATP (Weusthuis *et al.* 1993). Therefore, an improved free-energy conservation can be achieved when the proton-symport system is replaced by a transporter that catalyses facilitated diffusion (SUF, **Figure 4.1D**). 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.* 2014a) .

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 that lacks all native sucrose proton-symporters and hydrolases and that remained sucrose-negative upon strong selective pressures (Marques *et al.* 2017), was used as a platform strain 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.

4.2 Methods

Strains, growth conditions and storage

The *S. cerevisiae* strains used in this study (**Table 4.1**) share the CEN.PK genetic background (Entian and Kötter 2007; Nijkamp *et al.* 2012) with the exception of BY4742 which is derived from S288C (Brachmann *et al.* 1998). Cultures were grown at 30°C in 500 mL shake flasks containing 100 mL synthetic medium (SM) (Verduyn *et al.* 1992) with 20 g/L glucose as a carbon source in an Innova incubator shaker (Eppendorf, Hamburg, Germany) set at 200 rpm. Frozen stocks were prepared by addition of glycerol (30% v/v) to exponentially growing shake-flask cultures of *S. cerevisiae* and stored aseptically in 1 mL aliquots at -80°C.

Table 4.1: 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

Plasmid construction

All plasmids used in this study are listed in **Table 4.2**. Plasmid pUDC156 was assembled by *in vivo* homologous recombination (Kuijpers *et al.* 2013b; Marques *et al.* 2017), in strain IMK291 resulting in strain IMZ570 (**Table 4.1**). 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 S4.1**). The p426TEF backbone was amplified with primers 6845 & 6846 and ligation was done via Gibson assembly.

Plasmid pUDE379 was constructed via Gibson assembly of the *MAL11* ORF amplified from pUDI035 (De Kok *et al.* 2011) using primers 8379 & 8380 and a vector backbone amplified from plasmid p426TEF-*amdSYM* using primers 7998 & 7999. Plasmid pUDE432 was constructed via Gibson assembly of a *MAL11* expression cassette, amplified from pUDE379 using primers 9043 & 9044 and a vector backbone linearized from p426GPD (Mumberg, 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 4.1**) 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 4.1**). 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 4.1**). Plasmids pUDE367, pUDE368, pUDE369, pUDE370 and pUDE374

Table 4.2: 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

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

from pUDE369, pUDE370, pUDE374, pUDE367 and pUDE368 respectively with primers 9043 & 9044 and the p426GPD vector backbone digested with KpnI and SacI.

For fluorescent tagging of Mal11 and PvSUF1 with the YPet fluorescent protein (Nguyen and Daugherty 2005), first plasmid pRHA00 was made by amplifying the backbone of pFB001 (Bianchi *et al.* 2016) using primers 5273 & 5274 and the *MAL11* gene from the genomic DNA of *S. cerevisiae* strain BY4742 (EUROSCARF, Accession No. Y10000 (Brachmann *et al.* 1998)) with primers 5271 & 5272 followed by *in vivo* assembly. *MAL11* from BY4742 is identical to *MAL11* from CEN.PK-derived strains. Then, the *URA3* marker was omitted from pRHA00 via PCR with primers 5437 & 5438 and replaced with the *LEU2* gene, amplified from pRS315 (Sikorski and Hieter 1989) with primers 5435 & 5436 via *in vivo* assembly. pR151 was then made by Gibson assembly of three fragments; the plasmid backbone and *MAL11* gene amplified from pRHA00L with primers 5959 & 6324 and 5961 & 5272 respectively and the *TEF1* promoter amplified from pUDE379 with primers 4995 & 5960. Plasmid pUDE453 was made via Gibson assembly of a *MAL11*-YPet expression cassette, amplified from pR151 using primers 6717 & 580 and vector backbone amplified from pUDE413 with primers 5921 & 7812. For construction of plasmid pUDE471 via Gibson assembly, the *MAL11* gene from pUDE453 was replaced by *PvSUF1* via amplification of the plasmid backbone, including the YPet-tag, from pUDE453 with primers 5921 & 9772 and amplification of the *PvSUF1* ORF from pUDE413 with primers 6717 & 9763 before assembly of both fragments.

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 4.1**). 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.* 2016a) 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.

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 S4.1**. 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.* 2013b) , 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. 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 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 20 g/L 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 PvSUF1

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

4.3 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 4.1**) 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 4.3**). Therefore, IMZ616 and 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 by an *in vitro* sucrose phosphorylase activity of 0.90 µmol/mg protein/min (**Table 4.3**). 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 4.3**). 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). These results demonstrate that *Lm*SPase was functionally expressed in IMZ665. Additionally, a reference strain expressing *MAL11* combined with a native glucosidase

Table 4.3: Specific growth rates on sucrose and enzyme activities of sucrose hydrolase and sucrose phosphorylase in *S. cerevisiae* strains expressing either the sucrose transporter Mal11 or PvSUF1 in combination with either the sucrose hydrolase Mal12 or sucrose phosphorylase *Lm*SPase in anaerobic bioreactors. Average growth rates were determined from triplicate experiments and based on CO_2 production in anaerobic batch fermentations at pH 5.0 and 25 g/L sucrose. Enzyme activities represent the average of measurements on duplicate steady-state chemostat cultures at pH 5.0, 25 g/L sucrose and a dilution rate of 0.030 h^{-1} (IMZ664, IMZ665 and IMZ636) or 0.07 h^{-1} (IMZ709 and IMZ696). IMZ616 was incubated in aerobic shake flasks with 20 g/L sucrose for growth assessment and 20 g/L ethanol and 20 g/L sucrose for enzyme activity assays.

Strain	Relevant Genotype	Growth rate (h^{-1})	Enzyme activity (µmol/mg protein/min)		
			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/mg protein/min)
[a] Measured in duplicate
[b] No growth observed after 2 months

(*MAL12*) was constructed (IMZ664, **Table 4.1**). This strain grew anaerobically on sucrose, at a specific growth rate of 0.19 ± 0.01 h^{-1} (**Table 4.3**). 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 4.1B**). Replacement of sucrose hydrolysis by sucrose phosphorylase increases the theoretical yield to 4 ATP per sucrose (**Figure 4.1C**). 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 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 4.3**), 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 4.4**). This observed difference of 31% is close to the theoretical value of 33%. Increased free-energy conservation in the catabolic pathway dictates that less sucrose needs to be fermented to ethanol and CO_2 to provide the same amount of ATP required for biomass formation. Accordingly, the biomass specific uptake rate of sucrose was 32% lower (1.21 ± 0.00 vs 1.59 ± 0.05 mmol/g biomass/h) and rates of ethanol and CO_2 production were 39% (4.17 ± 0.01 vs 5.81 ± 0.15 mmol/g biomass/h) and 34% (4.17 ± 0.01 vs 5.81 ± 0.15 mmol/g biomass/h) lower in strain IMZ655 (*MAL11*, *LmSPase*) as compared to those in IMZ664 (*MAL11*, *MAL12*). Replacement of sucrose hydrolysis by phosphorolysis also resulted in a decrease in the residual sucrose concentration from 0.12 ± 0.02 g/L to 0.07 ± 0.01 g/L (**Table 4.4**).

Table 4.4: 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 rates are shown with the denotation $q_{metabolite}$. Averages, mean deviations and standard deviations were obtained from duplicate (IMZ664, IMZ665 and IMZ636) or triplicate (IMZ709 and IMZ696) experiments.

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

N.D. = not determined

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

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 sucrose facilitator genes *PsSUF1*, *PsSUF4*, *PvSUF1*, *OsSWEET11* and *AtSWEET12* 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 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 4.2**). 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 4.3**). 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 *Lm*SPase-expressing strain IMZ636 was subjected to a further characterization.

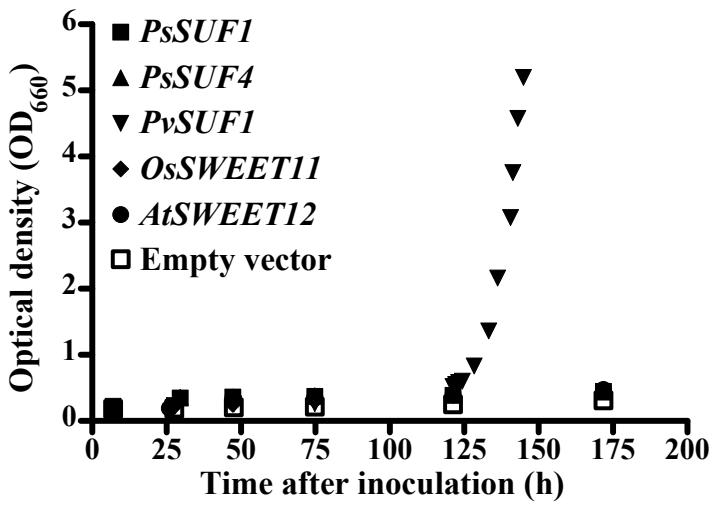


Figure 4.2: Growth curves of *S. cerevisiae* strains expressing *LmSPase* in combination with either *PsSUF1* (IMZ683 (■)), *PsSUF4* (IMZ682 (▲)), *PvSUF1* (IMZ636 (▼)), *OsSWEET11* (IMZ677 (◆)), *AtSWEET12* (IMZ678 (●)) or an empty vector (IMZ692 (□)) in aerobic shake flasks containing synthetic medium with sucrose as the sole carbon source. One representative culture of duplicate cultivations is shown in the figure. All the symbols overlap, except for ▼ from 125 h onwards.

Cellular localisation of both endogenous YPet-tagged Mal11 (IMX1272) and YPet-tagged PvSUF1 (IMX1273) was investigated microscopically (**Figure 4.3**). Both strains showed a similar fluorescence at the periphery of cytosol, confirming that both Mal11 and PvSUF1 were indeed targeted to the plasma membrane. However, distribution of the remainder of the fluorescence differed significantly between the two strains. Whereas intracellular fluorescence in strain IMX1272 (Mal11) was predominantly associated with vacuoles (**Figure 4.3A**), fluorescence of IMX1273 (PvSUF1) appeared to be distributed over multiple smaller intracellular compartments, probably reflecting its occurrence in endoplasmic reticulum (ER), Golgi complex and/or other cellular compartments (**Figure 4.3C**).

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 4.1D**). 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 *Lm*SPase and PvSUF1, strain IMZ636 (*PvSUF1*, *LmSPase*) was grown in anaerobic sucrose-limited

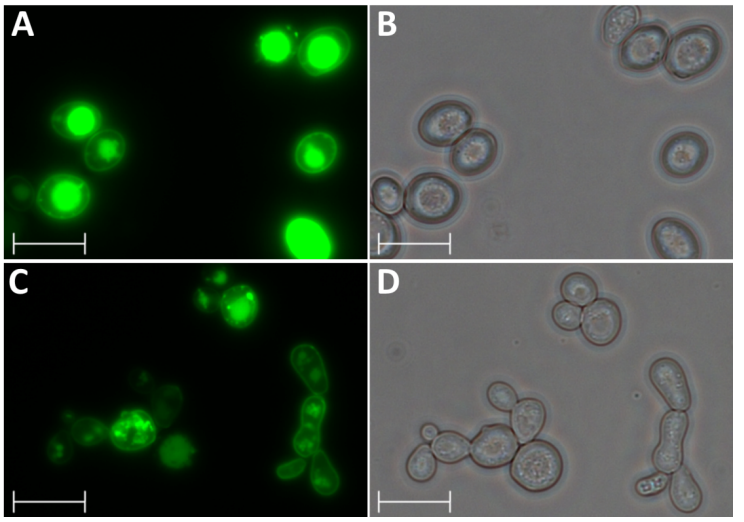


Figure 4.3: Fluorescent and phase-contrast pictures of *S. cerevisiae* strains IMX1272 (*MAL11-YPet, LmSPase*, **A** & **B**) and IMX1273 (*PvSUF1-YPet, LmSPase*, **C** & **D**). Cells were collected from aerobic, steady-state, sucrose-limited chemostat cultures grown at a dilution rate of 0.030 h^{-1}. The scale bar represents 10 μm.

chemostat cultures at a dilution rate of 0.030 h^{-1}. The observed anaerobic biomass yield on sucrose was increased by 8% from 0.069 ± 0.000 g/g glucose equivalent for strain IMZ655 (*MAL11*, *LmSPase*) to 0.075 ± 0.000 g/g glucose equivalent for IMZ636 (*PvSUF1*, *LmSPase*) (**Table 4.4**). In line with an increased ATP yield, the biomass specific sucrose uptake rate (1.11 ± 0.00 vs 1.21 ± 0.00 mmol/g biomass/h), specific ethanol production rate (3.69 ± 0.06 vs 4.17 ± 0.01 mmol/g biomass/h) and CO_2 production rate (3.79 ± 0.05 vs 4.29 ± 0.13 mmol/g biomass/h) decreased for IMZ636 (*PvSUF1*, *LmSPase*) compared to IMZ665 (*MAL11*, *SPase*). The observed 8% increase in the anaerobic biomass yield was lower than the predicted 25%. Mislocalization and/or increased protein turnover of PvSUF1 could have increased the maintenance energy requirement and thereby resulted in a lower-than-expected increase in the biomass yield. The impact of the cellular maintenance energy requirements on the biomass yield decreases with an increase in the specific growth rate (Leuenberger 1971). 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 *LmSPase*-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 4.3**). 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 4.3**). The higher maximum specific growth rates of *PGM2*-expressing strains allowed for a chemostat-based, quantitative evaluation

of the impact of the combined expression of PvSUF1 and *Lm*SPase at higher dilution rates and an additional set of steady-state chemostat cultures was performed at a dilution rate of 0.07 h^{-1}.

As expected, 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 4.4**). However, the difference in biomass yield between the strains was identical (8%) to the difference observed at 0.030 h^{-1}.

4.4 Discussion

The native sucrose hydrolases of *S. cerevisiae* were functionally replaced by a prokaryotic phosphorylase. The resulting increase in the anaerobic biomass yield on sucrose (31%) is very close to the theoretically expected difference (33%, i.e. from 3 to 4 moles of ATP per mole of sucrose consumed), proving the energetic benefit of replacing sucrose hydrolysis by phosphorolysis. Besides the energetic benefit for the cleavage of intracellular sucrose via SPase, also the kinetics of *L. mesenteroides* SPase, K_m = 5.7 mM and k_{cat} = 165 s^{-1} (Goedl *et al.* 2007), are better than those of Mal12, K_m = 12 mM and k_{cat} = 0.45 s^{-1} (Voordeckers *et al.* 2012), which could explain the lower residual sucrose concentrations observed under nutrient limitation (**Table 4.4**). This provides an additional advantage for fed-batch or continuous industrial applications by lowering the fraction of unused substrate. Other potential benefits 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 4.1A**). An engineered strain using proton-coupled uptake (e.g. Mal11) and intracellular phosphorolysis has an identical ATP yield (**Figure 4.1C**), 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 *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. This indicates that the conversion of glucose-1-P to glucose-6-P was rate limiting in sucrose-grown *Lm*SPase-expressing *S. cerevisiae* strains and co-expression of a phosphoglucomutase is essential for faster sucrose conversion.

Replacement of a hydrolase by a phosphorylase has previously been demonstrated for maltose and cellobiose (De Kok *et al.* 2011; Sadie *et al.* 2011). However,

only by combining the energetic benefits of disaccharide uniport and phosphorolysis can the energetic efficiency of wild type strains be exceeded. In this study, combined expression of *Lm*SPase and PvSUF1 resulted in an 8% increase in the biomass yield on sucrose compared to the Mal11/*Lm*SPase-based reference strain and 41% compared to the Mal11/Mal12-based reference strain (**Figure 4.1**, **Table 4.4**). Although promising, the observed 8% increase in biomass yield was below the theoretical prediction of 25%. Three 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 and/or sucrose hydrolysis activity. Cellular maintenance energy requirements have been shown to be growth rate independent in anaerobic, sugar-limited *S. cerevisiae* cultures (Boender *et al.* 2009). Especially at low specific growth rates, changes in genotype or experimental conditions that lead to changes in maintenance-energy requirements can strongly affect biomass yield (Leuenberger 1971). We hypothesized that the abundance of fluorescence originating from YPet-tagged PvSUF1 observed in various intracellular compartments (**Figure 4.3C**) could indicate increased protein turnover and/or maintenance in PvSUF1-expressing strains. However, the observation that the difference in the biomass yield between strains expressing *Lm*SPase with either Mal11 or PvSUF1 was independent of the dilution rate (**Table 4.4**) indicated that a difference in maintenance energy requirements was probably not responsible for the lower-than-predicted increase in biomass yield. Alternatively, a change in biomass composition could result in a lower biomass yield on sucrose in PvSUF1 expressing strains. Since protein synthesis is the main contributor to the energetic cost of biomass formation (Stouthamer 1973), the protein content of IMZ664 (*MAL11*, *MAL12*), IMZ665 (*MAL11*, *LmSPase*) and IMZ636 (*PvSUF1*, *LmSPase*) was determined in steady-state chemostat cultures at a dilution rate of 0.030 h^{-1}. No significant differences in cellular protein content were observed between the steady state chemostat cultures of the relevant strains grown at 0.030 h^{-1} (**Table 4.4**). Lastly, sucrose hydrolytic activity catalysed by *Lm*SPase has been described (Goedl *et al.* 2010), which has the potential to lower the biomass yield on sucrose due to competition with phosphorolytic cleavage. A low but significant (0.06 µmol/mg protein/min, **Table 4.3**) 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/mg protein/min, **Table 4.3**) and no activity could be detected in a strain void of either SPase or Mal12 (IMZ616, **Table 4.3**) 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.

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.* 2012). In addition to sucrose facilitators, a putative maltose facilitator from *Arabidopsis thaliana* (Rost, Frank and Beck 1996; Niittylä *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.* 2004b; De Kok *et al.* 2012a; 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.

4.5 Acknowledgements

We thank Professor Dr. John W. Patrick from the School of Environmental & Life Sciences (The University of Newcastle, Australia) for sending the plasmids containing *PsSUF1* and *PsSUF4* expression cassettes. We thank Ryan Henderson and Bert Poolman for stimulating discussions on the expression of heterologous transporters in yeast and Ryan for the construction of plasmid pR151. We thank our colleagues Marijke Luttik, Mark Bisschops and Xavier Hakkaart for assistance and advice regarding the fluorescence microscopy pictures. We thank Mandy Hulst, Yannick Buys, Hein van der Wall, Jordi Geelhoed and Annabel Giezekamp, who contributed to this work as part of their BSc/MSc studies, for their involvement in screening for functional sucrose facilitators. This work was supported by the BE-Basic R&D Program (FS6.003) (http://www.be-basic.org/), which was granted a FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I) and by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo, Brazil), grant numbers 2012/05548-1 and 2012/16630-0. The work of Wesley Marques was carried out as part of a Dual Degree PhD project under the agreement between UNICAMP and Delft University of Technology. The image on the title page (page 91) was modified from “Lightning And Dark Clouds” by axelle b.

Supplementary material

Table S4.1: Primers used in this study.

Primer	**Sequence (5' → 3')**	**Purpose**
3093	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGCC AGATCATCAATAGGCACCTTCGTACGCTGCAGGTCGAC	*amdSYM* amplification
3094	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCATAGCCA TGCCTTCACATATAGTGACGGATCGCTTGCCTGTAAC	*amdSYM* amplification
6845	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCATAGCCA TGCCTTCACATATAGTACAGGCAACACGCAGATATAGG	p426TEF backbone amplification
6846	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATTCGACCAG CCTAAGAATGTTCAACGGCCCACTACGTGAACCATC	p426TEF backbone amplification
8379	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGATCCATGA AAAATATCATTTCATTGGTAAGCAAGAAG	*MAL11* ORF amplification
8380	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAGCTTCCTT AACATTTATCAGCTGCATTTAATTC	*MAL11* ORF amplification
7998	AAGCTTATCGATACCGTCGACC	p426TEF *amdSYM* backbone amplification
7999	GGATCCACTAGTTCTAGAAAACTTAGATTAG	p426TEF *amdSYM* backbone amplification
9043	TCACAGAGGGATCCCGTTACCCATCTATGCTGAAGATTTATCAT ACTATTCCTCCGCTCGGCGATCGCGTGTGGAAGAAC	*MAL11* expressing cassette amplification
9044	CGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCGCC GCAAATTAAAGCCTTCG	*MAL11* expressing cassette amplification
10303	TTAAATCTATAACTACAAAAAACACATACATAAACTAAAAATGT CATTTCAAATTGAAACG	*PGM2* ORF amplification
10304	ACTACAATATAAAAAAACTATACAAATGACAAGTTCTTGATTAA GTACGAACCGTTGGTTCTTCAG	*PGM2* ORF amplification
6486	TTTTAGTTTATGTATGTGTTTTTTGTAGTTATAGATTTAAGCAAG	pUDE206 backbone amplification
9719	TCAAGAACTTGTCATTTGTATAG	pUDE206 backbone amplification
10305	GGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTAGT GTGAGCGGGATTTAAACTGTGAGG	*PGM2* expressing cassette amplification
10306	GTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGACAG TATAGCGACCAGCATTC	*PGM2* expressing cassette amplification
10307	AACAGCTATGACCATGATTA	pUDE432 or pUDE413 backbone amplification
10308	TCCTGTGTGAAATTGTTATC	pUDE432 or pUDE413 backbone amplification
8018	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGATCCCTAG AATGGATAATCCTTCCACCAATG	Transporter expression cassette amplification
8019	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAGCTTCTAA TGAAATCCACCCGCAATTGG	Transporter expression cassette amplification
8020	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGATCCAATG CCGAATCCCGACTCTTC	Transporter expression cassette amplification
8021	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAGCTTGTCG ACTCATACTGGGTTTCTAGG	Transporter expression cassette amplification
8022	CATAGCAATCTAATCTAAGTTTTCTAGAACTAGTGGATCCATGG AAGCCCCATCCCCAAC	Transporter expression cassette amplification
8023	ACTAATTACATGACTCGAGGTCGACGGTATCGATAAGCTTACTT AATGAAAACCACCTGCTACCATAC	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	CAAGGAGAAAAAACCCCGGATTCTAGAACTAGTGGATCCCCCA TGAAAAATATCATTTCATTGGTAAG	*MAL11* amplification from BY4742
5272	GAATAATTCTTCACCTTTAGAACCTTGAAAATATAAATTTTCCCC TCCACATTTATCAGCTGCATTTAATTC	*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	TCTAGAACTAGTGGATCCCAAAATGAAAAATATCATTTCATTGG	*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	AAAACTTAGATTAGATTGCTATGCTTTCTTTCTAATGAGC	pUDE413 backbone amplification or YPet-tag amplification from pUDE453
7812	TCATGTAATTAGTTATGTCACGCTTACATTC	pUDE413 backbone amplification
9772	CGAAGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTCATGGA GGGGAAAATTTATATTTTCAAGGTTCTAAAG	YPet-tag amplification from pUDE453
9763	ATGAAAACCACCTGCTACCATAC	Cassette amplification from pUDE413
9355	TGTAAATATCTAGGAAATACACTTGTGTATACTTCTCGCTTTTCT TTTATTTTTTTTTGTAGTTTATCATTATCAATACTCGCCATTTC	*LmSPase* or *MAL12* integration (*SGA1* homology)
9356	TTTACAATATAGTGATAATCGTGGACTAGAGCAAGATTTCAAAT AAGTAACAGCAGCAAAGTGTGGAAGAACGATTACAACAG	*MAL12* or *LmSPase* integration (*SGA1* homology)

LmSPase codon optimized:

ATGGAAATCCAAAACAAGGCTATGTTGATCACTTACGCTGACTCTTTGGGTAA
GAACTTGAAGGACGTTCACCAAGTTTTGAAGGAAGACATCGGTGACGCTATCGGT
GGTGTTCACTTGTTGCCATTCTTCCCATCTACTGGTGACAGAGGTTTCGCTCCAGC
TGACTACACTAGAGTTGACGCTGCTTTCGGTGACTGGGCTGACGTTGAAGCTTTGG
GTGAAGAATACTACTTGATGTTCGACTTCATGATCAACCACATCTCTAGAGAATCT
GTTATGTACCAAGACTTCAAGAAGAACCACGACGACTCTAAGTACAAGGACTTCT
TCATCAGATGGGAAAAGTTCTGGGCTAAGGCTGGTGAAAACAGACCAACTCAAGCT
GACGTTGACTTGATCTACAAGAGAAAGGACAAGGCTCCAACTCAAGAAATCACT
TTCGACGACGGTACTACTGAAAACTTGTGGAACACTTTCGGTGAAGAACAAATCGA
CATCGACGTTAACTCTGCTATCGCTAAGGAATTCATCAAGACTACTTTGGAAGACAT
GGTTAAGCACGGTGCTAACTTGATCAGATTGGACGCTTTCGCTTACGCTGTTAAGAA
GGTTGACACTAACGACTTCTTCGTTGAACCAGAAATCTGGGACACTTTGAACGAA
GTTAGAGAAATCTTGACTCCATTGAAGGCTGAAATCTTGCCAGAAATCCACGAA
CACTACTCTATCCCAAAGAAGATCAACGACCACGGTTACTTCACTTACGACTTCGCT
TTGCCAATGACTACTTTGTACACTTTGTACTCTGGTAAGACTAACCAATTGGCTAA
GTGGTTGAAGATGTCTCCAATGAAGCAATTCACTACTTTGGACACTCACGACGG

TATCGGTGTTGTTGACGCTAGAGACATCTTGACTGACGACGAAATCGACTACGCT
TCTGAACAATTGTACAAGGTTGGTGCTAACGTTAAGAAGACTTACTCTTCTGCT
TCTTACAACAACTTGGACATCTACCAAATCAACTCTACTTACTACTCTGCTTTGG
GTAACGACGACGCTGCTTACTTGTTGTCTAGAGTTTTCCAAGTTTTCGCTCCAG
GTATCCCACAAATCTACTACGTTGGTTTGTTGGCTGGTGAAAACGACATCGCT
TTGTTGGAATCTACTAAGGAAGGTAGAAACATCAACAGACACTACTACACTA
GAGAAGAAGTTAAGTCTGAAGTTAAGAGACCAGTTGTTGCTAACTTGTTGAA
GTTGTTGTCTTGGAGAAACGAATCTCCAGCTTTCGACTTGGCTGGTTCTATCACT
GTTGACACTCCAACTGACACTACTATCGTTGTTACTAGACAAGACGAAAACGGT
CAAAACAAGGCTGTTTTGACTGCTGACGCTGCTAACAAGACTTTCGAAATCGTT
GAAAACGGTCAAACTGTTATGTCTTCTGACAACTTGACTCAAAACTAA

***PvSUF1* codon optimized:**

ATGGAAGCCCCATCCCCAACAAAGCCTATCGACCCAACCAAACCAAGTATTACCA
CATTATCCGTAGAAGGTAGTCAAGGTGAACCATCTCCATTAAGAAAAATGTTCGC
CGTTGCTTCAATAGCTGCAGGTATCCAATTTGGTTGGGCTTTGCAATTATCTTTGT
TAACCCCATATGTCCAATTGTTAGGTGTACCTCATGCCGCTGCATCCTTTATATGGT
TATGTGGTCCAATCAGTGGTTTGGTTGTCCAACCTATCGTTGGTTACTACTCTGAT
AGATCTACTTCTAGATACGGTAGAAGAAGACCTTTTATTTTAGGTGGTGCAGTCGC
CGTAGCTATCGCAGTTTTCTTGATTGGTTATGCCGCTGATATTGGTTACTCAGCT
GGTGACGACATAACCAAAAAGACTAGACCAAGAGCCGTTGCTGTCTTCGTAATTG
GTTTCTGGATCTTGGACGTTGCAAACAACATGTTGCAAGGTCCTTGCAGAGCCT
TTTTGGCTGATTTGGCAGCCGGAGATCAAAGAAAGACTAGAATCGCAAACGGT
TTCTTTTCTTTCTTTATGGCCGTTGGTAACGTTTTAGGTTATGCTGCAGGTTCT
TTTTCAGGTTTGCACAAAATTTTCCCTTTTACTCAAACAAAGGCATGTGATGT
TTTCTGCGCCAATTTGAAGTCTTGTTTCTTTTTCTCTATCTTGTTGTTGTTAT
TTTTGTCCACAGTTGCTTTGATCTACGTCAAAGATAAGCCAGTAGCCGCTAGAGC
CGTTCAAGAAGACGCTCAACCTTCTTGCTTCTTTCAATTGTTCGGTGCTTTGAAG
GAATTGAAGAGACCAATGTGGATGTTGATGTTAGTCACCGCAGTAAATTGGATTG
GTTGGTTTCCTTATTTCTTGTTTGATACTGACTGGATGGGTAGAGAAGTTTATGGT
GGTACAGCTGGTGAAGATGCATACGCCGAAGGTGTTAGAGTCGGTTCCTTGGGT
TTAATGATTAACGCTGTAGTTTTGGGTTTTATGAGTTTAGCAGTTGAACCATTGG
GTAGAATGGTTGGTGGTGTCAAGAGATTGTGGGGTATCGTTAACTTCATCTTGGC
TATCGGTTTCGGTATGACAGTCGTAATAACCAAAATGGCAGAACATCAAAGACACT
TAAACCCAGCAGCCGTTGGACATCCTTCTGATGGTGTCAAGATTGGTTCAATGG
TATTCTTTGCTGTATTGGGTGTTCCATTAGCAATCACATTCTCTGTTCCTTTTGCT
TTGGCATCCATCTATTCTTCAGCAAGTGGTGCCGGTCAAGGTTTGTCATTAG
GTGTTTTGAATTTGGCTATTGTTGTCCCACAAATGGTAGTTTCCGCCTTAAGTG
GTCCTTGGGACGCTTTATTCGGTGGTGGTAACTTGCCAGCTTTTATGGTTGGTGCTG
CAGCCGCTGCATTGTCAGCTATCATGGCAATTGTCTTGTTACCTACCCCTAAGC
CAGCCGACGAAGCCAAAGCCGCCAGTATGGTAGCAGGTGGTTTTCATTAA

Chapter 5

A CRISPR/Cas9-based exploration into the elusive mechanism for lactate export in *Saccharomyces cerevisiae*



Robert Mans, Else-Jasmijn Hassing, Melanie Wijsman, Annabel Giezekamp, Jack T. Pronk, Jean-Marc Daran and Antonius J.A. van Maris

Abstract

CRISPR/Cas9-based genome editing allows rapid, simultaneous modification of multiple genetic loci in *Saccharomyces cerevisiae*. Here, this technique was used in a functional analysis study aimed at identifying the hitherto unknown mechanism of lactate export in this yeast. First, an *S. cerevisiae* strain was constructed with deletions in 25 genes encoding transport proteins, including the complete aqua(glycero)porin family and all known carboxylic-acid transporters. The 25-deletion strain was then transformed with an expression cassette for *Lactobacillus casei* lactate dehydrogenase (*LcLDH*). In anaerobic, glucose-grown batch cultures, this strain exhibited a lower specific growth rate (0.15 vs. 0.25 h^{-1}) and biomass-specific lactate production rate (0.7 vs. 2.4 mmol (g biomass)$^{-1}$ h^{-1}) than an *LcLDH*-expressing reference strain. However, a comparison of the two strains in anaerobic glucose-limited chemostat cultures (dilution rate 0.10 h^{-1}) showed identical lactate production rates. These results indicate that, although deletion of the 25 transporter genes affected the maximum specific growth rate, it did not impact lactate export rates when analysed at a fixed specific growth rate. The 25-deletion strain provides a first step towards a 'minimal transportome' yeast platform, which can be applied for functional analysis of specific (heterologous) transport proteins as well as for evaluation of metabolic engineering strategies.

5.1 Introduction

Based on current estimates, membrane transport in *Saccharomyces cerevisiae* involves approximately 300 proteins, of which 139 occur in the plasma membrane (Brohée *et al.* 2010). In cases where membrane translocation of a compound is mediated by one or a few transporter protein(s), accurate determination of mutants in which the corresponding structural gene(s) have been inactivated can aid the identification of transporter function and kinetics (Roman 1956; Grenson *et al.* 1966; Fisher 1969; Silver and Eaton 1969; Whelan, Gocke and Manney 1979). When transport of a single metabolite involves multiple transporters whose functions are not fully redundant, disruption of a single gene can cause a reduced fitness. In such cases, laboratory evolution can (partially) recover the reduced fitness. Subsequent analysis of the responsible suppressor mutations can then help to identify alternative transporters (De Kok *et al.* 2012b).

When an organism harbours a large number of transporters that are able to transport a single compound, single-gene deletion experiments are of very limited value for functional analysis (Delneri *et al.* 2000). The paradigm for addressing such a challenge in yeast research is the study by Wieczorke et al (1999) on the hexose-transporter (*HXT*) gene family in *S. cerevisiae*. These authors showed that sequential deletion of the hexose-transporter genes *HXT1*, *HXT2*, *HXT4*, *HXT5* and *HXT8-16/17* did not cause a distinct growth defect on glucose, fructose or mannose. Instead, growth on glucose was only completely abolished after subsequent deletion of the remaining *HXT3*, *HXT6* and *HXT7* genes (Wieczorke *et al.* 1999). The resulting 'HXT null' strain platform has become an invaluable platform for functional analysis of native and heterologous hexose and pentose transporter genes (for examples see Buziol *et al.* 2002, Wieczorke *et al.* 2003 and Subtil and Boles 2011, for a complete overview see Solis-Escalante *et al.* 2014). At the time of its construction, deletion of so many genes represented a herculean task. Later analysis showed that using multiple rounds of Cre-*LoxP*-based marker-gene recovery had, in fact, created several chromosomal translocations (Solis-Escalante *et al.* 2015).

In contrast to the advanced state of research on *HXT* genes in *S. cerevisiae*, research on the transport of other simple metabolites in this yeast is still in its infancy (Kell *et al.* 2015) and the mechanism by which lactate is exported from yeast cells is a particularly elusive metabolic conundrum. In *S. cerevisiae*, import of lactate can be catalysed by the major facilitator superfamily (MFS) lactate transporter Jen1 and the acetate transporter Ady2 (Cássio, Leao and Van Uden 1987; Casal *et al.* 1999; Paiva *et al.* 2004; Ramos, Sychrová and Kschischo 2016) and combined deletion of *JEN1* and *ADY2* eliminates growth on lactate as the sole carbon source at pH 5.0 (De Kok *et al.* 2012b). While a role of Jen1 in lactate export has been proposed (Porro *et al.* 1999; Branduardi *et al.* 2006), the combined deletion of *JEN1* and *ADY2* does not affect lactate-production rates by *S. cerevisiae* strains expressing a heterologous NAD^+-linked lactate dehydrogenase (*LDH*) (Pacheco *et al.* 2012).

Understanding the mechanism of lactate export in *S. cerevisiae* is of fundamental interest and, moreover, highly relevant for industrial production of lactic acid with engineered yeast strains (Dequin and Barre 1994; Porro *et al.* 1995; Van Maris *et al.* 2004a). Engineered 'homolactic' strains of *S. cerevisiae*, in which the three pyruvate decarboxylase genes have been replaced by a heterologous *LDH* gene, strictly require aeration for growth (Van Maris *et al.* 2004b; Abbott *et al.* 2009a). This oxygen requirement indicates that conversion of glucose to (extracellular) lactate does not result in a net formation of ATP and has been interpreted as reflecting a net ATP requirement for lactate export (**Figure 5.1**) (Van Maris *et al.* 2004b; Abbott *et al.* 2009a). Already before the gene deletion study by Pacheco *et al.* (2012), Jen1 and Ady2 were unlikely candidates for a role as lactate exporter. Both transporters are reported to mediate electroneutral symport of a carboxylate anion with a proton (Cássio, Leao and Van Uden 1987; Casal and Cardoso 1996; Casal *et al.* 1999), which is not compatible with a net stoichiometric requirement for ATP (Van Maris *et al.* 2004a; Abbott *et al.* 2009a).

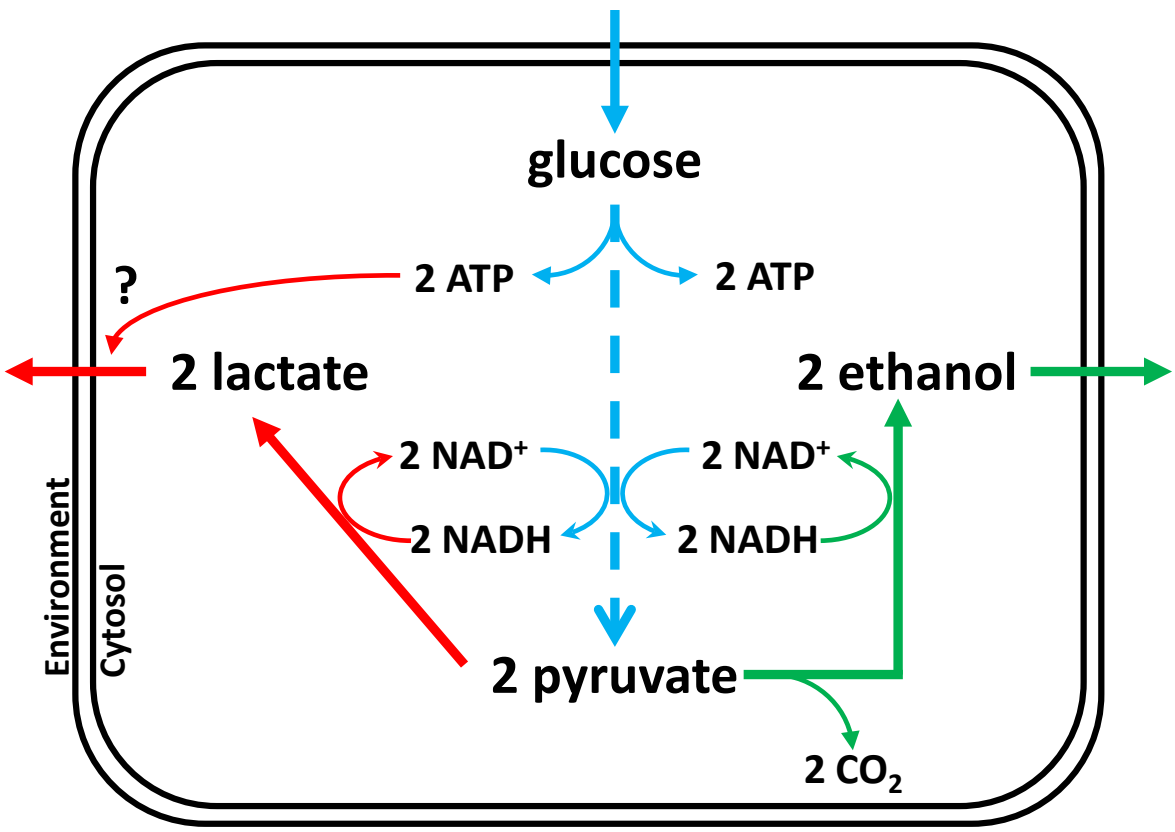


Figure 5.1: Schematic representation of fermentation of glucose to ethanol or lactate. Uptake of glucose proceeds via facilitated diffusion and subsequent conversion by the Embden-Meyerhof-Parnas (EMP) pathway leads to the formation of 2 ATP, 2 NADH and 2 pyruvate (blue). Conversion of 2 pyruvate to 2 ethanol via pyruvate decarboxylase and alcohol dehydrogenase activity leads to the re-oxidation of 2 NADH to 2 NAD^+ and diffusion of ethanol out of the cell is energy-neutral (green). In homolactic engineered *S. cerevisiae* strains, the conversion of 2 pyruvate to 2 lactate by lactate dehydrogenase activity also leads to the re-oxidation of 2 NADH to 2 NAD^+. However, inability of homolactic strains to grow anaerobically indicates that the ATP formed by the EMP-pathway is used for lactate export (red), thereby leaving no ATP for anaerobic growth or cellular maintenance.

At least three distinct mechanisms can be envisaged for energy-dependent transport of lactate (Van Maris *et al.* 2004a); i) transport via an ABC transporter that couples hydrolysis of ATP to export of the undissociated lactic acid or the lactate anion or ii) (facilitated) diffusion of the lactate anion accompanied by export of the associated proton via the plasma-membrane proton ATPase (which, in *S. cerevisiae*, has a stoichiometry of 1 H^+ per ATP; Van Leeuwen *et al.* 1992 and Weusthuis *et al.* 1993), or iii) antiport of lactic acid or lactate with a cation (most likely H^+ or Na^+) and subsequent cation-extrusion via a P-type ATPase to maintain proton-motive force and intracellular pH homeostasis.

The ABC transporter Pdr12 is involved in export of lipophilic carboxylic acids (Piper *et al.* 1998; Holyoak *et al.* 1999; Bauer *et al.* 2003; Hazelwood *et al.* 2006; Ullah *et al.* 2013; Nygård *et al.* 2014). However, a role of Pdr12 or other ABC transporters in export of lactate has not been demonstrated. Similarly, although the aquaglyceroporin Fps1 mediates diffusion of other carboxylic acids over the yeast plasma membrane (Mollapour and Piper 2007), a similar transport mechanism for the lactate anion has not been described. Additionally, the strongly reduced uptake of acetic acid via Fps1 at high pH values suggests that the undissociated carboxylic acid rather than the anion is the substrate(Mollapour and Piper 2007), which would not be compatible with a net ATP requirement for lactate export via this aquaglyceroporin.

Polar carboxylic acid anions such as lactate are generally assumed not to freely diffuse over membrane lipid bilayers at physiologically relevant rates (Cássio, Leao and Van Uden 1987; Lambert and Stratford 1999). Based on this assumption, at least one lactate transporter, responsible for energy-coupled lactate export, remains to be identified (Van Maris *et al.* 2004b; Abbott *et al.* 2009a). Hirasawa *et al.* (2013) addressed this challenge by transforming 4802 single- gene deletion mutants of *S. cerevisiae* with an expression cassette for an *LDH* gene. Subsequently, these authors quantified the effect of the deletion on the lactate production rates. Only one transporter gene deletion, in *PDR12*, caused a > 90 % reduction of final lactate titers. However, this effect was probably an artefact caused by the use of an auxotrophic strain background (Bauer *et al.* 2003) and the other single-gene deletions of transporter encoding genes did not have a similarly strong impact on lactate export. Apparently, the lactate exporter gene was not amongst the 4802 genes included in the screening or, alternatively, multiple, functionally redundant transporters mediate lactate export.

The recent development and implementation of CRISPR/Cas9-based genome editing has facilitates the simultaneous introduction of genetic modifications in multiple yeast genes (at least up to six; Bao *et al.* 2015; Horwitz *et al.* 2015; Mans *et al.* 2015). The aim of the present study was to investigate the elusive molecular basis for lactate export in *S. cerevisiae*. Candidate lactate transporters were selected based on their ability to transport other carboxylic acids, involvement in carboxylic-acid tolerance and similarity to known carboxylic acid transporter proteins. Additional target genes were selected because their deletion was reported to affect lactate production in a strain expressing *LDH* (Hirasawa *et al.* 2013). The selected transporters were then all deleted in

a single strain by CRISPR-Cas9-mediated genome editing. Subsequently, an *LDH* gene was introduced and the biomass-specific lactate production rate of the resulting strain was compared to that of an isogenic control strain containing the same *LDH* expression cassette but no transporter deletions.

5.2 Methods

Strains, growth conditions and storage

The *S. cerevisiae* strains used in this study (**Table 5.1**) share the CEN.PK genetic background (Entian and Kötter 2007; Nijkamp *et al.* 2012). Shake flask cultures were grown at 30°C in 500 mL flasks containing 100 mL synthetic medium (SM) (Verduyn *et al.* 1992) with 20 g L^{-1} glucose in an Innova incubator shaker (Eppendorf, Hamburg, Germany) set at 200 rpm. After introduction of the lactate dehydrogenase gene, a mixture of 2% (v/v) glycerol and 3% (v/v) ethanol was used as a carbon source during strain construction. When required, auxotrophic requirements were complemented via addition of 150 mg L^{-1} uracil, 100 mg L^{-1} histidine, 500 mg L^{-1} leucine, 75 mg L^{-1} tryptophan (Pronk 2002) or by growth in YP medium (demineralized water, 10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone). For plate cultivation 2% (w/v) agar was added to the medium prior to heat sterilization. Frozen stocks were prepared by addition of sterile glycerol (30% v/v) to exponentially growing shake-flask cultures of *S. cerevisiae* or overnight cultures of *E. coli* and 1 mL aliquots were stored aseptically at -80 °C .

General molecular biology techniques

PCR amplification with Phusion Hot Start II High Fidelity Polymerase (Thermo Fisher Scientific, Waltham, MA) was performed 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 to confirm successful deletions can be found in **Table S5.1**. Yeast genomic DNA was isolated according to Lõoke *et al* (Lõoke, Kristjuhan and Kristjuhan 2011). DNA fragments obtained by PCR were separated by gel electrophoresis on 1% (w/v) agarose gels (Thermo Fisher Scientific) in TAE buffer (Thermo Fisher Scientific) at 100 V for 30 min. Fragments were excised from gel and purified by gel purification (Zymoclean D2004, Zymo Research, Irvine, CA). Plasmids were isolated from *E. coli* with Sigma GenElute Plasmid kit (Sigma-Aldrich) according to the supplier's manual. Restriction analysis was performed using FastDigest enzymes (Thermo Scientific) according to the manufacturer's manual. Plasmid assembly was performed using the Gibson Assembly Cloning kit (New England Biolabs, Ipswich, MA) according to the suppliers protocol, but downscaled to 5 µL total volume. *E. coli* DH5α (18258-012, Thermo Fisher Scientific) or XL1-Blue (GE Healthcare Life Sciences, Uppsala, Sweden) was used for chemical transformation (T3001, Zymo Research) or for electroporation. Chemical transformation

Table 5.1: *Saccharomyces cerevisiae* strains used in this study.

Name	Relevant genotype	Parental strain	Origin
CEN.PK113-7D	MATa URA3 TRP1 LEU2 HIS3		P. Kötter
IMX672	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2*	CEN.PK2-1C	(Mans *et al.* 2015)
IMX717	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ* pUDR002 (2μm ampR *TRP1* gRNA-*MCH1* gRNA-*MCH2*) pUDR004 (2μm ampR *HIS3* gRNA-*MCH5* gRNA-*AQY1*) pUDR005 (2μm ampR *URA3* gRNA-*ITR1* gRNA-*PDR12*)	IMX672	(Mans *et al.* 2015)
IMX878	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ*	IMX717	This study
IMX880	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ* pUDR007 (2μm ampR *URA3* gRNA-*MCH3* gRNA-*MCH4*) pUDR008 (2μm ampR *HIS3* gRNA-YIL166C gRNA-*HXT1*)	IMX878	This study
IMX902	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ jen1Δ ady2Δ* pUDR007 (2μm ampR *URA3* gRNA-*MCH3* gRNA-*MCH4*) pUDR008 (2μm ampR *HIS3* gRNA-YLL053C gRNA-*HXT1*) pUDR126 (2μm ampR *KlLEU2* gRNA-*JEN1* gRNA-*ADY2*)	IMX880	This study
IMX903	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ jen1Δ ady2Δ*	IMX902	This study
IMX945	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ* pUDR151 (2μm ampR *KlLEU2* gRNA-*AQR2* gRNA-*THI73*) pUDR152 (2μm ampR *HIS3* gRNA-*FPS1* gRNA-*AQY2*-YLL053C)	IMX903	This study
IMX973	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ*	IMX945	This study
IMX987	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ* pUDR169 (2μm ampR *HIS3* gRNA-*AQY3* gRNA-*TPO2*)	IMX973	This study
IMX999	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ yro2Δ azr1Δ*	IMX987	This study
IMX1000	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ yro2Δ azr1Δ yhl008cΔ tpo3Δ*	IMX999	This study
IMX1052	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2* pUDE411 (2μm ampR *URA3 TRP1 LEU2 HIS3 LcLDH*)	IMX672	This study
IMX1053	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2* pUDE412 (2μm ampR *URA3 TRP1 LEU2 HIS3*)	IMX672	This study
IMX1066	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ yro2Δ azr1Δ yhl008cΔ tpo3Δ* pUDE411 (2μm ampR *URA3 TRP1 LEU2 HIS3 LcLDH*)	IMX1000	This study
IMX1067	*MAT*a *ura3-52 trp1-289 leu2-3,112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt11Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ yro2Δ azr1Δ yhl008cΔ tpo3Δ* pUDE412 (2μm ampR *URA3 TRP1 LEU2 HIS3*)	IMX1000	This study

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 DH5α cells were prepared according to BioRad's protocol, except for the use of LB medium without NaCl when pre-growing the cells.

Plasmid construction

For CRISPR/Cas9-based deletion of the transporter genes, plasmids containing two guide RNA (gRNA) sequences were constructed as described previously (Mans *et al.* 2015). **Table S5.1** lists the primers used for the amplification of the 2µm fragment for each of the double gRNA ("pUDR*") plasmids and the plasmid template used for the amplification of the backbone containing the selection marker with primer 6005 (Mans *et al.* 2015). The two fragments were combined using Gibson assembly, followed by chemical transformation to *E. coli* for storage and plasmid propagation (**Table 5.2**).

Plasmids containing the marker genes to complement the uracil, leucine, histidine and tryptophan auxotrophies were assembled *in vitro*, using Gibson assembly of a plasmid backbone containing the *URA3* marker and a 2µm origin of replication, with three fragments containing the *TRP1*, *LEU2* and *HIS3* marker genes. All fragments were obtained using Phusion Polymerase. The plasmid backbone was amplified from YepLDH#1 (Van Maris *et al.* 2004b) with primers 8996 & 9000 (all primers are listed in **Table S5.2**) and the *LEU2* fragment was obtained from pRS425 (Christianson *et al.* 1992) with primers 5892 & 9161. The *TRP1* and *HIS3* fragments were obtained from the genomic DNA of CEN.PK113-7D with primers 5891 & 8999 and 2335 & 2336 respectively. Correct assembly of the plasmid was confirmed via restriction analysis and PCR. The resulting plasmid (pUDE412, **Table 5.2**) was transformed to *E. coli* via electroporation for storage and plasmid propagation.

Table 5.2: Plasmids used in this study.

Name	Relevant characteristics	Origin
pROS10	2µm ampR *URA3* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	(Mans *et al.* 2015)
pROS14	2µm ampR *KlLEU2* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	(Mans *et al.* 2015)
pROS16	2µm ampR *HIS3* gRNA-*CAN1*.Y gRNA-*ADE2*.Y	(Mans *et al.* 2015)
pUDR007	2µm ampR *URA3* gRNA-*MCH3* gRNA-*MCH4*	This study
pUDR008	2µm ampR *HIS3* gRNA-YLL053C gRNA-*HXT1*	This study
pUDR126	2µm ampR *KlLEU2* gRNA-*JEN1* gRNA-*ADY2*	This study
pUDR151	2µm ampR *KlLEU2* gRNA-*AQR2* gRNA-*THI73*	This study
pUDR152	2µm ampR *HIS3* gRNA-*FPS1* gRNA-*AQY2*-YLL053C	This study
pUDR155	2µm ampR *URA3* gRNA-*ATO2* gRNA-*ATO3*	This study
pUDR168	2µm ampR *URA3* gRNA-*YRO2* gRNA-*AZR1*	This study
pUDR169	2µm ampR *HIS3* gRNA-*AQY3* gRNA-*TPO2*	This study
pUDR170	2µm ampR *KlLEU2* gRNA-YHL008C gRNA-*TPO3*	This study
pUDE411	2µm ampR *URA3 TRP1 LEU2 HIS3 LcLDH*	This study
pUDE412	2µm ampR *URA3 TRP1 LEU2 HIS3*	This study
pRS425	Template for amplification of *LEU2*	(Christianson *et al.* 1992)
YepLDH#1	Template for amplification of 2µm ampR *URA3 LcLDH* Template for amplification of 2µm ampR *URA3*	(Van Maris *et al.* 2004b)

For the construction of the lactate dehydrogenase (*LDH*) expression plasmid (pUDE411, **Table 5.2**), the approach described above was used with the exception that for the plasmid backbone primers 8996 and 8997 and for the *LEU2* fragment, primers 5892 and 9160 were used instead. In addition to the *URA3* marker and 2µm origin of replication, the Phusion PCR reaction using primers 8996 and 8997 also included the lactate dehydrogenase (*LDH*) gene in the plasmid backbone. Primers 5892 & 9160 were used for the *LEU2* fragment to adapt the homologous sequences flanking the *LEU2* fragment (required for Gibson assembly) to the *LDH*-containing plasmid backbone.

Strain construction

S. cerevisiae strains were transformed as described previously (Gietz and Woods 2002). Mutants were selected on synthetic medium plates supplemented with appropriate auxotrophic requirements (Verduyn *et al.* 1992; Pronk 2002). To minimize evolutionary pressure on lactate export during strain construction, a mixture of ethanol and glycerol was used as a carbon source to lower the flux through pyruvate with the aim to prevent excessive lactate formation. Gene deletions were performed as described previously (Mans *et al.* 2015) and were confirmed by colony PCR on randomly picked colonies, using the diagnostic primers listed in **Table S5.2**.

Strain IMX878 was obtained via removal of pUDR002, pUDR004 and pUDR005 from IMX717 (Mans *et al.* 2015), by growing in non-selective conditions as described previously (Mans *et al.* 2015). Transformation of IMX878 with pUDR007 and pUDR008 resulted in IMX880 (IMX878 + *mch3Δ mch4Δ yil166cΔ hxt1Δ*). IMX880 was then transformed with pUDR126, resulting in IMX902 (IMX880 + *jen1Δ ady2Δ*). The removal of pUDR007, pUDR008 and pUDR126 from IMX902 resulted in IMX903, which was then transformed with pUDR151 and pUDR152, resulting in IMX945 (IMX903 + *aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ*). IMX945 was then transformed with pUDR155 and after confirmation of the deletion of *ATO2* and *ATO3*, pUDR151, pUDR152 and pUDR155 were removed, resulting in IMX973 (IMX945 + *ato2Δ ato3Δ*). IMX973 was then transformed with pUDR169, resulting in IMX987 (IMX973 + *AQY3Δ tpo2Δ*) and transformation of IMX987 with pUDR168 and subsequent removal of both pUDR-plasmids resulted in IMX999 (IMX987 + *yro2Δ azr1Δ*). IMX999 was then transformed with pUDR170 and subsequent removal of this plasmid resulted in IMX1000 (IMX999 + *yhl008cΔ tpo3Δ*).

IMX1052 and IMX1066 were made via transformation of pUDE411 to IMX672 and IMX1000 respectively, using 200 ng of plasmid DNA in the transformation reaction. IMX1053 and IMX1067 were made via transformation of pUDE412 to IMX672 and IMX1000, respectively, using 300 ng of plasmid DNA. Transformants for all 4 strains were selected on SM plates without auxotrophic requirements and subjected to quality control as described above.

Media and cultivation

Precultures for batch cultivation in bioreactors were grown in a Bactron Anaerobic Chamber (Sheldon Manufactoring, Cornelius, OR) on synthetic medium with 20 g L^{-1} glucose and the anaerobic growth factors ergosterol (10 mg L^{-1}) and Tween80 (420 mg L^{-1}) dissolved in ethanol. Strain characterization was carried out at 30°C in 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 synthetic medium (Verduyn *et al.* 1992) was supplemented with heat sterilized (120°C for 20 min) Antifoam Emulsion C (Sigma) to a final concentration of 0.2 g L^{-1}, heat sterilized (110°C for 20 min) glucose to a final concentration of 20 g L^{-1}, the anaerobic growth factors ergosterol (10 mg L^{-1}) and Tween80 (420 mg L^{-1}) dissolved in ethanol and vitamins (Verduyn *et al.* 1992). The culture pH was maintained at 5.0 by automated addition of 2 M KOH and were stirred at 800 rpm. To maintain anaerobic conditions, the bioreactors were sparged with 500 mL N_2 min^{-1} (<10 ppm O_2) and equipped with Norprene tubing. After the batch phase, the medium pumps were switched on, resulting in the continuous addition of 100 mL min^{-1} of synthetic medium (25 g L^{-1} glucose) to the culture. The working volume was kept constant at 1.0 L using an effluent pump controlled by an electric level sensor, resulting in a dilution rate of 0.10 h^{-1} for the chemostat phase. The exact working volume was 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 2 additional volume changes.

Analytical methods

The optical density of cultures was monitored using a Libra S11 spectrophotometer (Biochrom, Cambridge, United Kingdom) at 660 nm. Culture dry weights were determined via filtration of 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 again using demineralized water and then 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 quenched using cold stainless-steel beads (Mashego *et al.* 2003). HPLC-analysis of the supernatant and residual sugar samples was performed as described previously (De Kok *et al.* 2011) and ethanol concentrations were corrected for ethanol evaporation (Guadalupe-Medina *et al.* 2010). Off-gas was cooled in a condenser and dried with a Perma Pure Dryer (Permapure, Lakewood, NJ) before CO_2 concentrations were measured with a NGA 2000 Rosemount gas analyser (Emerson, St. Louis, MO). To determine intracellular lactate concentrations, 50 mL of culture broth was spun down and the supernatant was discarded. The pellet was then resuspended in demineralized water and boiled for 20 minutes at 100°C. The supernatant from the boiled sample was collected via centrifugation and analysed via HPLC. To calculate

the intracellular concentrations, the sample volume, pellet volume and the volume of demineralized water were quantified based on weight, assuming 1 g = 1 mL. To determine the biomass weight in the pellet and the extracellular concentration of lactate, a second sample was taken at the same time point, on which dry weight determination and HPLC-analysis were performed as described previously. For conversion of g lactate (g dry cell weight)$^{-1}$ to estimated intracellular lactate concentrations, a cell volume of 2 mL (g dry cell weight)$^{-1}$ was assumed (Kresnowati, Groothuizen and Van Winden 2007).

Determination of in vitro lactate dehydrogenase activity

For enzymatic activity measurements, culture samples corresponding to 62.5 mg dry weight were harvested from steady-state chemostat cultures. Cell extracts were prepared by sonication and centrifugation (Postma *et al.* 1989). Lactate dehydrogenase activity was measured at 30°C with a reaction mixture consisting of 2 mM $MnCl_2$, 1 mM fructose-1,6-diphosphate and 0.15 mM NADH in 0.1 M imidazole at pH 6.5. Reactions were started by addition of 10 mM potassium pyruvate (Van Maris *et al.* 2004b).

5.3 Results

Identification of candidate genes for knock-out study

The currently available knowledge on lactate export by *S. cerevisiae* suggests the involvement of multiple transporters in this process. To try and capture multiple redundant transporters, a set of candidate membrane-protein-encoding genes was defined and deleted in a single *S. cerevisiae* strain. A set of 25 candidate lactate export genes was compiled based on the following criteria (**Table 5.3**): (i) a proposed involvement in lactate transport or (ii) a proposed involvement in lactate tolerance or (iii) a proposed role in carboxylic acid transport or (iv) carboxylic acid resistance.

First, genes that were previously shown to be involved in lactate uptake were selected (**Table 5.3**). *JEN1* and *ADY2* have both been described to be involved in the uptake of lactate (De Kok *et al.* 2012b; Pacheco *et al.* 2012). *ATO2* and *ATO3* were also selected, based on the sequence similarities of their encoded proteins to Ady2 (protein BLAST *e*-values of ≤ 10^{-164} and ≤ 10^{-36} respectively). Subsequently, 4 transporter genes (*HXT1*, *ITR1*, *PDR12* and *AQY1*) were selected based on a single-gene deletion screening in which their deletion was reported to reduce lactate production by >50% (Hirasawa *et al.* 2013). Aqua(glycero)porins have been shown to transport lactate in several organisms (Tsukaguchi *et al.* 1998; Choi and Roberts 2007; Bienert *et al.* 2013) and large strain-to-strain variations of aqua(glycero)porin genes occur in yeasts (Sabir, Loureiro-Dias and Prista 2016). Therefore, the aqua(glycero)porins *FPS1*, *AQY1*, *AQY2*, *AQY3* and YLL053C (contiguous with *AQY2*) (Coury *et al.* 1999; Mollapour and Piper 2007; Sabir, Loureiro-Dias and Prista 2016) were selected for gene deletion. Another group of transporter-encoding genes, consisting of the Haa1p-regulated genes *TPO2*, *TPO3* and *YRO2* (**Table 3**), was selected based on their involvement in resistance to lactic acid stress at low pH

Table 5.3: Membrane-protein-encoding *S. cerevisiae* genes, included in a systematic deletion study aimed at identifying membrane transporters involved in lactate export and motivation for their selection.

Gene	Std. name	Motivation for selection	Reference(s)
Group i) genes (potentially) involved in lactate transport			
JEN1	YKL217W	Known lactate transporter	(De Kok *et al.* 2012b; Pacheco *et al.* 2012)
ADY2	YCR010C	Known lactate transporter	(De Kok *et al.* 2012b; Pacheco *et al.* 2012)
ATO2	YNR002C	Similar to Ady2 (BLASTp e-value of $\leq 10^{-164}$)	This study
ATO3	YDR384C	Similar to Ady2 (BLASTp e-value of $\leq 10^{-36}$)	This study
HXT1	YHR094C	Target from knock-out screening	(Hirasawa *et al.* 2013)
ITR1	YDR497C	Target from knock-out screening	(Hirasawa *et al.* 2013)
PDR12	YPL058C	Target from knock-out screening	(Hirasawa *et al.* 2013)
AQY1	YPR192W	Aqua(glycero)porin & target from knock-out screening	(Coury *et al.* 1999; Sabir, Loureiro-Dias and Prista 2016)
FPS1	YLL043W	Aqua(glycero)porin involved in acetic acid uptake	(Mollapour and Piper 2007)
AQY2	YLL052C	Aqua(glycero)porin	(Coury *et al.* 1999; Sabir, Loureiro-Dias and Prista 2016)
AQY3	YFL054C	Aqua(glycero)porin	(Coury *et al.* 1999; Sabir, Loureiro-Dias and Prista 2016)
	YLL053C	Aqua(glycero)porin	(Coury *et al.* 1999; Sabir, Loureiro-Dias and Prista 2016)
Group ii) genes (potentially) involved in lactic acid resistance			
TPO2	YGR138C	Expression controlled by the Haa1p transcriptional regulator	(Abbott *et al.* 2009a)
TPO3	YPR156C	Expression controlled by the Haa1p transcriptional regulator	(Abbott *et al.* 2009a)
YRO2	YBR054W	Expression controlled by the Haa1p transcriptional regulator	(Abbott *et al.* 2009a)
Group iii) genes (potentially) involved in transport of other carboxylic acids			
MCH1	YDL054C	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
MCH2	YKL221W	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
MCH3	YNL125C	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
MCH4	YOL119C	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
MCH5	YOR306C	Similarity with mammalian monocarboxylate transporter	(Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010)
THI73	YLR004C	Plasma membrane anion:cation symporter with unknown substrate specificity	(Paulsen *et al.* 1998; Mojzita and Hohmann 2006; Hellborg *et al.* 2008)
	YIL166C	Plasma membrane anion:cation symporter with unknown substrate specificity	(Paulsen *et al.* 1998; Mojzita and Hohmann 2006; Hellborg *et al.* 2008)
	YHL008C	Putative acetic acid transporter	(Paulsen *et al.* 1998)
Group iv) genes (potentially) involved in resistance to other carboxylic acids			
AQR1	YNL065W	Resistance to short-chain monocarboxylic acids	(Tenreiro *et al.* 2002; Velasco *et al.* 2004)
AZR1	YGR224W	Resistance to acetic acid	(Tenreiro *et al.* 2000; Bauer *et al.* 2003)

(Abbott *et al.* 2008).

As a third category, genes were selected that have not directly been implicated in lactate transport or tolerance, but that have been linked to transport of other carboxylic acids. *MCH1, MCH2, MCH3, MCH4, MCH5* were selected based on their similarities

with the mammalian monocarboxylate transporters (MCT), some of which are known to transport lactate (Poole and Halestrap 1993; Nelissen, De Wachter and Goffeau 1997; Brohée *et al.* 2010). *THI73* and YIL166C are members of the anion:cation symporter subfamily (all members are putative weak-acid permeases) with a suggested plasma membrane localization and were selected based on their unknown substrate specificity (Paulsen *et al.* 1998; Mojzita and Hohmann 2006; Hellborg *et al.* 2008). Additionally, the putative acetic-acid transporter encoding gene YHL008C (Paulsen *et al.* 1998) was selected for deletion. As a last category, *AQR1* and AZR1 were selected in view of their reported involvement in tolerance of *S. cerevisiae* to short-chain monocarboxylic acids (Tenreiro *et al.* 2000, 2002; Bauer *et al.* 2003; Velasco *et al.* 2004).

Deletion of 25 transporter genes

To investigate whether any of the selected candidate genes are involved in the export of lactate, the complete set of 25 genes was deleted in the CEN.PK yeast background. Strain IMX717, which contains deletions in six of the candidate genes (*MCH1*, *MCH2*, *MCH5*, *AQY1*, *ITR1* and *PDR12;* Mans *et al.* 2015), was used as a starting point to construct a single strain that contained 25 transporter gene deletions. The deletion of all 25 transporter genes in strain IMX1000 was investigated by colony PCR, using primers that bind outside each of the deleted open reading frames (**Figure 5.2**). As a control, the same PCR reactions were performed on the DNA of the reference strain CEN.PK113-7D in which all 25 genes were intact according to a published whole-genome sequence (Nijkamp *et al.* 2012). Size analysis of the PCR products on gel confirmed successful deletion of all 25 candidate genes in IMX1000 (**Figure 5.2, Table S5.3**).

Lactate formation in anaerobic bioreactor batch cultures

To investigate whether the combined deletion of the 25 membrane-protein encoding candidate genes had a significant effect on the lactate production rate, an expression cassette for a lactate dehydrogenase gene from *Lactobacillus casei* (*LcLDH*) was introduced on a multi-copy plasmid, resulting in strains IMX1052 (reference + *LcLDH*) and IMX1066 (25 deletions + *LcLDH*). Lactate-dehydrogenase activities in cell extracts of these two strains were not significantly different (5.6 ± 0.3 and 5.6 ± 0.1 μmol min^{-1} mg of $protein^{-1}$ for IMX1052 and IMX1066 respectively). Since a homolactic strain with abolished lactate transport is may be inhibited by intracellular accumulation of lactate, the alcoholic fermentation pathway in these strains was left intact. This approach allows a distribution of pyruvate between the formation of lactate and ethanol (**Figure 5.1**), resulting in the formation of both products (Dequin and Barre 1994; Brambilla *et al.* 1999; Hirasawa *et al.* 2013). Another advantage of leaving the alcoholic fermentation pathway intact is that it allows for net conservation of free-energy in the form of ATP from substrate-level phosphorylation, thereby enabling anaerobic growth.

Both strains were grown in pH-controlled anaerobic batch bioreactors and formation of ethanol and lactate were monitored (**Figure 5.3**). *LDH* overexpression in

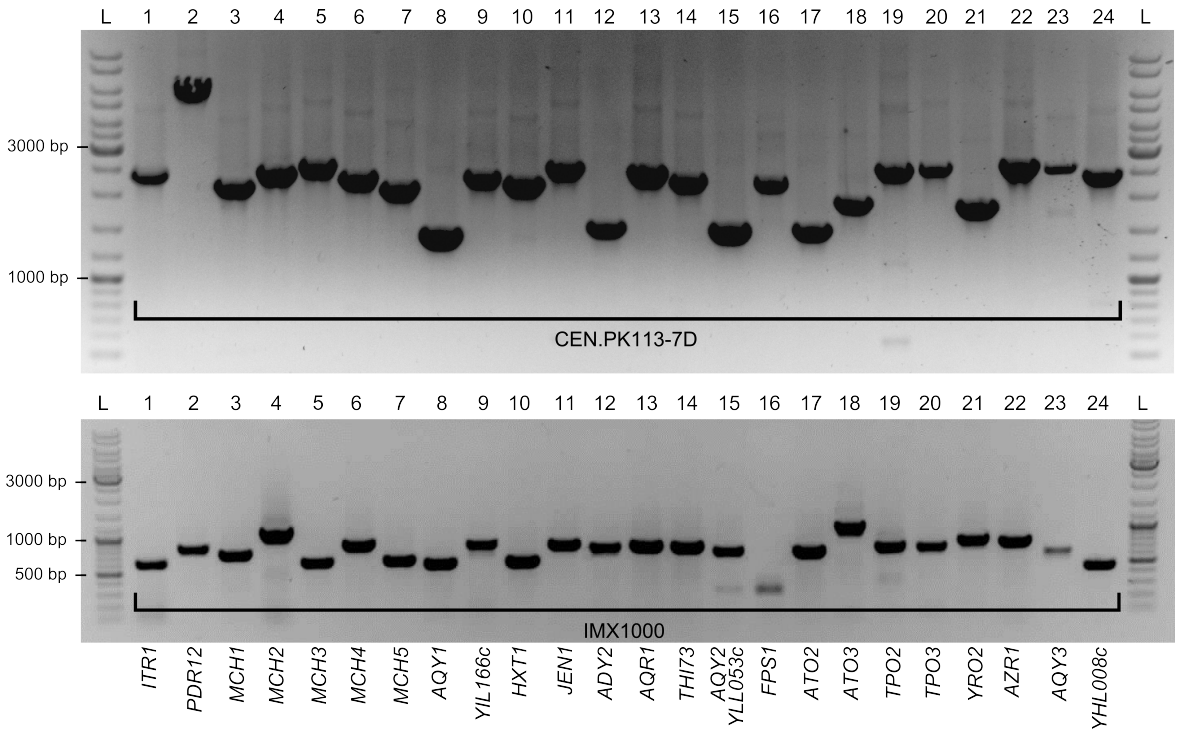


Figure 5.2: Diagnostic gel for verification of the correct deletion of 25 membrane-protein-encoding genes in *S. cerevisiae* strain IMX1000. The left and right lanes(L) contain GeneRuler DNA Ladder Mix. Lanes 1-24 were loaded with PCR fragments amplified from genomic DNA of *S. cerevisiae* strains CEN.PK113-7D (reference) or IMX1000 with oligonucleotide primers that bind outside of the target ORF. Lane 15 shows PCR fragments obtained with primers that bind outside both *AQY2* and YLL053C (contiguous with *AQY2*) and which were used to verify both deletions simultaneously. Primers used for the PCR and expected band sizes are listed in **Table S5.3**.

the reference strain IMX1052 indeed resulted in formation of lactate and ethanol as the major fermentation products. Upon glucose depletion (34.5 h), 152.4 ± 0.5 mM ethanol and 14.3 ± 0.0 mM lactate (ratio 11 : 1) had been formed. Under identical conditions, the 25-deletion strain IMX1066 accumulated 156.7 ± 0.3 mM ethanol and 7.7 ± 0.2 mM lactate (ratio 20 : 1) after 43.5 h (**Figure 5.3**). The almost twofold reduction in lactate formation could indicate a reduced capacity for lactate export, leading to higher intracellular lactate concentrations which might in turn favour the formation of ethanol from pyruvate in strain IMX1066. Not only the lactate-ethanol ratio, but also the biomass specific lactate (0.67 ± 0.01 mmol (g of biomass)$^{-1}$ h^{-1}) and ethanol production rate (11.9 ± 0.0 mmol (g of biomass)$^{-1}$ h^{-1}) of IMX1066 were significantly lower than that of the reference strain (2.38 ± 0.02 mmol lacate and 22.7 ± 0.6 mmol ethanol (g of biomass)$^{-1}$ h^{-1}). However, also the specific growth rate of IMX1066 was substantially lower (0.15 ± 0.00 h^{-1}) than that of the isogenic reference strain IMX1052 (0.25 ± 0.00 h^{-1}), Therefore, an effect of specific growth rate on the distribution of pyruvate over ethanol and lactate could not be excluded. The lower specific growth rate of the 25-deletion strain IMX1066 was not related to overexpression of *LDH* in this genetic background, as the isogenic 25-deletion control strain IMX1067 (25 deletions, no *LcLDH*) grew with the same specific rate of 0.16 ± 0.00 h^{-1} under identical conditions (**Figure 3**).

In contrast to batch cultivation, chemostat cultivation enables comparison between strains at identical, predefined specific growth rates. To eliminate a possible impact of specific growth rate on the comparison of lactate production in the 25-deletion and reference strains, a quantitative comparison of lactate and ethanol production between IMX1052 and IMX1066 was made in anaerobic glucose-limited chemostat cultures grown at a fixed dilution rate of 0.10 h^{-1} (**Table 5.4**). At a specific growth rate of 0.10 h^{-1}, both strains showed identical biomass-specific rates of ethanol and lactate production. Moreover, no significant differences were observed in any of the other measured parameters (**Table 5.4**). In line with the hypothesis that at low growth rates, the formation of ethanol is favoured over the formation of lactate, a distribution of 20 ethanol : 1 lactate was observed, which is similar to the distribution found for IMX1066 in anaerobic batch cultures at a specific growth rate of 0.15 h^{-1} (**Figure 5.3**). In addition, intracellular lactate concentrations in the two strains were not significantly different (**Table 5.4**).

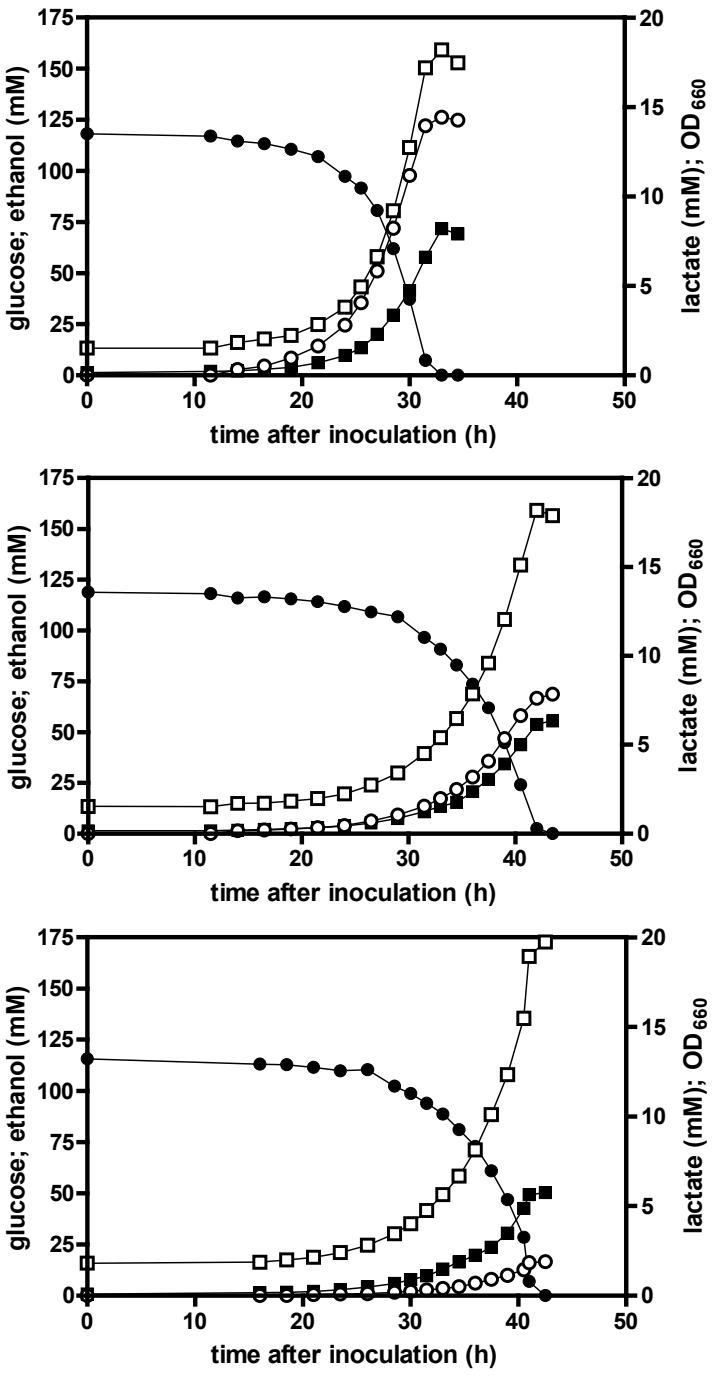


Figure 5.3 – Concentrations of biomass and metabolites in anaerobic batch cultures of *S. cerevisiae* IMX1052 (top, reference strain expressing *LcLDH*), IMX1066 (middle, 25 deletions in membrane-protein-encoding genes and expressing *LcLDH*) and MX1067 (bottom, 25 deletions in membrane-protein-encoding genes without expression of *LcLDH*) when grown in anaerobic bioreactors in on a synthetic medium containing 20 g L^{-1} glucose at 30°C and at pH 5.0. Symbols: ●, glucose; ■, optical density at 660 nm; ○, lactate; □, ethanol. Each graph shows representative data from one of two independent replicates.

Table 5.4: Physiological parameters of the *L. casei* lactate dehydrogenase (*LcLDH*)-expressing reference *S. cerevisiae* strain IMX1052 and the *LcLDH*-expressing strain IMX1066 (25 deletions in selection membrane-protein-encoding genes) in anaerobic glucose-limited chemostat cultures grown at a dilution rate of 0.10 h^{-1}. Averages and mean deviations were obtained from independent duplicate experiments of each strain.

Strain	IMX1052	IMX1066
Relevant genotype	Isogenic reference + *LcLDH*	25 transporters deleted + *LcLDH*
Dilution rate	0.10 ± 0.00	0.10 ± 0.00
$q_{glucose}$ (mmol g biomass^{-1} h^{-1})	6.8 ± 0.1	6.6 ± 0.2
q_{CO_2} (mmol g biomass^{-1} h^{-1})	10.5 ± 0.2	10.2 ± 0.1
$q_{ethanol}$ (mmol g biomass^{-1} h^{-1})	10.0 ± 0.5	10.0 ± 0.2
$q_{lactate}$ (mmol g biomass^{-1} h^{-1})	0.48 ± 0.02	0.47 ± 0.01
$q_{glycerol}$ (mmol g biomass^{-1} h^{-1})	0.93 ± 0.00	0.85 ± 0.02
$q_{pyruvate}$ (mmol g biomass^{-1} h^{-1})	0.01 ± 0.00	0.01 ± 0.00
$q_{acetate}$ (mmol g biomass^{-1} h^{-1})	0.04 ± 0.00	0.03 ± 0.00
Residual glucose (mmol L^{-1})	0.63 ± 0.02	0.54 ± 0.08
Intracellular lactate (mmol L^{-1})	20.6 ± 0.8	21.4 ± 1.4
Extracellular lactate (mmol L^{-1})	10.5 ± 0.1	10.6 ± 0.1

5.4 Discussion

The present study, in which 25 membrane-protein-encoding genes were inactivated in a single *S. cerevisiae* strain by multiple cycles of CRISPR/Cas9-mediated gene deletion, illustrates the potential of this genome-editing technique for functional analysis. In total, 8 transformations and 5 marker recycling steps were used for the construction of IMX1000 (25 deletions) from the parental strain IMX672 (Mans *et al.* 2015). Since, at the outset of the strain construction efforts, lethality of some combinations of mutations could not be excluded, the number of simultaneous deletions in most transformations was limited to 2 or 4. This decision implied that the full potential of the combination of Cas9 and gRNA-plasmids used in this study (at least 6 simultaneous mutations) was not used. Nevertheless and despite the relatively low specific growth rate of *S. cerevisiae* on ethanol (Van Dijken *et al.* 2000), the combined introduction of 25 gene deletions could be completed in 90 ± 5 days of strain construction (3.6 days per deletion). In the absence of laboratory automation, this lowers strain construction times by more than twofold compared to Cre-*LoxP* (9 days per deletion) or Green monster (9 days per deletion) strategies for genome editing (Delneri *et al.* 2000; Suzuki *et al.* 2011; Bao *et al.* 2015).

In addition to insights into the mechanism of lactate transport, the experiments performed in this study also provide insight into (homo)lactic acid fermentation in *S. cerevisiae*. The observed specific lactate production rate of strain IMX1052 (2.4 ± 0.0 mmol (g of biomass)$^{-1}$ h^{-1}) is substantially higher than reported for a homolactic pyruvate-decarboxylase-negative *S. cerevisiae* incubated under anaerobic conditions (0.8 mmol g of biomass^{-1} h^{-1}, van Maris *et al.* 2004b). Under anaerobiosis, homolactic *S. cerevisiae* strains have been shown to rapidly deplete intracellular ATP, resulting in extremely low adenylate energy charges within 30 min after a switch to anaerobic conditions (Abbott *et al.* 2009a). The high anaerobic lactate production rate observed in

cultures of strain IMX1052 therefore probably reflects availability of additional ATP from alcoholic fermentation (**Figure 5.1**).

The lower growth rate and the lower summed biomass-specific fluxes of ethanol and lactate of strain IMX1066 in anaerobic batch cultures compared to the reference strain IMX1052 indicate a lower glycolytic flux (required to provide ATP and metabolites for biomass formation) relative to the reference strain. It has previously been shown that, in *S. cerevisiae*, lower glycolytic fluxes correlate with lower intracellular concentrations of glycolytic intermediates (Canelas *et al.* 2010). Considering the lower K_m of *S. cerevisiae* pyruvate decarboxylase for pyruvate (0.65 mM, (Kresze and Ronft 1981)) compared to *L. casei* lactate dehydrogenase (1.0 mM, (Gordon and Doelle 1976)), lower intracellular pyruvate concentrations at lower glycolytic flux, might favour alcoholic fermentation over lactate fermentation (**Figure 5.1**). This hypothesis is consistent with the observation that both strains exhibited a similar flux distribution in chemostat cultures grown at identical growth rates of 0.10 h^{-1} (**Table 5.4**).

The absence of a significant impact of the 25 deletions on lactate production rates in the anaerobic, glucose-limited chemostats could, in theory, reflect a situation in which a reduced lactate export capacity was exactly compensated for by an increased thermodynamic driving force or by increased saturation of exporters with intracellular substrate. In both cases, this would require an increased intracellular lactate concentration. Since the 25-deletion strain did not show an increased intracellular lactate concentration (**Table 5.4**), the experimental data support the conclusion that deletion of these 25 (putative) transporter genes did not significantly affect lactate export capacity in IMX1066 under the experimental conditions.

The question remains which transporter(s) or transport mechanisms are responsible for lactate export in strain IMX1066. The observation that a mutant lacking both *JEN1* and *ADY2* is unable to grow on lactate (De Kok *et al.* 2012b) and the conclusion that lactate export is in all likelihood energy coupled (Van Maris *et al.* 2004b; Abbott *et al.* 2009a), suggest a unidirectional (export only) transport mechanisms under the conditions studied (extracellular pH 5.0). These considerations leave three plausible mechanisms for lactate transport: i) primary transport catalysed by an ABC-transporter, ii) antiport of lactic acid with a proton or other ion, iii) (facilitated) diffusion of the lactate anion and extrusion of the accompanying proton via the H^+-ATPase.

PDR12, encoding the only ABC-transporter with a confirmed role in transport of the weak-acid anions (Piper *et al.* 1998; Holyoak *et al.* 1999), was deleted in this study. Of the remaining 11 ABC transporters with transmembrane domains and localisation in the plasma membrane (Kerr 2004; Zhao *et al.* 2004, 2006; Paumi *et al.* 2009), only Pdr5, Pdr10 and Yor1 are known to be induced by more lipophilic weak acids, although a transport function of these proteins has not been unequivocally demonstrated (Mira, Teixeira and Sá-Correia 2010). Based on the measured intracellular lactate concentration (**Table 5.4**), antiport of lactic acid with an ion (such as H^+ or Na^+) and subsequent extrusion of the ion at the expense of 1 ATP via a P-type ATPase, is a thermodynamically

feasible transport mechanism. Lactic acid/proton-antiport would also be in agreement with the observation that combined deletion of *JEN1* and *ADY2* prevented growth on 55 mM lactate at an extracellular pH of 5.0, which would only allow a maximum achievable intracellular lactic-acid concentration of 0.2 mM (assuming a thermodynamic equilibrium and a constant proton-motive-force of -0,15 V and intracellular pH of 7.0; Van Maris *et al.* 2004a). The measured intra- and extracellular lactate concentrations (**Table 5.4**) in combination with a negative membrane potential, result in a driving force for (facilitated) diffusion of the lactate anion.

Ion channels in the plasma membrane of *S. cerevisiae* have been reported to facilitate the diffusion of small cations such as potassium, metals and ammonium (Ketchum *et al.* 1995; Marini *et al.* 1997; Eide 1998). To the best of our knowledge, no such mechanisms for anions have been described. Although free diffusion of charged molecules such as the anion thiocyanate (SCN^-) and the cation tetraphenylphosphonium (TPP) has been described, this is facilitated by their hydrophobicity and, in the case of TPP, charge delocalization by phenyl residues (Gutknecht and Walter 1982; Skulachev 2007; Trendeleva *et al.* 2012). Despite the generally accepted assumption that anions of polar carboxylic acids do not freely diffuse over the cell membrane (Cássio, Leao and Van Uden 1987; Lambert and Stratford 1999), it seems relevant to experimentally test this assumption in *in vitro* experiments.

The Yeast Transport Protein database (YTPdb) predicts a total number of 302 membrane transporter proteins in *S. cerevisiae* of which 139 have a predicted localization to the plasma membrane, 106 have a predicted localization to internal membrane and for the remaining 57 transporters the localization is unknown (Brohée *et al.* 2010). Of the genes deleted in IMX1000, 23 are classified as transporters according to YTPbd, 13 with a predicted plasma membrane localization, 3 with a predicted localization to internal membrane and 7 with unknown localization. Additionally, IMX1000 contains an integrated copy of Cas9 in the *CAN1* locus, thereby inactivating the plasma-membrane arginine transporter. The 25-deletion strain constructed in this study contains deletions of complete functional transporter groups, such as water transport (mediated by *AQY1*, *AQY2*, *AQY3* and *FPS1*, for an extensive review see (Ahmadpour *et al.* 2014)), glycerol export (mediated by *FPS1*) and all known carboxylic acid transporters (*JEN1*, *ADY2*, *FPS1* and *PDR12*) and provides a convenient platform for further studies. The search for the lactic-acid export mechanisms in *S. cerevisiae* might benefit from additional CRISPR/Cas9-based deletion of the remaining genes belonging to the 12 spanner drug:H^+-antiporters family (Sá-Correia and Tenreiro 2002; Mira, Teixeira and Sá-Correia 2010) and the remaining plasma-membrane located ABC-transporters, other than Pdr12. Systematic deletion of all members of the five transporter-encoding gene families that are non-essential for growth in synthetic laboratory media, could ultimately yield a 'minimal transportome yeast'. As proposed for other 'minimalist yeast' genome configurations and already demonstrated for the HXT-null and PDR-null strains (Wieczorke *et al.* 1999; Suzuki *et al.* 2011), such a strain could be highly valuable for

functional analysis of (heterologous) genes in *S. cerevisiae* (Oliver 1996). Additionally, a strain in which non-essential transporters have been systematically removed will be highly informative for strategies aimed at preventing protein crowding in membranes (Engelman 2005; Coskun and Simons 2010; Chavent, Duncan and Sansom 2016) of metabolically engineered yeast strains.

5.5 Acknowledgements

We thank Erik de Hulster for advice regarding the chemostat experiments and Antoon Backx for his involvement in selecting the transporter genes. We thank Ryan Henderson, Bert Poolman and Matteo Gabba for stimulating discussions on the mechanism of lactate transport over the yeast plasma membrane. This work was performed within the BE-Basic R&D Program (FS6.004; http://www.be-basic.org/), which was granted a FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I). The image on the title page (page 119) was modified from an image made by Phil (Flickr: _MG_6936) (own work), which is licenced under CC BY 2.0, via Wikimedia Commons.

Supplementary material

Table S5.1: Primers used for the amplification of 2µm fragments, using pROS10 as a template and the plasmid templates used for the amplification of the plasmid backbone containing the selection marker with primer 6005.

Plasmid	Target ORFs	Primers for 2µm fragment	Template for backbone
pUDR007	*MCH3* & *MCH4*	7160 & 7161	pROS10
pUDR008	*HXT1* & YLL053C	7162 & 7163	pROS16
pUDR126	*JEN1* & *ADY2*	8664 & 6262	pROS14
pUDR151	*AQR1* & *THI73*	8699 & 8704	pROS14
pUDR152	*FPS1* & *AQY2* & YLL053C	8709 & 8694	pROS16
pUDR155	*ATO2* & *ATO3*	8688 & 8691	pROS10
pUDR168	*YRO2* & *AZR1*	9125 & 9130	pROS10
pUDR169	*AQY3* & *TPO2*	9155 & 9140	pROS16
pUDR170	YHL008C & *TPO3*	9115 & 9291	pROS14

Table S5.2: Oligonucleotides used in this study.

Primer	Sequence (5'→ 3')	Purpose
6869	AGGTCTTCAATGCCGGGTTAG	Confirmation *ITR1* deletion
6870	GTATCAGCCGTCAGTAGTATCCAG	Confirmation *ITR1* deletion
253	CGGTATCACATTTTCTCGACGG	Confirmation *PDR12* deletion
3998	CGCGACAGACATTTGTTGG	Confirmation *PDR12* deletion
6862	GTCCAGGATTCTCCGAAGAACTC	Confirmation *MCH1* deletion
6863	ACGGCTGTTTGTCCGATATTGC	Confirmation *MCH1* deletion
6864	GCACGACTTTGCAGGCTTTC	Confirmation *MCH2* deletion
6865	CACCGAACCAACATTAGGTAGC	Confirmation *MCH2* deletion
7175	GTTTTTCCTGGATCCGCAACAC	Confirmation *MCH3* deletion
7176	CGGGCTATCTCGGCAATCTG	Confirmation *MCH3* deletion
7177	ATCTCACGGCAGCACCAAG	Confirmation *MCH4* deletion
7178	CAAACCTCCAGCGGGTAGTC	Confirmation *MCH4* deletion
6866	GAAGACTGACGGGCACTTTG	Confirmation *MCH5* deletion
6867	GCCCTAGGCGGTATTGTATGAG	Confirmation *MCH5* deletion
6868	GATGTCCTTCCACCCTCTTACAC	Confirmation *AQY1* deletion
5593	CGTGTATTGGTGTACGGATGAGTC	Confirmation *AQY1* deletion
1119	GCGCTAGGTTCGTCTTCTAC	Confirmation YLL053C deletion
1120	ACGCTGAAGCTATGCTATGC	Confirmation YLL053C deletion
7179	GTTGAAGCAGCAGCGTTGTC	Confirmation *HXT1* deletion
7180	CATGAGCTGCTGCGACATTTG	Confirmation *HXT1* deletion
8599	TTTCTCCATCTCAGAGCCGC	Confirmation *JEN1* deletion
8600	GCCCATTCAGTGCAAGAACC	Confirmation *JEN1* deletion
1728	GGGCGGCTATTTCGGTATTG	Confirmation *ADY2* deletion
1729	GCAACATCTTCTCCGCTAACC	Confirmation *ADY2* deletion
8702	TTCAGACGAGAAGCCGTTCC	Confirmation *AQR1* deletion
8703	ACTCGCAGACTTTGACCCAG	Confirmation *AQR1* deletion
8707	GTTTGGTTGGTTCGAGTGCC	Confirmation *THI73* deletion
8708	GGTGAACTTGCTGGAAGTGC	Confirmation *THI73* deletion
8697	CACGGCTCAAATTACTGCGG	Confirmation *AQY2* and YLL053C deletion
8698	GCATACTTCTGCCGCTTACG	Confirmation *AQY2* and YLL053C deletion
881	GTACGCTCGAGGGTACATTC	Confirmation *FPS1* deletion
882	GACCAGGCTGAGTTCATGTC	Confirmation *FPS1* deletion
2383	TAAATCGGCGGAGGGAGAAC	Confirmation *ATO2* deletion
2384	CTCCATGGCCAAGTTGGTTAAG	Confirmation *ATO2* deletion
2387	ACCGCCGGCTACAAACATAAC	Confirmation *ATO3* deletion
2388	TTCGCCTGCTAGGTCATCC	Confirmation *ATO3* deletion
9143	ACCGAAGAACACTGCCATCC	Confirmation *TPO2* deletion
9144	AAGAGACATTGGCCACGAGG	Confirmation *TPO2* deletion
9294	TTTTGTCCTCACCGGTAGCG	Confirmation *TPO3* deletion
9295	TGCAACCTCTCGTAGTGATTCC	Confirmation *TPO3* deletion
9128	TGGTTTTCCGCCCTTTTCTTC	Confirmation *YRO2* deletion
9129	GTTTTCTACTTAGCCTGCGCG	Confirmation *YRO2* deletion
9133	TTCCGTACCCTCTTGCAACG	Confirmation *AZR1* deletion
9134	GATAGCCGCTCAGGTGAAGG	Confirmation *AZR1* deletion
9158	ACTCACAATCACCCTCTGCG	Confirmation *AQY3* deletion
9159	ACCTAGTTGATGGTGCCAGG	Confirmation *AQY3* deletion
5115	GAGGAGAACGAGGAAACAAG	Confirmation YHL008C deletion
5116	TGAACGAGGGCATACTGAAG	Confirmation YHL008C deletion
8996	AGCTTGGCGTAATCATGGTC	Amplification of 2µm-*URA3* backbone
8997	CGGGATCCTCGAGAACGTC	Amplification of 2µm-*URA3*-*LcLDH* backbone
9000	CTAGAGTCGACCCGGGTATC	Amplification of 2µm-*URA3*(-*LcLDH*) backbone
5892	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATTCGACCA GCCTAAGAATGTTCAACTATCACGAGGCCCTTTCGTC	Amplification of *LEU2* cassette

9161	CTGAGCTCGAGATATCATGCGTAGTCAGGCACATCATACGGAT ACCCGGGTCGACTCTAGGTTCACGTAGTGGGCCATCG	Amplification of *LEU2* cassette
9160	CTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGAC GTTCTCGAGGATCCCGGTTCACGTAGTGGGCCATCG	Amplification of *LEU2* cassette
5891	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCATAGCC ATGCCTTCACATATAGTTGCCTCACTTGTCGCTTATG	Amplification of *TRP1* cassette
8999	AATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGA CCATGATTACGCCAAGCTGAGACCGAGTTAGGGACAGTTAGAG	Amplification of *TRP1* cassette
2335	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGC CAGATCATCAATAGGCACCTCTTGGCCTCCTCTAGTACACTC	Amplification of *HIS3* cassette
2336	GTTGAACATTCTTAGGCTGGTCGAATCATTTAGACACGGGCAT CGTCCTCTCGAAAGGTGTACGGAATACCACTTGCCACCTATC	Amplification of *HIS3* cassette

Table S5.3: Primers used for the verification of the 25 gene deletions in IMX1000, using CEN.PK113-7D as a control. Expected sizes of the resulting PCR fragments when the ORF is still present or absent.

Target ORF	Primers	Expected size when gene is	
		Present (bp)	Absent (bp)
ITR1	6869 & 6870	2368	613
PDR12	253 & 3998	5361	705
MCH1	6862 & 6863	2190	729
MCH2	6864 & 6865	2040	618
MCH3	7175 & 7176	2608	586
MCH4	7177 & 7178	2374	828
MCH5	6866 & 6867	2162	596
AQY1	6868 & 5593	1469	551
YLL053C	1119 & 1120	2418	789
HXT1	7179 & 7180	2285	572
JEN1	8599 & 8600	2649	798
ADY2	1728 & 1729	1595	743
AQR1	8702 & 8703	2556	795
THI73	8707 & 8708	2359	787
AQY2 + YLL053C	8697 & 8698	1560	701
FPS1	881 & 882	2269	259
ATO2	2383 & 2384	1568	779
ATO3	2387 & 2388	1945	1186
TPO2	9143 & 9144	2613	768
TPO3	9294 & 9295	2607	738
YRO2	9128 & 9129	1872	837
AZR1	9133 & 9134	2637	794
AQY3	9158 & 9159	2552	611
YHL008C	5115 & 5116	2362	462

Outlook

The research described in this thesis demonstrates the power and potential of genetic engineering using CRISPR/Cas9. The method described in **Chapter 2** was used in **Chapter 5** to construct a *S. cerevisiae* strain carrying deletions of 25 genes in approximately 90 days. The same method was also applied in **Chapter 3**, where transformation of one gRNA expression plasmid resulted in the deletion of 6 copies of isomaltase coding genes, an exercise that would have required at least 2 months of genetic engineering when using a conventional method. Given the relatively short development time of the CRISPR/Cas9 system for use as a genetic engineering tool and the tremendous improvements over more classical methods that have already been achieved, there is no doubt that the future will bring more impressive CRISPR/Cas9 based applications. For use in *S. cerevisiae*, it will be will be highly relevant for future studies to develop a method for the simultaneous introduction of many (>10) gRNA coding sequences and to further elucidate which gRNA target sequences lead to the most efficient restriction by Cas9. Other CRISPR-based nucleases, such as Cpf1, have recently been identified and exhibit interesting properties for genetic engineering. For example, DNA restriction by Cpf1 results in sticky ends, compared to blunt ends with Cas9 restriction. Cpf1 is also smaller than Cas9, recognises a different PAM sequence and naturally uses a single RNA molecule for targeting. These differences of the CRISPR/Cpf1 system might make it a promising alternative to CRISPR/Cas9 for specific genetic engineering goals, for example when a suitable PAM sequence for Cas9 restriction is not present. Moreover, the shorter RNA molecules used for targeting of Cpf1 are cheaper to engineer.

The research described in **Chapters 3 and 4** demonstrates the energetic benefit of combined engineering of disaccharide uptake and cleavage in *S. cerevisiae*. Although a promising increase in the anaerobic biomass yield on sucrose was observed, the energy-efficient *S. cerevisiae* strain grew slowly on sucrose under anaerobic conditions ($0.08 \pm 0.00\ h^{-1}$). For industrial applications, yeast strains with high substrate conversion rates are necessary and therefore, future research should first focus on increasing the rate of sucrose utilization. In the engineered strain it is likely that uptake of sucrose via PvSUF1 is rate-limiting and efforts to improve the rate of facilitated sucrose uptake are necessary. As a starting point, *S. cerevisiae* strains expressing the sucrose facilitators *PvSUF1*, *PsSUF1*, *PsSUF4*, *OsSWEET11* and *AtSWEET12*, could be subjected to mutagenesis and/or laboratory evolution to improve the rate of sucrose uptake by these transporters in yeast. Follow-up studies could then focus on engineering energy-efficient yeast strains in which fast facilitated diffusion of sucrose and intracellular phosphorolysis are combined. Ultimately, ethanol formation can be abolished via deletion of the pyruvate decarboxylase genes and (heterologous) product pathways can

be introduced. In the resulting energy-efficient platform strain, anaerobic production of chemicals whose current formation from substrate is energy neutral is expected to be enabled with the increased free-energy conservation. The use of the aforementioned strategy is not limited to the disaccharide sucrose, but can also be applied to increase free-energy conservation for other oligosaccharides, such as maltose and cellodextrin, for which putative facilitators and phosphorylases have also been described.

Based on the results discussed in **Chapter 5** it is likely that the 25 gene deletions did not target (all of) the lactic acid transporters in *S. cerevisiae*. A directed follow-up approach could start with a detailed study towards elucidation of the lactic acid export mechanism, which might provide additional insight into which family of transporters is responsible for lactic acid transport in yeast. For example, studying the export of lactic acid in the presence of ionophore uncouplers, which selectively dissipate ion gradients across biological membranes, might elucidate whether lactic acid transport is a secondary transport mechanism. An alternative or complementary approach could be the deletion of additional transporter genes in the strain platform developed in **Chapter 5**. Deletion of the remaining plasma-membrane ABC-transporters, the complete family of anion:cation symporters and/or the remaining genes belonging to the 12-spanner drug:H^+-antiporter family could be part of such a study. Additionally, the transporter deletion strain can provide a convenient platform for further studies on transport of, for example, water, glycerol and carboxylic acids other than lactic acid, as the majority of the transporters described to be involved in these functions have already been deleted. A more ambitious goal could be the construction of a 'minimalist' transporter yeast strain in which all transporter genes that are non-essential for growth in synthetic medium are removed. Such a strain could facilitate functional analysis of transporters and, indeed, it would be surprising if no previously unknown transport functions were identified during the construction of such a strain. Although only a small fraction of the 302 described transporter genes of *S. cerevisiae* were deleted in this thesis, the strain constructed in **Chapter 5**, combined with the gene deletion strategy developed in **Chapter 2**, may be considered as a modest start towards the ambitious goal of developing such a 'minimalist' transporter yeast.

Vooruitblik

Het in dit proefschrift beschreven onderzoek demonstreert de kracht en de potentie van het maken van genetische modificaties met CRISPR/Cas9. De in **Hoofdstuk 2** omschreven methode werd in het onderzoek beschreven in **Hoofdstuk 5** gebruikt om een *S. cerevisiae* stam te construeren waarin 25 genen in ongeveer 90 dagen waren uitgeschakeld. Dezelfde methode werd ook gebruikt in het onderzoek beschreven in **Hoofdstuk 3**, waar de transformatie met een enkel gRNA-expressie plasmide de deletie van 6 kopieën van isomaltase-coderende genen mogelijk maakte. Het verwijderen van deze 6 genen zou bij gebruik van een conventionele methode minimaal twee maanden in beslag genomen hebben. Met het oog op de relatief korte ontwikkelingstijd van het CRISPR/Cas9 systeem als gereedschap voor het maken van genetische modificaties en de enorme verbeteringen ten opzichte van de meer klassieke methodes die er al gemaakt zijn, is er geen twijfel mogelijk dat er in de toekomst nog meer indrukwekkende CRISPR/Cas9-gebaseerde toepassingen ontwikkeld worden. Voor gebruik in *S. cerevisiae* is het ontwikkelen van een methode voor het simultaan introduceren van vele (>10) gRNA-coderende sequenties en het verder definiëren van de beste gRNA-targets voor Cas9 van groot belang voor toekomstige onderzoeken. Alternatieve, recent ontdekte, CRISPR-gebaseerde nucleases, zoals Cpf1, hebben een aantal interessante eigenschappen voor het gebruik bij het maken van genetische modificaties. Het knippen van DNA met Cpf1 resulteert bijvoorbeeld in 'sticky' DNA uiteinden, vergeleken met de 'blunt' uiteinden die ontstaan bij het knippen van DNA met Cas9. Cpf1 is ook kleiner dan Cas9, herkent een andere PAM-sequentie en gebruikt van nature maar een enkel gRNA-molecuul. Deze eigenschappen maken het CRISPR/Cpf1 systeem tot een veelbelovend alternatief voor CRISPR/Cas9, met name voor specifieke doeleinden, zoals in situaties waar geen geschikte PAM-sequentie voor Cas9 aanwezig is. Verder zijn de kortere RNA-moleculen voor het sturen van Cpf1 goedkoper om te synthetiseren en aan te passen.

Het in de **Hoofdstukken 3 en 4** beschreven onderzoek demonstreert de energetische voordelen van de gecombineerde aanpassing van sacharose-opname en -splitsing in *S. cerevisiae*. Hoewel een veelbelovende verhoging van de anaërobe biomassaopbrengst werd waargenomen, groeide de energetisch efficiëntere *S. cerevisiae* stam onder anaërobe condities maar langzaam op sacharose (0,08 ± 0.00 uur^{-1}). Voor industriële toepassingen zijn giststammen met hoge omzettingssnelheden van het substraat nodig. Daarom zou toekomstig onderzoek zich moeten richten op het verhogen van de omzettingssnelheid van sacharose. In de gemodificeerde stam is de opname van sacharose via PvSUF1 waarschijnlijk de stap die de omzettingssnelheid beperkt. Het is daarom wenselijk om de opnamesnelheid van sacharose te verbeteren. Een eerste aanpak zou kunnen zijn om *S. cerevisiae* stammen, die de sacharosefacilitators *PvSUF1*, *PsSUF1*, *PsSUF4*, *OsSWEET11* en *AtSWEET12* tot expressie brengen, te onderwerpen

aan mutagenese en/of laboratoriumevolutie om zo de snelheid van sacharoseopname via deze alternatieve transporters in gist te verhogen. Vervolgstudies zouden zich dan kunnen richten op het construeren van een giststam waarin snelle en gefaciliteerde opname van sacharose gekoppeld is aan intracellulaire fosforolyse. Uiteindelijk zou de formatie van ethanol geblokkeerd kunnen worden door de pyruvaatdecarboxylasegenen te verwijderen en vervolgens kunnen (heterologe) productroutes geïntroduceerd worden. Een uit zulk onderzoek voortkomende energetisch efficiënte platformstam zou, door een verhoogde conservering van vrije energie, de anaërobe productie mogelijk moeten maken van chemicaliën waarvan de vorming uit substraat nu nog energetisch neutraal is. De in dit proefschrift beschreven strategie is niet beperkt tot de omzetting van sacharose, maar kan ook toegepast worden om de conservering van vrije energie te verhogen voor andere oligosachariden, zoals maltose en cellodextrine, waarvoor mogelijke facilitators en fosforylases al beschreven zijn.

Gebaseerd op de resultaten beschreven in **Hoofdstuk 5** is het aannemelijk dat de 25 gemaakte gen-deleties geen, of niet alle, melkzuurtransporters in *S. cerevisiae* omvatten. Een gerichte vervolgstudie zou kunnen beginnen met een gedetailleerde analyse van het mechanisme van melkzuurexport, om een beter beeld te krijgen over welk type transporter er verantwoordelijk is voor transport van dit molecuul in gist. Het bestuderen van het effect van ionoforen, die selectief ionengradiënten over het membraan kunnen afbreken, zou uitsluitsel kunnen geven over de mogelijkheid dat melkzuurtransport in *S. cerevisiae* gekatalyseerd wordt door een secundair transportmechanisme. Een alternatieve strategie zou kunnen zijn om nog meer transporters uit te schakelen in de in **Hoofdstuk 5** beschreven platformstam. De deletie van de overige ABC-transporters in het plasmamembraan, van de complete familie van anion:kationsymporters en/of de overige genen behorende tot de drug:H^+-antiporterfamilie zou(den) onderdeel kunnen zijn van een dergelijke studie. Verder zou de transporter-deletie stam ook een nuttig platform kunnen zijn voor toekomstige studies naar transport van bijvoorbeeld water, glycerol en van carbonzuren anders dan lactaat, omdat de meerderheid van de transporters die betrokken zijn bij deze functies al zijn verwijderd. Een ambitieuzer doel is de constructie van een 'minimale transportergist', een gist met deleties in alle transportergenen die niet nodig zijn voor groei in synthetisch medium. Een dergelijke stam zou functionele analyse van transporters kunnen vereenvoudigen en het zou verwonderlijk zijn als er tijdens de constructie van een dergelijke stam geen nieuwe transportfuncties aan genen kunnen worden toegekend. Ondanks dat er maar een fractie van de 302 beschreven transportergenen in *S. cerevisiae* zijn verwijderd in de in **Hoofdstuk 5** beschreven stam, zou dit onderzoek, samen met de in **Hoofdstuk 2** beschreven gendeletiemethode, kunnen worden omschreven als een bescheiden start richting het ambitieuze doel van het maken van een dergelijke 'minimale transportergist'.

Bibliography

Abbott DA, Van den Brink J, Minneboo IMK *et al.* Anaerobic homolactate fermentation with *Saccharomyces cerevisiae* results in depletion of ATP and impaired metabolic activity. *FEMS Yeast Res* 2009a;**9**:349–57.

Abbott DA, Suir E, Van Maris AJA *et al.* Physiological and transcriptional responses to high concentrations of lactic acid in anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 2008;**74**:5759–68.

Abbott DA, Zelle RM, Pronk JT *et al.* Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and challenges. *FEMS Yeast Res* 2009b;**9**:1123–36.

Aerts D, Verhaeghe TF, Roman BI *et al.* Transglucosylation potential of six sucrose phosphorylases toward different classes of acceptors. *Carbohydr Res* 2011;**346**:1860–7.

Ahmadpour D, Geijer C, Tamás MJ *et al.* Yeast reveals unexpected roles and regulatory features of aquaporins and aquaglyceroporins. *Biochim Biophys Acta - Gen Subj* 2014;**1840**:1482–91.

Alamäe T, Pärn P, Viigand K *et al.* Regulation of the *Hansenula polymorpha* maltase gene promoter in *H. polymorpha* and *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2003;**4**:165–73.

Alberty RA. *Thermodynamics of Biochemical Reactions*. Hoboken, NJ, USA: John Wiley & Sons, Inc., 2003.

Alexander JK. Purification and specificity of cellobiose phosphorylase from *Clostridium thermocellum*. *J Biol Chem* 1968;**243**:2899–904.

Alves SL, Herberts RA, Hollatz C *et al.* Molecular analysis of maltotriose active transport and fermentation by *Saccharomyces cerevisiae* reveals a determinant role for the *AGT1* permease. *Appl Environ Microbiol* 2008;**74**:1494–501.

Annaluru N, Muller H, Mitchell LA *et al.* Total synthesis of a functional designer eukaryotic chromosome. *Science* 2014;**344**:55–8.

Bachmann H, Pronk JT, Kleerebezem M *et al.* Evolutionary engineering to enhance starter culture performance in food fermentations. *Curr Opin Biotechnol* 2015;**32**:1–7.

Badotti F, Dário MG, Alves SL *et al.* Switching the mode of sucrose utilization by *Saccharomyces cerevisiae*. *Microb Cell Fact* 2008;**7**:4.

Bakker BM, Overkamp KM, van Maris AJA *et al.* Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 2001;**25**:15–37.

Bali M, Zhang B, Morano K a. *et al.* The Hsp90 molecular chaperone complex regulates maltose induction and stability of the *Saccharomyces MAL* gene transcription activator Mal63p. *J Biol Chem* 2003;**278**:47441–8.

Bao Z, Xiao H, Liang J *et al.* Homology-integrated CRISPR-Cas (HI-CRISPR) system for one-step multigene disruption in *Saccharomyces cerevisiae*. *ACS Synth Biol* 2015;**4**:585–94.

Barnett J. A history of research on yeasts 2: Louis Pasteur and his contemporaries, 1850–1880. *Yeast* 2000.

Barrangou R, Fremaux C, Deveau H *et al.* CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 2007;**315**:1709–12.

Basso TO, De Kok S, Dario M *et al.* Engineering topology and kinetics of sucrose metabolism in *Saccharomyces cerevisiae* for improved ethanol yield. *Metab Eng* 2011;**13**:694–703.

Batista AS, Miletti LC, Stambuk BU. Sucrose fermentation by *Saccharomyces cerevisiae* lacking hexose transport. *J Mol Microbiol Biotechnol* 2005;**8**:26–33.

Bauer BE, Rossington D, Mollapour M *et al.* Weak organic acid stress inhibits aromatic amino acid uptake by yeast, causing a strong influence of amino acid auxotrophies on the phenotypes of membrane transporter mutants. *Eur J Biochem* 2003;**270**:3189–95.

Becker JVW, Armstrong GO, Van der Merwe MJ *et al.* Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res* 2003;**4**:79–85.

Beekwilder J, Van Rossum HM, Koopman F *et al.* Polycistronic expression of a β-carotene biosynthetic pathway in *Saccharomyces cerevisiae* coupled to β-ionone production. *J Biotechnol* 2014:DOI: 10.1016/j.jbiotec.2013.12.016.

Belocopitow E, Maréchal LR. Trehalose phosphorylase from *Euglena gracilis*. *Biochim Biophys Acta - Enzymol* 1970;**198**:151–4.

Van den Berg MA, De Jong-Gubbels P, Kortland CJ *et al.* The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. *J Biol Chem* 1996;**271**:28953–9.

Van Berkel R. Cleaner production for process industries. *Plenary Lect Perth WA7 Chemeca* 2000.

Bertani G. Studies on lysogenesis I.: The mode of phage liberation by lysogenic *Escherichia coli*. *J Bacteriol* 1951;**62**:293–300.

Bianchi F, Van 't Klooster JS, Ruiz SJ *et al.* Asymmetry in inward- and outward-affinity constant of transport explain unidirectional lysine flux in *Saccharomyces cerevisiae*. *Sci Rep* 2016;**6**:31443.

Bienert GP, Desguin B, Chaumont F *et al.* Channel-mediated lactic acid transport: a novel function for aquaglyceroporins in bacteria. *Biochem J* 2013;**454**:559–70.

Boeke JD, La Croute F, Fink GR. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet MGG* 1984;**197**:345–6.

Boender LGM, De Hulster EAF, Van Maris AJA *et al.* Quantitative physiology of *Saccharomyces cerevisiae* at near-zero specific growth rates. *Appl Environ Microbiol* 2009;**75**:5607–14.

Borodina I, Nielsen J. Advances in metabolic engineering of yeast *Saccharomyces cerevisiae* for production of chemicals. *Biotechnol J* 2014;**9**:609–20.

Botstein D, Fink GR. Yeast: an experimental organism for 21st Century biology. *Genetics* 2011;**189**:695–704.

Brachmann CB, Davies A, Cost GJ *et al.* Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 1998;**14**:115–32.

Braglia P, Percudani R, Dieci G. Sequence context effects on oligo(dT) termination signal recognition by *Saccharomyces cerevisiae* RNA polymerase III. *J Biol Chem* 2005;**280**:19551–62.

Brambilla L, Bolzani D, Compagno C *et al.* NADH reoxidation does not control glycolytic flux during exposure of respiring *Saccharomyces cerevisiae* cultures to glucose excess. *FEMS Microbiol Lett* 1999;**171**:133–40.

Branduardi P, Sauer M, De Gioia L *et al.* Lactate production yield from engineered yeasts is dependent from the host background, the lactate dehydrogenase source and the lactate export. *Microb Cell Fact* 2006;**5**:4.

Van den Brink J, Akeroyd M, Van der Hoeven R *et al.* Energetic limits to metabolic flexibility: Responses of *Saccharomyces cerevisiae* to glucose-galactose transitions. *Microbiology* 2009;**155**:1340–50.

Brohée S, Barriot R, Moreau Y *et al.* YTPdb: A wiki database of yeast membrane transporters. *Biochim Biophys Acta - Biomembr* 2010;**1798**:1908–12.

Brouns SJJ, Jore MM, Lundgren M *et al.* Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 2008;**321**:960–4.

Brown CA, Murray AW, Verstrepen KJ. Rapid expansion and functional divergence of subtelomeric gene families in yeasts. *Curr Biol* 2010;**20**:895–903.

Buchholz K, Collins J. The roots—a short history of industrial microbiology and biotechnology. *Appl Microbiol Biotechnol* 2013.

Bud R. *The Uses of Life: A History of Biotechnology*., 1994.

Bud R. *Penicillin: Triumph and Tragedy*., 2007.

Buziol S, Becker J, Baumeister A *et al.* Determination of *in vivo* kinetics of the starvation-induced Hxt5 glucose transporter of *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2002;**2**:283–91.

Canelas AB, Harrison N, Fazio A *et al.* Integrated multilaboratory systems biology reveals differences in protein metabolism between two reference yeast strains. *Nat Commun* 2010;**1**:145.

Carlson M, Botstein D. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. *Cell* 1982;**28**:145–54.

Carlson M, Botstein D. Organization of the *SUC* gene family in *Saccharomyces*. *Mol Cell Biol* 1983;**3**:351–9.

Carlson M, Osmond BC, Botstein D. Mutants of yeast defective in sucrose utilization. *Genetics* 1981;**98**:25–40.

Carroll D. Genome engineering with zinc-finger nucleases. *Genetics* 2011;**188**:773–82.

Casal M, Cardoso H. Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. *Microbiology* 1996;**142**:1385–90.

Casal M, Paiva S, Andrade RP *et al.* The lactate-proton symport of *Saccharomyces cerevisiae* is encoded by *JEN1*. *J Bacteriol* 1999;**181**:2620–3.

Casini A, MacDonald JT, De Jonghe J *et al.* One-pot DNA construction for synthetic biology: the Modular Overlap-Directed Assembly with Linkers (MODAL) strategy. *Nucleic Acids Res* 2014;**42**:e7.

Caspeta L, Chen Y, Ghiaci P *et al.* Altered sterol composition renders yeast thermotolerant. *Science* 2014;**346**:75–8.

Cássio F, Leao C, Van Uden N. Transport of lactate and other short-chain monocarboxylates in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 1987;**53**:509–13.

Celiker H, Gore J. Competition between species can stabilize public-goods cooperation within a species. *Mol Syst Biol* 2012;**8**:621.

Chavent M, Duncan AL, Sansom MS. Molecular dynamics simulations of membrane proteins and their interactions: from nanoscale to mesoscale. *Curr Opin Struct Biol* 2016;**40**:8–16.

Chee MK, Haase SB. New and redesigned pRS Plasmid shuttle vectors for genetic manipulation of *Saccharomyces cerevisiae*. *G3 (Bethesda)* 2012;**2**:515–26.

Chen L-Q, Hou B-H, Lalonde S *et al.* Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature* 2010;**468**:527–32.

Chen L-Q, Qu X-Q, Hou B-H *et al.* Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. *Science* 2012;**335**:207–11.

Chen X, Nielsen KF, Borodina I *et al.* Increased isobutanol production in *Saccharomyces cerevisiae* by overexpression of genes in valine metabolism. *Biotechnol Biofuels* 2011;**4**:21.

Choi WG, Roberts DM. *Arabidopsis* NIP2;1, a major intrinsic protein transporter of lactic acid induced by anoxic stress. *J Biol Chem* 2007;**282**:24209–18.

Christian M, Cermak T, Doyle EL *et al.* Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 2010;**186**:757–61.

Christianson TW, Sikorski RS, Dante M *et al.* Multifunctional yeast high-copy-number shuttle vectors. *Gene* 1992;**110**:119–22.

Coghill RD. The development of penicillin strains. *Hist penicillin Prod Chem Eng gprogress New York* 1970:15–21.

Cohen S, Chang A, Boyer H. Construction of biologically functional bacterial plasmids *in vitro*. *Proc* 1973.

Connstein W, Lüdecke K. Über Glycerin-Gewinnung durch Gärung. *Eur J Inorg* 1919.

Coskun Ü, Simons K. Membrane rafting: From apical sorting to phase segregation. *FEBS Lett* 2010;**584**:1685–93.

Coury LA, Hiller M, Mathai JC *et al.* Water transport across yeast vacuolar and plasma membrane-targeted secretory vesicles occurs by passive diffusion. *J Bacteriol* 1999;**181**:4437–40.

Cueto-Rojas HF, Van Maris AJA, Wahl SA *et al.* Thermodynamics-based design of microbial cell factories for anaerobic product formation. *Trends Biotechnol* 2015;**33**:534–46.

Dai Z, Liu Y, Guo J *et al.* Yeast synthetic biology for high-value metabolites. *FEMS Yeast Res* 2015;**15**:1-11.

Della-Bianca BE, Basso TO, Stambuk BU *et al.* What do we know about the yeast strains from the Brazilian fuel ethanol industry? *Appl Microbiol Biotechnol* 2013;**97**:979–91.

Della-Bianca BE, De Hulster E, Pronk JT *et al.* Physiology of the fuel ethanol strain *Saccharomyces cerevisiae* PE-2 at low pH indicates a context-dependent performance relevant for industrial applications. *FEMS Yeast Res* 2014;**14**:1196–205.

Delneri D, Tomlin GC, Wixon JL *et al.* Exploring redundancy in the yeast genome: an improved strategy for use of the cre-*lox*P system. *Gene* 2000;**252**:127–35.

Deltcheva E, Chylinski K, Sharma CM *et al.* CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 2011;**471**:602–7.

Deng X, Petitjean M, Teste M-A *et al.* Similarities and differences in the biochemical and enzymological properties of the four isomaltases from *Saccharomyces cerevisiae*. *FEBS Open Bio* 2014;**4**:200–12.

Dequin S, Barre P. Mixed lactic acid–alcoholic fermentation by *Saccharomyes cerevisiae* expressing the *Lactobacillus casei* L(+)–LDH. *Bio/Technology* 1994;**12**:173–7.

DiCarlo JE, Conley AJ, Penttilä M *et al.* Yeast oligo-mediated genome engineering (YOGE). *ACS Synth Biol* 2013a;**2**:741–9.

DiCarlo JE, Norville JE, Mali P *et al.* Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res* 2013b;**41**:4336–43.

Van Dijken J., Bauer J, Brambilla L *et al.* An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb Technol* 2000;**26**:706–14.

Dijken JP, Scheffers WA. Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol Lett* 1986;**32**:199–224.

Doudoroff M. Disaccharide phosphorylases. 1955, 225–31.

Eide DJ. The molecular biology of metal ion transport in *Saccharomyces cerevisiae*. *Annu Rev Nutr* 1998;**18**:441–69.

Engelman DM. Membranes are more mosaic than fluid. *Nature* 2005;**438**:578–80.

Entian K-D, Kötter P. Yeast genetic strain and plasmid collections. *Methods in Microbiology*. Vol 36. 2007, 629–66.

Ereky K. Biotechnologie der Fleisch-, Fett-und Milcherzengung im landwirtschaftlichen Großbetriebe für naturwissenschaftlich gebildete landwirte, verfasst. 1919.

Feng Z, Zhang B, Ding W *et al.* Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res* 2013;**23**:1229–32.

Fisher CR. Enzymology of the pigmented adenine-requiring mutants of *Saccharomyces* and *Schizosaccharomyces*. *Biochem Biophys Res Commun* 1969;**34**:306–10.

Gao Y, Zhao Y. Self-processing of ribozyme-flanked RNAs into guide RNAs *in vitro* and *in vivo* for CRISPR-mediated genome editing. *J Integr Plant Biol* 2014;**56**:343–9.

Gavrilescu M, Chisti Y. Biotechnology - A sustainable alternative for chemical industry. *Biotechnol Adv* 2005;**23**:471–99.

Gibson AW, Wojciechowicz LA, Danzi SE *et al.* Constitutive mutations of the *Saccharomyces cerevisiae* mal-activator genes *MAL23*, *MAL43*, *MAL63*, and *MAL64*. *Genetics* 1997;**146**:1287–98.

Gibson DG, Benders GA, Andrews-Pfannkoch C *et al.* Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science* 2008;**319**:1215–20.

Gietz DR, Woods RA. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* 2002;**350**:87–96.

Goedl C, Sawangwan T, Wildberger P *et al.* Sucrose phosphorylase: a powerful transglucosylation catalyst for synthesis of α-D-glucosides as industrial fine chemicals. *Biocatal Biotransformation* 2010;**28**:10–21.

Goedl C, Schwarz A, Minani A *et al.* Recombinant sucrose phosphorylase from *Leuconostoc mesenteroides*: Characterization, kinetic studies of transglucosylation, and application of immobilised enzyme for production of α-D-glucose 1-phosphate. *J Biotechnol* 2007;**129**:77–86.

Goffeau A, Barrell BG, Bussey H *et al.* Life with 6000 Genes. *Science* 1996;**274**:546–67.

Gombert AK, Van Maris AJ. Improving conversion yield of fermentable sugars into fuel ethanol in 1st generation yeast-based production processes. *Curr Opin Biotechnol* 2015;**33**:81–6.

González F, Zhu Z, Shi Z-D *et al.* An iCRISPR platform for rapid, multiplexable, and inducible genome editing in human pluripotent stem cells. *Cell Stem Cell* 2014;**15**:215–26.

González-Ramos D, Van den Broek M, Van Maris AJ *et al.* Genome-scale analyses of butanol tolerance in *Saccharomyces cerevisiae* reveal an essential role of protein degradation. *Biotechnol Biofuels* 2013;**6**:48.

González-Ramos D, Gorter de Vries AR, Grijseels SS *et al.* A new laboratory evolution approach to select for constitutive acetic acid tolerance in *Saccharomyces cerevisiae* and identification of causal mutations. *Biotechnol Biofuels* 2016;**9**:173.

Gordon GL, Doelle HW. Purification, properties and immunological relationship of l(+)-Lactate dehydrogenase from *Lactobacillus casei*. *Eur J Biochem* 1976;**67**:543–55.

Gore J, Youk H, Van Oudenaarden A. Snowdrift game dynamics and facultative cheating in yeast. *Nature* 2009;**459**:253–6.

Gratz SJ, Cummings AM, Nguyen JN *et al.* Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. *Genetics* 2013;**194**:1029–35.

Greig D, Travisano M. The Prisoner's Dilemma and polymorphism in yeast *SUC* genes. *Proc R Soc B Biol Sci* 2004;**271**:S25–6.

Grenson M, Mousset M, Wiame JM *et al.* Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. *Biochim Biophys Acta - Gen Subj* 1966;**127**:325–38.

De Groeve MRM, De Baere M, Hoflack L *et al.* Creating lactose phosphorylase enzymes by directed evolution of cellobiose phosphorylase. *Protein Eng Des Sel* 2009;**22**:393–9.

Grote A, Hiller K, Scheer M *et al.* JCat: A novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res* 2005;**33**:526–31.

Guadalupe-Medina V, Almering MJH, Van Maris AJA *et al.* Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. *Appl Environ Microbiol* 2010;**76**:190–5.

Guadalupe-Medina V, Wisselink HW, Luttik MA *et al.* Carbon dioxide fixation by Calvin-Cycle enzymes improves ethanol yield in yeast. *Biotechnol Biofuels* 2013;**6**:125.

Gueldener U, Heinisch J, Koehler GJ *et al.* A second set of *loxP* marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res* 2002;**30**:e23.

Güldener U, Heck S, Fielder T *et al.* A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* 1996;**24**:2519–24.

Gutknecht J, Walter A. SCN– and HSCN transport through lipid bilayer membranes. *Biochim Biophys Acta - Biomembr* 1982;**685**:233–40.

Ha S-J, Galazka JM, Joong Oh E *et al.* Energetic benefits and rapid cellobiose fermentation by *Saccharomyces cerevisiae* expressing cellobiose phosphorylase and mutant cellodextrin transporters. *Metab Eng* 2012, DOI: 10.1016/j.ymben.2012.11.005.

Hansen EH, Moller BL, Kock GR *et al. De novo* biosynthesis of vanillin in fission yeast (*Schizosaccharomyces pombe*) and baker's yeast (*Saccharomyces cerevisiae*). *Appl Environ Microbiol* 2009;**75**:2765–74.

Hawthorne DC. The genetics of alpha-methyl-glucoside fermentation in *Saccharomyces*. *Heredity (Edinb)* 1958;**12**:273–84.

Hazelwood LA, Tai SL, Boer VM *et al.* A new physiological role for Pdr12p in *Saccharomyces cerevisiae* : export of aromatic and branched-chain organic acids produced in amino acid catabolism. *FEMS Yeast Res* 2006;**6**:937–45.

Hegemann JH, Heick SB. Delete and repeat: a comprehensive toolkit for sequential gene knockout in the budding yeast *Saccharomyces cerevisiae*. *Methods Mol Biol* 2011;**765**:189–206.

Hellborg L, Woolfit M, Arthursson-Hellborg M *et al.* Complex evolution of the *DAL5* transporter family. *BMC Genomics* 2008;**9**:164.

Helmstetter CE, Cooper S. DNA synthesis during the division cycle of rapidly growing *Escherichia coli*. *J Mol Biol* 1968;**31**:507–18.

Hirasawa T, Takekuni M, Yoshikawa K *et al.* Genome-wide identification of the targets for genetic manipulation to improve L-lactate production by *Saccharomyces cerevisiae* by using a single-gene deletion strain collection. *J Biotechnol* 2013;**168**:185–93.

Holyoak CD, Bracey D, Piper PW *et al.* The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy- dependent mechanism. *J Bacteriol* 1999;**181**:4644–52.

Hong KK, Nielsen J. Metabolic engineering of *Saccharomyces cerevisiae*: A key cell factory platform for future biorefineries. *Cell Mol Life Sci* 2012;**69**:2671–90.

Hong K-K, Vongsangnak W, Vemuri GN *et al.* Unravelling evolutionary strategies of yeast for improving galactose utilization through integrated systems level analysis. *Proc Natl Acad Sci U S A* 2011;**108**:12179–84.

Horák J. Regulations of sugar transporters: insights from yeast. *Curr Genet* 2013;**59**:1–31.

Horwitz AA, Walter JM, Schubert MG *et al.* Efficient multiplexed integration of synergistic alleles and metabolic pathways in yeasts via CRISPR-Cas. *Cell Syst* 2015;**1**:88–96.

Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 2014;**157**:1262–78.

Hsu PD, Scott DA, Weinstein JA *et al.* DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 2013;**31**:827–32.

Hu Z, Nehlin JO, Ronne H *et al. MIG1*-dependent and *MIG1*-independent glucose regulation of *MAL* gene expression in *Saccharomyces cerevisiae*. *Curr Genet* 1995;**28**:258–66.

Hughes TR, Roberts CJ, Dai H *et al.* Widespread aneuploidy revealed by DNA microarray expression profiling . *Nat Genet* 2000;**25**:333–7.

Hwang WY, Fu Y, Reyon D *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 2013;**31**:227–9.

Jacobs JZ, Ciccaglione KM, Tournier V *et al.* Implementation of the CRISPR-Cas9 system in fission yeast. *Nat Commun* 2014;**5**:5344.

Jung WS, Yoo YJ, Park JW *et al.* A combined approach of classical mutagenesis and rational metabolic engineering improves rapamycin biosynthesis and provides insights into methylmalonyl-CoA precursor supply pathway in *Streptomyces hygroscopicus* ATCC 29253. *Appl Microbiol Biotechnol* 2011;**91**:1389–97.

Kawasaki H, Nakamura N, Ohmori M *et al.* Screening for bacteria producing sucrose phosphorylase and characterization of the enzymes. *Biosci Biotechnol Biochem* 1996;**60**:319–21.

Kell DB, Swainston N, Pir P *et al.* Membrane transporter engineering in industrial biotechnology and whole cell biocatalysis. *Trends Biotechnol* 2015;**33**:237–46.

Kennedy M. The evolution of the word "Biotechnology." *Trends Biotechnol* 1991;**9**:218–20.

Kerr ID. Sequence analysis of twin ATP binding cassette proteins involved in translational control, antibiotic resistance, and ribonuclease L inhibition. *Biochem Biophys Res Commun* 2004;**315**:166–73.

Ketchum KA, Joiner WJ, Sellers AJ *et al.* A new family of outwardly rectifying potassium channel proteins with two pore domains in tandem. *Nature* 1995;**376**:690–5.

Kishore S, Alexander JK. Cellodextrin phosphorylase from *Clostridium thermocellum*. *Biochim Biophys Acta - Gen Subj* 1967;**148**:808–10.

De Kok S, Kozak BU, Pronk JT *et al.* Energy coupling in *Saccharomyces cerevisiae*: Selected opportunities for metabolic engineering. *FEMS Yeast Res* 2012a;**12**:387–97.

De Kok S, Nijkamp JF, Oud B *et al.* Laboratory evolution of new lactate transporter genes in a *jen1Δ* mutant of *Saccharomyces cerevisiae* and their identification as *ADY2* alleles by whole-genome resequencing and transcriptome analysis. *FEMS Yeast Res* 2012b;**12**:359–74.

De Kok S, Yilmaz D, Suir E *et al.* Increasing free-energy (ATP) conservation in maltose-grown *Saccharomyces cerevisiae* by expression of a heterologous maltose phosphorylase. *Metab Eng* 2011;**13**:518–26.

Koopman F, Beekwilder J, Crimi B *et al. De novo* production of the flavonoid naringenin in engineered *Saccharomyces cerevisiae*. *Microb Cell Fact* 2012;**11**:155.

Kosuri S, Church GM. Large-scale *de novo* DNA synthesis: technologies and applications. *Nat Methods* 2014;**11**:499–507.

Kozak BU, Van Rossum HM, Benjamin KR *et al.* Replacement of the *Saccharomyces cerevisiae* acetyl-CoA synthetases by alternative pathways for cytosolic acetyl-CoA synthesis. *Metab Eng* 2014a;**21**:46–59.

Kozak BU, Van Rossum HM, Luttik MAH *et al.* Engineering acetyl coenzyme A supply : Functional expression of a bacterial pyruvate dehydrogenase complex in the cytosol of *Saccharomyces cerevisiae*. *MBio* 2014b;**5**:e01696-14.

Kresnowati MTAP, Groothuizen MK, Van Winden WA. Measurement of fast dynamic intracellular pH in *Saccharomyces cerevisiae* using benzoic acid pulse. 2007;**97**:86–98.

Kresze G-B, Ronft H. Pyruvate dehydrogenase complex from baker's yeast. 1. Purification and some kinetic and regulatory properites. *Eur J Biochem* 1981;**119**:573–9.

Krieble VK. Activities and the hydrolysis of sucrose with concentrated acids. *J Am Chem Soc* 1935;**57**:15–9.

Kuijpers NGA, Chroumpi S, Vos T *et al.* One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2013a;**13**:769–81.

Kuijpers NGA, Solis-Escalante D, Bosman L *et al.* A versatile, efficient strategy for assembly of multi-fragment expression vectors in *Saccharomyces cerevisiae* using 60 bp synthetic recombination sequences. *Microb Cell Fact* 2013b;**12**:47.

Kunes S, Botstein D, Fox SM. Transformation of yeast with linearized plasmid DNA. Formation of inverted dimers and recombinant plasmid products. *J Mol Biol* 1985;**184**:375–87.

Kuyper M, Hartog MMP, Toirkens MJ *et al.* Metabolic engineering of a xylose-isomerase-expressing strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res* 2005;**5**:399–409.

Lagunas R. Sugar transport in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 1993;**10**:229–42.

Lambert RJ, Stratford M. Weak-acid preservatives: Modelling microbial inhibition and response. *J Appl Microbiol* 1999;**86**:157–64.

Langmead B, Trapnell C, Pop M *et al.* Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* 2009;**10**:R25.

Lee J-H, Moon Y-H, Kim N *et al.* Cloning and expression of the sucrose phosphorylase gene from *Leuconostoc mesenteroides* in *Escherichia coli*. *Biotechnol Lett* 2008;**30**:749–54.

Lee KJ, Skotnicki ML, Tribe DE *et al.* Kinetic studies on a highly productive strain of *Zymomonas mobilis*. *Biotechnol Lett* 1980;**2**:339–44.

Lee SY, Kim HM, Cheon S. Metabolic engineering for the production of hydrocarbon fuels. *Curr Opin Biotechnol* 2015;**33**:15–22.

Van Leeuwen CCM, Weusthuis RA, Postma E *et al.* Maltose/proton co-transport in *Saccharomyces cerevisiae*. Comparative study with cells and plasma membrane vesicles. *Biochem J* 1992;**284**:441–5.

Leuenberger HGW. Cultivation of *Saccharomyces cerevisiae* in continuous culture - I. Growth kinetics of a respiratory deficient yeast strain grown in continuous culture. *Arch Mikrobiol* 1971;**79**:176–86.

Lian J, Li Y, HamediRad M *et al.* Directed evolution of a cellodextrin transporter for improved biofuel production under anaerobic conditions in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 2014;**111**:1521–31.

Lin IW, Sosso D, Chen L-Q *et al.* Nectar secretion requires sucrose phosphate synthases and the sugar transporter SWEET9. *Nature* 2014a;**508**:546–9.

Lin Y, Cradick TJ, Brown MT *et al.* CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. *Nucleic Acids Res* 2014b;**42**:7473–85.

Linn S, Arber W. Host specificity of DNA produced by *Escherichia coli*, X. *In vitro* restriction of phage fd replicative form. *Proc Natl Acad* 1968.

Liu G, Yong MYJ, Yurieva M *et al.* Gene essentiality is a quantitative property linked to cellular evolvability. *Cell* 2015;**163**:1388–99.

Lodolo EJ, Kock JLF, Axcell BC *et al.* The yeast *Saccharomyces cerevisiae* - the main character in beer brewing. *FEMS Yeast Res* 2008;**8**:1018–36.

Lõoke M, Kristjuhan K, Kristjuhan A. Extraction of genomic DNA from yeasts for PCR-based applications. *Biotechniques* 2011;**50**:325–8.

Lorenz R, Bernhart SH, Höner Zu Siederdissen C *et al.* ViennaRNA Package 2.0. *Algorithms Mol Biol* 2011;**6**:26.

Lowry OH, Rosebrough NJ, Farr AL *et al.* Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193**:265–75.

Luong JHT, Volesky B. Determination of the heat of some aerobic fermentations. *Can J Chem Eng* 1980;**58**:497–504.

Maiorella BL, Blanch HW, Wilke CR. Eeconomic evaluation of alternative ethanol fermentation processes. *Biotechnol Bioeng* 1984;**26**:1003–25.

Mans R, Van Rossum HM, Wijsman M *et al.* CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2015;**15**

Marini AM, Soussi-Boudekou S, Vissers S *et al.* A family of ammonium transporters in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1997;**17**:4282–93.

Van Maris AJA, Abbott DA, Bellissimi E *et al.* Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: Current status. *Antonie van Leeuwenhoek, Int J Gen Mol Microbiol* 2006;**90**:391–418.

Van Maris AJA, Konings WN, Dijken JP Van *et al.* Microbial export of lactic and 3-hydroxypropanoic acid: Implications for industrial fermentation processes. *Metab Eng* 2004a;**6**:245–55.

Van Maris AJA, Winkler AA, Porro D *et al.* Homofermentative lactate production cannot sustain anaerobic growth of engineered *Saccharomyces cerevisiae*: Possible consequence of energy-dependent lactate export. *Appl Environ Microbiol* 2004b;**70**:2898–905.

Marques WL, Mans R, Marella ER *et al.* Elimination of sucrose transport and hydrolysis in *Saccharomyces cerevisiae*: a platform strain for engineering sucrose metabolism. *FEMS Yeast Res* 2017:fox006.

Marques WL, Raghavendran V, Stambuk BU *et al.* Sucrose and *Saccharomyces cerevisiae*: a relationship most sweet. *FEMS Yeast Res* 2015:1–16.

Marraffini LA, Sontheimer EJ. CRISPR interference limits horizontal gene transfer in *Staphylococci* by targeting DNA. 2008;**322**:1843–5.

Mashego MR, Van Gulik WM, Vinke JL *et al.* Critical evaluation of sampling techniques for residual glucose determination in carbon-limited chemostat culture of *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 2003;**83**:395–9.

Maxam A, Gilbert W. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol* 1980.

Meadows AL, Hawkins KM, Tsegaye Y *et al.* Rewriting yeast central carbon metabolism for industrial isoprenoid production. *Nature* 2016;**537**:694–7.

Mertz J, Davis R. Cleavage of DNA by R1 restriction endonuclease generates cohesive ends. *Natl Acad Sci* 1972.

Mieczkowski PA, Lemoine FJ, Petes TD. Recombination between retrotransposons as a source of chromosome rearrangements in the yeast *Saccharomyces cerevisiae*. *DNA Repair (Amst)* 2006;**5**:1010–20.

Miller JC, Tan S, Qiao G *et al.* A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol* 2011;**29**:143–8.

Mira NP, Teixeira MC, Sá-Correia I. Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae* : A genome-wide view. *Omi A J Integr Biol* 2010;**14**:525–40.

Mitchell D. *A Note on Rising Food Prices*., 2008.

Mojzita D, Hohmann S. Pdc2 coordinates expression of the *THI* regulon in the yeast *Saccharomyces cerevisiae*. *Mol Genet Genomics* 2006;**276**:147–61.

Mollapour M, Piper PW. Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Mol Cell Biol* 2007;**27**:6446–56.

Montague TG, Cruz JM, Gagnon JA *et al.* CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Res* 2014;**42**:W401-7.

Mullis K, Faloona F, Scharf S *et al.* Specific enzymatic amplification of DNA *in vitro*: The Polymerase Chain Reaction. *Cold Spring Harb Symp Quant Biol* 1986;**51**:263–73.

Mumberg D, Müller R, Funk M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 1995;**156**:119–22.

Mussolino C, Morbitzer R, Lütge F *et al.* A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res* 2011;**39**:9283–93.

Nathan F. The manufacture of acetone. *J Soc Chem Ind* 1919;**38**:271T–273T.

Naumoff DG. Hierarchical classification of glycoside hydrolases. *Biochemistry* 2011;**76**:622–35.

Naumoff DG, Naumov GI. Discovery of a novel family of α glucosidase *IMA* genes in yeast *Saccharomyces cerevisiae*. *Dokl Biochem Biophys* 2010;**432**:549–51.

Nelissen B, De Wachter R, Goffeau A. Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 1997;**21**:113–34.

Nguyen AW, Daugherty PS. Evolutionary optimization of fluorescent proteins for intracellular FRET. *Nat Biotechnol* 2005;**23**:355–60.

Nicholls DG, Ferguson SJ. Bioenergetics 3. *Biochem* 2004;**69**:818–9.

Nielsen J, Larsson C, Van Maris A *et al.* Metabolic engineering of yeast for production of fuels and chemicals. *Curr Opin Biotechnol* 2013;**24**:398–404.

Niittylä T, Messerli G, Trevisan M *et al.* A previously unknown maltose transporter essential for starch degradation in leaves. *Science* 2004;**303**:87–9.

Nijkamp JF, Van den Broek M, Datema E *et al. De novo* sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN. PK113-7D, a model for modern industrial biotechnology. *Microb Cell Fact* 2012;**11**:36.

Nygård Y, Mojzita D, Toivari M *et al.* The diverse role of Pdr12 in resistance to weak organic acids. *Yeast* 2014;**31**:219–32.

Oh D, Hopper JE. Transcription of a yeast phosphoglucomutase isozyme gene is galactose inducible and glucose repressible. *Mol Cell Biol* 1990;**10**:1415–22.

Oliver SG. From DNA sequence to biological function. *Nature* 1996;**379**:597–600.

Orr-Weaver TL, Szostak JW. Yeast recombination : The association between double-strand gap repair and crossing-over. *Proc Natl Acad Sci* 1983;**80**:4417–21.

Orr-Weaver TL, Szostak JW, Rothstein RJ. Genetic applications of yeast transformation with linear and gapped plasmids. *Methods Enzymol* 1983;**101**:228–45.

Oud B, Guadalupe-Medina V, Nijkamp JF *et al.* Genome duplication and mutations in *ACE2* cause multicellular, fast-sedimenting phenotypes in evolved *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 2013;**110**:E4223-31.

Oud B, Van Maris AJA, Daran J-M *et al.* Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast. *FEMS Yeast Res* 2012;**12**:183–96.

Pacheco A, Talaia G, Sá-Pessoa J *et al.* Lactic acid production in *Saccharomyces cerevisiae* is modulated by expression of the monocarboxylate transporters Jen1 and Ady2. *FEMS Yeast Res* 2012;**12**:375–81.

Paddon CJ, Westfall PJ, Pitera DJ *et al.* High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature* 2013;**496**:528–32.

Paiva S, Devaux F, Barbosa S *et al.* Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. *Yeast* 2004;**21**:201–10.

Pasteur L. Mémoire sur la fermentation appelée lactique. *Comptes Rendus Hebd des Seances l'Académie des Sci Paris* 1857a;**45**:913–6.

Pasteur L. Mémoire sur la fermentation alcoolique. *Comptes Rendus Hebd des Seances l'Académie des Sci Paris* 1857b;**45**:1032–6.

Pasteur L. Mémoire sur la fermentation appelée lactique. *Mémoires la Société Impériale des Sci l'Agriculture des Arts Lille IIe série* 1859;**5**:13–26.

Pasteur L. Improvement in the manufacture of beer and yeast. 1873.

Pasteur L. *Etudes Sur La Bière, Ses Maladies Avec Une Théorie Nouvelle de La Fermentation.*, 1876.

Paulsen IT, Sliwinski MK, Nelissen B *et al.* Unified inventory of established and putative transporters encoded within the complete genome of *Saccharomyces cerevisiae*. *FEBS Lett* 1998;**430**:116–25.

Paumi CM, Chuk M, Snider J *et al.* ABC transporters in *Saccharomyces cerevisiae* and their interactors: New technology advances the biology of the ABCC (MRP) subfamily. *Microbiol Mol Biol Rev* 2009;**73**:577–93.

Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002;**30**:e36.

Pfeiffer T, Schuster S, Bonhoeffer S. Cooperation and competition in the evolution of ATP-producing pathways. *Science* 2001;**292**:504–7.

Piper MDW, Daran-Lapujade P, Bro C *et al.* Reproducibility of oligonucleotide microarray transcriptome analyses. An interlaboratory comparison using chemostat cultures of Saccharomyces cerevisiae. *J Biol Chem* 2002;**277**:37001–8.

Piper P, Mahé Y, Thompson S *et al.* The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. *EMBO J* 1998;**17**:4257–65.

Podbielniak WJ, Kaiser HR, Ziegenhorn GJ. The History of Penicillin Production. *J Pharm Sci* 1970;**59**:1697.

Poole RC, Halestrap AP. Transport of lactate and other monocarboxylates across mammalian plasma membranes. *Am J Physiol* 1993;**264**:C761-82.

Porro D, Bianchi M, Ranzi B *et al.* Yeast strains for the production of lactic acid. 1999.

Porro D, Brambilla L, Ranzi BM *et al.* Development of metabolically engineered *Saccharomyces cerevisiae* cells for the production of lactic acid. *Biotechnol Prog* 1995;**11**:294–8.

Postma E, Verduyn C, Scheffers WA *et al.* Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 1989;**55**:468–77.

Pougach K, Voet A, Kondrashov FA *et al.* Duplication of a promiscuous transcription factor drives the emergence of a new regulatory network. *Nat Commun* 2014;**5**:4868.

Primrose S, Twyman R. *Principles of Gene Manipulation and Genomics*., 2013.

Pronk JT. Auxotrophic yeast strains in fundamental and applied research. *Appl Environ Microbiol* 2002;**68**:2095–2010.

Pronk JT, Wenzel TJ, Luttik MAH *et al.* Energetic aspects of glucose metabolism in a pyruvate-dehydrogenase-negative mutant of *Saccharomyces cerevisiae*. *Microbiology* 1994;**140**:601–10.

Ramos J, Sychrová H, Kschischo M eds. *Yeast Membrane Transport*. Cham: Springer International Publishing, 2016.

Ran F, Bali M, Michels CA. Hsp90/Hsp70 chaperone machine regulation of the *Saccharomyces MAL*-activator as determined *in vivo* using noninducible and constitutive mutant alleles. *Genetics* 2008;**179**:331–43.

Reidel EJ, Turgeon R, Cheng L. A maltose transporter from apple is expressed in source and sink tissues and complements the *Arabidopsis* maltose export-defective mutant. *Plant Cell Physiol* 2008;**49**:1607–13.

Reis ALS, De Fátima Rodrigues de Souza R, Baptista Torres RRN *et al.* Oxygen-limited cellobiose fermentation and the characterization of the cellobiase of an industrial *Dekkera/Brettanomyces bruxellensis* strain. *Springerplus* 2014;**3**:38.

Ro D-K, Paradise EM, Ouellet M *et al.* Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 2006;**440**:940–3.

Roberts RJ. How restriction enzymes became the workhorses of molecular biology. *Proc Natl Acad Sci U S A* 2005;**102**:5905–8.

Rogers PL, Lee KJ, Tribe DE. Kinetics of alcohol production by *Zymomonas mobilis* at high sugar concentrations. *Biotechnol Lett* 1979;**1**:165–70.

Roman H. Studies of gene mutation in *Saccharomyces*. *Cold Spring Harb Symp Quant Biol* 1956;**21**:175–85.

Van Rossum HM, Kozak BU, Niemeijer MS *et al.* Alternative reactions at the interface of glycolysis and citric acid cycle in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2016a;**16**:1–13.

Van Rossum HM, Kozak BU, Pronk JT *et al.* Engineering cytosolic acetyl-coenzyme A supply in *Saccharomyces cerevisiae*: Pathway stoichiometry, free-energy conservation and redox-cofactor balancing. *Metab Eng* 2016b;**36**:99–115.

Rost S, Frank C, Beck E. The chloroplast envelope is permeable for maltose but not for maltodextrins. *Biochim Biophys Acta - Gen Subj* 1996;**1291**:221–7.

Ryan OW, Skerker JM, Maurer MJ *et al.* Selection of chromosomal DNA libraries using a multiplex CRISPR system. *Elife* 2014:e03703.

Sabir F, Loureiro-Dias MC, Prista C. Comparative analysis of sequences, polymorphisms and topology of yeasts aquaporins and aquaglyceroporins. Nielsen J (ed.). *FEMS Yeast Res* 2016;**16**:fow025.

Sá-Correia I, Tenreiro S. The multidrug resistance transporters of the major facilitator superfamily, 6 years after disclosure of *Saccharomyces cerevisiae* genome sequence. *J Biotechnol* 2002;**98**:215–26.

Sadie CJ, Rose SH, Den Haan R *et al.* Co-expression of a cellobiose phosphorylase and lactose permease enables intracellular cellobiose utilisation by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 2011;**90**:1373–80.

Saiki R, Gelfand D, Stoffel S *et al.* Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988.

Sandoval CM, Ayson M, Moss N *et al.* Use of pantothenate as a metabolic switch increases the genetic stability of farnesene producing *Saccharomyces cerevisiae*. *Metab Eng* 2014;**25**:215–26.

Sanger F, Nicklen S. DNA sequencing with chain-terminating inhibitors. *Proc* 1977.

Santos E, Rodriguez L, Elorza MV *et al.* Uptake of sucrose by *Saccharomyces cerevisiae*. *Arch Biochem Biophys* 1982;**216**:652–60.

Sauer U. Evolutionary engineering of industrially important microbial phenotypes. 2001, 129–69.

Schmitt ME, Brown TA, Trumpower BL. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res* 1990;**18**:3091–2.

Shao Z, Zhao H, Zhao H. DNA assembler, an *in vivo* genetic method for rapid construction of biochemical pathways. *Nucleic Acids Res* 2009;**37**:e16.

Siewers V. An overview on selection marker genes for transformation of *Saccharomyces cerevisiae*. *Methods Mol Biol* 2014;**1152**:3–15.

Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 1989;**122**:19–27.

Silver JM, Eaton NR. Functional block of the ad1 and ad2 mutants of *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* 1969;**34**:301–5.

Silverstein R, Voet J, Reed D *et al.* Purification and mechanism of action of sucrose phosphorylase. *J Biol Chem* 1967;**242**:1338–46.

Sirenko OI, Ni B, Needleman RB. Purification and binding properties of the Mal63p activator of *Saccharomyces cerevisiae*. *Curr Genet* 1995;**27**:509–16.

Skulachev VP. A biochemical approach to the problem of aging: "Megaproject" on membrane-penetrating ions. The first results and prospects. *Biochem* 2007;**72**:1385–96.

Solis-Escalante D, Van den Broek M, Kuijpers NGA *et al.* The genome sequence of the popular hexose-transport-deficient *Saccharomyces cerevisiae* strain EBY.VW4000 reveals *Lox*P/Cre-induced translocations and gene loss. *FEMS Yeast Res* 2015;**15**

Solis-Escalante D, Kuijpers NGA, Bongaerts N *et al. amdSYM*, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2013;**13**:126–39.

Solis-Escalante D, Kuijpers NGA, Van der Linden FH *et al.* Efficient simultaneous excision of multiple selectable marker cassettes using I-SceI-induced double-strand DNA breaks in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2014;**14**:741–54.

Sprenger GA. Carbohydrate metabolism in *Zymomonas mobilis* : a catabolic highway with some scenic routes. *FEMS Microbiol Lett* 1996;**145**:301–7.

Stambuk BU, Batista AS, De Araujo PS. Kinetics of active sucrose transport in *Saccharomyces cerevisiae*. *J Biosci Bioeng* 2000;**89**:212–4.

Stambuk BU, Silva MA, Panek AD *et al.* Active α-glucoside transport in *Saccharomyces cerevisiae*. 1999;**170**:73–8.

Steen EJ, Kang Y, Bokinsky G *et al.* Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* 2010;**463**:559–62.

Storici F, Coglievina M, Bruschi C V. A 2-microm DNA-based marker recycling system for multiple gene disruption in the yeast *Saccharomyces cerevisiae*. *Yeast* 1999;**15**:271–83.

Storici F, Lewis LK, Resnick MA. *In vivo* site-directed mutagenesis using oligonucleotides. *Nat Biotechnol* 2001;**19**:773–6.

Stouthamer AH. A theoretical study on the amount of ATP required for synthesis of microbial cell material. *Antonie Van Leeuwenhoek* 1973;**39**:545–65.

Subtil T, Boles E. Improving L-arabinose utilization of pentose fermenting *Saccharomyces cerevisiae* cells by heterologous expression of L-arabinose transporting sugar transporters. *Biotechnol Biofuels* 2011;**4**:38.

Suzuki Y, St Onge RP, Mani R *et al.* Knocking out multigene redundancies via cycles of sexual assortment and fluorescence selection. *Nat Methods* 2011;**8**:159–64.

Swinnen S, Klein M, Carrillo M *et al.* Re-evaluation of glycerol utilization in *Saccharomyces cerevisiae*: characterization of an isolate that grows on glycerol without supporting supplements. *Biotechnol Biofuels* 2013;**6**:157.

Tedokon M, Suzuki K, Kayamori Y *et al.* Enzymatic assay of inorganic phosphate with use of sucrose phosphorylase and phosphoglucomutase. *Clin Chem* 1992;**38**:512–5.

Tenreiro S, Nunes PA, Viegas CA *et al. AQR1* gene (ORF *YNL065w*) encodes a plasma membrane transporter of the major facilitator superfamily that confers resistance to short-chain monocarboxylic acids and quinidine in *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* 2002;**292**:741–8.

Tenreiro S, Rosa PC, Viegas CA *et al.* Expression of the *AZR1* gene (ORF *YGR224w*), encoding a plasma membrane transporter of the major facilitator superfamily, is required for adaptation to acetic acid and resistance to azoles in *Saccharomyces cerevisiae*. *Yeast* 2000;**16**:1469–81.

Terentiev Y, Pico AH, Böer E *et al.* A wide-range integrative yeast expression vector system based on *Arxula adeninivorans*-derived elements. *J Ind Microbiol Biotechnol* 2004;**31**:223–8.

Teste M-A, Duquenne M, François JM *et al.* Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*. *BMC Mol Biol* 2009;**10**:99.

Teste M-A, François JM, Parrou J-L. Characterization of a new multigene family encoding isomaltases in the yeast *Saccharomyces cerevisiae*, the *IMA* family. *J Biol Chem* 2010;**285**:26815–24.

Trendeleva TA, Rogov AG, Cherepanov DA *et al.* Interaction of tetraphenylphosphonium and dodecyltriphenylphosphonium with lipid membranes and mitochondria. *Biochem* 2012;**77**:1021–8.

Tsukaguchi H, Shayakul C, Berger U V. *et al.* Molecular characterization of a broad selectivity neutral solute channel. *J Biol Chem* 1998;**273**:24737–43.

Ullah A, Chandrasekaran G, Brul S *et al.* Yeast adaptation to weak acids prevents futile energy expenditure. *Front Microbiol* 2013;**4**:1–10.

Urnov FD, Rebar EJ, Holmes MC *et al.* Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 2010;**11**:636–46.

Velasco I, Tenreiro S, Calderon IL *et al. Saccharomyces cerevisiae* Aqr1 is an internal-membrane transporter involved in excretion of amino acids. *Eukaryot Cell* 2004;**3**:1492–503.

Verduyn C, Postma E, Scheffers WA *et al.* Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. *J Gen Microbiol* 1990;**136**:395–403.

Verduyn C, Postma E, Scheffers WA *et al.* Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* 1992;**8**:501–17.

Verduyn C, Stouthamer AH, Scheffers WA *et al.* A theoretical evaluation of growth yields of yeasts. *Antonie Van Leeuwenhoek* 1991;**59**:49–63.

Voordeckers K, Brown CA, Vanneste K *et al.* Reconstruction of ancestral metabolic enzymes reveals molecular mechanisms underlying evolutionary innovation through gene duplication. *PLoS Biol* 2012;**10**, DOI: 10.1371/journal.pbio.1001446.

Walker JE. The ATP synthase: the understood, the uncertain and the unknown. *Biochem Soc Trans* 2013;**41**:1–16.

Wang H, Yang H, Shivalila CS *et al.* One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 2013;**153**:910–8.

Wang Q, Wang L. New methods enabling efficient incorporation of unnatural amino acids in yeast. *J Am Chem Soc* 2008;**130**:6066–7.

Wang Y, Halls C, Zhang J *et al.* Stepwise increase of resveratrol biosynthesis in yeast *Saccharomyces cerevisiae* by metabolic engineering. *Metab Eng* 2011;**13**:455–63.

Watson J, Crick F. Uma Estrutura para o Ácido Desoxirribonucléico. *Nature* 1953.

Weinhandl K, Winkler M, Glieder A *et al.* Carbon source dependent promoters in yeasts. *Microb Cell Fact* 2014;**13**:5.

Weiss B, Richardson CC. Enzymatic breakage and joining of deoxyribonucleic acid, I. Repair of single-strand breaks in DNA by an enzyme system from *Escherichia coli* infected with T4 bacteriophage. *Proc Natl Acad Sci* 1967;**57**:1021–8.

Weizmann C, Anthony HG. Fermentation process for the production of acetone and butyl alcohol. 1920.

Westfall PJ, Pitera DJ, Lenihan JR *et al.* Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proc Natl Acad Sci* 2012;**109**:E111–8.

Weusthuis RA, Adams H, Scheffers WA *et al.* Energetics and kinetics of maltose transport in *Saccharomyces cerevisiae*: a continuous culture study. *Appl Environ Microbiol* 1993;**59**:3102–9.

Weusthuis RA, Lamot I, Van der Oost J *et al.* Microbial production of bulk chemicals: Development of anaerobic processes. *Trends Biotechnol* 2011;**29**:153–8.

Whelan WL, Gocke E, Manney TR. The *CAN1* locus of *Saccharomyces cerevisiae*: Fine-structure analysis and forward mutation rates. *Genetics* 1979;**91**:35–51.

Wieczorke R, Dlugai S, Krampe S *et al.* Characterisation of mammalian GLUT glucose transporters in a heterologous yeast expression system. *Cell Physiol Biochem* 2003;**13**:123–34.

Wieczorke R, Krampe S, Weierstall T *et al.* Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. *FEBS Lett* 1999;**464**:123–8.

Winge O, Roberts C. The relation between the polymeric genes for maltose raffinose, and sucrose fermentation in yeasts. *Cr Trav Lab Carlsberg Ser Physiol* 1952;**25**:141–71.

Winkler JD, Kao KC. Recent advances in the evolutionary engineering of industrial biocatalysts. *Genomics* 2014;**104**:406–11.

Winzeler EA, Shoemaker DD, Astromoff A *et al.* Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* 1999;**285**:901 LP-906.

Wittmann C, Liao J, Lee S *et al. Industrial Biotechnology: Microorganisms.*, 2017.

Wolfenden R, Yuan Y. Rates of spontaneous cleavage of glucose, fructose, sucrose, and trehalose in water, and the catalytic proficiencies of invertase and trehalase. *J Am Chem Soc* 2008;**130**:7548–9.

Wood EJ. The oxford dictionary of biochemistry and molecular biology. *Biochem Educ* 1997;**25**:251.

Yamamoto K, Nakayama A, Yamamoto Y *et al.* Val216 decides the substrate specificity of alpha-glucosidase in *Saccharomyces cerevisiae*. *Eur J Biochem* 2004;**271**:3414–20.

Zelle RM, Trueheart J, Harrison JC *et al.* Phosphoenolpyruvate carboxykinase as the sole anaplerotic enzyme in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 2010;**76**:5383–9.

Zhang F. CRISPR-Cas systems and methods for altering expression of gene products. 2014:US patent no. 8,697,359 B1.

Zhang G, Kong II, Kim H *et al.* Construction of a quadruple auxotrophic mutant of an industrial polyploidy *Saccharomyces cerevisiae* using RNA-guided Cas9 nuclease. *Appl Environ Microbiol* 2014;**80**:7694–701.

Zhao Z, Fang LL, Johnsen R *et al.* ATP-binding cassette protein E is involved in gene transcription and translation in *Caenorhabditis elegans*. *Biochem Biophys Res Commun* 2004;**323**:104–11.

Zhao Z, Thomas JH, Chen N *et al.* Comparative genomics and adaptive selection of the ATP-Binding-Cassette gene family in *Caenorhabditis* species. *Genetics* 2006;**175**:1407–18.

Zhou Y, Grof CP, Patrick JW. Proof of concept for a novel functional screening system for plant sucrose effluxers. *J Biol Methods* 2014;**1**:1–6.

Zhou Y, Qu H, Dibley KE *et al.* A suite of sucrose transporters expressed in coats of developing legume seeds includes novel pH-independent facilitators. *Plant J* 2007;**49**:750–64.

Zimmerman SB, Little JW, Oshinsky CK *et al.* Enzymatic joining of DNA strands: a novel reaction of diphosphopyridine nucleotide. *Proc Natl Acad Sci* 1967;**57**:1841–8.

Acknowledgements

The years I have spent working on my PhD project have been accompanied by various emotions towards my daily work: excitement from obtaining experimental results, relief when one of those stubborn PCRs finally worked, boredom during over-night experiments and the rewarding feeling when presenting for various audiences. Science rarely proceeds as planned and to get to the end of this thesis, frustrating (experimental) hurdles had to be overcome. Looking back at the last four years, no matter how frustrating and disappointing the research, I do not think there has been a single day that I did not have fun while working in the Industrial Microbiology (IMB) group. Although it is impossible to address all the people I have had the pleasure of sharing my time at IMB with, there are a few people in particular that I would like to thank.

First of all I would like to thank Victor and Gabriele, the daily supervisors of my Bachelor and Master thesis. Your scientific enthusiasm has shaped my scientific enthusiasm and made the choice to come back to IMB to pursue a scientific career as a PhD student much easier.

During my PhD, I have enjoyed excellent supervision from Jack, Ton and Jean-Marc. Jean-Marc, your myriad of ideas and exceptional gut-feeling for molecular biology has, on many occasions, helped my project further when everything else failed. I enjoyed our scientific discussions and I appreciate your very approachable nature. Ton, thank you for your excellent guidance and supportive and critical attitude towards my PhD research. From day one you showed confidence in me by letting me pursue various research lines simultaneously, a challenge I very happily accepted. Jack, your calm and reassuring nature has helped ease my mind on more than one occasion, especially during the more difficult times over the last four years. Your enthusiasm whenever I walked into your office has been very motivational. Furthermore, I really enjoyed teaching a course for second year students side-by-side with you. I would also like to thank Pascale for the reassuring and motivational discussions we had throughout my time as a PhD student and for also being vital for making IMB such a great place to work.

I would also like to thank three former office mates. Barbara, your very precise work in the lab and structural approach towards planning experiments have been a big inspiration. Also, thank you for keeping me in check in the office with a water gun when necessary ;-). Niels, thank you for answering a million questions regarding molecular biology, less is definitely more. The pranks back and forth were a welcome distraction from science. Do not think I forgot our bet, which I won (I'm still waiting for the beers). Pauline, thanks a lot for all the fun during and after working hours. I enjoyed exploring the variation of cocktails available at the Luna together with you. Also, thank you very much for being a great listener and your advice during the more challenging times of my PhD.

There is not a single colleague in IMB that did not contribute to my work in one way or another, but there are a few I would like to thank in particular. Harmen, I felt a great synergy while working and writing together on the CRISPR research, which was a lot of fun. With the overlap in our sense of humour, I had a lot of fun discussing science and virtually any other topic with you. Wesley, it has been a pleasure to work together on the sucrose research. Although we sometimes approach science from a different angle, I am very proud of the work we have published together. I'm convinced "Apple juice" and "cold steel balls" will always bring back good memories of our work in the lab. Jasmine, if you ever want to discuss crazy and exciting start-up ideas, you know where to find me! Xavier and Arthur, thank you for the insightful (scientific) discussions. I enjoyed our talks about the group, the people around us and general attitude towards work. At times it was a great relief to vent some frustrations with either one of you. It's a pleasure, honour and slightly scary to have the both of you as my paranymphs. Xavier, I enjoyed my short stay in "the Gazelle-geit" with you and, for the sake of Wijb and Maarten, please keep the word-jokes coming! I like to thank Matthijs, Hannes and Nicolo for being part of the "energetics team" and our fruitful scientific discussions.

Work in the lab where (PhD) students come and go would be chaotic without having the right people to facilitate the (experimental) work. I'm very happy to have worked with extremely talented and helpful staff members. Jannie, Apilena and Astrid, from the moment I first walked into the medium kitchen you made me feel right at home. Your happy and positive attitude and general interest in my wellbeing inside and outside of the lab were very much appreciated. Erik and Marijke, thank you for the help with fermentation and enzymatic experiments. I appreciate how both of you are very easy to approach and almost immediately make time to fix whatever needs fixing. I have yet to encounter a problem with equipment or experimental setups that you are unable to solve. Marcel, thank you for the help with whole-genome sequencing data and being a great neighbour in the office during the last year of my PhD research. Pilar and Susan, both of you joined the group just before the start of my project and I feel very lucky to still have both of you as great colleagues. Thank you Pilar for your positive attitude, all the serious and funny discussions and the impressive way you managed to keep a highly enjoyable working atmosphere in the very busy molecular lab.

I also thank the colleagues from other universities that I had the pleasure of working together with. Bert Poolman, thank you very much for sharing your insights regarding metabolite transport and inviting us over to Groningen on so many occasions. Ryan, it has been a pleasure to regularly discuss science with you and to share a week of experimental work with you in Groningen. I hope the pizza delivery guy is okay ;-)! Matteo, thank you for your efforts regarding metabolite diffusion experiments. I also like to express my gratitude towards Mariel and Mariana from the National University of Rosario in Argentina. It has been a pleasure working together with you on the elucidation of the function of plant malic enzyme, a topic which provided a nice variation to my other research. Although not featured in this thesis, I'm very proud of the work we

have published together. Also big thanks to Andreas in Brazil for sharing your insights regarding the sucrose-work. I'm grateful for the financial support from the BE-Basic R&D program, which was granted a FES subsidy from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I) that enabled the research described in this thesis.

During my PhD I have had the pleasure of supervising many very motivated Bachelor (BEP) and Master (MEP) students, which made invaluable contributions to my PhD thesis presented here. Jonna, I consider myself very lucky to have been able to supervise you as my first student during your BEP. Even when research was not easy, you showed incredible perseverance and I have never seen anyone type a thesis as fast as you did. I'm very happy to see you return to IMB as a PhD student! Alicia, from all the students I have supervised there is no doubt I learned the most about myself during your stay for your MEP. Even when we were making very long hours with our slow-growing strains, it was always a pleasure to share the work with you because of your great sense of humour. Antoon, your BEP work provided us with the basis for all the work with CRISPR/Cas9 and your great independent planning of experimental work made my job as a supervisor very easy and enjoyable. Melanie, with your efficient way of working in the lab, you managed to singlehandedly perform almost all of the experimental work described in chapter 2 of this thesis and made great contributions to chapter 5. I enjoyed our talks at the coffee machine and I am very grateful that you re-joined the group as an excellent technician in molecular biology after your MEP. Hein, it was a pleasure to supervise your BEP and the results you obtained have been the basis for an entire chapter worth of research featured in this thesis (chapter 3). I apologize for making you go after the *Lm*SPase gene via PCR ;-)! Yannick, Mandy and Jordi, your contributions and insights have been of great value for the work on sucrose described in chapter 4 of this thesis. Yannick, I have never seen a BEP student perform a complicated chemostat experiment with the precision and ease as you did. Mandy and Jordi, your advice and insights regarding the expression of PvSUF1 were indispensable. Vicky, your scientific work and the high standard you set for yourself made for a welcome additional challenge as your MEP supervisor. You regarded the manual calculation of growth rates as "feutenwerk" and I would like to thank you for the MATLAB tool you made to automatically calculate these rates. Annabel, thank you for being a great colleague during your MEP and your flexibility to work on three topics (sucrose, CRISPR and lactic acid) simultaneously! Jasmijn, I'm very grateful of having had the pleasure to supervise you as my last MEP student. You were the first (and probably last) student I have encountered that spent the majority of the deadline-day laughing. Your suspiciously sharp awareness of the locations of free food and gossip were greatly appreciated and very useful. I'm happy to have had the chance to welcome you back as fellow PhD in IMB. Roy, thank you very much for your enthusiasm during your work on sucrose and I would have never been able to finish all of the experimental work if you did not volunteer to come back to help out after finishing your MEP. Also, thank you for helping me reflect during my "random moments of apparent brilliance", I have

decided to not put a -80°C freezer in my house.

Special thanks go to Anne, Jolanda, Yvonne, Bert, Emmy, Bas, Xavier, Jeroen and Arthur for carefully proofreading various parts of this PhD thesis. Jolanda, thank you very much for turning my manuscripts into this thesis.

Tot slot wil ik graag mijn vrienden en familie bedanken voor alle steun buiten de kantooruren om. Yvonne en Bert, jullie hebben mijn nieuwsgierigheid voor de wetenschap van jongs af aan aangewakkerd en zonder jullie onvoorwaardelijke steun en motivatie zou ik mezelf nooit genoeg hebben uitgedaagd om het zo ver geschopt hebben. Bas, zo lang ik me kan herinneren, hebben wij elkaar altijd gemotiveerd om te blijven verbeteren. Ik kijk ontzettend op tegen jouw precisie en methodische aanpak voor welk probleem dan ook, dit is een enorme drijfveer geweest om het maximale uit mezelf te halen. Mijn (oud) huisgenoten Mo en Booker, bedankt voor de steun en het begrip tijdens de dagen dat ik nauwelijks thuis was door drukte in het lab. Ik durf me geen voorstelling te maken van de hoeveelheid junkfood dat ik naar binnen zou hebben gewerkt als er niet regelmatig een heerlijke maaltijd voor me klaarstond als ik laat thuis kwam (of zelfs op werk werd langsgebracht!). Anne, Laurike, Enny, Junnes, MJ en Freyr, bedankt voor alle gezellige avonden en de interesse in mijn onderzoek. Jolanda, na een lange dag in de avond thuiskomen en ontspannen met jou hebben een wereld van verschil gemaakt het afgelopen jaar, zeker tijdens stressvolle tijden in het lab. Heel erg bedankt voor alle steun en liefde, ik hou ontzettend veel van je!

Curriculum vitae

Robert Mans was born on May 4, 1989 in Amsterdam, The Netherlands. After following pre-university education (VWO) at the Interconfessionele Scholengroep Westland (ISW) in Naaldwijk and graduating in 2007, he enrolled in the BSc Life Science and Technology programme, shared between Leiden University and the Delft University of Technology (TU Delft). Robert performed his BSc research project at the Industrial Microbiology group at TU Delft, under the supervision of dr. Victor Guadalupe-Medina. In this project he worked on evolutionary engineering of a *Saccharomyces cerevisiae* strain, unable to produce glycerol, for a better osmotolerance. Robert then enrolled in the MSc programme of Life Science and Technology at TU Delft, where he followed the tracks Biocatalysis, Cell Factory and Biochemical Engineering. In his MSc research project in the Industrial Microbiology group, Robert worked under the supervision of dr. Gabriele Romagnoli and dr. Jean-Marc Daran on the elucidation of arginine catabolism in *Kluyveromyces lactis*. As the final part of his MSc study, Robert performed an internship on the re-wiring of central carbon metabolism of industrial yeast strains at Amyris, a California-based biotech company. After obtaining his MSc studies (in 2012, with honours), Robert was elected TU Delft Student of the Year 2012. He subsequently started a PhD project at the Industrial Microbiology group, supervised by prof. dr. ir. Ton van Maris and prof. dr. Jack Pronk. This project, the results of which are described in this thesis, focussed on engineering free-energy conservation in *S. cerevisiae*.

List of publications

1. Guadalupe-Medina V, Metz B, Oud B, van der Graaf C, **Mans R**, Pronk JT, van Maris AJA. Evolutionary engineering of a glycerol-3-phosphate dehydrogenase-negative, acetate-reducing *Saccharomyces cerevisiae* strain enables anaerobic growth at high glucose concentrations. *Microbial Biotechnology* 2014;7:44–53.

2. Romagnoli G, Verhoeven MD, **Mans R**, Fleury Rey Y, Bel-Rhlid R, van den Broek M, Maleki Seifar R, Ten Pierick A, Thompson M, Müller V, Wahl SA, Pronk JT, Daran JMG. An alternative, arginase-independent pathway for arginine metabolism in *Kluyveromyces lactis* involves guanidinobutyrase as a key enzyme. *Molecular Microbiology* 2014;93:369-389.

3. **Mans R**, van Rossum HM, Wijsman M, Backx A, Kuijpers NGA, van den Broek M, Daran-Lapujade P, Pronk JT, van Maris AJA, Daran JMG. CRISPR/Cas9: A molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. *FEMS Yeast Research* 2015;15:1–15.

4. Meadows AL, Hawkins KM, Tsegaye Y, Antipov E, Kim Y, Raetz L, Dahl RH, Tai A, Mahatdejkul-Meadows T, Xu L, Zhao L, Dasika MS, Murarka A, Lenihan J, Eng D, Leng JS, Liu C, Wenger JW, Jiang H, Chao L, Westfall P, Lai J, Ganesan S, Jackson P, **Mans R**, Platt D, Reeves CD, Saija PR, Wichmann G, Holmes VF, Benjamin K, Hill PW, Gardner TS, Tsong AE. Rewriting yeast central carbon metabolism for industrial isoprenoid production. *Nature* 2016;537:694-697

5. Badia MB, **Mans R**, Lis AV, Tronconi MA, Arias CL, Maurino VG, Andreo CS, Drincovich MF, van Maris AJA, Gerrard Wheeler MC. Specific *Arabidopsis thaliana* malic enzyme isoforms can provide anaplerotic pyruvate carboxylation function in *Saccharomyces cerevisiae*. *FEBS Journal* 2017;284:654-665

6. Marques WL, **Mans R**, Marella ER, Cordeiro RL, van den Broek M, Daran JMG, Pronk JT, Gombert AK, van Maris AJA. Elimination of sucrose transport and hydrolysis in *Saccharomyces cerevisiae*: a platform strain for engineering sucrose metabolism. *FEMS Yeast Research* 2017;17:fox006

7. Schumacher R, van Berkum M, **Mans R**, Teusink B, Wahl SA. Droplet based cultivation of *S. cerevisiae* for selection of phenotypes with increased biomass yield. Manuscript in preparation.

8. Marques WL, **Mans R**, Marella ER, ter Horst J, de Hulster E, Daran JMG, Pronk J, Gombert AK, van Maris AJA. Combined engineering of disaccharide transport and phosphorolysis for enhanced ATP yield from sucrose fermentation in *Saccharomyces cerevisiae*. Submitted for publication.

9. **Mans R**, Hassing EJ, Wijsman M, Giezekamp A, Pronk JT, Daran JMG, van Maris AJA. A CRISPR/Cas9-based exploration into the elusive mechanism for lactate export in *Saccharomyces cerevisiae*. Submitted for publication.