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Quantitative analysis of *Saccharomyces cerevisiae*'s growth and metabolism on sucrose

Dissertation

for the purpose of obtaining the degree of doctor

at Delft University of Technology

by the authority of the Rector Magnificus prof.dr.ir. T.H.J.J. van der Hagen,

chair of the Board for Doctorates

to be defended publicly on

Monday 10 May 2021 at 15:00 o'clock

by

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Even before I started college, my mom told me I would become a doctor one day. At that moment and for many years ahead, I did not consider it. Oh well, I guess moms are always right!

Dedicated to my mom, Edna,

Who planted the Ph.D. seed in my mind

and covertly cultivated it.

Errata for Ph.D. dissertation

"Quantitative analysis of *Saccharomyces cerevisiae*'s growth and metabolism on sucrose"

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- Incorrect language used on page ii:

The english equivalent for Fundação de Amparo à Pesquisa do Estado de São Paulo is São Paulo Research Foundation.

- Missing information on page ii:

Where it reads "The project was financed by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo, Brazil)" should read "The project was financed by São Paulo Research Foundation (FAPESP, São Paulo, Brazil; grant numbers 2016/07285-9, 2017/18206-5, and 2017/08464-7) and by the BE-BASIC/BIO-EN program (project F06.006).

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Summary

In recent decades, there has been an increase demand for renewable sources of energy and chemicals in replacement of their fossil-based counterparts to tackle the economic, social, and environmental issues associated with the processing and use of petrochemicals by humanity. Sucrose has proven to be a suitable alternative feedstock to substitute petroleum for the commercial manufacture of not only fuel ethanol but also higher value-added compounds, such as *trans* β-farnesene and polyethylene. And there is a great potential to expand this portfolio. Besides its low market price, sucrose is also advantageous to industrial applications owing to its ready-to-use property that results in reduced overall production costs. Industrial sucrose-based microbial fermentation is feasible to a great extent due to the yeast *Saccharomyces cerevisiae*'s natural ability to metabolize this sugar at high rates. Also, yeast's robustness under harsh industrial conditions, its simple nutritional requirements and the availability of modern genetic tools for the engineering of taylor-made strain has made it an appropriate catalyst in a wide range of bioprocesses. In spite of all this, *S. cerevisiae*'s physiology on sucrose, as well as the regulatory mechanisms triggered by this disaccharide in yeast are still rather under-researched topics.

Our current understanding on sucrose metabolism in *S. cerevisiae*, in particular the knowledge gained using well-controlled cultivation conditions, is extremely limited. The hitherto reported studies focused on laboratory strains or on a particular strain that was pre-adapted to growth on sucrose for several generations, which changed its original physiology. To our knowledge, no published study has investigated several strains simultaneously for their physiology on sucrose, at least not under well-controlled conditions, such as those encountered in a bioreactor. Previous experiments indicated that *S. cerevisiae*'s physiology on sucrose can vary dramatically among different strains. One trait that stood out in the latter study is the maximum specific growth rate on sucrose, which was higher, equal, or lower than the corresponding value on glucose, depending on the strain. Since these previous experiments had been performed under not-so-well-controlled conditions (microtiter plate cultivations with growth monitored by light-scattering-based methods), and due to the importance of many biotechnological processes that employ *S. cerevisiae* and sucrose at their core, the work presented in this thesis aimed at deepening our knowledge on this yeast's physiology on this important disaccharide.

Because determining the maximum specific growth rate (μ_{MAX}) of a microorganism under a defined condition is of utmost relevance in bioprocess development, **chapter 2** illustrates the challenges to be overcome before an appropriate calculation and interpretation of this parameter can be accomplished. The μ_{MAX} of different *S. cerevisiae* strains was calculated for growth on glucose in microplates, shake-flasks, or bioreactors as cultivation systems. The evaluation of several calibration methods showed that the translation of absorbance (Abs) into dry cell mass concentration is rather inaccurate. Statistical analysis exemplified that the errors associated to μ_{MAX} depended on the regression method used, namely either fitting one regression line to data of each independent replicate analyzed all together or fitting one regression line per independent replicate (i.e., data from independent replicates are analyzed separately),

and might alter the statistical outcomes. Moreover, the divergent μ_{MAX} values obtained during cultivations with complex medium compared to defined medium for any individual strain, or during cultivations with the same medium but in different systems endorsed the influence of both medium and system on this growth parameter. Experiments with sucrose or fructose were carried out and the μ_{MAX} values obtained under these conditions were compared to the values on glucose. This comparative analysis showed that the growth capability of a strain on a specific carbon source can be either lower, equivalent, or higher than in another substrate depending on the cultivation system used. This practical example illustrates the implications of carefulness on the interpretation of μ_{MAX}, and is particularly relevant in screening procedures.

C**hapter 3** describes a fundamental study of the quantitative aerobic physiology of the yeast *S. cerevisiae* during growth on sucrose as sole carbon and energy source. The laboratory strain CEN.PK113-7D, the fuel ethanol industrial strain JP1, and the wild isolate UFMG-CM-Y259 were characterized during well-controlled aerobic batch bioreactor cultivations. Quantification of extracellular metabolite concentrations, dry mass, and periplasmic invertase activity allowed for a comprehensive analysis of relevant physiological parameters. The growth capacity on sucrose was different amid the three strains and this was accompanied by distinct extracellular hexose concentration and invertase activity profiles. The lowest maximum specific growth rate on sucrose (μ_{MAX} = 0.21 h^{-1}) was achieved by the CEN.PK113-7D strain, for which the lowest periplasmic invertase activity (0.04 to 0.09 U mg_{DM}^{-1}) and extracellular hexose concentrations were also found. These observations suggest that under the evaluated conditions invertase activity was a constraint for sucrose metabolism in this laboratory strain. Furthermore, cultivations with glucose or fructose alone, or in an equimolar mixture, were performed. These experiments enabled comparative physiological analyses that indicated combined mechanisms of sucrose utilization by the industrial strain JP1, and exposed the ability of the indigenous strain UFMG-CM-Y259 to grow faster on sucrose than on glucose under well-controlled conditions. Besides, for the latter strain, the physiology on fructose was shown to be more similar to that on sucrose than on glucose.

As a follow up of the remarkable observation of higher μ_{MAX} on sucrose than on glucose displayed by the UFMG-CM-Y259 strain, **chapter 4** focuses on comparative quantitative proteomics of these two growth conditions for the same three strains. A label free quantification technique was employed to reveal the changes in protein abundance for the sucrose-glucose pairwise comparisons. ANOVA was applied on the observed differences to test for significance. The abundance of invertase (Suc2p) was shown significantly unchanged in all strains. Besides, no changes in transcription factor (TF) levels were common to the three strains, which indicates that no protein in this category responded to sucrose levels. The protein kinase A (PKA) regulatory subunit, Bcy1p, was up- and down-regulated in CEN.PK113-7D and UFMG-CM-Y259, respectively, suggesting that PKA influences the growth capability on

sucrose. With the JP1 strain, neither the up- nor the down-regulated proteins promoted the overrepresentation of any gene ontology (GO) biological process or the enrichment of any pathway according to the KEGG database. This was attributed to the similar physiology of this strain on sucrose compared with glucose (chapter 3). A reverse pattern of overrepresented GO biological processes and enriched KEGG pathways was shown for the CEN.PK113-7D and UFMG-CM-Y259 strains. A negative correlation between growth rate on sucrose and ribosome enrichment was observed, which corroborates a previous theory on a trade-off between ATP yield per protein mass and the respiratory capacity. According to this theory, higher respiratory capacity demands more ribosomes, and, therefore, lower ATP yield per protein mass is achieved by the cells, which has consequences on $\mu_{MAX.}$

This study demonstrates the potential for improving sucrose-based industrial bioprocesses using *S. cerevisiae* as a cell factory. Additionally, the knowledge acquired with the work reported in this thesis provides the basis for further research on strain improvement.

Samenvatting

Translated by Maxime den Ridder

In de afgelopen decennia is de vraag naar hernieuwbare energiebronnen en chemicaliën ter vervanging van hun fossiele tegenhangers toegenomen om de economische, sociale en milieu kwesties aan te pakken die verband houden met de verwerking en het gebruik van petrochemicaliën door de mensheid. Sucrose blijkt een geschikte alternatieve grondstof om petroleum (gedeeltelijk) te vervangen voor de commerciële productie van niet alleen brandstof ethanol maar ook voor hogere toegevoegde waarde verbindingen, zoals trans β-farneseen en polyethyleen. En er bestaat een grote mogelijkheid om deze portfolio uit te breiden. Naast de lage marktprijs is sucrose ook voordelig voor industriële toepassingen, omdat de totale productiekosten laag blijven door de gebruiksklare eigenschap van deze grondstof. Industriële, op sucrose-gebaseerde microbiële fermentatie is in hoge mate mogelijk dankzij het natuurlijke vermogen van de gist Saccharomyces cerevisiae om deze suiker met hoge snelheden te metaboliseren. Daarnaast is deze gist een geschikte katalysator in een breed scala aan bio-processen door zijn robuustheid onder zware industriële omstandigheden, de eenvoudige voedingsbehoeften en de beschikbaarheid van moderne genetische instrumenten voor de engineering van een op maat gemaakte stam. Desondanks zijn de fysiologie van S. cerevisiae op sucrose, evenals de regulerende mechanismen die door deze disacharide in gist worden geactiveerd, nog steeds vrij weinig onderzochte onderwerpen.

Ons huidige begrip van het sucrosemetabolisme in S. cerevisiae is uiterst beperkt, in het bijzonder de kennis die is opgedaan onder goed gecontroleerde cultivatieomstandigheden. De tot nu toe gerapporteerde onderzoeken waren gericht op laboratoriumstammen of op een bepaalde stam die vooraf was aangepast aan groei op sucrose gedurende verschillende generaties, waardoor de oorspronkelijke fysiologie was veranderd. Voor zover ons bekend, heeft geen enkele gepubliceerde studie verschillende stammen tegelijkertijd onderzocht op hun fysiologie op sucrose, althans niet onder goed gecontroleerde omstandigheden, zoals die worden aangetroffen in een bioreactor. Eerdere experimenten gaven aan dat de fysiologie van S. cerevisiae op sucrose dramatisch kan variëren tussen verschillende stammen. De maximale specifieke groeisnelheid op sucrose was hier opvallend, aangezien deze hoger, gelijk of lager was dan de overeenkomstige waarde voor glucose, afhankelijk van de stam. Maar deze eerdere experimenten waren uitgevoerd onder niet-zo-goed gecontroleerde omstandigheden (cultivaties in microtiter platen met groei gevolgd door methoden gebaseerd op lichtverstrooiing). Daarnaast zijn sucrose en S. cerevisiae de kern ingrediënten in vele biotechnologische processen. Het werk dat in dit proefschrift wordt gepresenteerd is hierdoor gericht op het verdiepen van onze kennis over de fysiologie van deze gist op dit belangrijke disacharide.

Omdat het bepalen van de maximale specifieke groeisnelheid (μ_{MAX}) van een micro-organisme onder een gedefinieerde conditie van het grootste belang is bij de ontwikkeling van bio-processen, illustreert **hoofdstuk 2** de uitdagingen die moeten worden overwonnen voordat een geschikte

berekening en interpretatie van deze parameter kan worden bereikt. De μ_{MAX} van verschillende *S. cerevisiae*-stammen werd berekend voor groei op glucose in microplaten, schudkolven of bioreactoren als cultivatiesystemen. De evaluatie van verschillende kalibratiemethoden toonde aan dat de vertaling van absorptie (Abs) naar droge celmassaconcentratie nogal onnauwkeurig is. Statistische analyse illustreerde dat de fouten geassocieerd met μ_{MAX} afhingen van de gebruikte regressiemethode, namelijk ofwel het passen van één regressielijn aan de gegevens van elk onafhankelijke replica in één gezamenlijke analyse, ofwel het passen van één regressielijn per onafhankelijke replica (d.w.z. gegevens van onafhankelijke replica's werden afzonderlijk geanalyseerd), en kan de statistische uitkomsten beïnvloeden. Bovendien onderschreven de divergerende μ_{MAX}-waarden die werden verkregen tijdens cultivaties met complex medium in vergelijking met gedefinieerd medium voor elke individuele stam, of tijdens cultivaties met hetzelfde medium maar in verschillende cultivatiesystemen, de invloed van zowel medium als systeem op deze groeiparameter. Experimenten met sucrose of fructose werden uitgevoerd en de μ_{MAX}-waarden die onder deze omstandigheden werden verkregen, werden vergeleken met de waarden op glucose. Deze vergelijkende analyse toonde aan dat het groei-vermogen van een stam op een specifieke koolstofbron lager, equivalent of hoger kan zijn dan in een ander substraat, afhankelijk van de gebruikte cultivatiemethode. Dit praktische voorbeeld illustreert de implicaties van zorgvuldigheid bij de interpretatie van μ_{MAX}, en is met name relevant bij screeningprocedures.

Hoofdstuk 3 beschrijft een fundamentele studie van de kwantitatieve aerobe fysiologie van de gist S. cerevisiae tijdens groei op sucrose als enige koolstof- en energiebron. De laboratorium-stam CEN.PK113-7D, de brandstof-ethanol industriële stam JP1 en het wilde isolaat UFMG-CM-Y259 werden gekarakteriseerd tijdens goed gecontroleerde aerobe batch-bioreactor cultivaties. Kwantificering van extracellulaire metabolietconcentraties, droge massa en periplasmatische invertase-activiteit maakte een uitgebreide analyse van relevante fysiologische parameters mogelijk. De groeicapaciteit op sucrose was verschillend tussen de drie stammen en dit ging gepaard met verschillende extracellulaire hexose-concentraties en invertase-activiteitsprofielen. De laagste maximale specifieke groeisnelheid op sucrose (μ_{MAX} = 0.21 h^{-1}) werd behaald door de CEN.PK113-7D stam, waarvoor ook de laagste periplasmatische invertase-activiteit (0.04 tot 0.09 U mg_{DM}^{-1}) en extracellulaire hexose-concentraties werden gevonden. Deze waarnemingen suggereren dat onder de geëvalueerde omstandigheden de invertase-activiteit beperkend was voor het sucrosemetabolisme in deze laboratoriumstam. Daarnaast werden gist cultivaties uitgevoerd met glucose of fructose alleen, of in een equimolair mengsel. Deze experimenten maakten vergelijkende fysiologische analyses mogelijk die duidden op gecombineerde mechanismen van sucrosegebruik door de industriële stam JP1, en lieten het vermogen van de inheemse stam UFMG-CM-Y259 zien om onder goed gecontroleerde omstandigheden sneller te groeien op sucrose dan op glucose. Bovendien bleek voor de laatste stam de fysiologie op fructose meer vergelijkbaar te zijn met die op sucrose dan op glucose.

Als vervolg op de opmerkelijke waarneming van een hogere μ_{MAX} op sucrose dan op glucose, weergegeven door de UFMG-CM-Y259-stam, richt **hoofdstuk 4** zich op vergelijkende kwantitatieve proteomics van deze twee groeiomstandigheden voor dezelfde drie stammen. Een labelvrije kwantificeringstechniek werd gebruikt om de veranderingen in eiwit hoeveelheid te bepalen voor de paarsgewijze vergelijkingen van sucrose en glucose. ANOVA werd toegepast op de waargenomen verschillen om te testen op significantie. De hoeveelheid invertase (Suc2p) bleek significant onveranderd te zijn in alle stammen, wat suggereert dat in JP1 en UFMG-CM-Y259 dit eiwit ook kan worden gecodeerd door een ander SUC-gen dan SUC2. Bovendien waren er geen veranderingen in transcriptiefactor (TF) niveaus gemeenschappelijk voor de drie stammen, wat aangeeft dat geen enkel eiwit in deze categorie reageerde op sucrose niveaus. De regulerende sub-eenheid van proteïnekinase A (PKA), Bcy1p, werd op- en neerwaarts gereguleerd in respectievelijk CEN.PK113-7D en UFMG-CM-Y259, wat suggereert dat PKA het groeivermogen op sucrose beïnvloedt. Voor de JP1-stam, bevorderden noch de op- noch de neerwaarts-gereguleerde eiwitten de oververtegenwoordiging van enig biologisch proces van genontologie (GO) of de verrijking van welke route dan ook volgens de KEGG-database. Dit werd toegeschreven aan de vergelijkbare fysiologie van deze stam op sucrose in vergelijking met glucose (hoofdstuk 3). Een omgekeerd patroon van oververtegenwoordigde biologische GO-processen en verrijkte KEGG-routes werd aangetoond voor de CEN.PK113-7D- en UFMG-CM-Y259-stammen. Een negatieve correlatie tussen groeisnelheid op sucrose en ribosoomverrijking werd waargenomen, wat een eerdere theorie bevestigt over een afweging tussen ATP-opbrengst per eiwitmassa en de respiratoire capaciteit. Volgens deze theorie vereist een hogere respiratoire capaciteit meer ribosomen en daarom wordt een lagere ATP-opbrengst per eiwitmassa bereikt door de cellen, wat gevolgen heeft voor μ_{MAX}.

Deze studie toont de mogelijkheid aan voor het verbeteren van op sucrose-gebaseerde industriële bio-processen door S. cerevisiae als cel fabriek te gebruiken. Bovendien vormt de kennis die is opgedaan met het werk dat in dit proefschrift wordt beschreven, de basis voor verder onderzoek naar stamverbetering.

Resumo

Nas últimas décadas, tem havido um aumento da demanda por fontes renováveis de energia e químicos como alternativa aos materiais petroquímicos, a fim de amenizar os impactos econômicos, sociais e ambientais oriundos do processamento e uso de petroquímicos pela humanidade. A sacarose destaca-se como uma matéria-prima alternativa para substituir o petróleo na manufatura de etanol combustível, bem como de outros compostos de alto valor agregado, como trans-β-farneseno e polietileno. E, há um grande potential para expansão deste portifólio. Além do seu baixo valor de mercado, a sacarose é vantajosa para aplicações industriais pelo benefício de não necessitar de pré-tratamento, o que reduz o custo total do processo de produção. A fermentação industrial microbiana à base de sacarose é possível em grande parte devido à abilidade natural da levedura *Saccharomyces cerevisiae* para metabolizar este açúcar com velocidade alta. Ainda, a robustês desta levedura frente às drásticas condições industriais, requerimentos nutricionais simples e a disponibilidade de ferramentas genéticas para o engenheiramento de linhagens viabilizam o uso deste biocatalizador em uma ampla gama de bioprocessos. Apesar das vantagens mencionadas, a fisiologia da levedura *S. cerevisiae* em sacarose, bem como os mecanismos regulatórios desencadeados por este dissacarídeo, não foram suficientemente estudados pela comunidade científica.

Nosso conhecimento sobre o metabolismo da sacarose em *S. cerevisiae*, em particular sob condições de cultivo bem controlodas, é extremamente limitado. Os estudos já reportados focaram ou em linhagens laboratoriais ou em uma linhagem particular pré-adaptada para crescimento em sacarose por muitas gerações, o que modificou sua fisiologia original. Até onde sabemos, nenhum estudo publicado investigou várias linhagens simultaneamente quanto à fisiologia em sacarose, em condições bem controladas, como as encontradas em um biorreator. Experimentos anteriores indicaram que a fisiologia da *S. cerevisiae* em sacarose pode variar dramaticamente entre diferentes linhagens. Uma característica que se destacou neste estudo prévio é a velocidade máxima específica de crescimento em sacarose, que foi maior, igual ou menor do que o valor correspondente em glicose, dependendo da linhagem. Uma vez que estes experimentos anteriores foram realizados em condições não tão bem controladas (cultivos em microplacas com crescimento monitorado por métodos baseados em espalhamento de luz), e devido à importância de muitos processos biotecnológicos que utilizam *S. cerevisiae* e sacarose, o trabalho apresentado nesta tese objetivou aprofundar nosso conhecimento sobre a fisiologia desta levedura neste importante dissacarídeo.

Uma vez que determinar a velocidade máxima específica de crescimento (μ_{MAX}) de um microrganismo sob uma condição definida é de extrema relevância no desenvolvimento de bioprocessos, o **capítulo 2** ilustra as precauções que devem ser tomadas para que o cálculo e interpretação deste parâmetro possam ser realizados apropriadamente. A μ_{MAX} de diferentes linhagens de *S. cerevisiae* foi calculada para o crescimento em glicose utilizando microplacas, frascos agitados, ou biorreatores como sistemas de cultivo. A avaliação de vários métodos de calibração mostrou que a tradução da absorbância

(Abs) em concentração de massa seca celular é bastante imprecisa. Análises estatísticas exemplificaram que os erros associados ao cálculo da μ_{MAX} dependem do método de regressão usado — ajuste de uma linha de regressão aos dados de cada réplica analisados em conjunto, ou ajuste de uma linha de regressão por réplica (ou seja, os dados de réplicas independentes são analisados separadamente) —, e podem alterar os resultados estatísticos. Além disso, os valores divergentes das μ_{MAX}s obtidos durante os cultivos com meio complexo em comparação com os cultivos com meio definido para qualquer linhagem, ou durante os cultivos com o mesmo meio, mas em sistemas diferentes, endossaram a influência do meio e do sistema neste parâmetro de crescimento. Experimentos com sacarose ou frutose foram realizados e os valores das μ_{MAX}s obtidos nessas condições foram comparados com os valores em glicose. Esta análise comparativa mostrou que a capacidade de crescimento de uma linhagem em uma fonte de carbono específica pode ser menor, equivalente ou maior do que em outro substrato, dependendo do sistema de cultivo utilizado. Este estudo de caso ilustra as implicações da precaução na interpretação da μ_{MAX} e é particularmente relevante em procedimentos de triagem.

O **capítulo 3** descreve um estudo fundamental da fisiologia aeróbia quantitativa da levedura *S. cerevisiae* durante o crescimento em sacarose como fonte única de carbono e energia. A linhagem laboratorial CEN.PK113-7D, a linhagem da indústria de etanol combustível JP1 e a linhagem selvagem UFMG-CM-Y259 foram caracterizadas durante cultivos aeróbios em modo batelada em biorreatores bem controlados. A quantificação das concentrações de metabólitos extracelulares, massa seca e atividade da invertase periplasmática permitiu uma análise abrangente de parâmetros fisiológicos relevantes. A capacidade de crescimento em sacarose foi diferente entre as três linhagens e isso foi acompanhado por perfis distintos de concentração extracelular de hexose e atividade da invertase. A menor velocidade máxima específica de crescimento em sacarose (μ_{MAX} = 0,21 h^{-1}) foi atingida pela linhagem CEN.PK113-7D, para a qual as menores atividades da invertase periplasmática (0,04 a 0,09 U mg_{DM}^{-1}) e as menores concentrações de hexose extracelular também foram observadas. Estas observações sugerem que, nas condições avaliadas, a atividade da invertase restringiu o metabolismo da sacarose nesta linhagem laboratorial. Além dos cultivos em sacarose, foram realizados cultivos apenas com glicose ou frutose, ou com uma mistura equimolar destes monosacarídeos. Esses experimentos permitiram análises fisiológicas comparativas que indicaram mecanismos combinados de utilização de sacarose pela linhagem industrial JP1 e expuseram a capacidade da linhagem selvagem UFMG-CM-Y259 de crescer mais rápido em sacarose do que em glicose em condições bem controladas. Além disso, para esta última linhagem, a fisiologia em frutose se mostrou mais semelhante àquela em sacarose do que em glicose.

Para melhor investigar os mecanismos por trás da maior μ_{MAX} em sacarose do que em glicose exibida pela linhagem UFMG-CM-Y259, o **capítulo 4** aborda um estudo comparativo da proteômica quantitativa entre estas duas condições de crescimento para as mesmas três linhagens. A técnica de

quantificação *label-free* foi empregada para revelar as alterações no perfil proteômico dos cultivos em sacarose comparados aos cultivos em glicose. ANOVA foi aplicada nas diferenças observadas para testar a significância estatística. A expressão da invertase (Suc2p) mostrou-se significativamente inalterada em todas as linhagens. Além disso, nenhuma alteração nos níveis de expressão de algum fator de transcrição (TF) foi comum às três linhagens, o que indica que nenhuma proteína desta categoria tem sua expressão regulada pela sacarose. A subunidade reguladora da proteína quinase A (PKA), Bcy1p, teve sua expressão aumentada e diminuída com as linhagens CEN.PK113-7D e UFMG-CM-Y259, respectivamente, sugerindo que a PKA influencia a capacidade de crescimento em sacarose. Com a linhagem JP1, nem as proteínas cuja expressão foi aumentada nem aquelas cuja expressão foi diminuída promoveram a super-representação de qualquer processo biológico com base na categorização da Ontologia Gene (GO) ou o enriquecimento de qualquer via de acordo com o banco de dados KEGG. Isso foi atribuído à fisiologia semelhante dessa linhagem em sacarose em comparação com a glicose (capítulo 3). Um padrão reverso de processos biológicos super-representados e vias enriquecidas foi observado para as linhagens CEN.PK113-7D e UFMG-CM-Y259. Observou-se ainda uma correlação negativa entre a velocidade de crescimento em sacarose e o enriquecimento de ribossomo, o que corrobora uma teoria anterior sobre um *trade-off* entre o rendimento de ATP por massa de proteína e a capacidade respiratória. Segundo esta teoria, maior capacidade respiratória demanda mais ribossomos e, portanto, menor rendimento de ATP por massa de proteína é alcançado pelas células, o que tem consequências no μ_{MAX}.

Este estudo demonstra o potencial para melhorar os bioprocessos industriais à base de sacarose usando *S. cerevisiae* como *cell factory*. Além disso, o conhecimento adquirido com o trabalho relatado nesta tese fornece a base para novas pesquisas sobre melhoramento de linhagens.

1

General Introduction

Sucrose, the industrial feedstock

Sucrose (α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside) is a disaccharide formed by a glycosidic linkage between the reducing ends of the monomers glucose and fructose, that is available from agricultural crops like sugar cane or sugar beet. Over 110 countries worldwide — from tropical and temperate zones — cultivate these crops [1]. In the 2019/2020 crop year, the global sugar production reached 166.2 million tons [2], of which 18% was produced from sugar cane in Brazil. From the beet sugar side, the European Union production alone accounted for 17.3 million tons during the same crop year [2]. The high and reliable availability accompanied by the low market price of sucrose make this sugar an interesting feedstock for biotechnology, where the economics are highly driven by the raw material costs.

Furthermore, when compared to glucose, sucrose is advantageous because sugarcane, its principal source, is more profitable and environmentally friendly than maize, the main source of glucose [3]–[5]. As an example, in the industrial production of first generation fuel ethanol, nearly 2,000 liters more are produced per hectare from sugarcane as compared to maize, depending on the cultivation conditions ($Y_{ethanol/sugarcane}$ = 6,471 $l.ha^{-1}$, $Y_{ethanol/maize}$ = 4,182 $l.ha^{-1}$; [4]). From the environmental perspective, sugarcane ethanol can reduce life-cycle greenhouse gas (GHG) emissions in a range of 40 to 62%, against 19-10% from corn-based fuel, relative to petroleum gasoline [3]. On the other hand, the ethanol yield from sugar beet has been reported to be 95 $l.ton^{-1}$ [6]. With a productivity of 57.2 $ton.ha^{-1}$ $year^{-1}$ [7], sugar beet ethanol yielded 5,434 $l.ha^{1}$ in 2018, which makes this crop also an attractive alternative to maize. However, due to the high costs and energy demand, sugar beet processing requires extra efforts towards the feasibility of its industrial applications [8], [9].

The production of fuel and other chemicals from sucrose is a promising strategy to meet the policies for reducing the environmental impact from the fossil-based manufactures. In Brazil, the Biofuels National Policy (Renovabio) seeks to expand the contribution of fuel ethanol and biodiesel in 67% and 225%, respectively, in the national fuel matrix by 2029. This comes along with at least 10% decrease in the carbon footprint from this sector [10], [11]. The European Union is currently also facing a reformulation of its energy matrix. The Renewable Energy Directive (RED) asks for renewable energy to make up to 32% of the overall energy consumed in the Union by 2030 [12].

The transition to bio-based processes nowadays expands beyond the food and fuel sectors. Large-volume markets, such as surfactants [13], organic acids [14]–[16] and bio-based polymers [17], [18] illustrate targets for sucrose conversion. Currently commercialized sucrose-based value-added products include I'm green™ Polyethylene (Braskem, Brazil) [19], *trans* β-farnesene (Amyris, USA) [20], and

the protein materials Brewed Protein™ (Spiber, Japan) [21] that can be processed into, for instance, delicate filament fibers for applications in the textile industry.

Sucrose, *Saccharomyces cerevisiae*'s food

The yeast *Saccharomyces cerevisiae* naturally metabolizes sucrose via two pathways [22]–[25]. One begins in the periplasmic space of the cell and comprises the cleavage of sucrose glycosidic linkages by the enzyme invertase (E.C. 3.2.1.2.6) expressed in its glycosylated form. The released monosaccharides, glucose and fructose, are transported into the cytosol via facilitated diffusion, mediated by hexose transporters (Hxtp), and are catabolized by glycolysis (**Figure 1.1A**).

In contrast to the periplasmic cleavage, the second pathway transports sucrose into the cell's cytosol coupled to one proton, via Malx1p, Agt1p and/or Mph2-3p transporters [23], [26] (**Figure 1.1B**). The intracellular invertase (Suc), a maltase (Malx2p) or an isomaltase (Ima1-5p) hydrolyze the disaccharide into glucose and fructose [27]. The proton coupled uptake of sucrose implies in a lower energy yield since the imported proton needs to be expelled to maintain the cell's pH homeostasis [25]. The H^+-ATPases pump the proton to the extracellular space at the expense of ATP, with a 1:1 stoichiometry. Although the extracellular route is predominant in sucrose-grown cultures of wild-type *S. cerevisiae* [22], [25], strains cultivated under glucose and/or fructose repressing levels, as well as strains evolved on sucrose, exhibit active uptake of the disaccharide [23], [28]–[30].

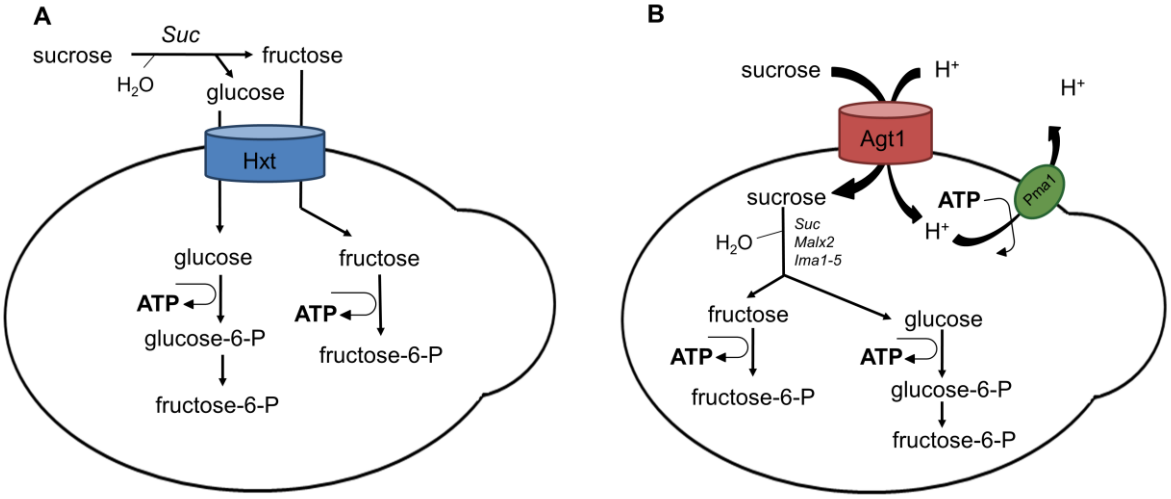

Figure 1.1 Scheme of sucrose metabolism in *Saccharomyces cerevisiae*. A) Extracellular hydrolysis of sucrose followed by hexose transport; B) Sucrose transport and intracellular hydrolysis.

Quantitative physiology of *S. cerevisiae* during growth on sucrose in aerobic and microaerobic batch cultivations

There are many open questions regarding the influence of sucrose on the cellular physiology. Especially, the growth of *S. cerevisiae* on this disaccharide as sole carbon and energy source in aerobic batch mode has not yet been sufficiently researched (**Table 1.1**). Presumably, the most detailed studies made available come from the research group led by Dr. J.P. Barford (Sidney University, Australia). This group mainly focused on the mechanisms of sucrose utilization, providing evidence, through different experiments, for the active uptake of sucrose using the *S. cerevisiae* 248 UNSW 703100 strain pre-adapted for 27 or 250 generations [28], [29], [31], [32]. Recently, the physiology of the Brazilian fuel ethanol strains FT858 and CAT-1 during shake-flask cultivations with sucrose as carbon and energy source was described by the group of Dr. G. Fonseca [33], [34]. Other researchers have limited their study to the quantification of the maximum specific growth rate (μ_{MAX}) [35], [36].

Saccharomyces cerevisiae's *SUC* gene family

The *SUC* gene family of the yeast *Saccharomyces* comprises nine structural genes (*SUC1-SUC5*, *SUC7-SUC10*) located at distinct loci on several chromosomes [38]–[41]. The *SUC* genotype varies from species to species, or even within representatives of the same species [42]. Each *SUC* gene encodes both a glycosylated and a non-glycosylated form of the enzyme invertase [38]. Thus, having a single *SUC+* allele in the genome is sufficient for a strain to carry the ability to metabolize sucrose. However, not all *Saccharomyces* yeasts carry positive alleles in their *SUC* genotype. But rather, naturally occurring negative alleles (suc^0) are present at some or all *SUC* loci in most yeasts from this genus[39].

In *Saccharomyces cerevisiae*, the *SUC2* gene, located at 14 kb from the subtelomeric region of the left arm of chromosome IX, is present in all studied strains of this yeast species [43]. Besides *SUC2*, it has been demonstrated that some industrial strains carry several other *SUC* genes [44].

A single *SUC2* allele originates two different messenger RNA, with 1.9 Kb and 1.8 Kb, that differ from each other only in the transcription start position (5' portion) [39], [45]. The longest mRNA includes a coding sequence for a signal peptide that allows for the secretion and glycosylation of the periplasmic form of invertase. The shortest one, on the other hand, is responsible for the production of the non-glycosylated cytosolic invertase. While the expression of the secreted form of invertase is controlled by glucose levels in the environment (**Figure 1.2**), the intracellular form is constitutively expressed [45].

Table 1.1 Overview of physiological parameters for *Saccharomyces cerevisiae* grown on sucrose in aerobic or microaerobic batch mode.

Strain	**Adaptation [*gen*]**	**Initial sucrose [$g.l^{-1}$]**	**μ_{MAX} [h^{-1}]**	**$Y_{X/S}$ [$g_{Biomass}.g_{sugar}^{-1}$]**	**q_S [$mmol_{GLCeq}.g_{Biomass}^{-1}.h^{-1}$]**	**q_{CO2} [$mmol.g_{Biomass}^{-1}.h^{-1}$]**	**q_{O2} [$mmol.g_{Biomass}^{-1}.h^{-1}$]**	**Reference**
BIOREACTOR								
UNSW 703 100	250	10.0	0.55	0.16	19.0	N.A.	N.A.	[28]
UNSW 703 100	250	12.5	0.50	N.A.	N.A.	N.A.	N.A.	[29]
UNSW 703 100	27	10.0	0.42	0.16	14.7	20.8	4.9	[37]
UNSW 703 100	250	10.0	0.54	0.16	19.0	18.3	4.3	[37]
SHAKE-FLASK								
CBS8066	0	10.0	0.42	N.A.	N.A.	N.A.	N.A.	[35]
BAY.17	0	10.0	0.42	N.A.	N.A.	N.A.	N.A.	[35]
X2180	0	10.0	0.34	N.A.	N.A.	N.A.	N.A.	[35]
CEN.PK122	0	10.0	0.38	N.A.	N.A.	N.A.	N.A.	[35]
FT858	0	10.0	0.43	0.14	N.A.	N.A.	N.A.	[33]
CAT-1	0	10.0	0.45	0.16	N.A.	N.A.	N.A.	[34]
MICROPLATE								
UFMG-CM-Y254	0	20.0	0.50	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y255	0	20.0	0.51	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y256	0	20.0	0.56	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y257	0	20.0	0.57	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y259	0	20.0	0.57	N.A.	N.A.	N.A.	N.A.	[36]

Strain	Adaptation [*gen*]	Initial sucrose [$g.l^{-1}$]	μ_{MAX} [h^{-1}]	$Y_{X/S}$ [$g_{Biomass}.g_{sugar}^{-1}$]	q_S [$mmol_{GLCeq}.g_{Biomass}^{-1}.h^{-1}$]	q_{CO2} [$mmol.g_{Biomass}^{-1}.h^{-1}$]	q_{O2} [$mmol.g_{Biomass}^{-1}.h^{-1}$]	Reference
Microplate								
UFMG-CM-Y260	0	20.0	0.49	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y262	0	20.0	0.49	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y263	0	20.0	0.48	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y264	0	20.0	0.49	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y266	0	20.0	0.53	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y267	0	20.0	0.40	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y455	0	20.0	0.55	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y636	0	20.0	0.42	N.A.	N.A.	N.A.	N.A.	[36]
UFMG-CM-Y643	0	20.0	0.51	N.A.	N.A.	N.A.	N.A.	[36]
CAT-1	0	20.0	0.45	N.A.	N.A.	N.A.	N.A.	[36]
PE-2	0	20.0	0.45	N.A.	N.A.	N.A.	N.A.	[36]
JP1	0	20.0	0.44	N.A.	N.A.	N.A.	N.A.	[36]
CEN.PK113-7D	0	20.0	0.38	N.A.	N.A.	N.A.	N.A.	[36]

N.A means non available.

Regulation of *SUC2* gene in *Saccharomyces cerevisiae*

The precise mechanism controlling the expression resp. repression of sucrose-related genes in the presence of glucose is not yet fully elucidated. Nonetheless, a set of proteins involved in the regulation of *SUC2* has been described (**Figure 1.2**) [46]–[48]. Briefly: when high extracellular glucose concentrations (0.5, 2-3.5 g l^{-1} for the *SUC2* gene[30], [47], [49], depending on the strain) are sensed by the glucose repression-resistant protein (Grr1p, not shown in **Figure 1.2**), the Reg1-Glc7 protein complex dephosphorylates the sucrose non-fermenting 1 kinase protein (Snf1p) complex by acting on Snf4p, resulting in the inactivation of Snf1p complex. Consequently, the transcriptional repressor Mig1p is free to bind a specific sequence in the promoter region of *SUC2*. Moreover, the general transcriptional co-repressor Cyc8p and the repressor Tup1p obstruct the attachment of the RNA polymerase to the DNA and effectively repress the transcription. On the other hand, under low (< 0.1%; [47]) or absent levels of glucose, the Snf1p complex is activated via phosphorylation by either Sak1p, Tos3p or Elm1p kinase. Once activated, this complex, in turn, phosphorylates Mig1p, which causes its migration to the cytosol, cancelling its repressor role.

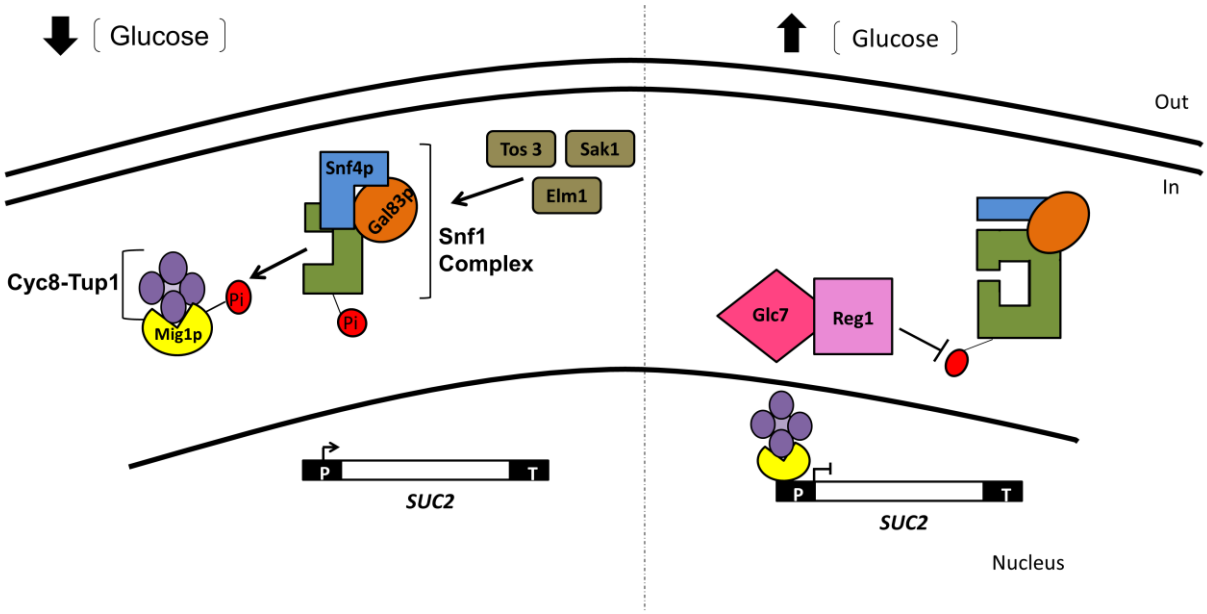

Figure 1.2 Scheme of *SUC2* gene regulation at different glucose concentrations. At high glucose concentration, Mig1p, a central component of the glucose repression mechanism, represses the transcription of the *SUC2* gene. At low glucose, the Snf1 protein complex phosphorylates Mig1p and the repression of *SUC2* is released. The Snf1 complex is activated by the redundant kinases Tos3p, Sak1p and Elm1p, whereas the regulatory subunit Reg1 of protein phosphatase 1 is responsible for the inactivation of this complex.

Regulation of cell growth by carbon sources in *Saccharomyces cerevisiae*

The growth rate of yeast cells is strongly sensitive to the given nutritional environment. As an example, a laboratory diploid strain belonging to the *Saccharomyces cerevisiae* species, named CEN.PK122, capable of aerobically growing on glucose at 1.69 generations per hour undergoes a growth rate decrease of nearly 70% when ethanol is the main carbon and energy source available [35].

Adapting to perturbations w.r.t. the nature and availability of nutrients requires energy and time for transcriptional reprogramming, which is mediated by different regulatory pathways [48], [50]–[54]. In yeasts, nutrients may function as both metabolites and signaling molecules, and understanding the link between these two roles, despite challenging, is essential to successfully unravel nutrient regulation.

Glucose is a preferred substrate and a key signaling molecule that controls the use of other carbon sources, as well as ethanol fermentation, a phenomenon known as glucose repression [55]. As a consequence of this, the yeast *S. cerevisiae* favors the consumption of glucose over any other saccharide or non-fermentable carbon source [55]. This means that in conditions with mixed substrates, the consumption of carbon sources other than glucose will occur upon depletion of the latter.

This preference for glucose is assured by allosteric regulation of several enzymes of the glycolytic and gluconeogenesis pathways, as well as by a complex transcriptional regulatory network responsive to detected levels of glucose in the extracellular environment. Under high levels of glucose, the expression of genes encoding proteins necessary for the metabolism of alternative carbon sources, such as sucrose (as discussed above), galactose, and maltose, are repressed. Genes related to the oxidative metabolism are repressed as well [48], [52], [54]–[56].

Next to the described repression, glucose also regulates cell growth via protein kinase A (PKA). This protein strongly impacts yeast cell growth versus quiescence (**Figure 1.3**) through the regulation of the expression of genes involved in cell mass accumulation (rProtein genes and ribosome biogenesis genes), as well as in stress response (*RIM15, MSN2 and MSN4*) [52]–[54], [57]. Sucrose has also been reported to play a role in activating PKA [58].

In most of the *S. cerevisiae* strains, the main signaling cascade leading to the activation of PKA is mediated by the cyclic adenosine monophosphate (cAMP) molecule and the Ras proteins. Briefly: glucose and/or sucrose transmits a signal to the enzyme adenylyl cyclase (Cyr1p) by a system that involves both the Gpr1 and Gpa2 proteins, as well as the Ras proteins [50], [53], [54], [59]. The latter exerts a more dominant effect on adenylyl cyclase than Gpr1p-Gpa2p, and its activity is stimulated by the presence of fructose-1,6-bisphosphate in the cytosol [57]. The Gpr1p-Gpa2p signalling system, which has higher affinity for sucrose in comparison to glucose, and is insensitive to fructose [58], is assumed to depend on the intracellular activation of Rasp. Adenylyl Cyclase promotes the conversion

of AMP into cAMP, which binds to the regulatory site of protein kinase A, termed Bcy1p. The binding of cAMP into Bcy1p promotes its disconnection from the catalytic site Tpkp, hence activating PKA [50], [53], [54], [59]. Once activated, PKA can hyperphosphorylate the guanine exchange factors Cdc25p and Sdc25p, resulting in their displacement from the plasma membrane to the cytosol and, consequently, reduction of their ability to activate Rasp.

Alternatively to the control exerted by cAMP, PKA activity is also regulated by the Kelch repeat proteins Krh1p and Krh2p [60], [61] and is inhibited by Sch9p, which regulates the localization and phosphorylation of Bcy1p. The Sch9 protein kinase is assumed to act on cell growth in response to glucose levels [53], [54], although the precise regulatory mechanism is still unkown. Furthermore, at least one *S. cerevisiae* strain, CEN.PK113-7D, is unable to produce cAMP. This particular strain presents mutations in the *CYR1* sequence, and, therefore, does not produce adenylyl cyclase [62]. The Krh1/Krh2 coding sequences are also mutated in CEN.PK11307D [62]. This means that the regulation of PKA occurs neither via cAMP nor via the Kelch repeat proteins in this strain. The mechanism by which protein kinase A is activated in CEN.PK113-7D cells remains to be revealed.

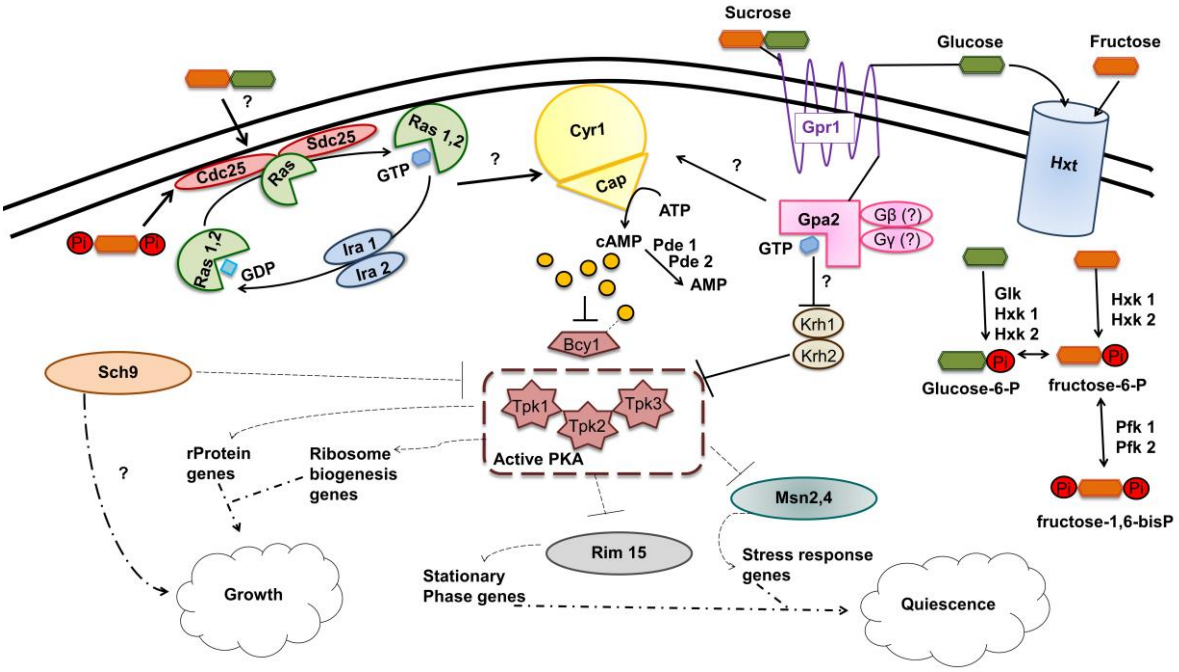

Figure 1.3 Overview of the transcriptional regulatory network sensitive to sucrose, glucose and fructose. Protein kinase A is involved in the regulation of genes related to growth and stress response in the yeast S. cerevisiae. The known mechanisms involved in the regulation of this kinase activity are either mediated by Ras proteins together with the G-protein couple receptor (Gpr1), via the kelch repeat proteins (Krh1p and Krh2p) or via Sch9p. The G-protein coupled receptor responds differently to the presence of sucrose, glucose or fructose in the extracellular environment. Ras proteins are activated by fructose-1,6-biphosphate. "?" indicates the steps in the PKA signaling cascade for which the mechanisms remain to be elucidated. Adapted from [53].

Regulation of cell growth and metabolism via PKA

The metabolic activity of cells supplies the building blocks and Gibbs free energy necessary for growth, leading to a tight coupling between metabolism and growth. Inability to adapt to nutrient transitions will generate growth deficits. In the yeast *S. cerevisiae*, the fluxes through central carbon metabolism have been reported to change significantly depending on the carbon source available [63]. For instance, the flux through pyruvate kinase was observed to be 37.2 ± 0.3 mol.Cmol^{-1}.h^{-1} when the strain CEN.PK113-7D was cultivated on glucose, and 40 ± 0.7, 4.6 ± 0, 4.9 ± 0.1 mol.Cmol^{-1}.h^{-1} when cultivations were performed with maltose, ethanol, or acetate as sole carbon and energy source, respectively. On the other hand, the C2-compounds triggered a higher flux through the enzymes acetyl-CoA synthase (94.5 ± 0.7 mol.Cmol^{-1}.h^{-1} with ethanol; 149 ± 1.5 mol.Cmol^{-1}.h^{-1} with acetate) and aconitase (59.3 ± 0.7 mol.Cmol^{-1}.h^{-1} with ethanol; 114.8 ± 1.4 mol.Cmol^{-1}.h^{-1} with acetate), as compared to the sugars glucose (acetyl-CoA synthase: 6.1 ± 0.1 mol.Cmol^{-1}.h^{-1}; aconitase: 21.6 ± 0.2 mol.Cmol^{-1}.h^{-1}) and maltose (acetyl-CoA synthase: 6.1 ± 0.1; aconitase: 24.5 ± 0.7). Nevertheless, such changes seem to hardly correlate to the expression levels of genes encoding enzymes that catalyzes the synthesis or degradation of the metabolic intermediates involved in the altered fluxes, resp. the changes in fluxes may not be fully attributed to changes in the expression of related genes [63]–[65]. Instead, post-translational modifications as well as allosteric control of specific enzymes are the main factors influencing short-term (seconds-time scale) metabolic regulation of yeast cell growth [66], [67].

Particularly, phosphorylation via protein kinase A has a significant role in regulating several metabolic activities, such as the synthesis and degradation of storage carbohydrates [68]–[70], the metabolic flux through the glycolytic pathway [71], [72], and gluconeogenesis [73].

Neutral trehalase (Nth1p) is activated through phosphorylation by PKA [68]. This kinase also acts on glycogen synthase (Gsy2p) via another kinase, named Pho85p, which is in fact the protein that directly regulates Gsy2p activity [74].

In glycolysis, PKA directly phosphorylates and activates two key enzymes: phosphofructokinase 2 (Pfk2p) [72] and pyruvate kinase 1 (Pyk1p) [71]. Pfk2p is involved in the synthesis of fructose-2,6-biphosphate, which is an allosteric activator of the enzyme phosphofructokinase 1 (Pfk1p), the glycolytic product of which is fructose-1,6-biphosphate (FBP). Following the glycolytic pathway, FBP binds to Pyk1p's allosteric site [75], thus regulating pyruvate production alternatively to the post-translational modification exerted by PKA on Pyk1p. The reactions catalyzed by either Pfk1p or Pyk1p are assumed rate limiting steps in glycolysis [76], [77].

Quantitative shotgun proteomics

Proteins are organic molecules that serve diverse functions in all biological processes in a living system. The overall protein content of a cell, tissue, organism, or a biofluid is referred to as proteome [78], [79]. Due to the dynamic nature of the protein expression regulatory systems, the proteomes are highly temporal and spatial [80], [81]. Thus, a complete characterization of the proteome is a multidimensional task that includes measurements of properties such as compartment-specific localization, post-translational modifications (PTM), interactions, isoform expression and turnover rate [81], [82]. In 1997, Peter James [83] introduced the term "proteomics", which encompasses the group of techniques applied for the identification and quantification of the proteome. Proteomics-based technologies are utilized in several research fields to observe *in-vivo* amount of proteins in a defined condition, aiming to answer a wide range of biological questions, from elucidation of disease mechanisms [84], [85], identification of biomarkers [86]–[88], drug target discovery [89]–[91], to alteration of expression patterns in microbes in response to different stimuli [92]–[94], and many more.

In the specific case of proteome research employing *Saccharomyces cerevisiae* as a model microorganism, the influence of carbon sources on the global proteome has been exploited before [92]–[95]. For instance, Paulo and colleagues, 2015 [93], investigated proteomic alterations in the BY4742 strain resulting from growth on galactose, glucose, or raffinose. These authors concluded that most of the changed proteins were involved in metabolic processes, and that these proteins can be found in plasma membrane and mitochondrion. In a latter study from this same group [94], alterations in protein abundance due to nine different carbon sources, including fermentable (maltose, trehalose, fructose, and sucrose) and non-fermentable (glycerol, acetate, pyruvate, lactic acid, and oleate) were quantified with respect to growth on glucose, again using strain BY4742. In this latter work, Paulo and co-workers reported that the proteomes during cultivations with fructose or sucrose were the most similar to those from cultivations with glucose, amid the investigated carbon sources. Again, major alterations were observed for proteins playing a role in metabolic pathway and mitochondrial proteins. In a recent work, Garcia-Albornoz and colleagues, 2020 [92], addressed the changes in global proteome of the *S. cerevisiae* CEN.PK113-7D cultivated in galactose, maltose, or trehalose, using growth on glucose as standard condition. The significant observations were used to build a carbon-source dependent genetic regulatory network, which demonstrated that stress defense, amino acid synthesis and carbohydrate metabolic process were the main affected pathway and biological functions.

One of the main challenges of proteomics is to enhance the speed, sensitivity and resolution with which proteins can be identified [86], [96]. To this end, bottom-up mass spectrometry-based (MS) proteomics has proven to be useful since it simplifies the analytical challenges by performing the analyses on peptides rather than on proteins themselves [86], [97], [98]. In the bottom-up approach, at

first, proteins undergo a proteolytic digestion, for instance using the enzyme trypsin, and then the resulting peptides are identified by a MS-based methodology (**Figure 1.4**). Tandem mass spectrometry (MS/MS) strategy, also referred to as shotgun proteomics, is particularly interesting for the identification process because it does not require protein separation prior to digestion, therefore enabling the identification of proteins components from a mixture [86], [97], [98]. MS/MS is usually coupled to high pressure liquid chromatography (LC-MS/MS) to separate peptide mixtures prior to the ionization process, thus minimizing precursor interference. The intact ionized peptides are then analyzed in the MS1 analysis. The MS1 spectrum relates the mass-to-charge ratio (m/z) and signal intensities of all peptide ions. Next in the shotgun proteomics workflow, the peptides are fragmented, and the product ions are analyzed in the second MS to acquire the tandem mass spectrum. The MS/MS spectrum is then compared against theoretical spectra of a database to identify matching peptides sequences that are reassembled to infer the proteins. Proteins are identified based on unique peptides.

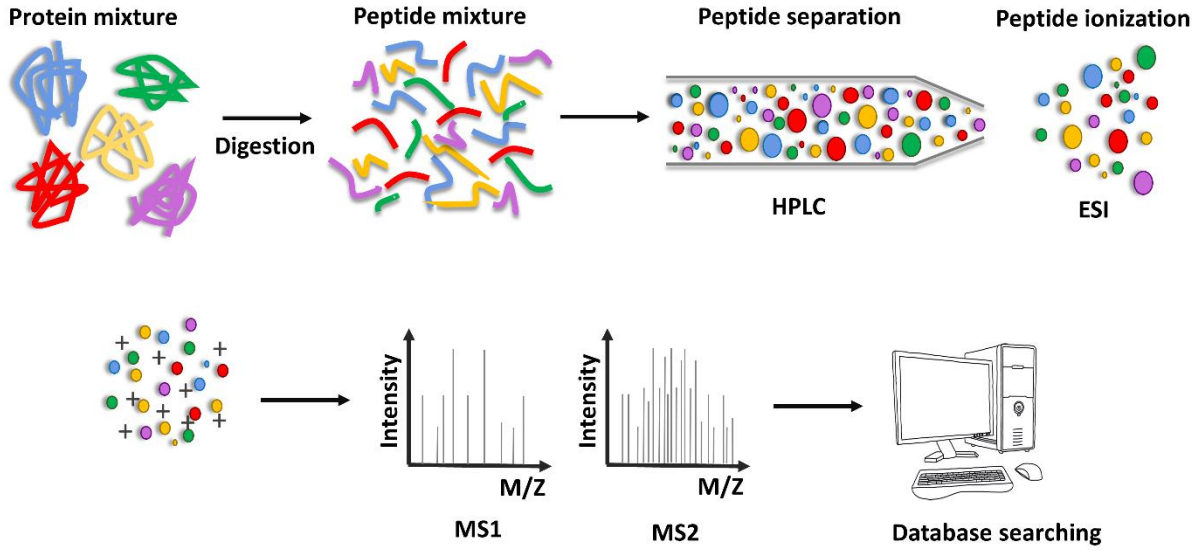

Figure 1.3 Schematic representation of the shotgun proteomics workflow.

There are several methodologies for the relative or absolute quantification of the abundances of the identified proteins [81], [82]. Particularly, the label free quantification (LFQ) is a cost-effective approach for measuring relative abundances, and requires simplified sample preparation [99], [100]. LFQ can be performed either by integrating the area under the curve (AUC) of chromatographic peaks for any given peptide in the LC-MS runs, or via counting the number of peptides assigned to a protein after tandem mass spectrometry analysis (spectrum counting) [99], [100]. A direct comparison between analyses of the tested conditions gives the fold change in protein abundance.

Aim and outline of this thesis

The aim of this thesis was to investigate the physiology of *S. cerevisiae* during growth on sucrose to generate a better understanding and enable improving sucrose-based bioprocesses. Therefore, quantitative approaches were applied, comparing different strains and environmental conditions. Especially, growth and metabolism under sucrose as sole carbon and energy source, was compared to related substrates glucose, fructose or an equimolar mixture of these hexoses. The systems biology approach also included quantitative proteomics to unravel intracellular regulation and expression of proteins under different conditions. The proteomic data was correlated with data obtained from the physiological study, namely the maximum specific growth rate, substrate uptake, and respiration capacity.

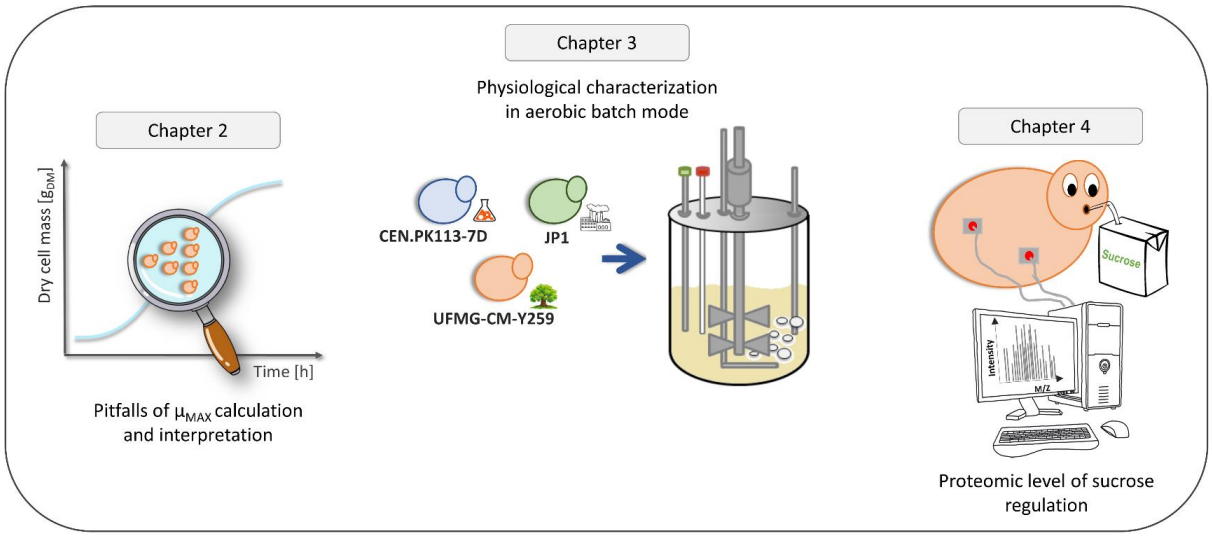

Figure 1.4 Schematic representation of the main chapters of this thesis.

The maximum specific growth rate (μ_{MAX}) of a microbe in a desired environment is presumably the most relevant physiological parameter for both fundamental and applied research. To proper determine and interpret this parameter, however, can be a very challenging task. In **Chapter 2**, a series of these challenges is discussed with emphasis on calibration methodology, statistical analysis, culture media, and cultivation system.

Chapter 3 presents a detailed study of the aerobic physiology of three *Saccharomyces cerevisiae* strains, originally from different environments, during batch growth on sucrose. The strain-specific peculiarities of sucrose metabolism are revealed, and insights on the mode of sucrose utilization and regulation are gained by a comparative analysis with the physiology on the related carbon sources glucose, fructose, and an equimolar mixture of these two monosaccharides.

The global proteome of each one of the three yeast strains characterized during growth on sucrose was then compared to that on glucose in **Chapter 4**, by applying the label free quantification technique. Identification of enhanced biological processes and pathways was performed, aiming at getting insights on the mechanisms underlying rapid or slow growth on the disaccharide.

Finally, the key contributions of this work, along with open questions for future work, are addressed in the outlook section.

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2

μ_{MAX} of *Saccharomyces cerevisiae*: so often used, so seldom put into perspective

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ABSTRACT

The maximum specific growth rate of a microbe in a given growth condition is of primary relevance for bioprocess development. In the case of the unicellular yeast *Saccharomyces cerevisiae*, this physiological parameter is routinely calculated in (almost) every laboratory, but this procedure conceals several challenges that are often neglected in scientific works, which might lead to misinterpretation of the reported data and of phenomena. We present here several pitfalls involved in μ_{MAX} calculation and interpretation, which was achieved through comparative analyses of: 1) the use of different methodologies for determining cell mass concentration, 2) different calibration procedures to correlate indirect (e.g. absorbance) to direct (e.g. dry cell mass) cell mass concentration measurements, 3) different statistical methods for determining the significance of μ_{MAX} differences, 4) the influence of culture media composition, and 5) the influence of the cultivation system (e.g. microplate, shake-flask or bioreactor). It becomes clear that each of these factors has a great influence on μ_{MAX} calculation and interpretation. We also present a case study involving five yeast strains and three different carbon sources, illustrating that opposite conclusions can be drawn in a screening procedure, if proper caution is not taken during data generation and analysis. Last but not least, we conclude this work with a series of recommendations that we believe could make experimental planning, data generation, μ_{MAX} calculation and interpretation more meaningful and scientifically sound, contributing to the improvement of yeast research and of microbiology in general.

INTRODUCTION

The growth of a microbial population is the increase in cell number or overall mass in the population [1]. Determining the rate at which a microbial population grows is one of the main interests of the fundamental microbiologist, as well as presumably the most important piece of information in an industrial bioprocess design. This aspect is captured in a parameter referred to as the specific growth rate, most commonly represented by the Greek letter μ [2]–[5]. Cell growth is an autocatalytic reaction, meaning that the catalyst itself is a product of the reaction [3]. Hence, the cell specific growth rate, rather than a simple growth rate, is the most appropriate parameter to describe microbial growth. For a microbial population growing in batch cultivations, the specific growth rate is defined as,

$$\mu = \frac{1}{X} * \frac{dX}{dt} \qquad \mathbf{1}$$

where X = cell mass concentration (e.g. in dry cell mass/volume) and t is the reaction time (e.g. in hours).

In terms of cell number concentration (N) (e.g. in cells/volume), microbial growth is mathematically expressed as

$$\mu_R = \frac{1}{N} * \frac{dN}{dt} \qquad \mathbf{2}$$

where μ_R is the specific replication rate [1].

From equation **1**, it can be observed that μ is similar to the kinetic constant of a 1st-order chemical reaction and has dimensions of time^{-1}. Other formulations for rates, such as total or volumetric rates, are scale-dependent and do not directly reflect catalyst performance.

Instead of μ, some professionals prefer to use the doubling (or generation) time (t_G) to quantify the rate of microbial growth. t_G is the time required for the microbial population to double its size , and is expressed as:

$$t_G = \frac{t}{\log \frac{N}{N_0}} * ln2 \qquad \mathbf{3}$$

where N is the final cell number concentration and N_0 is the initial cell number concentration in a given time t. t_G can also be expressed in terms of X, thus characterizing the doubling time of cell mass.

μ and t_G are intrinsically related by the following equation:

$$\mu = \frac{\ln 2}{t_G} \quad \textbf{4}$$

Because most studies in the context of bioprocess engineering report microbial specific growth rates considering μ rather than t_G, we will here only use μ for all our analyses and discussions.

μ cannot be directly measured. Nevertheless, measurements of cell concentration (X or N) at a minimum of two time points allow for the estimation/calculation of this parameter.

There are several methods to quantitate cell concentration, including direct cell count, dry cell mass, particle count and colony forming units, among other direct off-line methods [6]. Amid the direct methods, measurements of cell mass instead of cell number is the most frequent choice for physiology studies employing budding yeast at standard growth conditions (e.g. non-stressful conditions, no inductions, solids-free medium). Hence, we carried out our analysis based on cell mass concentration (X).

Cell concentration is also usually assessed by light-scattering measurements, such as those performed with the use of a spectrophotometer, a ubiquitous laboratory piece of equipment. Other terms used to designate this type of measurement are optical density (OD), turbidity, and absorbance. However, the results of such an indirect analysis need to be calibrated against a direct method, and this requires some caution. Calibration should be performed under a particular condition and applied to this circumstance only. Otherwise, the correlation could be compromised. Even analyses performed with cells from a single cultivation but collected at different growth phases represent a source of error due to inadequate calibration [7]. The possibly different cell morphologies in each growth phase affect deviation of light and compromise the translation of the indirectly assessed cell concentrations into real cell concentrations.

Further options for indirect determination of cell concentration rely on the measurement of a cell component, for instance protein [8] or nucleic acids [9]. In this case, calibration against a direct method such as dry cell mass is also necessary and, as discussed above, care should be taken in the sense that cell composition during growth might differ from the one employed during the calibration procedure. Furthermore, although online methods centered on turbidity, permittivity [10], or fluorescence can as well be used to assess cell concentration, as yet they have not fully substituted the above mentioned off-line methods, which require sampling, in many laboratories' workflow. One exception is the application of online monitoring of cell growth in high throughput systems, such as microplate [11]–[13].

The exponential growth phase (EGP) occurs very often both in research and in applied cases. During the EGP, cells encounter neither any nutrient limitation nor any inhibition. The population then grows

at the maximum possible rate (the maximum specific growth rate, μ_{MAX}) under the applied conditions, until one nutrient becomes growth-limiting or some compound achieves inhibitory concentrations.

Fermentation Technology and/or Bioprocess Engineering textbooks usually do not provide a discussion on how cell concentration measurements affect the calculation of the specific growth rate. Stanbury and coworkers [5], for instance, presented the specific growth rate without any connection to cell concentration determination methods. In one exception, Clarke [2] points out that "μ_{MAX} can vary significantly depending on the method used to measure the cell concentration".

There are basically two different approaches to calculate μ_{MAX} from cell concentration measurements. One of them is based on a first adjustment of a growth model to data from an entire batch cultivation, including all growth phases (lag, log, de-acceleration and stationary). Frequently used models include the logistic model, the Gompertz and the Richards models, among others [14]. The second method consists of the integration of equations **1** or **2** under the assumption that in the EGP μ is constant and equal to μ_{MAX}. While early researchers used a $\log_2$ or $\log_{10}$ transformation to linearize this equation [2], nowadays, the use of the natural logarithm is common practice:

$$lnX = lnX_0 + \mu_{MAX} * t \qquad \mathbf{5}$$

where X_0 = cell mass concentration at the beginning of the EGP, corresponding to t = 0.

This transformation allows us to calculate μ_{MAX} by plotting ln(X) values along time and taking the slope of the linear region as μ_{MAX}. This procedure also results in the identification of the duration of the EGP. Due to the use of the natural logarithm, μ_{MAX} represents the number of "*e*-fold" generations in a given time point t, or the exponential increase of biomass by a factor of *e* [15]. We will restrict our analysis and discussion here to this approach, because it is by far the most frequently employed in the context of yeast research.

μ_{MAX} is also a key parameter in kinetic models used in biological research and in bioprocess development. In its simplest form, it appears in the Monod equation that relates μ_{MAX} to the limiting substrate concentration S:

$$\mu = \mu_{MAX} * \frac{S}{S + K_S} \qquad \mathbf{6}$$

μ_{MAX} has also been termed the Malthusian parameter and used as a proxy for fitness by part of the scientific community, mainly those involved in population genetics or experimental evolution studies [16].

For the sake of completeness, it should be mentioned that there are methods to calculate μ_{MAX} using continuous cultivation data [17] and methods that take substrate and product concentrations into account [18], [19]. We will not discuss them here.

Finally, it is important to mention that not only the analytical method used to determine cell concentration influences μ_{MAX} calculations, but also other factors such as the cultivation system. Potvin et al [20] compared μ_{MAX} values obtained for *Lactobacillus plantarum* cells grown in an automated plate reader, in shake-flasks and in a bioreactor. Bioreactor cultivations led to higher μ_{MAX} values as compared to shake-flask cultivations, which the authors attributed to external pH control in bioreactors. These authors also showed that the μ_{MAX} calculated from direct absorbance measurements in an automated plate reader, without sample dilution, differed from the values obtained with samples from shake-flask cultivations that were diluted prior to the absorbance measurements. Although these observations seem obvious, this matter has only been given proper attention in few published works.

In the only report we identified involving yeast, Stevenson and co-workers [7] evaluated the relationship between optical density and cell counts both in *Escherichia coli* and *Saccharomyces cerevisiae* cultures with respect to particle size and shape, refractive index, spectrophotometer model, cell growth phase, among others. The authors concluded that the cell size effect on the calibration between OD and cell counts was stronger in bacteria than in yeast. This is because the size of the bacterial cells is closer to the wavelength of light (600 nm) used in the OD measurements. In this sense, the bigger size of yeast cells makes them more suitable than bacteria for the application of light scattering techniques at 600 nm or similar wavelengths. Moreover, they demonstrated that the difference between the refractive index of the medium and that of the cells influences the calibration curve. This has implications for yeast research, since sugars commonly used in yeast media, such as sucrose, change the refractive index of the medium significantly [7], [21].

This context motivated us to investigate how different cell concentration determination methods, statistical analyses, cultivation systems, and also culture media influence μ_{MAX} calculations during yeast cultivations performed with different strains, including wild isolates, laboratory and industrial ones.

MATERIAL AND METHODS

Yeast strains and preservation

Eight *S. cerevisiae* strains from indigenous, industrial or laboratory origin were used in this work (**Table 2.1**). Stock cultures were prepared by growing cells until stationary phase in 500-ml Erlenmeyer flasks containing 100 ml YPD (1% yeast extract, 2% peptone, and 2% glucose) medium. 20% (v/v, final

concentration) sterile glycerol was added and 1-ml aliquots were stored in 2-ml cryogenic vials in an ultra-freezer (ColdLab, Piracicaba, Brazil) at -80 °C until further use.

Cultivation media

Yeast cultivations were carried out using either a defined medium [22], the composition of which altered depending on the cultivation system (**Table 2.2**), or a complex medium (YPD). Microplate cultivations were performed using both media, whilst shake-flask and bioreactor cultivations were restricted to the defined medium. When needed, urea was used as the sole nitrogen source in replacement for ammonium sulphate, to avoid drastic changes in the broth's pH caused by proton release during ammonium consumption. Glucose was added as carbon and energy source to all cultivation media, unless otherwise stated. Each medium was sterilised either by autoclaving some of its components at 121 °C for 20 min or by filtration through 0.22-µm pore membranes. Carbon sources, vitamin and trace element solutions were always filter-sterilised to avoid Maillard reactions or thermal decomposition of the components.

Cultivations

Microplate cultivations

All eight strains were cultivated in 96-well microplates (CELLSTAR® flat bottom, mfr. No. 655161 - Greiner bio-one, Kremsmunster, Austria) using the plate reader Tecan Infinite M200 Pro. Initially, cells from the -80 °C stock were streaked onto solid YPD medium (with 2% agar) and incubated at 30 °C (502 Incubator, FANEM, São Paulo, Brazil) for 48 h. Cells from a single colony were then transferred to a 50-ml centrifuge tube filled with 3 ml of either a defined medium, which pH was adjusted to 6.0 by addition of 2 M KOH, or a complex medium, constituting the inoculum. The inoculum was placed in a shaker incubator (Innova 4430, New Brunswick Scientific, Edison, USA) operating at 200 rpm and 30 °C for 24 h. An aliquot of its content, enough to make 1 ml of a cell suspension with absorbance at 600 nm equal to 1, was then collected. The aliquot was centrifuged at 974 *g* for 5 min (MIKRO200 centrifuge, Hettich, Tuttlingen, Germany), the supernatant discarded and the pellet washed with 1 ml of fresh culture medium. This washing procedure was performed twice. Next, 10 µl of the cell suspension was transferred to one 234-µl well (working volume 20-200 µl) of a microplate that had already been filled with 90 µl of the same fresh culture medium used for inoculum growth. A desired number of wells were also filled with 100 µl of fresh culture medium only (blank). Once all the desired wells were filled with both medium and cell suspension, the microplate was sealed with PCR sealing film (AMPLlSeal™ - Greiner bio-one, Kremsmunster, Austria). The cultivation was carried out in quintuplicate (5 wells on the same plate) at 30 °C with orbital agitation amplitude of 3.5 mm and frequency of 198.4 rpm. Absorbance at 600 nm wavelength and 9 nm bandwidth was online measured every 15 min during 24

h. The measured absorbance values from wells containing culture broth were corrected by subtracting the average absorbance measured from wells containing the blank, as to take into account possible background absorption by components in the cultivation medium.

Table 2.1 Yeast strains used in this work.

Strain designation	Group	Ploidy	Precedence	References
CEN.PK113-7D	Laboratory	n	Dr. Peter Kötter (University of Frankfurt, Germany)	[23]
Fleischmann	Industrial (baking)	2n	Dr. L. C. Basso (USP, Brazil)	[24]
PE-2	Industrial (fuel ethanol)	2n	Dr. L. C. Basso (USP, Brazil)	[25]
CAT-1	Industrial (fuel ethanol)	2n	Dr. L. C. Basso (USP, Brazil)	[25]
JP1	Industrial (fuel ethanol)	2n	Dr. M. A. de Morais Jr (UFPE, Brazil)	[26]
UFMG-CM-Y257	Indigenous[1]	2n	Dr. C. A. Rosa (UFMG, Brazil)	[27]
UFMG-CM-Y259	Indigenous[1]	2n	Dr. C. A. Rosa (UFMG, Brazil)	[27]
UFMG-CM-Y267	Indigenous[2]	2n	Dr. C. A. Rosa (UFMG, Brazil)	[27]

[1]Originally from barks of *Quercus rubra*, located within the Brazilian Atlantic Forest biome.
[2]Originally from barks of *Tapira guaianenses*, located within the Brazilian Cerrado biome.

Table 2.2 Composition of the cultivation media used in this work.

Cultivation Medium	Components	Composition ($g\ l^{-1}$)	Cultivation System
Complex (YPD)	Yeast Extract	10.0	Microplate
	Peptone	20.0	
	Glucose	10.0	
Defined Adapted from [22]	K_2SO_4	6.6	Microplate
	CH_4N_2O	2.3	
	KH_2PO_4	3.3	
	$MgSO_4.7H_2O$	0.5	
	Trace Elements solution[1]	1.0	Shake-flask
	Vitamins solution[2]	1.0	
	Glucose, fructose or sucrose	10.0[3]	
Defined [22]	$(NH_4)_2SO_4$	5.0	Bioreactor
	KH_2PO_4	3.0	
	$MgSO_4.7H_2O$	0.5	
	Trace Elements solution	1.0	
	Vitamins solution	1.0	
	Glucose, fructose or sucrose	20.0[3]	

[1] Composition of trace elements solution (per litre):15 mg EDTA, 4.5 mg $ZnSO_4.7H_2O$, 1 mg $MnCl_2.4H_2O$, 0.30 mg $CoCl_2.6H_20$, 0.30 mg $CuSO_4.5H_2O$, 0.40 mg $Na_2MoO_4.2H_2O$, 4.5 mg $CaCl_2.2H_2O$, 3.0 mg $FeSO_4.7H_2O$, 1.0 mg H_3BO_3, 0.1 mg KI.

[2] Composition of vitamins solution (per litre): 0.05 mg biotin, 1 mg calcium pantothenate, 1 mg nicotinic acid, 25 mg inositol, 1 mg thiamine HCl, 1 mg pyridoxine HCl, and 0.2 mg para-aminobenzoic acid.

[3]In the case of cultivations with sucrose, 10 or 20 $g_{GLGequivalent}\ l^{-1}$ was used.

Shake-Flask cultivations

Shake-flask cultivations were performed with strains CEN.PK113-7D, PE-2, JP1, UFMG-CM-Y257, and UFMG-CM-Y259. First, an inoculum was prepared by transferring cells from one colony of each of the five strains into 500-ml baffled Erlenmeyer flasks containing 100 ml synthetic medium. The pH of the synthetic medium was adjusted to 6.0 upon preparation by addition of 2 M KOH. The inoculum was incubated in a shaker (Innova 4430, New Brunswick Scientific, Edison, USA) at 30 °C and 200 rpm for 24 h. Then, sufficient inoculum to begin the cultivation with an absorbance at 600 nm of 0.2 was centrifuged at 2153 *g* for 5 min (NT810 centrifuge, Nova Técnica, Piracicaba, Brazil). The supernatant was discarded, cells were washed twice and the cell pellet was resuspended in 1 ml synthetic

medium. This cell suspension was transferred to another 500-ml Erlenmeyer flask containing 100 ml fresh synthetic medium.

Samples of the cultivation broth were collected hourly and their absorbance at 600 nm measured in a spectrophotometer (Genesys 20, Thermo Fisher Scientific, Massachussets, USA), with cultivation medium used as blank. Whenever the absorbance of a sample was read above 0.3, sample dilutions were performed to ensure the measured absorbance would fall into the region of proportionality between cell mass concentration and absorbance. Sample pH was read using a pHmeter (DM21, Digimed, São Paulo, Brazil). The cultivations were stopped when the cells reached the stationary phase of growth, which was indicated by both constant OD measurements and an increase in pH.

Bioreactor batch cultivations

To prepare the inoculum for bioreactor cultivation, the content of one cryogenic vial was transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium, which was prepared as described for shake-flask cultivations. The pH of this pre-inoculum medium was adjusted to 6.0 by addition of 2 M KOH. Cells were propagated at 30 °C in a shaker (Certomat BS-1, Braun Biotech International, Berlin, Germany) under stirring speed of 200 rpm. After 24 h, 1 ml of the pre-inoculum was directly transferred to another shake-flask filled with fresh inoculum medium. Following a second round of growth in a shaker, an aliquot sufficient to start the batch cultivation with an absorbance of 0.2 at 600 nm was collected, centrifuged at 3500 *g* for 3 min, and the pellet resuspended in fresh cultivation medium. Afterwards, the cell suspension was transferred to a 2-l bioreactor (Applikon Biotechnology B.V., Delft, The Netherlands), making up an initial working volume of 1.2 l.

Cells were cultivated at 30 °C and 800 rpm until a decrease in the CO_2 molar fraction in the off-gas was observed. Aeration in the bioreactor occurred with compressed air at 0.5 l min^{-1} flow rate injected through a mass flow controller (Model 58505, Brooks Instrument B.V., Hatfield, USA). The pH of the medium was adjusted to 5.0 and kept constant by automatic addition of 0.5 M KOH solution. Whenever needed, a 10% (v/v) antifoam C emulsion (Sigma-Aldrich, Missouri, USA) was added manually to the broth. Samples of the broth were withdrawn approximately every hour to have their dry cell mass and absorbance measured. Absorbance was measured at 600 nm in a spectrophotometer (LibraS11, Biochrom, Cambridge, United Kingdom). Dry cell mass was determined according to [28], with a minor modification. Briefly, a desired volume of culture broth was collected, vortexed, and filtered through a 0.45 µm nitrocellulose membrane (SO-Pak filters, HAWP047S0 – Merck Millipore, Massachusetts, USA) that had been previously dried in an oven at 70 °C for 48 h and weighed (m_1). The cell pellet was washed twice with demineralized water. The filter containing the pellet was dried (70 °C for 48 h) then placed in a desiccator to cool down prior to being weighed (m_2). The dry cell mass (DCM) was calculated by subtracting the difference between the filter's mass after and before filtration. Cell mass

concentration was then calculated by dividing the dry cell mass by the sample volume filtered (V); $X = (m_2 – m_1)/V$. The result was expressed in $g_{DM}\ l^{-1}$.

Identification of the EGP

To identify the exponential growth phase (EGP), the natural logarithm of cell concentration values — that were determined either by means of measuring dry cell mass (bioreactor cultivations) or Abs_{600} (shake-flask and microplates) — were plotted against time. The time span corresponding to the linear region of this plot, which was visually identified, was considered to be the exponential growth phase.

Calculation of the maximum specific growth rates and statistical comparisons

The maximum specific growth rate (μ_{MAX}) corresponds to the slope of the linear regression fitted to the cell concentration data within the EGP (**Equation 5**). We applied two approaches, namely Method A and Method B, to perform the least-squares regression method in replicate experiments and, therefore, calculate the μ_{MAX} for each investigated condition.

In the first approach, using Microsoft Excel 365 (Redmond, USA), data from independent replicates were analyzed separately, each one yielding a μ_{MAX} value of its own fitted by the least-squares regression method. The average and the standard deviation of these μ_{MAX} values were then calculated **(Figure 2.1, Method A**). Significant changes in μ_{MAX} were evaluated using t-tests with 95% and 99% confidence levels.

In the second approach, using GraphPad Prism 8 (San Diego, USA), data from independent replicates of each experiment were analyzed together, generating one single μ_{MAX} value from one regression line also fitted by the least-squares method. This procedure generated the standard error of the slope (**Figure 2.1, Method B**). Significant changes in μ_{MAX} were evaluated using F-tests with 95% and 99% confidence levels.

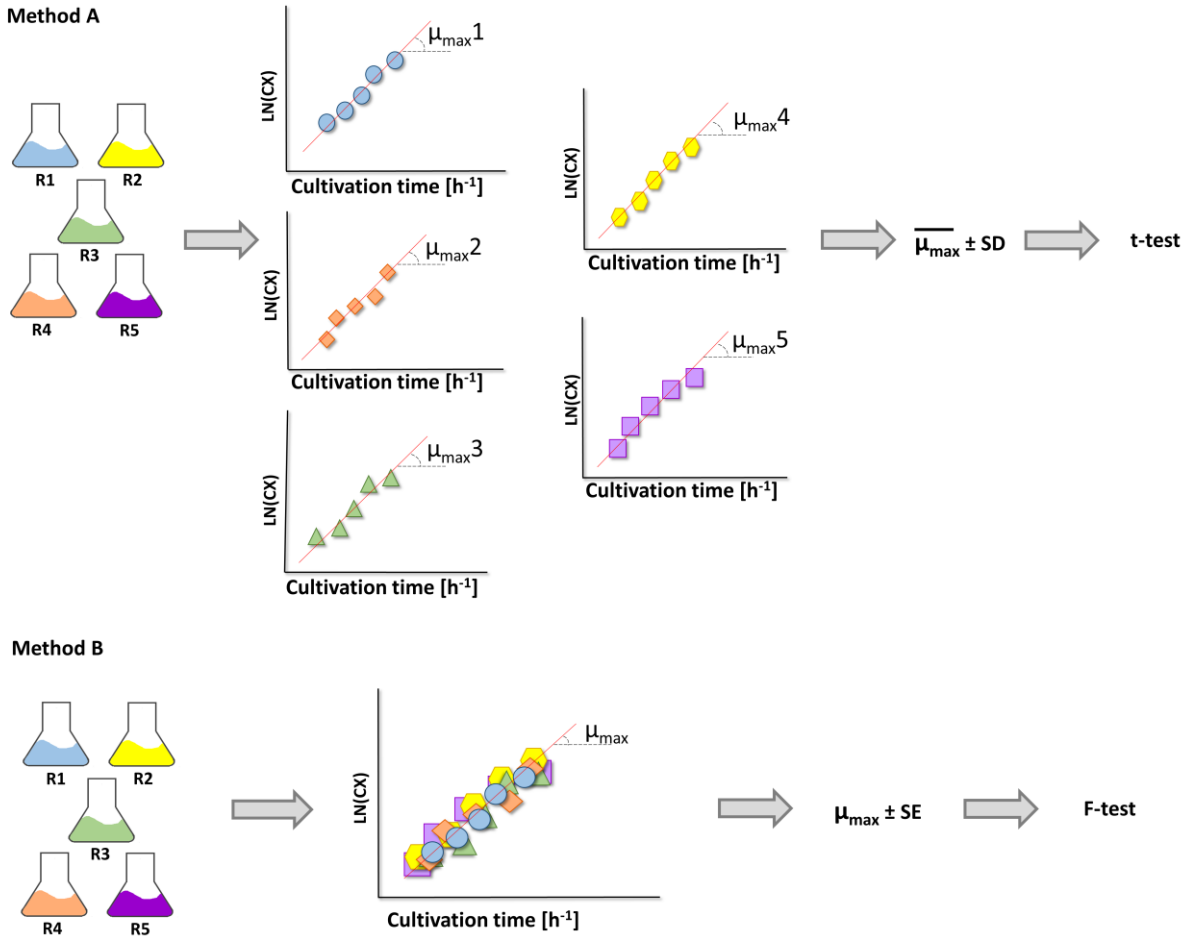

Figure 2.1 Methods used for calculating and comparing the slope of regression lines (μ_{MAX}). Method A yields an average μ_{MAX} and a standard deviation while Method B yields a unique μ_{max} and a standard error.

RESULTS AND DISCUSSION

Calculated μ_{MAX} values depend on the cell concentration determination and on the calibration with a direct method

In spite of being an indirect method for the determination of cell concentration, Absorbance (Abs) measurements are commonly used during yeast cultivations. Researchers frequently use these measurements to directly calculate μ_{MAX} by plotting ln(Abs) values against time, identifying the EGP as the linear region, performing a linear regression with the corresponding data and taking the slope as μ_{MAX}. In some cases, researchers report the calibration equation used to convert the Abs data into real cell concentrations without mentioning how (or under which conditions) it was obtained. Calibration can be performed in different ways and these might influence the calculation of μ_{MAX}. To illustrate this, let us consider the cell concentration data points X_1 and X_2 obtained at two time points (t_1 and t_2) during the EGP; from these data, μ_{MAX} can be calculated as:

$$\mu_{MAX} = \frac{\ln X_2 - ln X_1}{t_2 - t_1} \quad \textbf{7}$$

Taking a linear relation (calibration) between Abs measurements and a direct cell mass concentration (X) method, as follows:

$$X = a * Abs + b \quad \textbf{8}$$

and substituting **equation 8** into **7**, results in:

$$\mu_{MAX} = \frac{\ln(a * X_2 + b) - \ln(a * X_1 + b)}{t_2 - t_1} \quad \textbf{9}$$

It is clear from **equation 9** that only if the linear coefficient (intercept) b = 0, μ_{MAX} calculated from Abs or direct cell mass concentration measurements will be the same.

In our experience at least, b is usually different from zero (**Table 2.3**). We demonstrate this here with μ_{MAX} calculations from data obtained during bioreactor cultivations of three different yeast strains on glucose, namely CEN.PK113-7D, UFMG-CM-Y259, JP1 (**Table 2.4**). Samples taken throughout the cultivation had their absorbances measured and their cell mass concentration determined by a direct method (dry cell mass). μ_{MAX} was calculated using four different approaches: 1) directly from Abs data; 2) directly from dry cell mass data; 3) from calculated dry cell mass values obtained using a calibration equation established between the Abs and the dry cell mass data, including all data points in the cultivation; 4) from calculated dry cell mass values obtained using a calibration equation established

between the Abs and the dry cell mass data, including only data points in the EGP (as identified from the dry cell mass data used for calibration).

Table 2.3 Calibration curves between dry cell mass concentration (X) and absorbance (Abs_{600}) data* from aerobic bioreactor cultivations of *S. cerevisiae* CEN.PK113-7D, JP1, and UFMG-CM-Y259 strains on glucose. b values are highlighted. Calibrations curves were obtained using total least square regression.

Strain	Replicate 1		Replicate 2	
	Calibration curve	Pearson's correlation coefficient	Calibration curve	Pearson's correlation coefficient
	Using data points from the entire cultivation			
CEN.PK113-7D	$X = 0.1902*Abs_{600} + \mathbf{0.1996}$	0.9945	$X = 0.1867*Abs_{600} + \mathbf{0.2010}$	0.9950
JP1	$X = 0.2230*Abs_{600} + \mathbf{0.2383}$	0.9922	$X = 0.2306*Abs_{600} + \mathbf{0.3062}$	0.9965
UFMG-CM-Y259	$X = 0.1568*Abs_{600} + \mathbf{0.3657}$	0.9958	$X = 0.1774*Abs_{600} + \mathbf{0.3493}$	0.9956
	Using data points within the EGP			
CEN.PK113-7D	$X = 0.1928*Abs_{600} + \mathbf{0.1799}$	0.9946	$X = 0.1852*Abs_{600} + \mathbf{0.2108}$	0.9970
JP1	$X = 0.2401*Abs_{600} + \mathbf{0.1966}$	0.9948	$X = 0.2271*Abs_{600} + \mathbf{0.3185}$	0.9912
UFMG-CM-Y259	$X = 0.3094*Abs_{600} + \mathbf{0.3484}$	0.9947	$X = 0.1685*Abs_{600} + \mathbf{0.4363}$	0.9910

*Raw data is shown in **Supplementary Table S2.1**.

Table 2.4 μ_{MAX} values calculated using four different approaches for three *S. cerevisiae* strains cultivated in aerobic bioreactors with glucose as sole carbon and energy source. Data represents the slope of the linear regression and the standard error.

	Approach											
	1*			2*			3			4		
	CEN.PK113-7D											
Replicate 1	0.443	±	0.001	0.320	±	0.004	0.327	±	0.008	0.334	±	0.008
Replicate 2	0.415	±	0.000	0.290	±	0.005	0.325	±	0.008	0.321	±	0.007
	JP1											
Replicate 1	0.422	±	0.000	0.305	±	0.002	0.301	±	0.067	0.312	±	0.069
Replicate 2	0.385	±	0.001	0.259	±	0.005	0.298	±	0.026	0.295	±	0.026
	UFMG-CM-Y259											
Replicate 1	0.419	±	0.000	0.312	±	0.003	0.283	±	0.004	0.333	±	0.087
Replicate 2	0.450	±	0.000	0.291	±	0.006	0.318	±	0.026	0.294	±	0.028

* Weighted linear regressions were applied for approaches 1 and 2 as to considerer the measurement errors.

Approaches: 1) Directly from Abs data; 2) directly from dry cell mass data; 3) from calculated dry cell mass values obtained using a calibration equation established between the Abs and the dry cell mass data, including all data points in the cultivation; 4) from calculated dry cell mass values obtained using a calibration equation established between the Abs and the dry cell mass data, including only data points in the EGP.

Remarkably, μ_{MAX} values calculated based on approach 1 were in the range of 35 to 56% higher than those calculated from dry cell mass data (approach 2). Because the latter approach is based on a direct assessment of cell mass concentration, widely considered as an accurate analytical method (as long as the appropriate amount of biomass is weighed on the filtration membrane or in the centrifuge tube, [28]), we took this μ_{MAX} value as the reference.

On the other hand, μ_{MAX} values calculated using approaches 3 or 4 were much closer to the reference μ_{MAX} value. In the case of the 3rd approach, which includes data points from the lag, EGP and de-acceleration growth phases in the calibration procedure, the calculated μ_{MAX} values differed at most 15% from the reference μ_{MAX} value, even when the calibration had been established with data from a different strain (see **Supplementary Tables S2.2 and S2.3; Supplementary Figure S2.1**). Nevertheless, it should be noted that other approaches, such as a modified version of approach 3 to force the linear regression to an intercept of zero, or the establishment of a calibration curve between Abs and dry cell mass using the final data point in the cultivation only, lead to the same results as those obtained using approach 1 (data not shown). This latter option has a very practical aspect, since it allows for the use of shake-flask cultivations monitored by absorbance measurements (which require small sample volumes) along the whole cultivation, accompanied by dry cell mass determination (which requires larger sample volumes) in the final sample only.

Errors associated to μ_{MAX} values depend on the regression method and may alter statistical outcomes

Experiments in scientific research are often carried out in replicates, so that statistical comparisons can be performed. It is of interest, for instance, to verify how the μ_{MAX} of a given strain compares to that of another strain under the same conditions, or to the μ_{MAX} of the same strain under different conditions. The error associated to the calculated μ_{MAX} value is therefore critical, since it is the basis for statistical comparisons. One approach to determine the absolute error that affects μ_{MAX} was proposed by Borzani [29], [30], and it depends on both the relative error of the cell concentration measurements and the duration of the experiment. This methodology was not used here since often researchers do not know (or do not report) the relative error of the cell concentration measurement itself.

Also, we would like to stress that time-series data are not independent, meaning that the value of one data point depends on the value of previous data points. And, strictly speaking, linear regression could not be used when data are not independent [31]. However, data from microbial growth curves have

historically been treated as being independent. This is due to the assumption that "Whether one point is above or below the line is a matter of chance, and does not influence whether another point is above or below the line" [32]. Hence, we also proceeded this way in this work.

Using Abs values from exponential growth of strain CAT-1 in microplates, two methods for statistical comparison of μ_{MAX} on defined and complex media were evaluated (**Table 2.5 and Supplementary Tables S2.4 – S2.6**). Although the final μ_{MAX} values obtained from both methods were the same, each was linked to distinct deviation/error values representing the scattering of the same data.

Another analysis we carried out was the removal of outliers. After visual inspection, some data points appeared much more distant to the regression lines than others, with no apparent reason. The removal of outliers based on an informal, visual approach is not recommended; thus the ROUT (Robust regression followed by Outlier identification) method was used. This is an automatic routine, based only on the distance of the data point from the robust best-fit curve [33]. We evaluated all data points again in GraphPad Prism software using the ROUT method, set up to eliminate outliers with a coefficient Q = 1% [33]. We then calculated μ_{MAX} with the remaining data points (**Table 2.5, and Supplementary Tables S10-S12**) by Method B (**Figure 2.1)**. As expected, different μ_{MAX} values were calculated by Method B without outliers and their standard errors were lower than the ones obtained by Method B.

Table 2.5 Maximum specific growth rates (μ_{MAX}) for strains CAT-1 and UFMG-CM-Y259 grown in microplates in two cultivation media, calculated from Abs_{600} data using two different regression methods*.

Medium	Method A			Method B (all data)			Method B (without outliers)		
	μ_{MAX}	SD	n	μ_{MAX}	SE	n	μ_{MAX}	SE	N
CAT-1									
Defined	0.2588	0.0171	5	0.2588	0.0131	40	0.2516	0.0039	32
Complex	0.3221	0.0525	5	0.3221	0.0900	20	0.3436	0.0460	16
UFMG-CM-Y259									
Defined	0.2500	0.0068	5	0.2500	0.0069	40		N.A.	
Complex	0.2808	0.0253	5	0.2808	0.0081	30		N.A.	

*described in the Methods section. SD is the standard deviation; SE is the standard error of the slope; n is the number of observations. N.A. = not available. For this case, outliers were not identified.

Next, we performed statistical comparisons of the data from **Table 2.5** to check if the methods would yield the same results. Method A required a t-test to compare the averages from different treatments (in this case, the two cultivation media) and define whether their difference was statistically significant or

not. A two-tailed, pooled t-test was chosen because we assumed that both populations were independent and normally distributed, their variances were unknown but equal, and the sample sizes were small (n = 5 for each data set) [34]. Method B, on the other hand, relied on an F-test, which is equivalent to an Analysis of Covariance (ANCOVA). The F-value is based on the residual sum-of-squares of both the common and the pooled regressions, the number of regressions tested, and the degrees of freedom of the pooled regression (details in [35] and **Supplementary Material**). For both methods, the null hypothesis was H_0: $\mu_{MAX,1} = \mu_{MAX,2}$, and the alternative hypothesis was H_1: $\mu_{MAX,1} \neq \mu_{MAX,2}$. If the calculated p-value was less than the significance level α (0.05 or 0.01), we would reject the null hypothesis and the μ_{MAX} from the two cultivation media could be considered different at the significance level used (**Table 2.6**).

Depending on the method and the significance level applied, the outcomes of the comparison diverged, as shown by the resulting p-values. At $\alpha = 0.01$, both methods A and B (with the complete data set) agreed in that the μ_{MAX} values of strain CAT-1 in defined or complex media are not statistically different from each other. However, at $\alpha = 0.05$ the methods disagreed. A different result was observed for the *S. cerevisiae* UFMG-CM-Y259 strain. At $\alpha = 0.05$ both methods resulted in a significant difference between defined and complex media, whereas that was not the case at $\alpha = 0.01$. Other strains were also tested, but the same conclusions were achieved from both methods and significance levels (**Figure 2.2, Supplementary Tables S5 and S6**). After the removal of outliers, Method B resulted in completely different conclusions at both α for strain CAT-1, when compared to the same method using all data points.

Even though Method A is widely used due to its simplicity and straightforwardness, it may not be the best way to calculate the error associated to μ_{MAX} values. Each replicate μ_{MAX}, once calculated independently, already has its own error associated to the fitting of the regression line itself. But these errors are not taken into account by Method A as they are simply not calculated, differently from Method B. Additionally, we showed that the removal of outliers was decisive for the results. One can easily see that the comparison between μ_{MAX} values calculated using distinct methods is extremely discouraged. Poorly described statistics in microbial physiology papers makes it difficult to understand how data were obtained and even more difficult to know whether interlaboratory comparisons can be performed.

Table 2.6 Statistical comparison of μ_{MAX} values for strains CAT-1 and UFMG-CM-Y259 grown on defined or complex media, using data from Table 4. Depending on the statistical method and the significance level applied, distinct conclusions can be drawn.

	Test statistic	**p-value**	**Conclusion ($\alpha = 0.05$)**	**Conclusion ($\alpha = 0.01$)**
			CAT-1	
Method A	2.5632[a]	0.0335	different μ_{MAX}	same μ_{MAX}
Method B (all data)	1.1016[b]	0.3178	same μ_{MAX}	same μ_{MAX}
Method B (without outliers)	9.9324[b]	0.0029	different μ_{MAX}	different μ_{MAX}
			UFMG-CM-Y259	
Method A	2.6294[a]	0.0302	different μ_{MAX}	same μ_{MAX}
Method B (all data)	7.4850[b]	0.008	different μ_{MAX}	different μ_{MAX}

[a] t-test; [b] F-test

Influence of the type of medium on μ_{MAX} calculations

Researchers often report μ_{MAX} values of a yeast strain on a given carbon and energy source, such as glucose. However, whether this carbon source is provided in a synthetic defined medium or in a complex undefined medium will influence the growth rate of a microbial population. In principle, μ_{MAX} values should be higher in the latter environment, because cells benefit from compounds that can be taken up directly from the medium, instead of having to synthesize them from metabolic intermediates at the expense of energy. To verify to which extent μ_{MAX} values are influenced by these two types of media, we evaluated this physiological parameter for eight different *S. cerevisiae* strains cultivated in microplates (**Figure 2.2**).

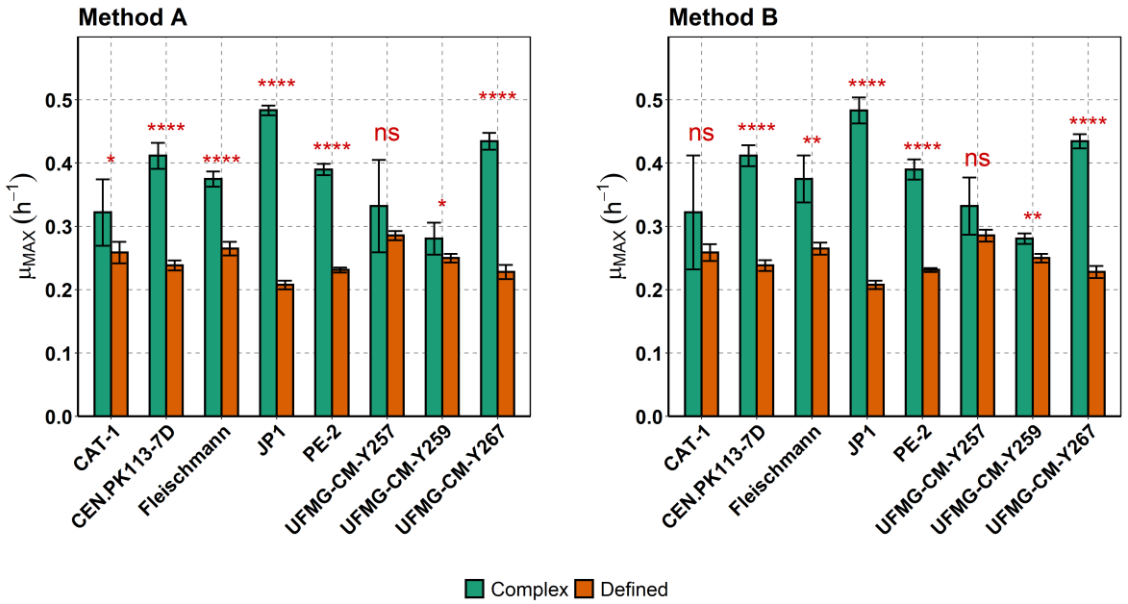

Figure 2.2 Maximum specific growth rates (μ_{MAX}) of strains grown on glucose in microplates in two cultivation media, calculated using two different regression methods (A and B). Depending on the statistical method and the significance level used, distinct conclusions can be drawn. * represent the p-value at which a significant difference between the treatments were observed; ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

Overall, the μ_{MAX} values were higher for a given strain in YPD medium than in defined Verduyn medium, as expected. Nevertheless, the level to which this occurs varies among strains (**Table 2.7**), and, for a few cases, the difference between the pair of μ_{MAX} values was not significant at 95% or higher confidence level. The complex/defined μ_{MAX} ratio ranged from 1.12 to 2.33, which is quite remarkable, considering that all strains belong to the same species and that both media employed here are commonly used in experimental research. We were not able to identify any trend in these data, e.g. whether the haploid CEN.PK113-7D strain would present a different behavior than the diploid ones, or whether industrial strains (CAT-1, JP-1, PE-2) would behave differently than the laboratory, the baker's or the wild isolates. This indicates that these results are probably related to cell morphology, which strongly influence Abs measurements [7], rather than to cells' metabolism or physiology, once again highlighting the importance of taking great care when calculating and/or interpreting μ_{MAX} values from such indirect, light-scattering-based methods.

Table 2.7 Ratio between μ_{MAX} in a complex medium (YPD) over μ_{MAX} in a defined medium with glucose as sole carbon and energy source. μ_{MAX} values were calculated from Abs_{600} data using either Method A or Method B. Both methods resulted in the same μ_{MAX} values.

	CAT-1	**CEN.PK113-7D**	**Fleischmann**	**JP1**	**PE-2**	**UFMG-CM-Y257**	**UFMG-CM-Y259**	**UFMG-CM-Y267**
μ_{Max}_complex/ μ_{Max}_defined	1.24	1.73	1.41	2.33	1.69	1.16	1.12	1.91

Although complex and defined media must contain all the essential nutrients for cell growth, Abelovska and colleagues [36] demonstrated that the amount of some compounds can vary up to 20 fold from one sort to another. These authors compared the elemental composition of complex (2% peptone, 1% yeast extract) and minimal media (yeast nitrogen base), and detected lower levels of important enzyme cofactors such as magnesium and manganese in the complex medium. However, for the cofactors iron and zinc, as well as for sodium and potassium ions, which are crucial elements in the generation of electrochemical potential across the cell membrane [37], the results turned out to be the opposite.

Influence of the cultivation system on μ_{MAX} calculations

We assessed how the cultivation system affects the calculation of μ_{MAX} by comparing the calculated values obtained from microplate, shake-flask, and bioreactor cultivations of three *S. cerevisiae* strains (**Figure 2.3**). The calculations were performed considering the Abs values of distinct samples from the EGP as described in the Material and Methods section (**Figure 2.1, Method B**). For any particular strain, the three systems led to different μ_{MAX} values, with the lowest values always being achieved using microplate cultivations. This is consistent with our expectations, and has been observed before with bacteria [20]. Cells are exposed to varying growth conditions in the three systems, such as dissolved O_2 and pH, which results from the different agitation, oxygen supply and pH control setups. This per se should lead to different physiologies.

However, the measuring peculiarities of each system also contribute to the observed differences in μ_{MAX}. While in microplates the absorbance is usually measured without prior dilution of the cell broth, in the other two setups, dilution is performed to assure the measured Abs values fall within the limits of proportionality with cell number or dry cell mass [37]. The real Abs is then calculated by multiplying the measured value by the dilution factor. Begot and co-workers [38] evaluated the growth of several *Listeria monocytogeneses* strains in both microplate and bioreactor systems, and showed that the range of proportionality between Abs and bacterial population (CFU/ml) depended on the apparatus used to measure Abs, which adds even more complexity and demands prior knowledge on the particular piece of equipment used.

In the case of the results shown here, the spectrophotometer used for measuring the absorbance during shake-flask cultivations was different from the one used for the bioreactor cultivations (see Material and Methods section for specifications), as these experiments were performed in different laboratories. Thus, one should also take the contribution of changing the equipment into account when interpreting these data. As an example of how different spectrophotometers can affect the measurements, Koch [39] demonstrated that the standard curves of apparent absorbance versus bacterial dry mass concentrations vary among different instruments under a selected range of wavelengths and aperture

widths. By apparent absorbance the author refers to the absorbance measured in non-ideal turbidimeters. Because the absorbance represents the logarithmic difference between the light transmitted by the light source and the light received by the detector, the slit width plays an important role in quantifying this parameter, as so does the wavelength [7].

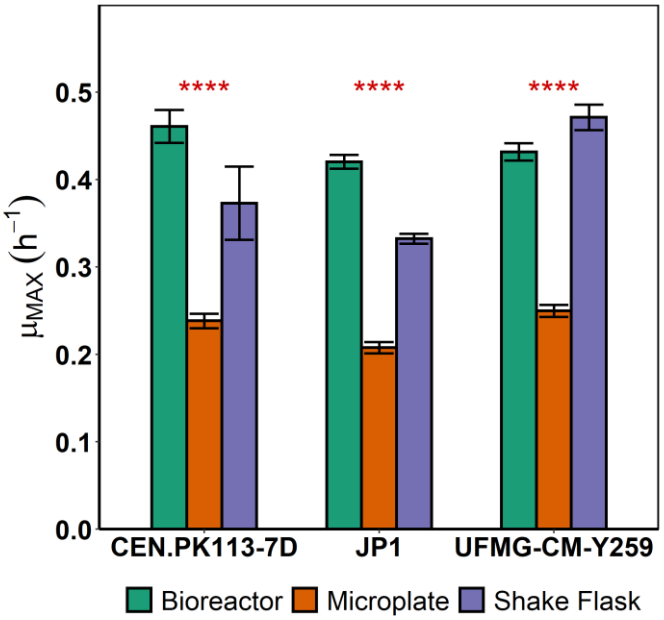

Figure 2.3 Maximum specific growth rates (μ_{max}) for three *S. cerevisiae* strains grown in a defined medium with glucose as sole carbon and energy source, in three different cultivation systems. Data from different systems were used to calculate and statistically compare μ_{MAX} values using Method B and GraphPad Prism software. This yielded a p-value ≤ 0.0001 (****) for all strains.

A practical example on how to misinterpret μ_{MAX} values

To further illustrate the importance of taking proper care while reporting or interpreting μ_{MAX} data, we calculated this parameter for some *S. cerevisiae* strains during cultivations in a defined medium containing a carbon and energy source other than glucose, namely sucrose or fructose. These μ_{MAX} values were then compared to the glucose data, both for microplate and shake-flask cultivations. As an example, a researcher could be interested in verifying on which of the three sugars yeast would grow with the highest μ_{MAX}, or one could be interested in screening several yeast strains for fructophilic behavior, which is a desirable feature in the wine industry, for instance, to overcome challenges with stuck fermentations [40]–[42].

The results obtained in microplates do not necessarily corroborate those obtained in shake-flask cultivations (**Figure 2.4)**. For instance, the UFMG-CM-Y259 strain displayed faster growth on sucrose in the microscale system, compared to its growth on either of the hexoses. In shake-flask cultivations, however, it grew with a smaller μ_{max} on sucrose, again compared to growth on glucose or fructose. The CEN.PK113-7D strain also displayed a higher μ_{MAX} on sucrose in microplate cultivations, but no significant difference was observed in the μ_{MAX} values on the three substrates during shake-flask cultivations.

When considering growth on fructose, in comparison to glucose only, the UFMG-CM-Y257 strain showed higher μ_{MAX} on glucose for cultivations using microplates, whereas equivalent growth rates on both substrates were observed during shake-flask cultivations. The opposite was observed for the JP1 strain. Resolving the mechanisms underlying such strain-specific behaviors is beyond the scope of this work. Here, the importance relies on the fact that one could easily miss the cultivation system-dependency of μ_{MAX} in *S. cerevisiae*, if a careful evaluation of the reported methodologies was not performed. In spite of this, comparisons with literature data are often reported without properly highlighting the differences in the experimental setup between the evaluated studies, which frequently leads to misinterpretation.

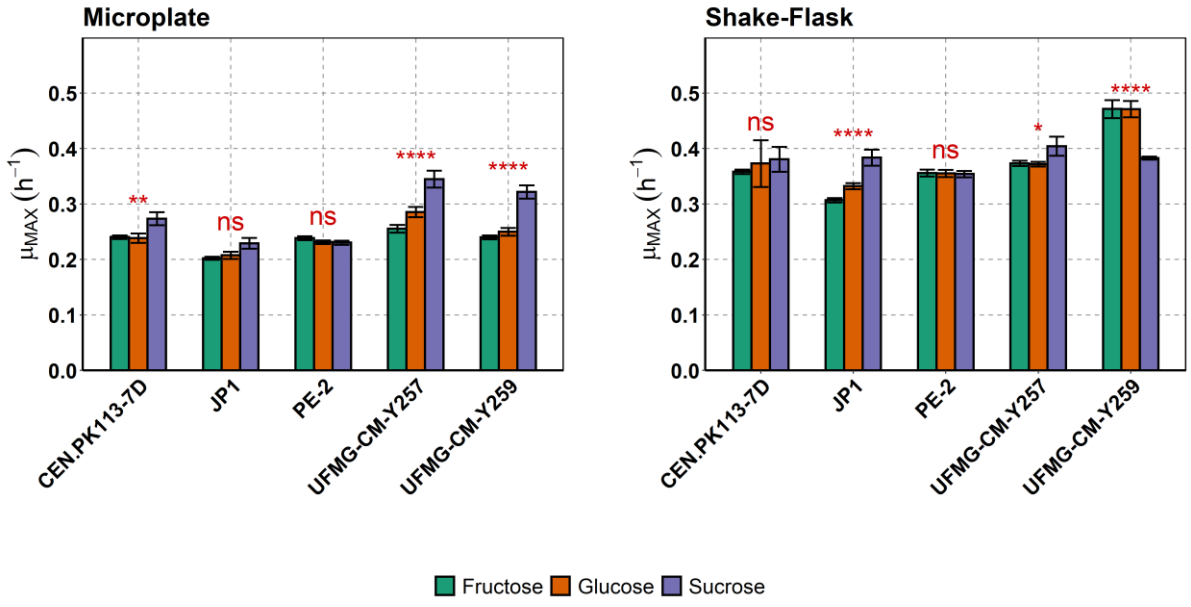

Figure 2.4 Maximum specific growth rates (μ_{MAX}) of *S. cerevisiae* strains grown in microplates or shake-flasks in a defined medium supplemented with sucrose, glucose or fructose as sole carbon and energy source, calculated by Method B from Abs_{600} measurements. * represent the p-value at which a significant difference between the treatments were observed; ns ($p > 0.05$); * ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$); **** ($p \leq 0.0001$).

FINAL REMARKS

Determining the maximum specific growth rate is routine in any microbiology laboratory, be it in industry or academia. The several different methods available for such purpose, however, add up to challenge this task. Most frequently, researchers report the μ_{MAX} values they calculate in a comparative manner, either with external publications or with those within their research group. The challenge of these comparative analyses is to assure that the evaluated cultivations, the analytical procedures, and data treatment have been executed in the exact same way, and under proper caution. We demonstrate here through a series of examples the implications on μ_{MAX} calculations when distinct cultivations setups or analytical methodologies are employed. We, therefore, would like to draw the attention of our fellow microbiologists to the following:

1) Avoid calculations of μ_{MAX} directly from Abs measurements. First convert the Abs data to real cell concentration values using a pre-established calibration equation, obtained under identical cultivation conditions, and only then calculate μ_{MAX}. This calibration equation can be established using data from an entire batch cultivation, but ideally only data points in the EGP should be used to avoid any eventual artifacts introduced by cell morphology changes.

2) When methodologies other than obtaining μ_{MAX} directly from Abs measurements are not an option, one should never think of the calculated values as absolute. Comparisons with data reported in different works should thus be made with utmost care.

3) Always make comparisons of your own calculated μ_{MAX} values with caution and explicitly report the conditions used by other authors or under which other experiments in the same lab were carried out.

4) Do not overstate findings related to μ_{MAX}, since its value can vary with any cultivation detail that is different, such as the geometry of the cultivation vessel, contaminants present in chemicals used to formulate media, rotation radius of the shaker incubator, method used to determine the cell concentration, etc.

5) Ideally, cell concentration determinations should be carried out in technical replicates to aid in statistical analysis.

6) Decide on a statistical method to use for comparisons between your own μ_{MAX} data and explicitly describe it. Report p-values rather than simply stating the statistical conclusion [43].

7) Describe all calculations in detail, even if they are quite obvious to some. Supplementary material in research articles or data repositories could be used for this purpose. This will make comparisons easier, more meaningful and scientifically more sound.

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SUPPLEMENTARY MATERIAL

Methods for determining cell concentration and calculating the maximum specific growth rate

Table S2.1 Absorbance and dry cell mass concentration experimental data for *S. cerevisiae* CEN.PK113-7D, JP1, and UFMG-CM-Y259 grown on glucose in aerobic batch bioreactors. Experiments were carried out in duplicate.

Replicate 1							Replicate 2						
	Approach[a]							Approach[a]					
Time (h)	1 Abs_{600}[b]			2 X[c]			Time (h)	1 Abs_{600}[b]			2 X[c]		
CEN.PK113-7D													
0.22	0.183	±	0.000	0.220	±	0.020	0.23	0.151	±	0.001	0.270	±	0.030
1.02	0.210	±	0.000	0.140	±	0.020	2.05	0.231	±	0.001	0.300	±	0.080
2.22	0.292	±	0.001	0.250	±	0.030	3.30	0.398	±	0.003	0.310	±	0.030
3.00	0.436	±	0.000	0.330	±	0.010	4.13	0.620	±	0.005	0.150	±	0.030
4.15	0.765	±	0.015	0.420	±	0.018	5.22	0.960	±	0.005	0.390	±	0.010
5.35	1.280	±	0.010	0.420	±	0.000	6.07	1.370	±	0.001	0.480	±	0.060
6.10	1.735	±	0.005	0.570	±	0.030	7.12	2.000	±	0.035	0.560	±	0.040
7.22	2.670	±	0.020	0.760	±	0.040	8.18	3.340	±	0.030	0.790	±	0.030
8.22	4.300	±	0.040	1.030	±	0.030	9.32	5.020	±	0.080	1.260	±	0.040
9.33	6.975	±	0.025	1.480	±	0.020	10.08	7.250	±	0.001	1.540	±	0.020
10.05	8.900	±	0.100	1.910	±	0.050	11.03	10.150	±	0.025	2.100	±	0.100
11.07	12.475	±	0.125	2.620	±	0.020	11.65	12.050	±	0.025	2.540	±	0.160
JP1													
0.13	0.154	±	0.000	0.370	±	0.050	0.67	0.182	±	0.001	0.320	±	0.020
2.05	0.231	±	0.002	0.370	±	0.050	2.19	0.254	±	0.001	0.380	±	0.020
3.05	0.381	±	0.001	0.370	±	0.010	3.20	0.383	±	0.003	0.390	±	0.010
4.13	0.655	±	0.025	0.340	±	0.010	4.27	0.630	±	0.010	0.440	±	0.040
5.05	0.910	±	0.010	0.410	±	0.010	5.60	0.995	±	0.005	0.530	±	0.010
6.05	1.400	±	0.020	0.550	±	0.010	8.46	3.450	±	0.010	1.120	±	0.010
7.13	2.073	±	0.003	0.780	±	0.040	10.17	6.045	±	0.005	1.740	±	0.050
8.05	3.205	±	0.005	0.960	±	0.020	11.85	11.800	±	0.001	2.690	±	0.080
9.22	5.050	±	0.070	1.400	±	0.002							
10.05	8.425	±	0.075	1.810	±	0.030							
11.00	9.713	±	0.013	2.460	±	0.020							
11.38	11.100	±	0.000	2.720	±	0.020							
UFMG-CM-Y259													
0.27	0.224	±	0.002	0.400	±	0.000	0.350	0.182	±	0.002	0.320	±	0.020
1.00	0.250	±	0.000	0.400	±	0.020	2.067	0.262	±	0.007	0.370	±	0.010
2.00	0.340	±	0.000	0.410	±	0.010	3.184	0.426	±	0.000	0.450	±	0.010

Replicate 1							Replicate 2						
	Approach[a]							Approach[a]					
Time (h)	1 Abs$_{600}$[b]			2 X[c]			Time (h)	1 Abs$_{600}$[b]			2 X[c]		
3.00	0.496	±	0.000				4.380	0.692	±	0.006	0.470	±	0.030
3.98	0.815	±	0.003	0.420	±	0.015	6.670	2.110	±	0.010	0.780	±	0.020
4.98	1.175	±	0.003	0.510	±	0.030	8.580	4.840	±	0.040	1.320	±	0.040
6.00	1.725	±	0.005	0.680	±	0.020	10.780	13.050	±	0.000	2.590	±	0.030
7.02	2.545	±	0.010	0.890	±	0.010	11.380	13.375	±	0.225	2.980	±	0.080
8.00	4.120	±	0.040	1.190	±	0.030							
9.00	6.200	±	0.025	1.650	±	0.070							
10.00	9.275	±	0.013	2.290	±	0.010							
10.98	12.500	±	0.100	2.930	±	0.050							

[a] Approaches: (1) Identify EGP and calculate μ_{MAX} from Dry Cell Mass concentrations (X) Data; (2) Identify EGP and calculate μ_{MAX} from Abs Data; [b] Absorbance measurements at 600 nm. [c] cell concentration in $g_{DM}.l^{-1}$.

Table S2.2 Cell concentration data for *S. cerevisiae* CEN.PK113-7D, JP1, and UFMG-CM-Y259 grown on glucose in aerobic batch bioreactors calculated applying different calibration equations that were established with the data presented in Table S2.1.

Replicate 1							Replicate 2						
	Approach[a]							Approach[a]					
Time (h)	3 X3	4 X4	5 X3b	6 X4b	7 X5a	8 X5b	Time (h)	3 X3	4 X4	5 X3b	6 X4b	7 X5a	8 X5b
CEN.PK113-7D													
0.22	0.234	0.215	0.235	0.245	0.241	0.360	0.23	0.229	0.239	0.228	0.209	0.353	0.233
1.02	0.240	0.220	0.240	0.250	0.247	0.366	2.05	0.244	0.254	0.244	0.224	0.371	0.252
2.22	0.255	0.236	0.256	0.265	0.267	0.385	3.30	0.275	0.285	0.275	0.257	0.409	0.292
3.00	0.283	0.264	0.282	0.292	0.301	0.418	4.13	0.317	0.326	0.318	0.299	0.459	0.345
4.15	0.345	0.327	0.344	0.352	0.380	0.492	5.22	0.380	0.389	0.382	0.365	0.537	0.427
5.35	0.443	0.427	0.440	0.448	0.504	0.609	6.07	0.457	0.465	0.460	0.444	0.630	0.526
6.10	0.530	0.514	0.525	0.532	0.613	0.713	7.12	0.574	0.581	0.580	0.566	0.773	0.677
7.22	0.707	0.695	0.699	0.705	0.838	0.925	8.18	0.825	0.829	0.835	0.824	1.077	0.999
8.22	1.017	1.009	1.004	1.007	1.229	1.295	9.32	1.138	1.141	1.154	1.148	1.459	1.402
9.33	1.526	1.525	1.503	1.503	1.871	1.903	10.08	1.555	1.554	1.579	1.578	1.965	1.937
10.05	1.892	1.896	1.863	1.859	2.333	2.340	11.03	2.096	2.091	2.130	2.137	2.624	2.634
11.07	2.572	2.585	2.530	2.521	3.192	3.152	11.65	2.451	2.442	2.492	2.503	3.055	3.090
JP1													
0.13	0.273	0.234	0.342	0.353	0.21	0.24	0.67	0.348	0.360	0.279	0.240	0.24	0.21
2.05	0.290	0.252	0.359	0.371	0.22	0.25	2.19	0.365	0.376	0.295	0.258	0.26	0.23
3.05	0.323	0.288	0.394	0.405	0.25	0.28	3.20	0.395	0.405	0.324	0.289	0.28	0.25
4.13	0.384	0.354	0.457	0.467	0.31	0.33	4.27	0.451	0.462	0.379	0.348	0.33	0.30
5.05	0.441	0.415	0.516	0.525	0.36	0.38	5.60	0.536	0.544	0.460	0.435	0.40	0.37
6.05	0.551	0.533	0.629	0.636	0.45	0.47	8.46	1.102	1.102	1.008	1.025	0.85	0.85
7.13	0.701	0.694	0.784	0.789	0.58	0.59	10.17	1.700	1.691	1.586	1.648	1.33	1.35
8.05	0.953	0.966	1.045	1.046	0.80	0.80	11.85	3.027	2.998	2.870	3.030	2.40	2.45
9.22	1.364	1.409	1.471	1.465	1.15	1.15							
10.05	2.404	2.529	2.546	2.524	2.05	2.01							

Replicate 1							Replicate 2						
Time (h)	Approach[a]						Time (h)	Approach[a]					
	3 X3	4 X4	5 X3b	6 X4b	7 X5a	8 X5b		3 X3	4 X4	5 X3b	6 X4b	7 X5a	8 X5b
11.00	2.117	2.219	2.249	2.232	1.80	1.77							
11.38	2.714	2.862	2.866	2.839	2.32	2.27							
UFMG-CM-Y259													
0.27	0.401	0.418	0.389	0.474			0.35	0.382	0.467	0.394	0.405		
1.00	0.405	0.426	0.394	0.478			2.07	0.396	0.480	0.407	0.429		
2.00	0.419	0.454	0.410	0.494			3.18	0.425	0.508	0.432	0.480		
3.00	0.443	0.502	0.437	0.520			4.38	0.472	0.553	0.474	0.563		
3.98	0.493	0.601	0.494	0.574			6.67	0.724	0.792	0.697	1.001		
4.98	0.550	0.712	0.558	0.634			8.58	1.208	1.252	1.125	1.846		
6.00	0.636	0.882	0.655	0.727			10.78	2.664	2.635	2.412	4.386		
7.02	0.765	1.136	0.801	0.865			11.38	2.722	2.690	2.463	4.487		
8.00	1.012	1.623	1.080	1.131									
9.00	1.338	2.267	1.449	1.481									
10.00	1.820	3.218	1.995	1.999									
10.98	2.326	4.216	2.567	2.543									

[a] Approaches: (3) Convert Abs to X (X3) using a calibration curve established with all data points, identify EGP and calculate μ_{MAX}; (4) Convert Abs to X (X4) using a calibration curve established with EGP-only data points, identify EGP and calculate μ_{MAX}; (5) Convert Abs to X (X3b) using a calibration curve established with all data points from the other replicate, identify EGP and calculate μ_{MAX}; (6) Convert Abs to X (X4b) using a calibration curve established with EGP-only data points from the other replicate, identify EGP and calculate μ_{MAX}; (7) Convert Abs to X (X5a) using a calibration curve with EGP-only data points from one replicate of a different strain (JP1 or CEN.PK113-7D), identify EGP and calculate μ_{MAX}; (8) Convert Abs to X (X5b) using a calibration curve with EGP-only data points from the other replicate of the different strain (JP1 or CEN.PK113-7D), identify EGP and calculate μ_{MAX}.

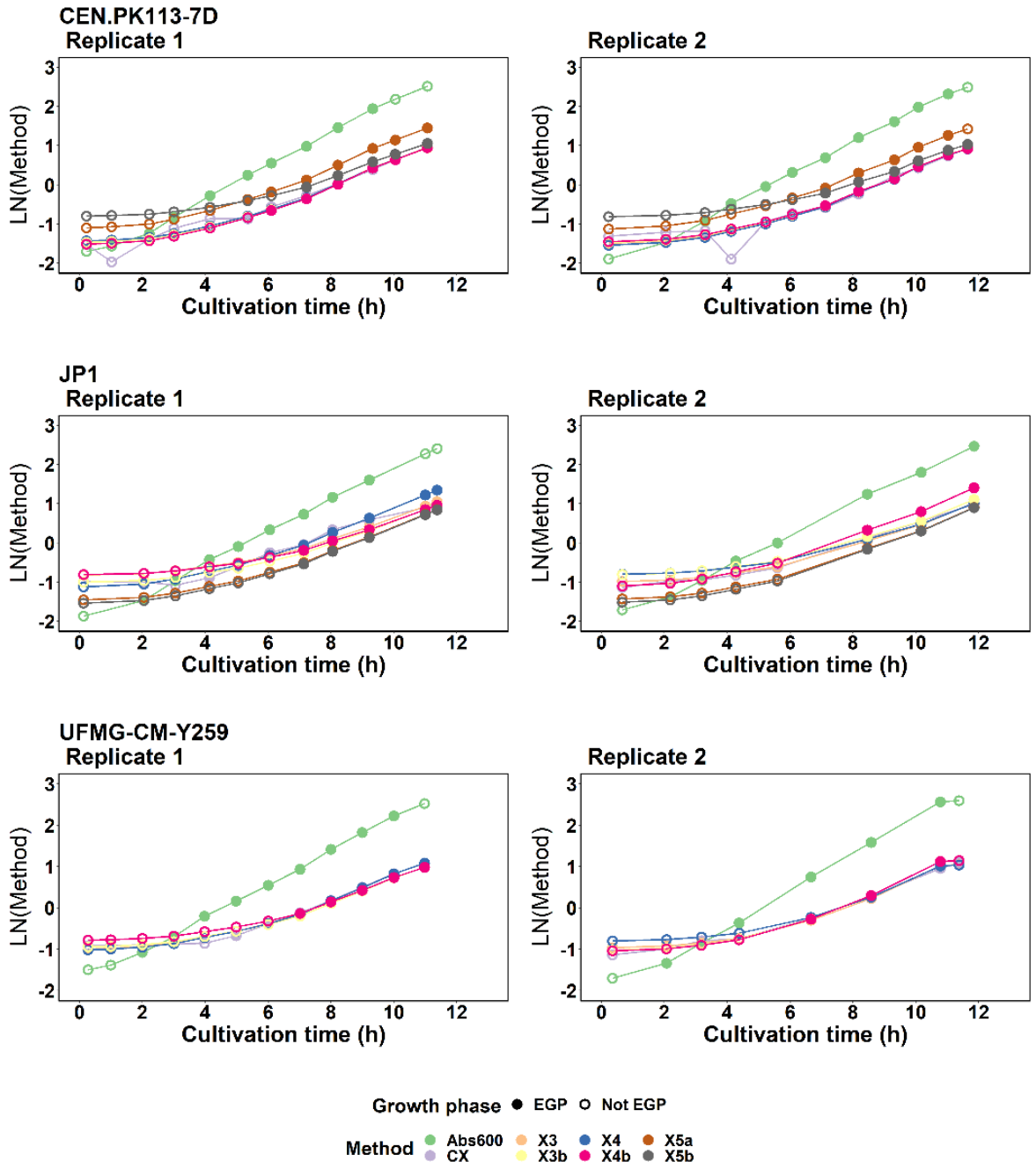

Figure S2.1 Illustration of the EGP identification for the eight different methods described in Tables S1 and S2, for *S. cerevisiae* strains CEN.PK113-7D, JP1, and UFMG-CM-Y259 cultivated in aerobic batch bioreactors with glucose as sole carbon and energy source.

Table S2.3 Maximum specific growth rate, calculated using different calibration approaches and applying Method A, of *S. cerevisiae* CEN.PK113-7D, JP1, and UFMG-CM-Y259 during growth on glucose in aerobic batch bioreactors.

Method	Replicate 1			Replicate 2		
	EGP (h)	μ_{MAX} (h^{-1})	Δ%	EGP (h)	μ_{MAX} (h^{-1})	Δ%
CEN.PK113-7D						
X	5.35 - 11.07	0.32	0%	5.22 - 11.65	0.29	0%
Abs600	2.22 - 9.33	0.44	40%	3.30 - 11.03	0.42	43%
X3	6.10 - 11.07	0.33	4%	7.12 - 11.65	0.33	13%
X4	6.10 - 11.07	0.33	3%	7.12 - 11.65	0.32	10%
X3b	6.10 - 11.07	0.33	4%	7.12 - 11.65	0.33	12%
X4b	6.10 - 11.07	0.32	2%	7.12 - 11.65	0.33	12%
X5a	5.35 - 11.07	0.33	5%	7.12 - 11.06	0.31	7%
X5b	7.22 - 11.07	0.32	2%	7.12 - 11.65	0.34	17%
JP1						
X	4.13 - 9.22	0.31	0%	5.60 - 11.85	0.26	0%
Abs600	2.05 - 9.22	0.42	36%	2.19 - 11.85	0.39	50%
X3	7.13 - 11.38	0.30	-3%	8.46 - 11.85	0.30	15%
X4	7.13 - 11.38	0.31	0%	8.46 - 11.85	0.30	15%
X3b	7.13 - 11.38	0.29	-6%	8.46 - 11.85	0.31	19%
X4b	7.13 - 11.38	0.29	-6%	8.46 - 11.85	0.32	22%
X5a	7.13 - 11.38	0.31	0%	8.46 - 11.85	0.31	18%
X5b	7.13 - 11.38	0.30	-3%	8.46 - 11.85	0.31	20%
UFMG-CM-Y259						
X	4.98 - 10.98	0.31	0%	6.67 -11.38	0.29	0%
Abs600	2.00 - 10.00	0.42	35%	2.07 - 10.78	0.45	56%
X3	7.02 - 10.98	0.28	-10%	6.67 - 10.78	0.32	11%
X4	7.02 - 10.98	0.33	6%	6.67 - 10.78	0.29	0%
X3b	7.02 - 10.98	0.30	-3%	6.67 - 10.78	0.30	4%
X4b	7.02 - 10.98	0.28	-8%	6.67 - 10.78	0.36	25%

EGP = exponential growth phase.
Δ = fold change with respect to the calculations using dry cell mass concentrations (X) data.
Blue shade indicates that the EGP comprised only three data points.

Regression methods and statistical outcomes

Method A

Table S2.4 Maximum specific growth rate (μ_{MAX}) for eight different *S. cerevisiae* strains grown on either defined or complex medium supplemented with glucose as sole carbon and energy source, using microplate as cultivation system. Experiments were carried out in five replicates, and for each replicate one μ_{MAX} was calculated from Abs_{600} data within the exponential growth phase (EGP).

	CAT-1	CEN.PK113-7D	Fleischmann	JP-1	PE-2	UFMG-CM-Y257	UFMG-CM-Y259	UFMG-CM-Y267
DEFINED MEDIUM								
replicate 1	0.2877	0.2509	0.2804	0.2139	0.2363	0.2969	0.2583	0.2262
replicate 2	0.2572	0.2323	0.2569	0.2152	0.2304	0.2868	0.2485	0.2144
replicate 3	0.2523	0.2314	0.2694	0.2056	0.2342	0.2857	0.2554	0.2255
replicate 4	0.2544	0.2400	0.2660	0.2051	0.2292	0.2799	0.2463	0.2286
replicate 5	0.2425	0.2374	0.2530	0.1986	0.2264	0.2788	0.2417	0.2459
COMPLEX MEDIUM								
replicate 1	0.2361	0.4242	0.3664	0.4823	0.3877	0.2060	0.3186	0.4141
replicate 2	0.3585	0.3825	0.3843	0.4744	0.3979	0.3667	0.2812	0.4328
replicate 3	0.3644	0.4201	0.3895	0.4782	0.3997	0.3925	0.2784	0.4409
replicate 4	0.3412	0.4328	0.3608	0.4883	0.3784	0.3526	0.2473	0.4506
replicate 5	0.3102	0.3988	0.3740	0.4935	0.3862	0.3427	0.2787	0.4344

Table S2.5 Comparative statistical analysis, based on method A[a], of the maximum specific growth rates showed in Table S2.4.

	CAT-1	CEN.PK113-7D	FLEISCHMANN	JP1	PE-2	UFMG-CM-Y257	UFMG-CM-Y259	UFMG-CM-Y267
DEFINED MEDIUM								
Average μ_{MAX} ($\overline{X_1}$)	0.2588	0.2384	0.2651	0.2077	0.2313	0.2856	0.2500	0.2281
STDEV[b] (S_1)	0.0171	0.0078	0.0108	0.0069	0.0040	0.0072	0.0068	0.0113
Observations (n_1)	5	5	5	5	5	5	5	5
COMPLEX MEDIUM								
Average μ_{MAX} ($\overline{X_2}$)	0.3221	0.4117	0.3750	0.4833	0.3900	0.3321	0.2808	0.4346
STDEV[b] (S_2)	0.0525	0.0206	0.0120	0.0077	0.0088	0.0729	0.0253	0.0134
Observations (n_2)	5	5	5	5	5	5	5	5
STATISTICAL COMPARISON								
S_p^2	0.0015	0.0002	0.0001	0.0001	0.0000	0.0027	0.0003	0.0002
t-test	-2.5632	-17.6071	-15.2321	-59.8752	-36.7226	-1.4181	-2.6294	-26.2777
p-value	0.0335	0.0000	0.0000	0.0000	0.0000	0.1939	0.0302	0.0000
Conclusion ($\alpha = 0.05$)	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 = \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$
Conclusion ($\alpha = 0.01$)	$\mu1 = \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 = \mu2$	$\mu1 = \mu2$	$\mu1 <> \mu2$

[a] Method A consists in the calculation of an average μ_{MAX} taking each replicate individual μ_{MAX}.
[b] STDEV = standard deviation.

Method B

Table S2.6 Comparative statistical analysis, based on method B[1], of the maximum specific growth rates (μ_{MAX}) of different *S. cerevisiae* strains grown on either defined or complex medium supplemented with glucose as sole carbon and energy source, using microplate as cultivation system. Experiments were performed in five replicates. One single μ_{MAX} was calculated from Abs_{600} data from all replicates.

	CAT-1	CEN.PK113-7D	FLEISCHMANN	JP1	PE-2	UFMG-CM-Y257	UFMG-CM-Y259	UFMG-CM-Y267
DEFINED MEDIUM								
Average μ_{MAX} (b_1)	0.2588	0.2384	0.2651	0.2077	0.2313	0.2856	0.2500	0.2281
ST Error (SE_1)	0.0131	0.0084	0.0094	0.0066	0.0030	0.0092	0.0069	0.0096
Observations (n_1)	40	45	35	45	50	35	40	40
COMPLEX MEDIUM								
Average μ_{MAX} (b_2)	0.3221	0.4117	0.3750	0.4833	0.3900	0.3321	0.2808	0.4346
ST Error (SE_2)	0.0900	0.0166	0.0371	0.0206	0.0160	0.0451	0.0081	0.0112
Observations (n_2)	20	30	15	30	35	20	30	20
STATISTICAL COMPARISON								
F-test	1.0160	88.5400	9.5010	231.0000	156.6000	1.8150	7.4850	73.3500
p-value	0.3178	0.0000	0.0035	0.0000	0.0000	0.1839	0.0080	0.0000
Conclusion ($\alpha = 0.05$)	$\mu1 = \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 = \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$
Conclusion ($\alpha = 0.01$)	$\mu1 = \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 = \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$

[1] Method B consists in the calculation of one single μ_{MAX} taking all data across replicates.

Exemplification of Method B methodology for calculating μ_{MAX} and performing comparative statistical analysis

Table S2.7 Raw Abs_{600} data from the exponential phase of growth of *S. cerevisiae* CAT-1 cultivated on either defined or complex medium supplemented with glucose as sole carbon and energy source, using microplate as cultivation system.

DEFINED MEDIUM						COMPLEX MEDIUM					
Time (h)	Replicate					Time (h)	Replicate				
	1	2	3	4	5		1	2	3	4	5
2.1746	0.2192	0.2570	0.2620	0.2558	0.2688	2.3016	0.4077	0.2621	0.2661	0.2943	0.3106
2.4852	0.2353	0.2743	0.2783	0.2751	0.2861	2.6303	0.4395	0.2909	0.2963	0.3265	0.3423
2.7959	0.2556	0.2946	0.2984	0.2942	0.3056	2.9593	0.4816	0.3305	0.3344	0.3684	0.3822
3.1066	0.2802	0.3195	0.3223	0.3197	0.3282	3.2880	0.5122	0.3720	0.3810	0.4108	0.4206
3.4172	0.3060	0.3462	0.3497	0.3454	0.3565						
3.7279	0.3375	0.3780	0.3823	0.3778	0.3848						
4.0385	0.3720	0.4116	0.4154	0.4088	0.4205						
4.3492	0.4049	0.4457	0.4479	0.4420	0.4514						

After linearizing (ln Abs) and arranging the data shown in Table S6 from replicates in only one column, the regression coefficients (μ_{MAX}) ± standard errors for each treatment are calculated using the following equations [32]:

- Regression coefficient or slope b:

$$\hat{Y}_i = a + bX_i$$

$$b = \frac{\sum xy}{\sum x^2} = \frac{sum\ of\ cross\ products}{sum\ of\ squares}$$

- Standard error SE of the slope:

$$SE = \sqrt{\frac{S_{YX}^2}{\sum x^2}}$$

$$S_{YX}^2 = \frac{residual\ SS}{residual\ DF}$$

$$residual\ SS = total\ SS - regression\ SS$$

$$residual\ DF = n - 2$$

Table S2.8 Calculations for testing for significant differences among slopes for the *S. cerevisiae* strain CAT-1.

	$\sum x^2$	$\sum xy$	$\sum y^2$	**residual SS**	**residual DF**
Regression 1	20.2665	5.2454	1.4897	0.13208075	38
Regression 2	2.7027	0.8705	0.6741	0.39374609	18
Pooled regression				0.52582684	56
Common regression	22.9692	6.1159	2.1638	0.53536684	57

The significant differences among slopes (b) of 2 simple linear regression lines are testing using ANCOVA, as described in [32]. For that, the null (H0) and alternative (H1) hypotheses are defined as H0: b1 - b2 = 0 (i.e. $b_{defined} = b_{complex}$), and H1: b1 - b2 ≠ 0 (i.e. $b_{defined} \neq b_{complex}$). The F parameter is calculated as follow:

$$F = \frac{\left(\frac{SS_c - SS_p}{k - 1}\right)}{\frac{SS_p}{DF_p}}$$

Where k = number of regressions; k-1 = numerator degrees of freedom, and DFp = denominator degrees of freedom.

Table S2.9 Summary of the statistical outcome of the F-test for the *S. cerevisiae* strain CAT-1.

	Average μ_{MAX} b_1	**ST Error SE_1**	**Observations n_1**
Defined medium	0.2588	0.0131	40
Complex medium	0.3221	0.0900	20
STATISTICAL COMPARISON			
F-test	**p-value**	**Conclusion α = 0.05**	**Conclusionα = 0.01**
1.016	0.3178	μ1 = μ2	μ1 = μ2

Method B no outliers

Table S2.10 Raw Abs600 data from the exponential phase of growth of *S. cerevisiae* CAT-1 cultivated on either defined or complex medium supplemented with glucose as sole carbon and energy source, using microplate as cultivation system. The crossed out data represent the outliers identified using ROUT option on GraphPad Prism software with Q = 1%.

DEFINED MEDIUM						COMPLEX MEDIUM					
Time (h)	Replicate					Time (h)	Replicate				
	~~1~~	2	3	4	5		~~1~~	2	3	4	5
2.1746	~~0.2192~~	0.2570	0.2620	0.2558	0.2688	2.3016	~~0.4077~~	0.2621	0.2661	0.2943	0.3106
2.4852	~~0.2353~~	0.2743	0.2783	0.2751	0.2861	2.6303	~~0.4395~~	0.2909	0.2963	0.3265	0.3423
2.7959	~~0.2556~~	0.2946	0.2984	0.2942	0.3056	2.9593	~~0.4816~~	0.3305	0.3344	0.3684	0.3822
3.1066	~~0.2802~~	0.3195	0.3223	0.3197	0.3282	3.2880	~~0.5122~~	0.3720	0.3810	0.4108	0.4206
3.4172	~~0.3060~~	0.3462	0.3497	0.3454	0.3565						
3.7279	~~0.3375~~	0.3780	0.3823	0.3778	0.3848						
4.0385	~~0.3720~~	0.4116	0.4154	0.4088	0.4205						
4.3492	~~0.4049~~	0.4457	0.4479	0.4420	0.4514						

Table S2.11 Calculations for testing for significant differences among slopes for the *S. cerevisiae* strain CAT-1 after removal of outliers.

	$\sum x^2$	$\sum xy$	$\sum y^2$	residual SS	residual DF
Regression 1	16.2132	4.0793	1.0338	0.00740682	30
Regression 2	2.1622	0.7428	0.3193	0.06405158	14
Pooled regression				0.07145840	44
Common regression	18.3753	4.8221	1.3530	0.08758911	45

Table S2.12 Summary of the statistical outcome of the F-test for the *S. cerevisiae* strain CAT-1 after removal of outliers.

	Average μ_{MAX} b_1	ST Error SE_1	Observations n_1
Defined medium	0.2516	0.0039	32
Complex medium	0.3436	0.0460	16
STATISTICAL COMPARISON			
F-test	**p-value**	**Conclusion α = 0.05**	**Conclusionα = 0.01**
9.9324	0.0029	μ1 <> μ2	μ1 <> μ2

Statistical outcome of the F-test for cultivation system as treatment

Table S2.13 Comparative statistical analysis, based on method B[1], of the maximum specific growth rates displayed by *S. cerevisiae* CEN.PK113-7D, JP1,and UFMG-CM-Y259 during growth on synthetic medium supplemented with glucose as sole carbon and energy source, using either microplate, shake-flask, or bioreactor as cultivation system.

	CEN.PK113-7D	JP1	UFMG-CM-Y259
MICROPLATE			
Average μ_{MAX} (b_1)	0.2384	0.2077	0.2500
ST Error (SE_1)	0.0084	0.0066	0.0069
Observations (n_1)	45	45	40
SHAKE-FLASK			
Average μ_{MAX} (b_2)	0.3730	0.3323	0.4710
ST Error (SE_2)	0.0421	0.0055	0.0146
Observations (n_2)	12	12	10
BIOREACTOR			
Average μ_{MAX} (b_3)	0.4608	0.4203	0.4316
Error (SE_3)	0.0189	0.0078	0.0099
Observations (n_3)	11	12	15
STATISTICAL COMPARISON			
F-test	29.7900	276.4000	86.6700
p-value	0.0000	0.0000	0.0000
Conclusion ($\alpha = 0.05$)	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$
Conclusion ($\alpha = 0.01$)	$\mu1 <> \mu2$	$\mu1 <> \mu2$	$\mu1 <> \mu2$

Statistical outcome of the F-test for carbon source as treatment

Table S2.14 Comparative statistical analysis, based on method B[1], of the maximum specific growth rates displayed by *S. cerevisiae* CEN.PK113-7D, JP1, PE-2, UFMG-CM-Y257, and UFMG-CM-Y259 during growth on synthetic medium supplemented with glucose, fructose, or sucrose as sole carbon and energy source, using either microplate or shake-flask as cultivation system.

	CEN.PK113-7D	JP1	PE-2	UFMG-CM-Y257	UFMG-CM-Y259
MICROPLATE					
GLUCOSE					
Average μ_{MAX} (b_1)	0.2384	0.2077	0.2313	0.2856	0.2500
ST Error (SE_1)	0.0084	0.0066	0.0030	0.0092	0.0069
Observations (n_1)	45	45	50	35	40
FRUCTOSE					

	CEN.PK113-7D	JP1	PE-2	UFMG-CM-Y257	UFMG-CM-Y259
Average μ_{MAX} (b_2)	0.2403	0.2022	0.2384	0.2556	0.2398
ST Error (SE_2)	0.0031	0.0028	0.0034	0.0070	0.0035
Observations (n_2)	40	40	60	30	50
SUCROSE					
Average μ_{MAX} (b_3)	0.2736	0.2292	0.2304	0.3449	0.3218
ST Error (SE_3)	0.0119	0.0099	0.0036	0.0153	0.0117
Observations (n_3)	40	55	60	30	30
STATISTICAL COMPARISON					
F-test	5.1060	2.9710	1.7440	15.4800	30.1400
p-value	0.0075	0.0546	0.1780	0.0000	0.0000
Conclusion ($\alpha = 0.05$)	μ1 <> μ2	μ1 = μ2	μ1 = μ2	μ1 <> μ2	μ1 <> μ2
Conclusion ($\alpha = 0.01$)	μ1 <> μ2	μ1 = μ2	μ1 = μ2	μ1 <> μ2	μ1 <> μ2
SHAKE-FLASK					
GLUCOSE					
Average μ_{MAX} (b_1)	0.3730	0.3323	0.3550	0.3718	0.4710
ST Error (SE_1)	0.0421	0.0055	0.0065	0.0042	0.0146
Observations (n_1)	12	12	14	16	10
FRUCTOSE					
Average μ_{MAX} (b_2)	0.3582	0.3069	0.3559	0.3733	0.4712
ST Error (SE_2)	0.0041	0.0039	0.0063	0.0047	0.0160
Observations (n_2)	14	14	14	16	10
SUCROSE					
Average μ_{MAX} (b_3)	0.3807	0.3835	0.3540	0.4043	0.3827
ST Error (SE_3)	0.0224	0.0144	0.0056	0.0173	0.0027
Observations (n_3)	18	10	16	10	14
STATISTICAL COMPARISON					
F-test	0.2309	24.2600	0.0250	3.4630	31.8300
p-value	0.7949	0.0000	0.9754	0.0421	0.0000
Conclusion ($\alpha = 0.05$)	μ1 = μ2	μ1 <> μ2	μ1 = μ2	μ1 <> μ2	μ1 <> μ2
Conclusion ($\alpha = 0.01$)	μ1 = μ2	μ1 <> μ2	μ1 = μ2	μ1 = μ2	μ1 <> μ2

[1] Method B consists in the calculation of a single μ_{MAX} from data points of all replicates.

Table S2.15 Cell concentration of *S. cerevisiae* CEN.PK113-7D, JP1, PE-2, UFMG-CM-Y257, and UFMG-CM-Y259 strains during aerobic growth on glucose using shake-flask as a cultivation system. Experimental data is highlighted.

Replicate 1					Replicate 2				
Time (h)	**pH**	**Abs_{600}**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})***	**Time (h)**	**pH**	**Abs_{600}**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})***
CEN.PK113-7D									
0	6.10	0.08		0.05	0	6.00	0.12		0.07
1	6.14	0.09		0.06	1	6.10	0.12		0.07
2	6.11	0.11		0.07	2	6.06	0.15		0.09
3	6.08	0.15		0.09	3	6.04	0.21		0.12
5	6.01	0.26		0.16	5	6.00	0.37		0.21
6	5.98	0.35		0.22	6	5.80	0.62		0.36
7	5.94	0.60		0.38	7	5.73	0.93		0.53
8	5.83	0.85		0.53	8	5.50	1.31		0.75
9	5.55	1.15		0.72	9	5.17	1.77		1.02
10	5.43	1.64		1.03	10	4.92	2.46		1.41
11	5.07	2.50		1.56	11	4.96	2.87		1.65
12	5.00	2.83		1.77	12	5.11	2.97		1.70
13	5.11	2.95		1.85	13	5.23	3.08		1.77
14	5.20	3.04	1.90	1.90	14	5.40	3.18	1.83	1.83
JP1									
0	5.99	0.27		0.17	0	6.01	0.27		0.15
1	5.93	0.34		0.21	1	5.92	0.33		0.19
2	5.83	0.44		0.28	2	5.82	0.44		0.25
3	5.71	0.64		0.40	3	5.78	0.64		0.37
4	5.51	0.87		0.55	4	5.50	0.85		0.49
5	5.35	1.26		0.80	5	5.36	1.28		0.73
6	5.15	1.64		1.03	6	5.16	1.76		1.01
7	5.01	2.21		1.39	7	5.03	2.24		1.28
8	5.22	2.67		1.68	8	5.22	2.68		1.54
9	5.37	2.71	1.71	1.71	9	5.42	2.74	1.57	1.57
PE-2									
0	6.02	0.19		0.10	0	5.99	0.19		0.11
1	5.95	0.21		0.12	1	5.93	0.20		0.12
2	5.92	0.23		0.12	2	5.90	0.25		0.14
3	5.86	0.37		0.20	3	5.83	0.36		0.21
5	5.65	0.82		0.44	5	5.62	0.78		0.45
6	5.63	1.08		0.58	6	5.57	1.10		0.63
7	5.46	1.54		0.83	7	5.45	1.55		0.89
8	5.24	2.08		1.12	8	5.18	2.03		1.17
9	5.13	2.86		1.54	9	5.09	2.99		1.72
10	5.32	3.46		1.86	10	5.30	3.63		2.09
11	5.45	3.71	2.00	2.00	11	5.46	3.67	2.11	2.11
UFMG-CM-Y257									
0	6.05	0.21		0.09	0	6.08	0.21		0.11
1	6.01	0.28		0.13	1	5.99	0.29		0.16
2	5.90	0.43		0.19	2	5.92	0.46		0.25
3	5.81	0.68		0.30	3	5.82	0.61		0.34

Replicate 1					Replicate 2				
Time (h)	**pH**	**Abs$_{600}$**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})***	**Time (h)**	**pH**	**Abs$_{600}$**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})***
4	5.73	0.88		0.39	4	5.72	0.89		0.49
5	5.55	1.40		0.63	5	5.50	1.30		0.72
6	5.27	1.91		0.85	6	5.23	1.89		1.04
7	5.08	2.75		1.23	7	5.00	2.70		1.49
8	5.35	3.03		1.35	8	5.31	2.98		1.64
9		3.26		1.46	9	5.56	3.13		1.73
10	6.04	3.40	1.52	1.52	10	6.03	3.39	1.87	1.87
UFMG-CM-Y259									
0	6.11	0.14		0.09	0	6.11	0.14		0.09
1	6.09	0.17		0.10	1	6.08	0.17		0.12
2	5.88	0.22		0.14	2	5.88	0.23		0.15
3	5.79	0.33		0.21	3	5.84	0.33		0.22
4	5.79	0.51		0.32	4	5.74	0.53		0.35
5	5.48	0.91		0.57	5	5.45	0.90		0.60
6	5.26	1.34		0.85	6	5.27	1.57		1.04
7	4.93	1.93		1.22	7	4.95	2.26		1.50
8	4.91	2.68		1.69	8	4.91	2.85		1.89
9	5.16	3.06		1.93	9	5.02	2.87	1.90	1.90
10	5.54	3.25	2.05	2.05					

Table S2.16 Cell concentration of *S. cerevisiae* CEN.PK113-7D, JP1, PE-2, UFMG-CM-Y257, and UFMG-CM-Y259 strains during aerobic growth on fructose using shake-flask as a cultivation system. Experimental data is highlighted.

Replicate 1					Replicate 2				
Time (h)	**pH**	**Abs$_{600}$**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})**	**Time (h)**	**pH**	**Abs$_{600}$**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})**
CEN.PK113-7D									
0	6.04	0.27		0.21	0	6.10	0.26		0.18
1	6.06	0.27		0.21	1	6.02	0.27		0.19
2	5.97	0.33		0.25	2	5.95	0.34		0.24
3	5.91	0.46		0.35	3	5.87	0.46		0.32
4	5.80	0.66		0.51	4	5.76	0.67		0.47
5	5.68	0.96		0.74	5	5.58	0.97		0.68
6	5.45	1.33		1.02	6	5.42	1.43		1.00
7	5.17	1.94		1.49	7	5.01	2.10		1.47
8	4.87	2.70		2.07	8	4.85	2.85		1.99
9	5.09	3.35		2.57	9	5.15	3.36		2.35
10	5.07	3.43		2.63	10	5.12	3.53		2.47
11	5.45	3.61	2.77	2.77	11	5.65	3.74	2.62	2.62
JP1									
0	6.03	0.23		0.12	0	6.02	0.23		0.11
1	6.01	0.30		0.15	1	5.99	0.29		0.15

Replicate 1					Replicate 2				
Time (h)	**pH**	**Abs_{600}**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})**	**Time (h)**	**pH**	**Abs_{600}**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})**
2	5.88	0.42		0.21	2	5.88	0.42		0.21
3	5.84	0.59		0.29	3	5.82	0.58		0.29
4	5.66	0.80		0.40	4	5.66	0.79		0.39
5	5.60	1.07		0.53	5	5.57	1.06		0.52
6	5.27	1.43		0.71	6	5.26	1.37		0.68
7	5.12	1.90		0.94	7	5.07	1.82	0.90	0.90
8	5.14	2.61		1.29					
9	5.37	2.99	1.48	1.48					
PE-2									
0	6.17	0.20		0.08	0	6.21	0.20		0.12
1	6.10	0.28		0.11	1	6.07	0.27		0.16
2	6.00	0.46		0.19	2	6.09	0.43		0.26
3	6.02	0.58		0.24	3	6.06	0.60		0.36
4	5.90	0.84		0.34	4	5.91	0.81		0.49
5	5.72	1.25		0.51	5	5.76	1.15		0.69
6	5.63	1.70		0.69	6	5.64	1.75		1.06
7	5.50	2.34		0.95	7	5.48	2.27		1.37
8	5.35	3.09		1.25	8	5.31	2.96		1.79
9	5.44	3.54		1.44	9	5.41	3.56		2.15
10	5.42	3.64	1.48	1.48	10	5.35	3.67	2.22	2.22
UFMG-CM-Y257									
0	5.99	0.20		0.12	0	6.00	0.20		0.11
1	5.95	0.23		0.14	1	5.92	0.23		0.13
2	5.85	0.30		0.18	2	5.86	0.29		0.16
3	5.74	0.49		0.30	3	5.75	0.48		0.27
4	5.59	0.71		0.43	4	5.59	0.70		0.39
5	5.48	1.00		0.61	5	5.43	0.99		0.55
6	5.22	1.47		0.90	6	5.25	1.42		0.79
7	4.98	2.17		1.33	7	5.01	2.11		1.18
8	5.12	2.97		1.82	8	5.06	2.95		1.65
9	5.51	3.30		2.03	9	5.48	3.31		1.85
10	5.93	3.47	2.13	2.13	10	5.86	3.52	1.97	1.97
UFMG-CM-Y259									
0	6.12	0.17		0.11	0	6.12	0.18		0.11
1	6.06	0.22		0.14	1	6.06	0.22		0.13
2	5.89	0.30		0.19	2	5.89	0.29		0.18
3	5.87	0.45		0.29	3	5.88	0.42		0.26
4	5.65	0.73		0.47	4	5.66	0.62		0.38
5	5.48	1.22		0.78	5	5.40	1.09		0.67
6	5.12	1.91		1.22	6	5.17	1.91		1.18
7	4.90	2.67		1.71	7	4.86	2.50		1.55
8	5.28	3.14		2.01	8	5.08	3.06		1.89

Replicate 1					Replicate 2				
Time (h)	pH	Abs_{600}	X (g_{DM} l^{-1})	X (g_{DM} l^{-1})	Time (h)	pH	Abs_{600}	X (g_{DM} l^{-1})	X (g_{DM} l^{-1})
9	5.57	3.13	2.00	2.00	9	5.39	3.30	2.04	2.04
					10	5.74	3.6		2.23

Table S2.17 Cell concentration of *S. cerevisiae* CEN.PK113-7D, JP1, PE-2, UFMG-CM-Y257, and UFMG-CM-Y259 strains during aerobic growth on sucrose using shake-flask as a cultivation system. Experimental data is highlighted.

Replicate 1					Replicate 2				
Time (h)	pH	Abs_{600}	X (g_{DM} l^{-1})	X (g_{DM} l^{-1})	Time (h)	pH	Abs_{600}	X (g_{DM} l^{-1})	X (g_{DM} l^{-1})
CEN.PK113-7D									
0	6.09	0.10		0.07	0	6.10	0.08		0.06
1	6.03	0.15		0.10	1	6.06	0.10		0.07
2	5.98	0.20		0.14	2	6.05	0.13		0.09
3	5.91	0.28		0.19	3	5.96	0.18		0.13
4	5.84	0.39		0.27	4	5.95	0.26		0.18
5	5.70	0.65		0.45	5	5.84	0.37		0.26
6	5.62	0.98		0.67	6	5.70	0.59		0.41
7	5.31	1.41		0.97	7	5.62	0.91		0.64
8	5.07	2.02		1.39	8	5.40	1.28		0.90
9	4.92	2.80		1.92	9	5.17	1.84		1.29
10	5.12	3.48		2.39	10	4.95	2.56		1.79
11	5.10	3.56		2.44	11	5.00	3.28		2.30
12	5.05	3.60	2.47	2.47	12	5.18	3.66		2.56
					13	5.02	3.70	2.59	2.59
JP1									
0	6.10	0.19		0.11	0	6.09	0.19		0.12
1	6.05	0.22		0.12	1	6.04	0.22		0.14
2	5.93	0.30		0.16	2	5.93	0.33		0.22
3	5.82	0.46		0.25	3	5.80	0.49		0.32
4	5.68	0.73		0.40	4	5.66	0.67		0.43
5	5.55	1.10		0.61	5	5.54	0.90		0.59
6	5.37	1.43		0.79	6	5.37	1.25		0.82
7	5.24	1.82		1.01	7		1.81		1.18
8	4.99	1.84		1.02	8	4.93	1.86		1.22
9	5.19	3.05	1.69	1.69	9	5.18	2.97	1.94	1.94
PE-2									
0	6.15	0.18		0.11	0	6.07	0.19		0.12
1	6.15	0.24		0.15	1	6.13	0.24		0.15
2	6.06	0.36		0.22	2	6.07	0.41		0.25
3	6.00	0.51		0.30	3	5.97	0.50		0.30
4	5.87	0.71		0.43	4	5.84	0.78		0.48

Replicate 1					Replicate 2				
Time (h)	**pH**	**Abs_{600}**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})**	**Time (h)**	**pH**	**Abs_{600}**	**X (g_{DM} l^{-1})**	**X (g_{DM} l^{-1})**
5	5.72	1.00		0.60	5	5.67	1.09		0.66
6	5.59	1.49		0.90	6	5.53	1.58		0.96
7	5.38	2.14		1.28	7	5.28	2.15		1.32
8	5.28	2.77		1.67	8	5.29	3.09		1.89
9	5.47	3.32		2.00	9	5.40	3.27		2.00
10	5.36	3.46	2.08	2.08	10	5.40	3.55	2.17	2.17
UFMG-CM-Y257									
0	6.11	0.20		0.13	0	6.11	0.21		0.14
1	6.05	0.24		0.16	1	6.04	0.23		0.16
2	5.91	0.38		0.26	2	5.91	0.32		0.21
3	5.75	0.60		0.40	3	5.74	0.53		0.35
4	5.63	0.86		0.58	4	5.58	0.74		0.49
5	5.42	1.27		0.86	5	5.38	1.11		0.74
6	5.13	1.76		1.19	6	5.11	1.62		1.08
7	4.97	2.25		1.53	7	4.93	2.33		1.55
8	5.20	2.30		1.56	8	5.20	2.63		1.75
9	5.58	3.38	2.29	2.29	9	5.58	3.63	2.42	2.42
UFMG-CM-Y259									
0	6.03	0.18		0.10	0	6.04	0.18		0.10
1	5.97	0.22		0.12	1	5.96	0.23		0.13
2	5.86	0.32		0.18	2	5.85	0.34		0.19
3	5.76	0.50		0.28	3	5.74	0.50		0.28
4	5.57	0.70		0.39	4	5.54	0.73		0.42
5	5.38	1.05		0.59	5	5.35	1.04		0.59
6	4.99	1.52		0.85	6	4.99	1.55		0.88
7	4.78	2.18		1.22	7	4.79	2.27		1.29
8	5.098	2.93		1.63	8	5.072	2.85		1.62
9	5.47	3.18		1.77	9	5.48	3.23		1.84
10		3.28	1.83	1.83	10		3.21	1.83	1.83

3

Aerobic growth physiology of *Saccharomyces cerevisiae* on sucrose is strain-dependent

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ABSTRACT

Present knowledge on the quantitative aerobic physiology of the yeast *Saccharomyces cerevisiae* during growth on sucrose as sole carbon and energy source is limited to either adapted cells or to the model laboratory strain CEN.PK113-7D. To broaden our understanding of this matter and open novel opportunities for sucrose-based biotechnological processes, we characterized three strains, with distinct backgrounds, during aerobic batch bioreactor cultivations. Our results reveal that sucrose metabolism in *S. cerevisiae* is a strain-specific trait. Each strain displayed a distinct extracellular hexose concentration and invertase activity profiles. Especially, the inferior maximum specific growth rate (0.21 h^{-1}) of the CEN.PK113-7D strain, with respect to that of strains UFMG-CM-Y259 (0.37 h^{-1}) and JP1 (0.32 h^{-1}), could be associated to its low invertase activity (0.04 to 0.09 U mg_{DM}^{-1}). Moreover, comparative experiments with glucose or fructose alone, or in combination, suggest mixed mechanisms of sucrose utilization by the industrial strain JP1, and points out the remarkable ability of the indigenous strain UFMG-CM-259 to grow faster on sucrose than on glucose in a well-controlled cultivation system. This work hints to a series of metabolic traits that can be exploited to increase the sucrose catabolic rates and process efficiency.

INTRODUCTION

Sucrose has long been used in the food industry as a substrate for the production of bakery goods and beverages. In the last decades, however, it has also been considered a valuable feedstock for the replacement of petrochemical derived materials, due to its low market price [1], [2]. Especially, sucrose feedstocks have a high land efficiency and vast availability of sugar-rich crops. Furthermore, sucrose does not require any pretreatment prior to its use in industrial fermentation. Whilst fuel ethanol is by far the prime product of the nonfood sucrose-based industry [3], with an annual production of 33.1 billion liters in Brazil alone in 2019 [4], the potential use of this disaccharide goes beyond fuel manufacturing, with value-added chemicals such as citric acid [5], [6], lactic acid [7], [8] and farnesene [9] also being successfully commercialized. Several additional chemical intermediates can be produced using sucrose as substrate, including 5-hydroxymethylfurfural [9], [10], 1-2-propylene glycol [10], [11], acrylic acid [9], and succinic acid [9], [11]–[13].

The yeast *Saccharomyces cerevisiae*, the primary workhorse of the biotechnology industry [14]–[16], naturally metabolizes sucrose either through its hydrolysis in the periplasmic space or through direct uptake via active transport of the disaccharide and its hydrolysis in the cytosol [17]. In the first mechanism, *S. cerevisiae* needs to express an invertase-encoding gene (*SUC2*, the most common one) and secrete the protein to the periplasmic space after oligomerization and post-translational modification [18]. The hydrolysis' products, namely glucose and fructose, can enter the cells by facilitated diffusion. In the other mechanism, sucrose is directly transported to the cytosol via a proton-symport mechanism mediated by the high-affinity (K_M = 7.9 ± 0.8 mM) transporter Agt1p or the low-affinity (K_M = 120 ± 20 mM) transporters encoded by *MALx1* genes (x denotes the locus number) [19], [20]. In this case, ATP is invested to expel the imported proton with a stoichiometry of 1:1, in order to keep cell's homeostasis and the proton motive force across the plasma membrane. Consequently, cells are expected to achieve a higher glycolytic rate to compensate for the lower energy efficiency in the overall metabolic process.

Aerobic sucrose metabolism by *S. cerevisiae* has been shown to be fast, with maximum specific growth rates ranging from 0.38 to 0.57 h^{-1} [21], [22], depending on the strain, conditions and analytical methods employed. In spite of this successful relationship between sucrose and *S. cerevisiae* [2], little attention has been given to the specific effects of this carbon source on the quantitative aerobic physiology of this yeast. The few studies involving a comprehensive quantitative aerobic physiological analysis were performed with strains pre-evolved on sucrose for 200 to 250 generations [23]–[25], or with the model laboratory strain CEN.PK113-7D [26], which limits our understanding of relevant phenomena. On the one hand, the use of pre-adapted strains changes their initial/natural physiology. On the other hand, the results published on a non-adapted strain refer to one particular laboratory strain,

meaning that we still do not have a good overview of the physiology of this yeast species during growth on sucrose and how this eventually varies among different strains. Relevant biological questions remain to be elucidated: does the strain background influence *S. cerevisiae*'s physiology on sucrose? Would a non-adapted fast sucrose-growing strain rely on direct uptake as a preferred natural mechanism of sucrose utilization? How does growth on sucrose compare to growth on glucose? A better understanding of *S. cerevisiae*'s growth on sucrose will not only aid in answering those questions but also open novel opportunities for the development of strain improvement strategies to enhance sucrose-based industrial bioprocesses, in terms of the yields, productivities, and/or titers required for the production of market goods in a competitive manner.

Here, we present the quantitative physiology of *S. cerevisiae* grown on sucrose as the sole carbon and energy source in aerobic batch bioreactor cultivations. To access the effects of the strain background on sucrose physiology, the experiments were performed with one laboratory (CEN.PK113-7D), one industrial (JP1) and one wild isolate (UFMG-CM-Y259) strain. While the CEN.PK113-7D strain serves as a reference for physiological studies [22], JP1 and UFMG-CM-Y259 were chosen due to their different behaviors on sucrose [21]. JP1 was isolated from fermenters used to produce fuel ethanol from sugarcane in Northeastern Brazil and is a relatively thermotolerant strain [27], [28], whereas UFMG-CM-Y259 presented the highest maximum specific growth rate (μ_{MAX}) on sucrose in a screening test made with 18 different *S. cerevisiae* strains. Its μ_{MAX} on sucrose was also ~20% higher than the corresponding value on glucose [21].

Additionally, in order to investigate the mechanisms underlying the diverging physiologies observed during growth on sucrose, cultivations were also performed on an equimolar mixture of glucose and fructose and on each one of these two monosaccharides separately.

MATERIAL AND METHODOLOGY

Yeast strains, preservation and pre-cultures

The *Saccharomyces cerevisiae* strains studied in this work comprise an indigenous strain, named UFMG-CM-Y259, isolated from barks of the tree *Quercus rubra* (Northern Red Oak) located in Santuário do Caraça (Minas Gerais, Brazil) within the Atlantic Forest biome [21], kindly provided by Dr. Carlos A. Rosa (Federal University of Minas Gerais, Belo Horizonte, Brazil); JP1, a Brazilian fuel ethanol industrial strain isolated from the Japungu Agroinsdustrial sugarcane-based distillery located in Northeastern Brazil [27], kindly provided by Dr. Marcos Morais Jr. (Federal University of Pernambuco, Recife, Brazil); and the laboratory strain CEN.PK113-7D (kindly provided by Dr. Petter Kötter, EUROSCARF, Germany), which is largely employed in physiological studies by the scientific community [22].

Stock cultures were prepared by growing cells until stationary phase in 500-ml Erlenmeyer flasks containing 100 ml YPD medium (per liter: 10.0 g **Y**east extract, 20.0 g **P**eptone and 20.0 g **D**extrose/Glucose), in an incubator shaker (Certomat BS-1, Braun Biotech International, Berlin, Germany) operating at 30°C and 200 rpm for 24 h. 20% (v/v, final concentration) glycerol was added and 1 ml aliquots were stored in 2-ml cryogenic vials in an ultra-freezer (CryoCube HEF, model F570h-86, Eppendorf, Hamburg, Germany) at -80°C until further use.

The pre-culture medium was prepared according to [29] with a few modifications. The medium consisted of (per liter): 3.0 g KH_2PO_4, 6.6 g K_2SO_4, 0.5 g $MgSO_4.7H_2O$, 2.3 g urea, 1 ml trace elements solution, 1 ml vitamins solution and 10 g_{GLCeq} carbon source (sucrose, glucose, fructose or an equimolar mixture of glucose and fructose). The initial pH of the pre-culture medium was adjusted to 6.0 using 2 mol/l KOH. Sterilization of the pre-culture medium occurred by filtration through 0.22 µm pore membranes (Millex-GV, Merck Millipore, Massachusetts, USA).

To prepare the inoculum for bioreactor cultivations, the content of one stock cryogenic vial was centrifuged at 867 *g* for 4 min, and cells were transferred to a 500-ml Erlenmeyer flask containing 100 ml of the pre-culture medium. The pre-inoculum was left in a shaker (Certomat BS-1, Braun Biotech International) set at 30°C and 200 rpm for 24 h preceding the direct transfer of 1 ml of its content to another shake flask with fresh pre-culture medium. After a second round of 24 h of growth in a shaker, operating at the same settings as before, an aliquot sufficient to start the bioreactor batch cultivation with an optical density of 0.2 at 600 nm was collected and washed. For the washing procedure, cells were centrifuged at room temperature and 3500 *g* for 3 min, the supernatant was discarded and fresh pre-culture medium was added to the cell pellet. This was then vortexed and centrifuged once again. At last, the cells were resuspended with cultivation medium and transferred to a proper flask that allows for an aseptic transfer to the bioreactor.

Bioreactor batch cultivations

A synthetic medium formulated as described in [29] was used in all bioreactor batch cultivations. The medium contains per liter: 3.0 g KH_2PO_4, 5.0 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 1 ml trace elements solution, 1 ml vitamins solution and 20 g (or equivalent in g_{GLCeq}) carbon and energy source ─ sucrose, glucose, fructose or an equimolar mixture of glucose and fructose. The initial pH was adjusted to 5.0 using 4 mol/l NaOH. Sterilization of the medium occurred by filtration through 0.22 µm pore membranes (Millex-GV, Merck Millipore, Massachusetts, USA).

A 2-liter bioreactor (Applikon Biotechnology B.V., Delft, The Netherlands), with a working volume of 1.2 l was used throughout this work. Cells were grown at 30°C and 800 rpm stirring speed. Aeration occurred with compressed air at 0.5 l min^{-1} flow rate using a mass flow controller (Model 58505, Brooks

Instrument, Hatfield, USA). The pH of the broth was controlled at 5.0 by automatic addition of a 0.5 mol/l KOH solution. A 10% (v/v) antifoam C emulsion (Sigma-Aldrich, Missouri, USA) was added manually to the broth upon necessity. Aliquots were collected manually at different sampling times to be analysed for extracellular metabolites concentrations, dry cell mass and invertase activity. For each collected sample, the exact mass withdrawn was determined.

The gas flowing out of the bioreactor had its CO_2 and O_2 molar fractions determined using a gas analyzer device (Rosemount NGA 2000, Emerson Electric Co., Ferguson, USA). Determination of O_2 molar fraction was performed through a paramagnetic detector, while an infrared detector was used to determine CO_2 molar fraction. Bioreactor volumetric rates were calculated taking into account variations in pressure and volume (e.g. due to sampling, base and antifoam addition).

Cultivations were finished when a decrease in the CO_2 molar fraction in the off-gas was observed.

Analytical Methods

Dry cell mass concentration

Cells were harvested and filtered through a 0.45 µm nitrocellulose membrane (SO-Pak filters, HAWP047S0 – Merck Millipore, Massachusetts, USA) that had been previously dried and weighed (m_1). The cell pellet was washed twice with demineralized water. The filter containing the pellet was dried in an oven at 70°C for 48 h and then placed in a desiccator to cool down prior to being weighed (m_2). The cell dry mass (X_{DM}) was calculated by dividing the difference between the filter's mass after and before filtration by the sample volume filtered (V); $X_{DM} = (m_2 – m_1)/V$. The result was expressed in $g_{DM}.l^{-1}$.

Concentration of extracellular metabolites

Sampling for extracellular metabolites followed the procedure described elsewhere [30]. Briefly, a defined volume of broth was rapidly collected in a syringe containing a calculated amount of cold metal beads (-20°C) – enough to cool the collected broth to 1°C –, and filtered through a 0.45 µm PVFD membrane (Millex - HV, Merck Millipore) directly into a tube. All sample tubes containing filtrate were immediately placed on ice and stored at -80°C until analysis.

The concentrations of residual sugars in the filtrate – sucrose, glucose and fructose – were obtained either enzymatically using the K-SURFG kit (Megazyme, Bray, Ireland), following the manufacturer instructions, or by means of ion chromatography coupled with pulsed electrochemical detection at gold electrode (CarboQuad pulse, AgCl reference). The chromatography system was a Dionex ICS – 5000 HPIC system with AS-AP sampler, SP pump (Thermal Sceintific, Waltham, USA) equipped with a Carbopac PA-20 3x150 mm column and Aminotrap 3x30 mm precolumn at 30°C, eluted at 0.5 ml min-

[1] with 5% NaOH 200 mM for 15 min, followed by a cleaning step with 20% sodium acetate solution 0.5 mol/l in 200 mmol/l NaOH for 5 min, and reequilibration for 15 min with Milli-Q water (eluents C and D). The temperature of the detector was kept at 15°C.

The concentrations of ethanol, glycerol and organic acids (lactate, succinate and acetate) were always determined by high performance liquid chromatography (HPLC) using a Bio-Rad Aminex HPX-87H column (Bio-Rad, USA), kept at 60°C and eluted with highly diluted phosphoric acid (60 ml min^{-1}) at a pH between 2 and 3, which was preheated before use. The samples were injected using an autosampler (Waters 717, USA). Detection of organic acids was performed via a UV detector (Waters 2489), while detection of ethanol or glycerol was performed using a refraction index detector (Waters 2414, USA). During cultivations without sucrose, residual glucose and fructose were determined in the same HPLC run used for the other metabolites, and the detection of these hexoses was performed using a refraction index detector (Waters 2414, USA). HPLC data were processed using Empower software (Waters Corporation, Milford, USA).

Extracellular invertase activity

Extracellular (or periplasmic) invertase activity was determined according to the approach described in [31], with a few modifications. Briefly, cells were centrifuged and resuspended in distilled water such that a 20 $g_{DM}.l^{-1}$ suspension was obtained. Next, the cells were treated with Succinate-Tris buffer (pH 5.0) containing sodium fluoride, which is an inhibitor of enolase. A sucrose solution was added to the reaction mixture and the glucose formed at 30°C due to the disaccharide hydrolysis was measured using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany). Invertase activity was reported as μmol of glucose produced per minute per milligram of cell dry mass (U mg_{DM}^{-1}).

Calculation of physiological parameters

Prior to calculating physiological parameters, the experimental data points were treated as follows. Extracellular metabolites concentrations and CO_2 and O_2 amounts were plotted against time and a polynomial was fitted to the data. Concentrations or amounts were calculated for each time point taking the polynomial equation. Glucose and fructose concentrations during experiments performed with either sucrose or an equimolar mixture of glucose and fructose were not adjusted to avoid concealing their actual consumption trend. Only data points within the exponential growth phase (EGP) were considered for the calculation of physiological parameters.

The specific growth rate during the EGP (μ_{MAX}) was calculated as the slope of the straight line adjusted to the linear region of an $\ln(X_{DM})$ *versus* time plot. The time span corresponding to this linear region was considered to be the EGP. Biomass ($Y_{X/S}$) and product yield ($Y_{P/S}$) on substrate, except for CO_2, were calculated as the absolute value of the slope of a biomass concentration (X_{DM}) *versus* substrate

concentration (S) plot, and of a product concentration (P) *versus* substrate concentration (S) plot, respectively. CO_2 yield on substrate was derived from the absolute value of the slope of an integrated CO_2 amount versus substrate amount plot. For cultivations with sucrose or a mixture of glucose and fructose, substrate concentration (S) was determined as the sum of the concentrations of all three carbohydrates in $g_{GLGeq}\,l^{-1}$. For that, 1 g of sucrose was considered to be equivalent to 1.0526 g of hexose.

The maximum specific substrate consumption rate (during the EGP) was calculated as the ratio between the maximum specific growth rate and the biomass yield on substrate; $q_{S,MAX} = \mu_{MAX}/Y_{X/S}$.

The maximum specific product formation rate (q_P) was calculated taking the ratio between the desired yield on substrate and biomass yield on substrate, and multiplying the result by the maximum specific growth rate; $q_{P,MAX} = \mu_{MAX} * Y_{P/S}/Y_{X/S}$.

For determining the specific oxygen consumption rate (q_{O2}), the integrated amount in mmol of oxygen consumed per gram of substrate consumed at each sampling time was first obtained by the absolute slope of the integrated O_2 *versus* substrate amount plot. Following, this value was divided by the biomass yield on substrate and multiplied by the maximum specific growth rate.

Calculation of the percentage of sugar metabolized via the fermentation pathway during the EGP

The specific rate of ethanol formation (q_{ETH}) was used to calculate the specific rate of CO_2 formed due to fermentation (q_{CO2_ferm}) in the EGP. This value was divided by 2 and considered to be the specific rate of sugar that was fermented (e.g. q_{GLC_ferm}), as a result of the stoichiometry of ethanolic fermentation ($C_6H_{12}O_6 \rightarrow 2\ C_2H_6O + 2\ CO_2$). This value, in turn, was divided by the (total) maximum specific substrate consumption rate (e.g. $q_{GLC,MAX}$) and multiplied by 100, resulting in the % fermented sugar.

RESULTS AND DISCUSSION

S. cerevisiae strains from different environments display different physiologies on sucrose

The physiology of the *S. cerevisiae* strains CEN.PK113-7D, UFMG-CM-Y259 and JP1 during growth on sucrose was assessed in controlled batch aerobic bioreactor cultivations (**Figures 3.1 and 3.2; Table 3.1; Supplementary Figures S3.1-S3.5, Supplementary Tables S3.1-S3.3**). The laboratory strain CEN.PK113-7D displayed the lowest maximum specific growth rate on sucrose (μ_{MAX} = 0.21 ± 0.01 h^{-1}), which is 56.8 % and 65.6 % of the corresponding rates presented by the indigenous strain UFMG-CM-Y259 and the industrial strain JP1, respectively (**Table 3.1**). Strain CEN.PK113-7D was also found to present the lowest specific substrate consumption rate (q_{SMAX} = -8.23 ± 0.37 $mmol_{GLCeq}$

g_{DM}^{-1} h^{-1}), when compared to the values displayed by UFMG-CM-Y259 (q_{SMAX} = -12.84 ± 0.21 $mmol_{GLCeq}$ g_{DM}^{-1} h^{-1}) and by JP1 (q_{SMAX} = -12.29 ± 0.12 $mmol_{GLCeq}$ g_{DM}^{-1} h^{-1}).

Accordingly, the relative amount of substrate metabolized through the fermentative pathway was higher for UFMG-CM-Y259 and JP1 strains (~ 80%; **Supplementary Figure S3.1**) than that of CEN.PK113-7D (~ 70%; **Supplementary Figure S3.1**). This could also be deduced by the respiratory quotient (**Table 3.1**). Allied to such observations, the ethanol yield on substrate was lower in the laboratory strain ($Y_{E/S}$ = 0.36 ± 0.01 g g_{GLCeq}^{-1}), as compared to the indigenous ($Y_{E/S}$ = 0.41 ± 0.01 g g_{GLCeq}^{-1}) and the industrial strain ($Y_{E/S}$ = 0.41 ± 0.00 g g_{GLCeq}^{-1}).

One of the main traits of the yeast *Saccharomyces cerevisiae* is its capability to perform aerobic fermentation [32]; in other words, even in the presence of oxygen, a higher relative amount of the substrate is subjected to fermentative catabolism rather than respiratory. A phenomenon that constrains the use of the respiratory pathway in yeast under circumstances of high sugar concentrations, as imposed in batch mode, is the so-called glucose repression (or carbon catabolite). High concentrations of this sugar trigger a signaling cascade that will culminate in the repression of the transcription of genes encoding for components of the electron transport chain and other respiratory proteins. [33]–[36]. This strategy results in an energetically less efficient metabolism as the ATP yield from fermentation is lower than that from respiration; for instance, in *S. cerevisiae* approximately 18 ATP are produced per mole of glucose via respiration against only 2 through fermentation [37], [38]. A few hypotheses have been proposed to explain how cells overcome the lower ATP yield from fermentation [39]–[41]. The so-called rate/yield trade-off hypothesis (RYT), for instance, proposes that the cells accelerate growth to rise energy production rates [39]. Through another perspective, the use of fermentation allows for a higher ATP yield per protein mass as compared to respiration [40]. Our data somehow fit with these theories, in the sense that the lower growth rate of CEN.PK113-7D on sucrose, as compared to the other two strains, correlates with a lower relative amount of substrate channeled to fermentation in this laboratory strain.

The different ways in which the three yeast strains investigated here consume sucrose is likely to reflect the ecological niche of each one individually, at least partially. Strain JP1 was isolated from a sugarcane-based distillery where sugarcane juice (rather than molasses, which is not as sucrose-rich) is used to prepare the fermentation medium [27], meaning that it is adapted to an anaerobic environment – in which fermentative metabolism is the only option – and to excess sucrose. On the other hand, CEN.PK113-7D is commonly employed in fundamental laboratory research on physiology [22], where glucose is the major substrate. The long-term exposure of a microbe to a specific environment may alter its regulatory mechanisms, as well as cause adaptation, strategies used to cope with stress and to enhance fitness. In this sense, JP1 holds great advantage over the CEN.PK113-7D strain when sucrose is the

carbon and energy source, due to its primary extensive contact with this sugar, which may have changed sucrose regulation in this lineage, or triggered mutations in genes encoding proteins that are essential to sucrose metabolism, resulting in an improved phenotype.

Furthermore, whether CEN.PK113-7D cells could evolve on sucrose and achieve a fitness comparable (or superior) to that of JP1 or UFMG-CM-Y259, remains to be explored. Previous laboratory evolution studies have demonstrated the potential of *S. cerevisiae* CEN.PK113-7D cells to improve its phenotype for maltose [42], galactose [43] and mixed-substrate consumption [44]. Especially when sucrose was the substrate of choice, evolved engineered *S. cerevisiae* strains were shown to increase sucrose transport capacity [19], [45]. Concerning the UFMG-CM-Y259 strain, it was originally isolated from the barks of a tree that was brought by Europeans to Brazil, namely *Quercus rubra* (Northern Red Oak tree) [21]. The characteristics of this niche are largely unknown. However, sucrose has been found in the barks of several other *Quercus* species from where *Saccharomyces* yeasts, including *S. cerevisiae*, have been isolated [46], suggesting that this disaccharide might also be present in Northern red oak tree's bark. Also, another study showed that a yeast strain isolated from an oak tree performed better than Ethanol Red (a widespread industrial strain used mainly in fuel ethanol production from corn) under mimicked industrial conditions in the laboratory [47].

Taken together, these observations led us to believe that the historical background plays a key role on the enhanced growth and sucrose consumption displayed by JP1 and UFMG-CM-Y259, over CEN.PK113-7D cells.

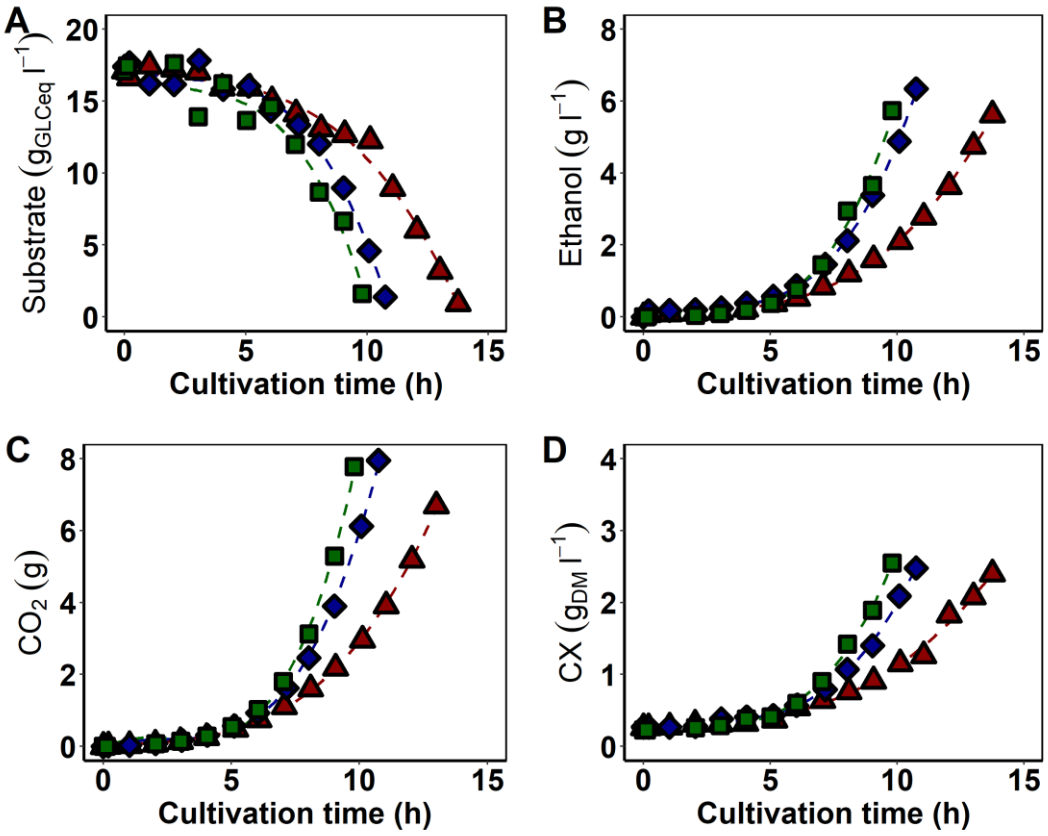

Figure 3.1 Substrate and metabolites concentrations/amounts during aerobic batch cultivation of *S. cerevisiae* CEN.PK113-7D (▲), JP1 (♦), and UFMG-CM-Y259 (■) cells with sucrose as sole carbon and energy source. Substrate represents the sum of sucrose, glucose and fructose concentrations. Dashed lines represent trend lines. One representative dataset of duplicate independent experiments is shown.

Table 3.1 Relevant physiological parameters of *S. cerevisiae* CEN.PK113-7D, UFMG-CM-Y259, and JP1 during aerobic batch cultivations with either sucrose or a glucose/fructose mixture as sole carbon and energy source. All parameters were calculated for the exponential growth phase. Residual substrate refers to the remaining concentration of substrate at the end of the experiment. The data represent the mean of two experiments and the average deviation.

	CEN.PK113-7D		**UFMG-CM-Y259**		**JP1**	
	Sucrose	**Glucose & Fructose**	**Sucrose**	**Glucose & Fructose**	**Sucrose**	**Glucose & Fructose**
μ_{MAX} (h^{-1})	0.21 ± 0.01	0.26 ± 0.01	0.37 ± 0.01	0.35 ± 0.03	0.32 ± 0.00	0.30 ± 0.02
$Y_{X/S}$ (g_{DM} $gGLC_{eq}^{-1}$)	0.14 ± 0.01	0.12 ± 0.00	0.16 ± 0.01	0.16 ± 0.01	0.14 ± 0.00	0.17 ± 0.01
$Y_{Ethanol/S}$ (g $gGLC_{eq}^{-1}$)	0.36 ± 0.01	0.37 ± 0.00	0.41 ± 0.01	0.38 ± 0.00	0.41 ± 0.00	0.44 ± 0.02
qS_{MAX} ($mmolGLC_{eq}$ g_{DM}^{-1} h^{-1})	-8.23 ± 0.37	-11.96 ± 0.02	-12.84 ± 0.21	-12.19 ± 0.63	-12.29 ± 0.12	-10.08 ± 0.39
qCO_2 (mmol g_{DM}^{-1} h^{-1})	14.48 ± 0.22	19.48 ± 0.08	22.50 ± 2.00	20.96 ± 1.55	21.74 ± 0.19	17.53 ± 1.80
qO_2 (mmol g_{DM}^{-1} h^{-1})	-2.96 ± 0.16	-2.56 ± 0.38	-3.34 ± 0.21	-2.88 ± 0.18	-2.19 ± 0.32	-2.15 ± 0.31
RQ ($mmolCO_2$ $mmolO_2^{-1}$)	4.91 ± 0.34	7.78 ± 1.19	6.81 ± 1.03	7.34 ± 1.00	10.13 ± 1.40	8.19 ± 0.33
Residual substrate ($gGLC_{eq}$ l^{-1})	0.10 ± 0.74	0.37 ± 0.94	1.03 ± 0.84	0.91 ± 0.40	1.79 ± 0.15	0.90 ± 0.11
Carbon recovery (%)	94.91 ± 2.86	93.75 ± 0.95	104.55 ± 2.95	98.80 ± 1.74	101.59 ± 0.11	107.25 ± 5.47
Electron balance (%)	95.35 ± 3.05	93.61 ± 0.44	107.25 ± 0.76	99.09 ± 0.96	102.13 ± 0.49	110.76 ± 5.34

During cultivations of *S. cerevisiae* on sucrose, higher glucose and fructose accumulation seem to correlate with higher growth rates

The sugar consumption profiles during aerobic growth on sucrose of the three strains reveal that the accumulation of the released monosaccharides in the cultivation broth occurs to different extents (**Figure 3.2**). The highest concentrations, 6.0 $g_{GLCeq}.l^{-1}$ of glucose and 6.1 $g_{GLCeq}.l^{-1}$ of fructose, were observed for the industrial strain JP1. For UFMG-CM-Y259, the levels of extracellular glucose and fructose reached 3.2 $g_{GLCeq}.l^{-1}$ and 3.3 $g_{GLCeq}.l^{-1}$, respectively. On the other hand, the accumulation of hexoses was minimal with the laboratory strain CEN.PK113-7D and did not surpass 0.69 g_{GLCeq} l^{-1} and 0.91 g_{GLCeq} l^{-1} of glucose and fructose, respectively. Since this was the strain with the lowest maximum specific growth rate on sucrose (**Table 3.1**), we speculate that hexose accumulation during growth on this disaccharide contributes to faster growth. This, in turn, could be caused by the regulatory mechanisms related to the extracellular glucose concentration – such as the repression of the transcription of respiratory-related genes, which is known to have pleiotropic effects on yeast's metabolism [35], [48]–[50].

Moreover, as the accumulation of either glucose or fructose in the cultivation broth is a result of the difference between invertase activity and hexose transport rate, the observed different profiles could indicate that (1) hexose transport occurs at different rates in the different strains and/or (2) the enzyme invertase has different activity in each strain.

The existence of low- and high-affinity hexose transporter systems in the yeast *S. cerevisiae* has been well documented and the expression of these proteins depends on the levels of their substrates in the environment [51]–[54]. For low-affinity hexose transporters, such as Hxt1p and Hxt3p, the K_M for glucose ranges from 15 to 20 mM, while the $K_{M(glucose)}$ of high-affinity hexose transporters, for instance Hxt2p and Hxt6p, is in the range of 1 to 2 mM [53]. Thus, one could assume that the cultivations on sucrose performed in this work triggered the expression of hexose transporters that differ from case to case, according to the concentration of glucose or fructose present in the broth. For instance, with the CEN.PK113-7D strain, for which the lowest levels of hexoses were observed (**Figure 3.2**), it is expected that high-affinity transporters are expressed. In a similar fashion, during cultivations with the UFMG-CM-Y259 or JP1 strains, low-affinity hexose transporters might be present. Transporter affinity and maximum velocity (V_{max}) of these transport systems are inversely correlated in *S. cerevisiae* [55]. This means that a high-affinity transporter system displays lower V_{max} as compared to low-affinity, high-rate transporter systems. The hypothesis that hexose transport occurs at different rates in the three scenarios investigated here is, therefore, supported by these previously described properties of hexose transporters in *S. cerevisiae*.

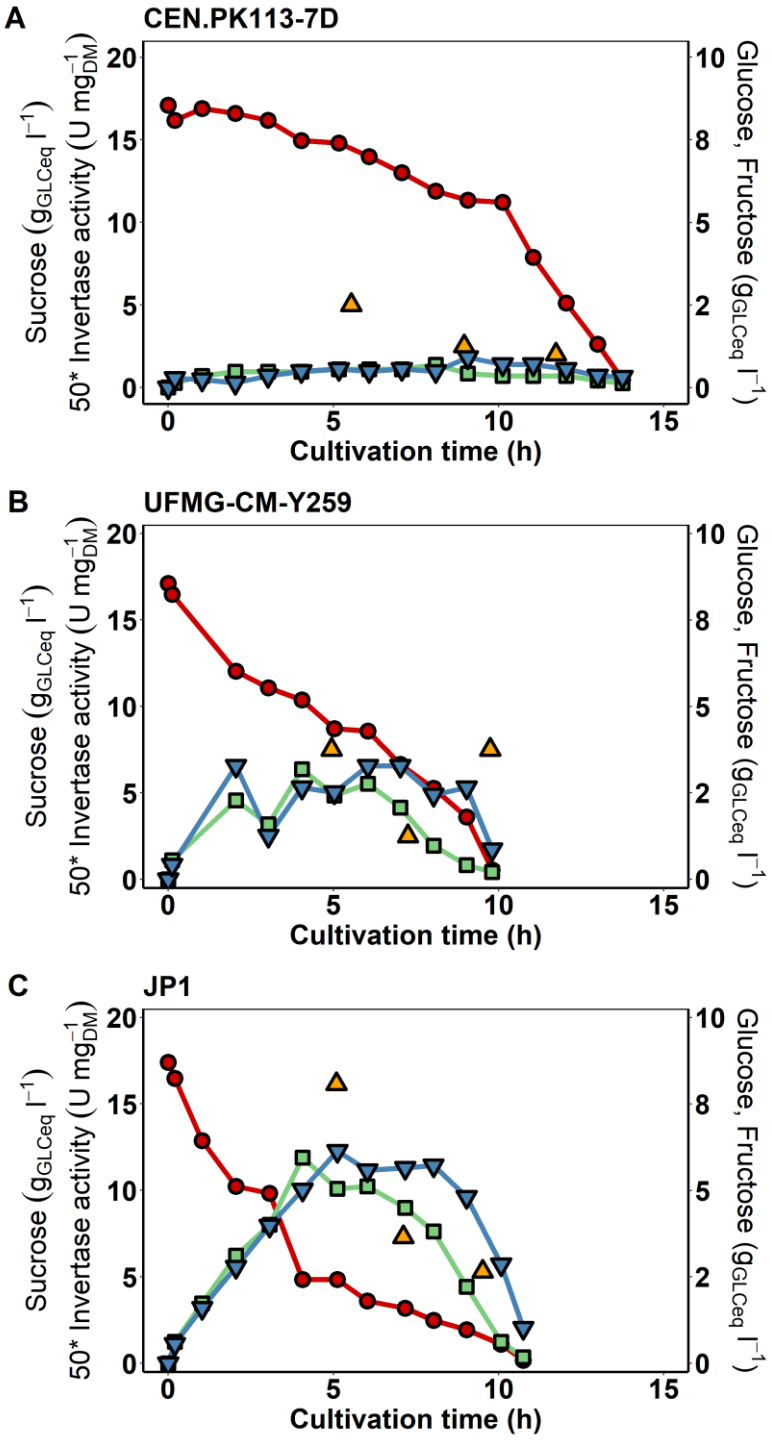

Figure 3.2. Sugar concentrations and periplasmic invertase activity during aerobic batch cultivation of *S. cerevisiae* CEN.PK113-7D (A), UFMG-CM-Y259 (B) and JP1 (C) cells on synthetic medium with sucrose as sole carbon and energy source. Sucrose (●); glucose (■); fructose (*closed* ▽); invertase (▲). Experiments were performed in duplicate. One representative dataset of duplicate independent experiments is shown.

Invertase activity might constrain *S. cerevisiae* CEN.PK113-7D's growth rate on sucrose

To investigate whether invertase activity contributes to the different hexose accumulation levels in the broth, we determined the specific activity of the periplasmic form of the enzyme in each of the three strains investigated here, during cultivations on sucrose, in the beginning, mid, and late exponential phase of growth.

In all samples, the periplasmic invertase of *S. cerevisiae* CEN.PK113-7D displays lower biomass specific activity, when compared to the UFMG-CM-Y259 and JP1 strains (**Figure 3.2**). The biomass specific periplasmic invertase activity achieved with the laboratory strain ranged from 0.04 ± 0.00 to 0.09 ± 0.00 U mg_{DM}^{-1}. In a previous study, Herwig and colleagues [26] reported biomass specific total invertase activity within 0.2 to 1.0 U mg_{DM}^{-1}, approximately, for the *S. cerevisiae* CEN.PK113-7D strain cultivated in aerobic batch bioreactors at 30°C and pH 5.0, with sucrose as sole carbon and energy source. For this particular strain, it is known that the cytoplasmic invertase displays much lower activity compared to that of the periplasmic invertase [19], [56], therefore the specific activity of the periplasmic form of this enzyme can be assumed to correspond roughly to total invertase activity. Hence, a remarkable difference of one order of magnitude can be observed between the above mentioned study and our data, obtained under similar conditions. This was accompanied by approximately 10 times higher levels of glucose and fructose in the broth observed by those authors, which is probably a consequence of the higher invertase activity achieved in their study. It is worth noticing that while our pre-cultivation was carried out with sucrose as sole carbon and energy source, their pre-culture was grown on glucose. This shift in the substrate from glucose in the pre-cultivation to sucrose in the cultivation itself probably explains the higher invertase activity observed by Herwig and colleagues, and is presumably a consequence of the cells being exposed to a sudden need for invertase.

The inferior biomass specific invertase activity combined with the lower levels of hexose accumulation in the broth (**Figure 3.2**) and the lower specific substrate consumption rate (**Table 3.1**), suggests that sucrose hydrolysis is a constraint for CEN.PK113-7D's growth on this carbon source. The two forms of the enzyme invertase (E.C. 3.2.1.2.6) present in *S. cerevisiae*'s cells are translated from genes of the *SUC* family that contains a total of nine structural genes (*SUC1-SUC5*, *SUC7-SUC10*) located in distinct loci of several chromosomes [57]–[59]. The *SUC2* gene, located in the subtelomeric region of chromosome IX [58], [59], is found in all strains of the *Saccharomyces cerevisiae* species, as well as in other yeasts from the same genus [58]. The observed differences in biomass specific invertase activity among the strains here analyzed could be due to mutations in the coding sequence for the *SUC2* gene of the UFMG-CM-Y259 and JP1 strains, as discussed above.

The enhanced glycolytic rate of *S. cerevisiae* JP1 on sucrose might be due to a combined mechanism of sucrose utilization

To elucidate whether the mechanism of sucrose utilization is responsible for the distinct substrate uptake rates observed in the sucrose-experiments, we carried out, under identical operational conditions, a physiological analysis of the studied strains on an equimolar mixture of glucose and fructose (**Figure 3.3; Table 3.1; Supplementary Figures S3.1-S3.5, Supplementary Tables S3.1-S3.3**). We hypothesized that if sucrose was being utilized exclusively via periplasmic hydrolysis, no difference in sugar uptake would be observed between the sucrose and the glucose/fructose mixture growth conditions, as long as no limitations in the hydrolysis step occurred.

Furthermore, since the metabolism of sucrose in the yeast *S. cerevisiae* differs from that of its monomers (or a mixture of them) only in the step of disaccharide breakdown, when compared to growth on glucose and/or fructose, it can be speculated that active transport of sucrose accelerates the sugar uptake rate, and, consequently, enhance the growth rate observed on the disaccharide. In fact, Barford and co-workers [24], [60] demonstrated that the superior growth of *S. cerevisiae* 248 UNSW 703100 – fully adapted to the culture medium (20 - 250 generations) – on sucrose, in comparison to a mixture of its monomers, was due to the direct uptake of sucrose molecules by actively growing yeast cells, which is faster than the passive transport of hexoses.

From our experiments, *S. cerevisiae* CEN.PK113-7D cells were more efficient in consuming the hexoses when they were provided directly ($q_{SMAX\_sucrose}$ = -8.23 ± 0.37 $mmol_{GLCeq}\ g_{DM}^{-1}\ h^{-1}$, q_{SMAX_G+F} = -11.96 ± 0.02 $mmol_{GLCeq}\ g_{DM}^{-1}\ h^{-1}$; **Table 3.1; Supplementary Table S3.1**); the opposite was observed with the industrial strain, JP1 ($q_{SMAX\_sucrose}$ = -12.29 ± 0.12 $mmol_{GLCeq}\ g_{DM}^{-1}\ h^{-1}$, q_{SMAX_G+F} = -10.08 ± 0.39 $mmol_{GLCeq}\ g_{DM}^{-1}\ h^{-1}$; **Table 3.1; Supplementary Table S3.3**), whereas the UFMG-CM-Y259 strain displayed equivalent sugar uptake rates for the sucrose experiment, as compared to the one with the glucose/fructose mixture ($q_{SMAX\_sucrose}$ = -12.84 ± 0.21 $mmol_{GLCeq}\ g_{DM}^{-1}\ h^{-1}$, q_{SMAX_G+F} = -12.19 ± 0.63 $mmol_{GLCeq}\ g_{DM}^{-1}\ h^{-1}$; **Table 3.1; Supplementary Table S3.2**).

The observation of immediate glucose and fructose formation in the broth as well as the measured biomass specific periplasmic invertase activity indicate that extracellular hydrolysis of sucrose is a mechanism of sucrose utilization in all experiments carried out on sucrose alone. However, the hypothesis that sucrose is not being actively transported likely holds true for the yeast strains UFMG-CM-Y259 and CEN.PK113-7D. In the latter case, as discussed above, there is evidence for a growth limitation caused by insufficient invertase activity. Because sucrose-grown JP1 cells displayed enhanced glycolytic rates, when compared to cells cultivated on the glucose/fructose mixture – which is evidenced both by the specific rates of substrate consumption and by the specific rates of products formation (**Table**

3.1; Supplementary Table S3.3) –, we believe that a combined mechanism of sucrose utilization is likely to take place when JP1 cells grow on sucrose alone. In other words, both periplasmic hydrolysis by invertase and active transport of sucrose occur in parallel.

This assumption is supported by a previous work from Barford at al. [61], in which a combination of direct sucrose uptake and extracellular hydrolysis with subsequent transport of its monomers was proposed to explain the higher glycolytic rate of the evolved *S. cerevisiae* 248 UNSW 703100 strain grown on sucrose, as compared to an equimolar mixture of the hexoses. Moreover, recently, Prado and co-workers [62] argued that a mixed mode of sucrose utilization by the *S. cerevisiae* strain LBGA-01 was responsible for its improved performance at high temperature.

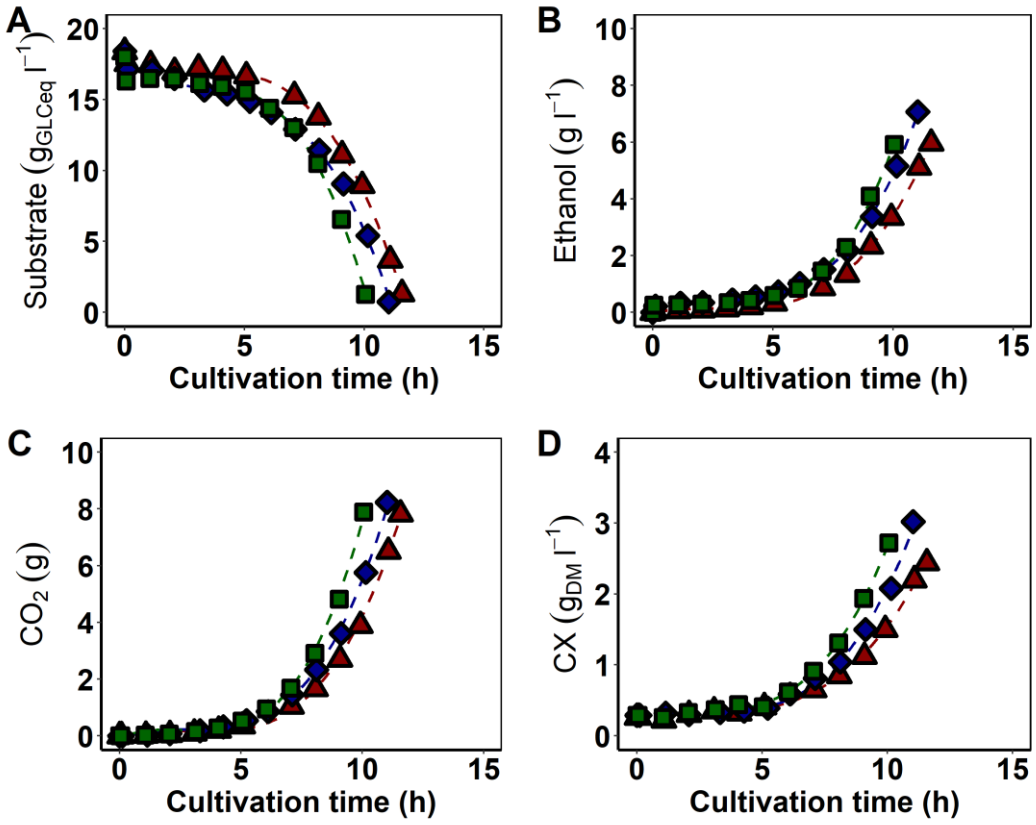

Figure 3.3 Substrate and metabolites concentrations/amounts during aerobic batch cultivation of *S. cerevisiae* CEN.PK113-7D (▲), JP1 (♦), and UFMG-CM-Y259 (■) cells with an equimolar mixture of glucose and fructose as sole carbon and energy source. Substrate represent the sum of glucose and fructose concentrations. Dashed lines represent trend lines. One representative dataset of duplicate independent experiments is shown.

Sucrose-grown *S. cerevisiae* UFMG-CM-Y259 cells display higher maximum specific growth rate than glucose-grown cells

A previous study from our group [21] revealed the capacity of some *S. cerevisiae* strains, including UFMG-CM-Y259, to grow faster on sucrose than on glucose through experiments carried out using microtiter plates as a cultivation system and optical density measurements for assessing cell concentration. As the calculation of the maximum specific growth rate can lead to different values depending on e.g. the cultivation system and the cell concentration measurements used [63], we sought to investigate whether such observations would hold in a well-controlled cultivation system, i.e. a bioreactor combined with direct cell concentration measurements via dry mass determinations. We performed cultivations of the three *S. cerevisiae* strains on glucose as the sole carbon and energy source **(Figure 3.4; Table 3.2; Supplementary Figures S3.1-S3.5, Supplementary Tables S3.1-S3.3)**, otherwise under identical conditions as the cultivations hitherto discussed. The UFMG-CM-Y259 strain displayed higher maximum specific growth rate on sucrose (μ_{MAX} = 0.37 ± 0.01 h^{-1}; **Table 3.1; Supplementary Table S3.2**) than on glucose (μ_{MAX} = 0.29 ± 0.00 h^{-1}; **Table 3.2; Supplementary Table S3.2**), which corroborates the aforementioned study. Results for the JP1 strain from both studies are also in agreement, in the sense that the strain presented a slightly higher μ_{MAX} on sucrose than on glucose. For the CEN.PK113-7D strain, while the results from the previous study indicated no statistical difference between μ_{MAX} on sucrose and on glucose, here the value on sucrose (0.21 h^{-1}) was lower than on glucose (0.31 h^{-1}).

In principle, the maximum specific growth rate on sucrose is not expected to exceed that on glucose because the yeast *Saccharomyces cerevisiae* is subjected to glucose repression [71], meaning that any carbon and energy source different from glucose eventually present in the medium will have their consumption delayed, as long as glucose is available. This preference mechanism suggests that the microbe will display a higher specific growth rate on glucose than on any other carbon and energy source. As previously discussed in this work, direct sucrose uptake could explain enhanced growth rates on this disaccharide over related carbon sources. Alongside the superior growth rate, we observed a slightly higher substrate uptake rate during sucrose cultivation ($q_{SMAX\_sucrose}$ = -12.84 ± 0.21 $mmol_{GLCeq}$ g_{DM}^{-1} h^{-1}; **Table 3.1; Supplementary Table S3.2**), as compared to growth on glucose alone ($q_{SMAX\_glucose}$ = -11.96 ± 0.43 $mmol_{GLCeq}$ g_{DM}^{-1} h^{-1}; **Table 3.2; Supplementary Table S3.2**) for the yeast UFMG-CM-Y259. The remaining physiological parameters were equivalent in both growth conditions.

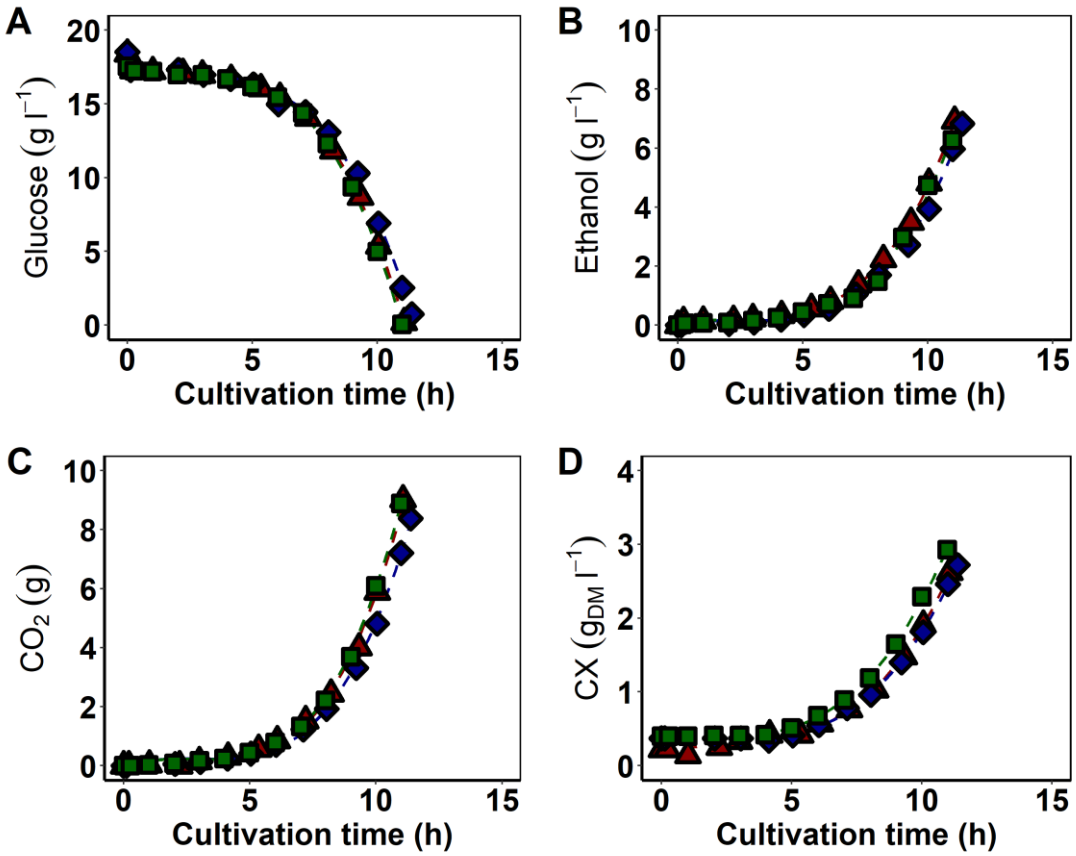

Figure 3.4 Substrate and metabolites concentrations/amounts during aerobic batch cultivation of *S. cerevisiae* CEN.PK113-7D (▲), JP1 (♦), and UFMG-CM-Y259 (■) cells with glucose as sole carbon and energy source. Dashed lines represent trend lines. One representative dataset of duplicate independent experiments is shown.

Through another perspective, glucose and sucrose differently impact signaling cascades in the cells, such as the one leading to the regulation of the protein kinase A (PKA) activity. A theory related to the PKA signaling cascade has also been proposed to explain high specific growth rates of *S. cerevisiae* on sucrose [2], [17]. This cascade is activated when the Gpr1-Gpa2 coupled receptor senses either glucose or sucrose, with the affinity of Gpr1p being higher for the latter sugar [65]. Once activated, the PKA protein can exert all its function, including regulating the synthesis and degradation of storage carbohydrates [66]–[68], the metabolic flux through the glycolytic pathway [69], [70], and gluconeogenesis [71].

To this point, the mechanisms behind faster growth on sucrose over glucose remains an open question. Further investigation, for instance by means of systems biology approaches, is needed to elucidate sucrose regulation in the yeast *S. cerevisiae*.

S. cerevisiae UFMG-CM-Y259 and JP1 display similar physiology during growth on sucrose or on fructose

At last, we performed aerobic batch cultivations with fructose as the sole carbon and energy source (**Figure 3.5; Table 3.2; Supplementary Figures S3.1-S3.5, Supplementary Tables S3.1-S3.3**), again otherwise under identical conditions when compared to all other cultivations described in this work. The physiological parameters were compared with those obtained from sucrose cultivations. The maximum specific growth rate between cultivations on the monosaccharide or on the disaccharide differed only for the CEN.PK113-7D strain, that grew faster when cultivated on the hexose ($\mu_{max\_Fructose} = 0.32 \pm 0.01$ h^{-1} and $\mu_{max\_Sucrose} = 0.21 \pm 0.01$ h^{-1}). The industrial (JP1) and the indigenous (UFMG-CM-Y259) strains' physiologies were equivalent when the carbon and energy sources sucrose or fructose are compared. Interestingly, the UFMG-CM-Y259 strain's physiology on fructose was more similar to that on sucrose than to the performance on glucose, contrasting the observations for the CEN.PK113-7D strain. This suggests that even the regulatory mechanisms triggered by hexoses in the yeast *S. cerevisiae* are strain-dependent.

Considering only glucose and fructose, transport has been pinpointed as a critical step for the different behaviors observed in the metabolism of such hexoses in *S. cerevisiae* [72], [73]. Among all the 20 proteins that constitute the group of hexose transporters and glucose sensors present in this microbe [52], [53], Hxt1p to Hxt7p are the main ones in the context of glucose and/or fructose catabolism [52], [73]–[76]. Expression of these proteins is strain dependent [75], [77] and their substrate affinities vary from one transporter to the other [54]. Enhanced fructose fermentation capacity has been demonstrated to be associated to the Hxt3p transporter [74]. In the latter study [74], the molecular basis behind a higher fructose utilization capacity displayed by the commercial wine *S. cerevisiae* Fermichamp was revealed, in comparison to that of standard *S. cerevisiae* wine strains, namely mutations in the *HXT3* DNA sequence were responsible for such phenotype.

Besides the transport step, hexose phosphorylation could also contribute to the contrasting growth physiologies exhibited by the UFMG-CM-Y259 strain on glucose or fructose, since it is the first step in yeast glycolysis. *In vitro* measurements have shown that the proteins hexokinase 1 and 2 target both monosaccharides (glucose or fructose), with lower affinity and higher relative maximum velocity of reaction (V_{max}) for fructose [78]. Glucokinase, on the other hand, is insensitive to fructose [78]. Among these isozymes, hexokinase 2 is the major kinase in this glycolytic step, as its absence dramatically changes *S. cerevisiae*'s physiology [79]. It is noteworthy that while the conversion of fructose into fructose-6-phosphate takes only one step, to convert glucose into the same metabolite two steps are required (phosphorylation and isomerization).

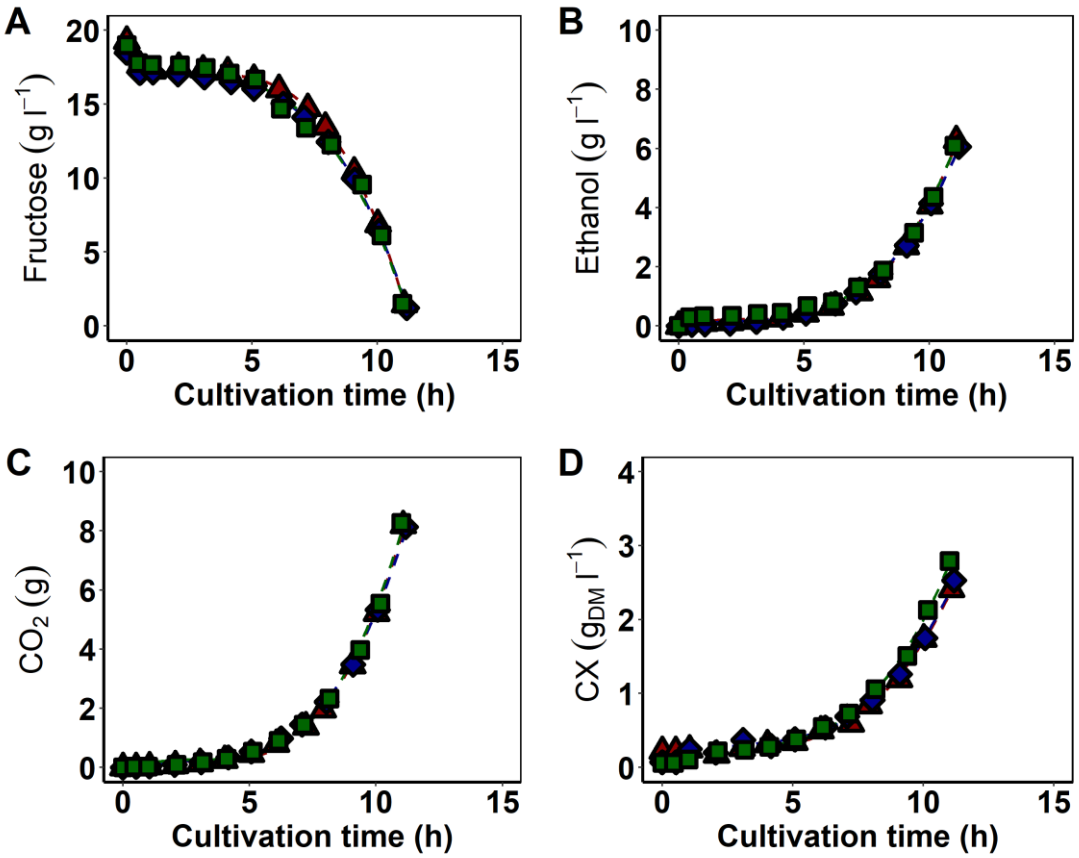

Figure 3.5 Substrate and metabolites concentrations/amounts during aerobic batch cultivation of *S. cerevisiae* CEN.PK113-7D (▲), JP1 (♦), and UFMG-CM-Y259 (■) cells with fructose as sole carbon and energy source. Dashed lines represent trend lines. One representative dataset of duplicate independent experiments is shown.

Table 3.2 Relevant physiological parameters of *S. cerevisiae* CEN.PK113-7D, UFMG-CM-Y259, and JP1 during aerobic batch cultivations with either glucose or fructose as sole carbon and energy source. All parameters were calculated for the exponential growth phase. Residual substrate refers to the remaining substrate concentration at the end of the experiment. The data represent the mean of two experiments and the average deviation.

	CEN.PK113-7D		**UFMG-CM-Y259**		**JP1**	
	Glucose	**Fructose**	**Glucose**	**Fructose**	**Glucose**	**Fructose**
μ_{MAX} (h^{-1})	0.31 ± 0.01	0.32 ± 0.01	0.29 ± 0.00	0.36 ± 0.02	0.28 ± 0.02	0.32 ± 0.02
$Y_{X/S}$ (g_{DM} $gGLC_{eq}^{-1}$)	0.13 ± 0.01	0.13 ± 0.00	0.14 ± 0.00	0.16 ± 0.00	0.13 ± 0.01	0.15 ± 0.01
$Y_{Ethanol/S}$ (g $gGLC_{eq}^{-1}$)	0.41 ± 0.01	0.38 ± 0.00	0.40 ± 0.03	0.37 ± 0.00	0.42 ± 0.00	0.38 ± 0.00
qS_{MAX} ($mmolGLC_{eq}$ g_{DM}^{-1} h^{-1})	-13.22 ± 0.23	-13.46 ± 0.51	-11.96 ± 0.43	-12.12 ± 0.84	-11.67 ± 0.45	-12.24 ± 0.07
qCO_2 (mmol g_{DM}^{-1} h^{-1})	23.57 ± 0.22	23.48 ± 0.34	20.77 ± 0.50	20.44 ± 1.39	19.75 ± 0.31	20.89 ± 0.05
qO_2 (mmol g_{DM}^{-1} h^{-1})	-2.45 ± 0.14	-2.84 ± 0.11	-2.63 ± 0.31	-3.03 ± 0.12	-2.12 ± 0.09	-2.43 ± 0.02
RQ ($mmolCO_2$ $mmolO_2^{-1}$)	9.66 ± 0.66	8.28 ± 0.19	8.01 ± 1.12	6.73 ± 0.20	9.31 ± 0.25	8.60 ± 0.04
Residual substrate ($gGLC_{eq}.l^{-1}$)	0.15 ± 0.12	2.72 ± 1.35	0.03 ± 0.03	2.28 ± 0.19	0.73 ± 0.08	1.34 ± 0.10
Carbon recovery (%)	100.73 ± 0.07	97.57 ± 0.66	103.25 ± 6.11	99.04 ± 0.94	101.44 ± 1.35	97.12 ± 0.51
Electron balance (%)	101.14 ± 0.70	97.41 ± 0.01	99.05 ± 2.05	100.00 ± 0.96	104.06 ± 0.53	97.25 ± 0.66

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SUPPLEMENTARY MATERIAL

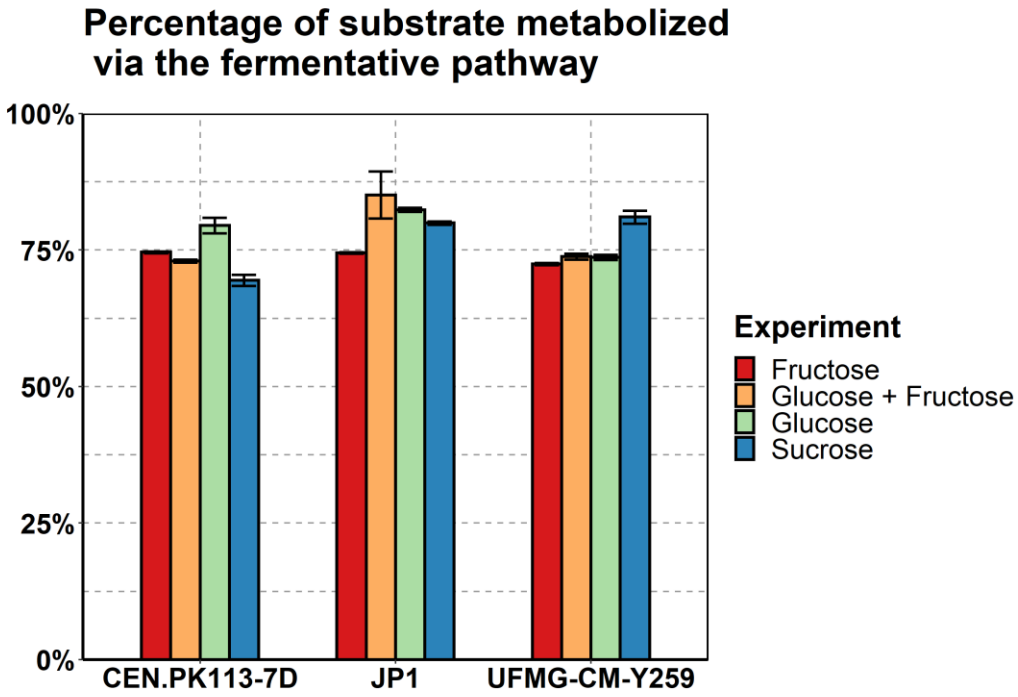

Figure S3.1 .Percentage of substrate metabolized via the fermentative pathway by *S. cerevisiae* CEN.PK113-7D, UFMG-CM-Y259, and JP1 during aerobic growth on sucrose as sole carbon and energy source. Error bars represent the average deviation of the values obtained from duplicate experiments.

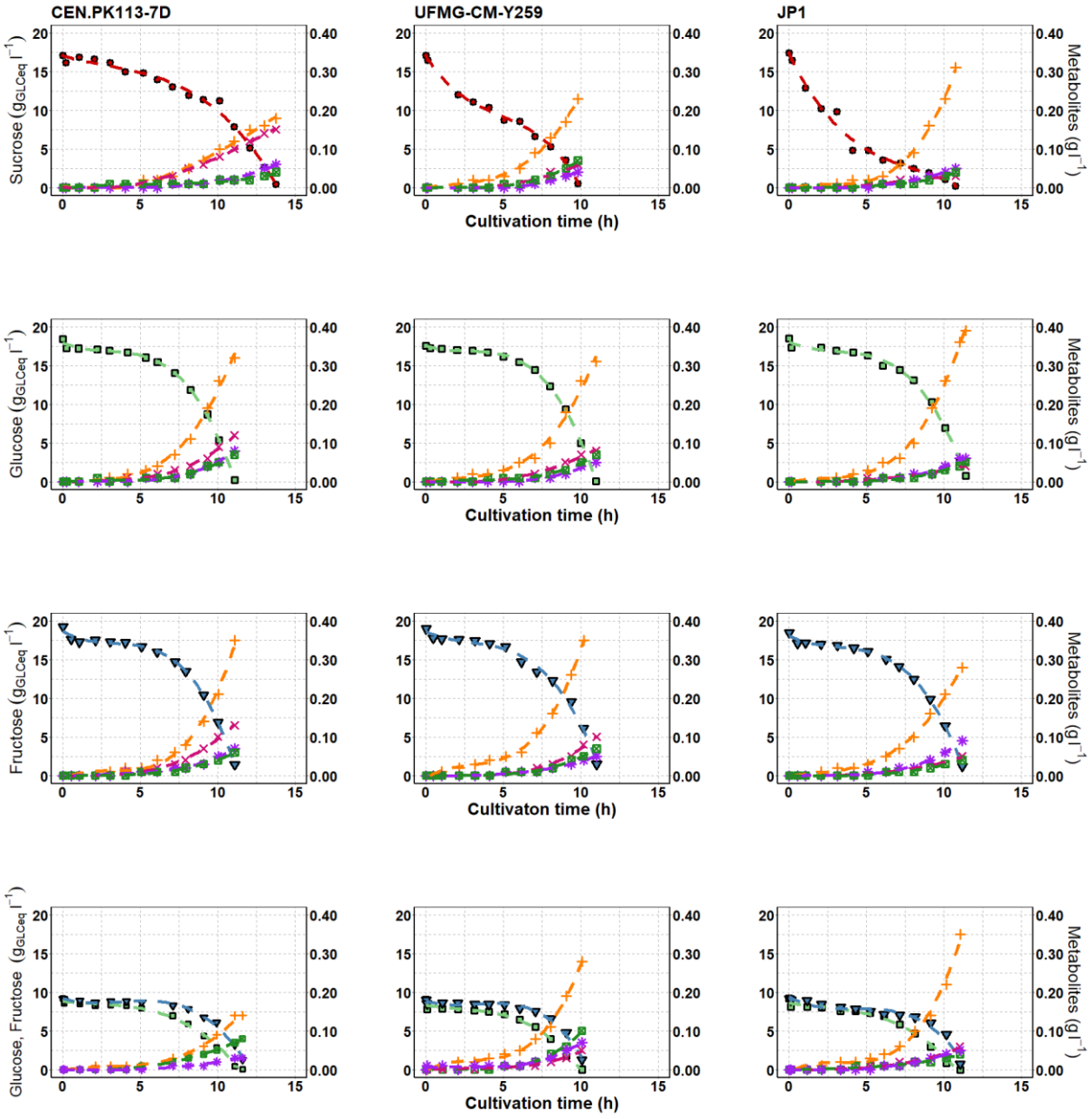

Figure S3.2 Substrate and metabolites concentrations during aerobic batch cultivation of *S. cerevisiae* CEN.PK113-7D, UFMG-CM-Y259 and JP1 with either sucrose, glucose, fructose or an equimolar mixture of glucose and fructose as sole carbon and energy source. Sucrose (●); Glucose (■), Fructose (*closed* ∇); Glycerol (+); Acetate (x); Succinate (*); Lactate (*crossed* □). Dashed lines represent trend lines. Experiments were performed in duplicate. Data shown in the plots represent from a single growth experiment.

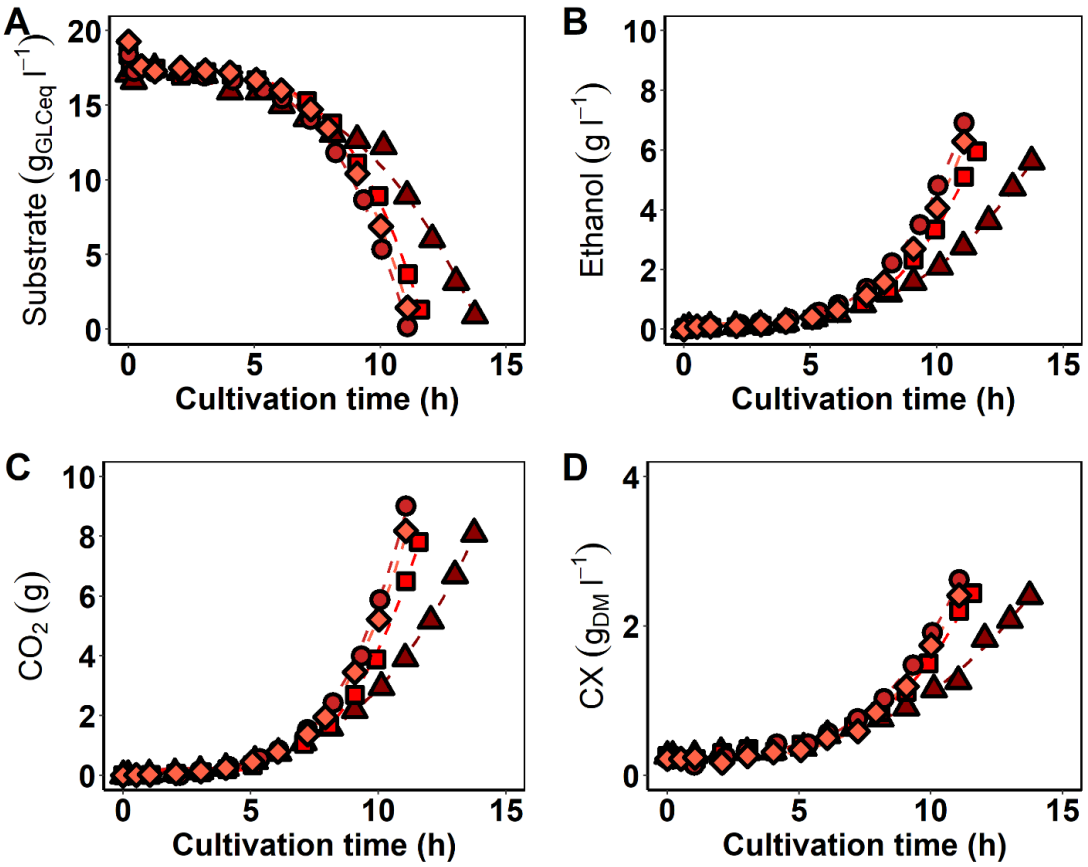

Figure S3.3 Substrate and metabolites concentrations/amounts during aerobic batch cultivation of *S. cerevisiae* CEN.PK113-7D with sucrose (▲), an equimolar mixture of glucose and fructose (■), glucose (●), or fructose (♦) as sole carbon and energy source. For the glucose + fructose experiment, substrate represent the sum of glucose and fructose concentrations. Dashed lines represent trend lines. Experiments were performed in duplicate. Data shown in the plots represent from a single growth experiment.

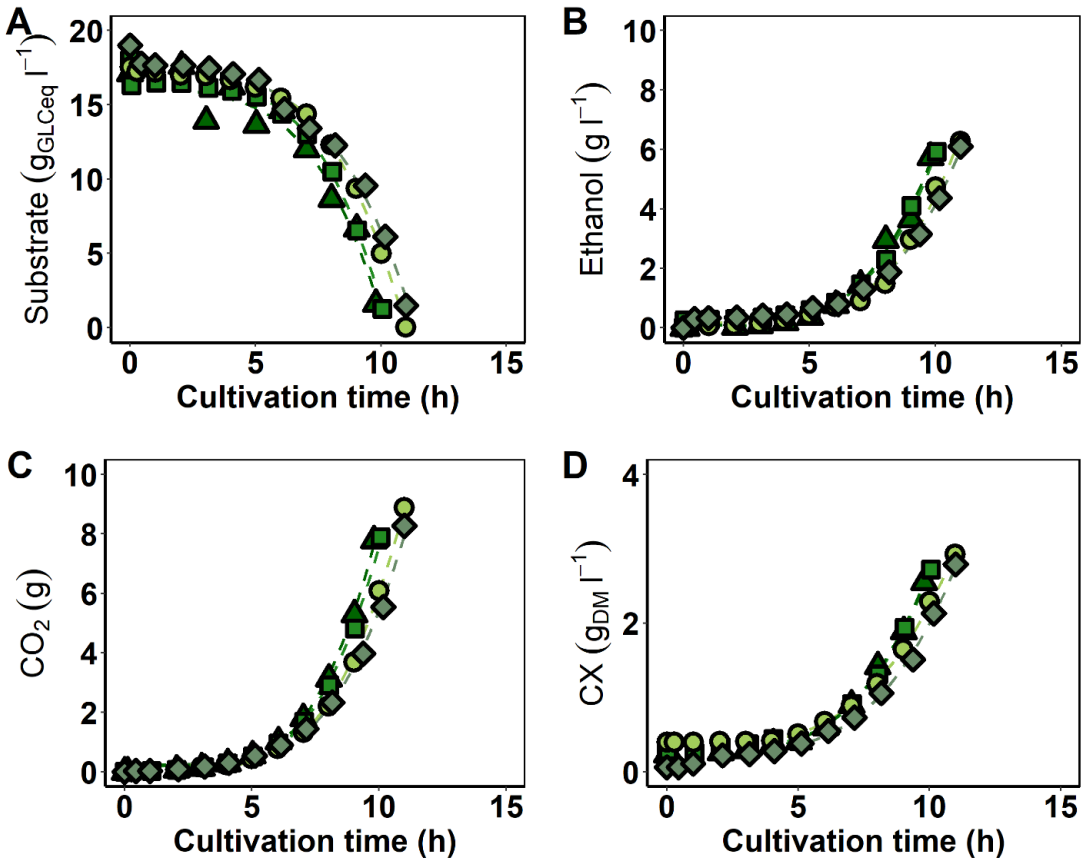

Figure S3.4 Substrate and metabolites concentrations/amounts during aerobic batch cultivation of *S. cerevisiae* UFMG-CM-Y259 with sucrose (▲), an equimolar mixture of glucose and fructose (■), glucose (●), or fructose (♦) as sole carbon and energy source. For the glucose + fructose experiment, substrate represent the sum of glucose and fructose concentrations. Dashed lines represent trend lines. Experiments were performed in duplicate. Data shown in the plots represent from a single growth experiment.

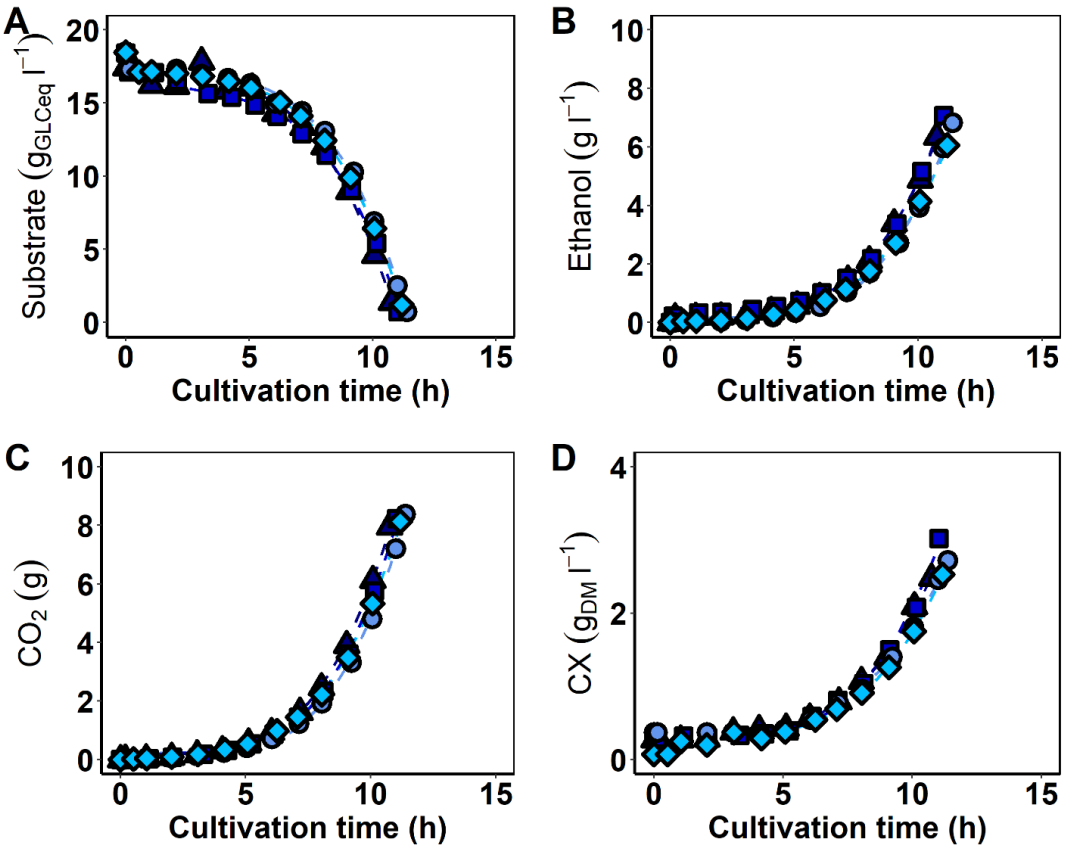

Figure S3.5 Substrate and metabolites concentrations/amounts during aerobic batch cultivation of *S. cerevisiae* JP1 with sucrose (▲), an equimolar mixture of glucose and fructose (■), glucose (●), or fructose (♦) as sole carbon and energy source. For the glucose + fructose experiment, substrate represent the sum of glucose and fructose concentrations. Dashed lines represent trend lines. Experiments were performed in duplicate. Data shown in the plots represent from a single growth experiment.

Table S3.1 Physiological parameters of *S. cerevisiae* CEN.PK113-7D during aerobic batch cultivations with either sucrose, glucose, fructose or an equimolar mixture of glucose and fructose as sole carbon and energy source. All parameters were calculated for the exponential growth phase. The data represent the mean of two experiments and the average deviation.

	Strain: CEN.PK113-7D			
	Glucose	Sucrose	Glucose & Fructose	Fructose
μ_{MAX} (h^{-1})	0.31 ± 0.01	0.21 ± 0.01	0.26 ± 0.01	0.32 ± 0.01
$Y_{X/S}$ (g_{DM} $gGLC_{eq}^{-1}$)	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.00	0.13 ± 0.00
$Y_{Ethanol/S}$ (g $gGLC_{eq}^{-1}$)	0.41 ± 0.01	0.36 ± 0.01	0.37 ± 0.00	0.38 ± 0.00
$Y_{CO2/S}$ (g $gGLC_{eq}^{-1}$)	0.44 ± 0.00	0.43 ± 0.01	0.40 ± 0.00	0.43 ± 0.01
$Y_{glycerol/S}$ (g $gGLC_{eq}^{-1}$)	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.00
$Y_{acetate/S}$ (g $gGLC_{eq}^{-1}$)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
$Y_{succinate/S}$ (g $gGLC_{eq}^{-1}$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
$Y_{lactate/S}$ (g $gGLC_{eq}^{-1}$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
qS_{MAX} ($mmolGLC_{eq}$ g_{DM}^{-1} h^{-1})	-13.22 ± 0.23	-8.23 ± 0.37	-11.96 ± 0.02	-13.46 ± 0.51
qCO_2 (mmol g_{DM}^{-1} h^{-1})	23.57 ± 0.22	14.48 ± 0.22	19.48 ± 0.08	23.48 ± 0.34
qO_2 (mmol g_{DM}^{-1} h^{-1})	-2.45 ± 0.14	-2.96 ± 0.16	-2.56 ± 0.38	-2.84 ± 0.11
RQ ($mmolCO_2$ $mmolO_2^{-1}$)	9.66 ± 0.66	4.91 ± 0.34	7.78 ± 1.19	8.28 ± 0.19
$q_{Ethanol}$ (mmol g_{DM}^{-1} h^{-1})	21.03 ± 0.74	11.43 ± 0.34	17.46 ± 0.09	20.08 ± 0.81
$q_{glycerol}$ (mmol g_{DM}^{-1} h^{-1})	0.53 ± 0.02	0.19 ± 0.01	0.37 ± 0.12	0.72 ± 0.01
$q_{acetate}$ (mmol g_{DM}^{-1} h^{-1})	0.21 ± 0.05	0.32 ± 0.06	0.28 ± 0.12	0.26 ± 0.03
$q_{succinate}$ (mmol g_{DM}^{-1} h^{-1})	0.08 ± 0.00	0.04 ± 0.01	0.05 ± 0.02	0.07 ± 0.02
$q_{lactate}$ (mmol g_{DM}^{-1} h^{-1})	0.13 ± 0.06	0.02 ± 0.02	0.05 ± 0.00	0.08 ± 0.01
Residual substrate ($gGLC_{eq}$ l^{-1})	0.15 ± 0.12	0.10 ± 0.74	0.37 ± 0.94	2.72 ± 1.35
Carbon recovery (%)	100.73 ± 0.07	94.91 ± 2.86	93.75 ± 0.95	97.57 ± 0.66
Electron balance (%)	101.14 ± 0.70	95.35 ± 3.05	93.61 ± 0.44	97.41 ± 0.01

Table S3.2 Physiological parameters of *S. cerevisiae* UFMG-CM-Y259 during aerobic batch cultivations with either sucrose, glucose, fructose or an equimolar mixture of glucose and fructose as sole carbon and energy source. All parameters were calculated for the exponential growth phase. The data represent the mean of two experiments and the average deviation.

	Strain: UFMG-CM-Y259			
	Glucose	Sucrose	Glucose & Fructose	Fructose
μ_{MAX} (h^{-1})	0.29 ± 0.00	0.37 ± 0.01	0.35 ± 0.03	0.36 ± 0.02
$Y_{X/S}$ (g_{DM} $gGLC_{eq}^{-1}$)	0.14 ± 0.00	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.00
$Y_{Ethanol/S}$ (g $gGLC_{eq}^{-1}$)	0.40 ± 0.03	0.41 ± 0.01	0.38 ± 0.00	0.37 ± 0.00
$Y_{CO2/S}$ (g $gGLC_{eq}^{-1}$)	0.45 ± 0.02	0.43 ± 0.05	0.42 ± 0.01	0.41 ± 0.00
$Y_{glycerol/S}$ (g $gGLC_{eq}^{-1}$)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01
$Y_{acetate/S}$ (g $gGLC_{eq}^{-1}$)	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
$Y_{succinate/S}$ (g $gGLC_{eq}^{-1}$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
$Y_{lactate/S}$ (g $gGLC_{eq}^{-1}$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
qS_{MAX} ($mmolGLC_{eq}$ g_{DM}^{-1} h^{-1})	-11.96 ± 0.43	-12.84 ± 0.21	-12.19 ± 0.63	-12.12 ± 0.84
qCO_2 (mmol g_{DM}^{-1} h^{-1})	20.77 ± 0.50	22.50 ± 2.00	20.96 ± 1.55	20.44 ± 1.39
qO_2 (mmol g_{DM}^{-1} h^{-1})	-2.63 ± 0.31	-3.34 ± 0.21	-2.88 ± 0.18	-3.03 ± 0.12
RQ ($mmolCO_2$ $mmolO_2^{-1}$)	8.01 ± 1.12	6.81 ± 1.03	7.34 ± 1.00	6.73 ± 0.20
$q_{Ethanol}$ (mmol g_{DM}^{-1} h^{-1})	17.63 ± 0.76	20.82 ± 0.65	18.00 ± 1.06	17.57 ± 1.26
$q_{glycerol}$ (mmol g_{DM}^{-1} h^{-1})	0.47 ± 0.07	0.48 ± 0.05	0.40 ± 0.07	0.60 ± 0.13
$q_{acetate}$ (mmol g_{DM}^{-1} h^{-1})	0.21 ± 0.03	0.15 ± 0.01	0.20 ± 0.03	0.30 ± 0.07
$q_{succinate}$ (mmol g_{DM}^{-1} h^{-1})	0.05 ± 0.00	0.05 ± 0.00	0.08 ± 0.03	0.07 ± 0.00
$q_{lactate}$ (mmol g_{DM}^{-1} h^{-1})	0.10 ± 0.02	0.12 ± 0.01	0.12 ± 0.03	0.09 ± 0.00
Residual substrate ($gGLC_{eq}$ l^{-1})	0.03 ± 0.03	1.03 ± 0.84	0.91 ± 0.40	2.28 ± 0.19
Carbon recovery (%)	103.25 ± 6.11	104.55 ± 2.95	98.80 ± 1.74	99.04 ± 0.94
Electron balance (%)	99.05 ± 2.05	107.25 ± 0.76	99.09 ± 0.96	100.00 ± 0.96

Table S3.3 Physiological parameters of *S. cerevisiae* JP1 during aerobic batch cultivations with either sucrose, glucose, fructose or an equimolar mixture of glucose and fructose as sole carbon and energy source. All parameters were calculated for the exponential growth phase. The data represent the mean of two experiments and the average deviation.

	Strain: JP1			
	Glucose	Sucrose	Glucose & Fructose	Fructose
μ_{MAX} (h^{-1})	0.28 ± 0.02	0.32 ± 0.00	0.30 ± 0.02	0.32 ± 0.02
$Y_{X/S}$ (g_{DM} $gGLC_{eq}^{-1}$)	0.13 ± 0.01	0.14 ± 0.00	0.17 ± 0.01	0.15 ± 0.01
$Y_{Ethanol/S}$ (g $gGLC_{eq}^{-1}$)	0.42 ± 0.00	0.41 ± 0.00	0.44 ± 0.02	0.38 ± 0.00
$Y_{CO2/S}$ (g $gGLC_{eq}^{-1}$)	0.41 ± 0.01	0.43 ± 0.00	0.42 ± 0.03	0.42 ± 0.00
$Y_{glycerol/S}$ (g $gGLC_{eq}^{-1}$)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
$Y_{acetate/S}$ (g $gGLC_{eq}^{-1}$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
$Y_{succinate/S}$ (g $gGLC_{eq}^{-1}$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
$Y_{lactate/S}$ (g $gGLC_{eq}^{-1}$)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
qS_{MAX} ($mmolGLC_{eq}$ g_{DM}^{-1} h^{-1})	-11.67 ± 0.45	-12.29 ± 0.12	-10.08 ± 0.39	-12.24 ± 0.07
qCO_2 (mmol g_{DM}^{-1} h^{-1})	19.75 ± 0.31	21.74 ± 0.19	17.53 ± 1.80	20.89 ± 0.05
qO_2 (mmol g_{DM}^{-1} h^{-1})	-2.12 ± 0.09	-2.19 ± 0.32	-2.15 ± 0.31	-2.43 ± 0.02
RQ ($mmolCO_2$ $mmolO_2^{-1}$)	9.31 ± 0.25	10.13 ± 1.40	8.19 ± 0.33	8.60 ± 0.04
$q_{Ethanol}$ (mmol g_{DM}^{-1} h^{-1})	19.23 ± 0.83	19.64 ± 0.26	17.18 ± 1.54	18.23 ± 0.14
$q_{glycerol}$ (mmol g_{DM}^{-1} h^{-1})	0.52 ± 0.08	0.48 ± 0.03	0.42 ± 0.00	0.39 ± 0.07
$q_{acetate}$ (mmol g_{DM}^{-1} h^{-1})	0.17 ± 0.09	0.08 ± 0.00	0.08 ± 0.00	0.10 ± 0.01
$q_{succinate}$ (mmol g_{DM}^{-1} h^{-1})	0.07 ± 0.00	0.06 ± 0.01	0.04 ± 0.02	0.05 ± 0.01
$q_{lactate}$ (mmol g_{DM}^{-1} h^{-1})	0.07 ± 0.01	0.05 ± 0.01	0.06 ± 0.00	0.07 ± 0.02
Residual substrate ($gGLC_{eq}$ l^{-1})	0.73 ± 0.08	1.79 ± 0.15	0.90 ± 0.11	1.34 ± 0.10
Carbon recovery (%)	101.44 ± 1.35	101.59 ± 0.11	107.25 ± 5.47	97.12 ± 0.51
Electron balance (%)	104.06 ± 0.53	102.13 ± 0.49	110.76 ± 5.34	97.25 ± 0.66

4

Comparative proteome analysis of different *Saccharomyces cerevisiae* strains during growth on sucrose and glucose

In collaboration with Maxime den Ridder, Martin Pabst, Andreas K. Gombert, and S.A. Wahl.

ABSTRACT

The aerobic physiology of the yeast *S. cerevisiae* on sucrose is strain-dependent, with some representatives displaying the ability to grow at higher specific rate on this carbon source compared to glucose. To investigate the mechanisms leading to such behavior, in this work, we performed a comparative analysis of the proteomes of the strains CEN.PK113-7D (laboratory), UFMG-CM-Y259 (indigenous), and JP1 (industrial) during growth on either sucrose or glucose. The strains were cultivated in well-controlled aerobic batch bioreactors, and the label free quantification technique was employed to assess the fold change in protein abundance between the two growth conditions. The observed differences were then tested for significance using ANOVA. The amount of up-regulated or down-regulated proteins, for each sucrose-glucose pairwise comparison, differed amid the strains. Only one protein was commonly up-regulated by the three strains, but no protein was shared down-regulated, after applying the biological and statistical threshold values of 1.2 for fold change and 5% for significance level. Moreover, Gene Ontology overrepresentation and KEGG pathway enrichment analyses were performed on the significant observations. Overall, a reversed pattern of enriched biological functions and pathways was observed for the strains CEN.PK113-7D and UFMG-CM-Y259, which have been previously shown to present opposite capacity of growth on sucrose compared to growth on glucose. A negative correlation between growth rate on sucrose and ribosome enrichment was observed, which corroborates with a previous theory on a trade-off between ATP yield per protein mass and the respiratory capacity.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* holds in its genome enzyme encoding genes that confer the ability to utilize different carbohydrates as carbon sources. The transcription of these genes is regulated according to the nutrient availability, through signaling pathways that mainly respond to the levels of glucose in the environment [1], [2]. A high glucose concentration triggers the catabolite repression response (CRR) that hampers the consumption of other substrates. Such preference for glucose suggests that this substrate should lead to higher maximum specific growth rates (μ_{MAX}) on glucose than on other carbon sources. However, previous physiology studies have demonstrated that representatives of this yeast species display a phenotype of faster growth on sucrose compared with glucose when microtiter plate was used as cultivation system [3], under anaerobic batch bioreactor cultivations [4], or using a strain adapted on sucrose for 250 generations [5]. This is especially surprising taking into account that sucrose is mainly degraded extracellularly by invertase to glucose and fructose, before these monosaccharides can be catabolized. Thus, this additional metabolic step requires the cells to synthesize and secret at least one additional protein.

In a recent study (chapter 3), we observed that the *S. cerevisiae* UFMG-CM-Y259 and JP1 strains presented higher μ_{MAX} on sucrose than on glucose during well-controlled aerobic batch cultivations. These findings highlight that our understanding of how *S. cerevisiae* tunes its regulatory machinery in response to glucose or sucrose, and how this impacts cellular operation is still incomplete. The precise regulatory mechanism governing rapid growth on the disaccharide, for instance, remains unresolved.

Faster growth is accompanied by an increased energy demand of the anabolic metabolism. Cells ensure that sufficient energy, in the form of adenosine triphosphate (ATP), is provided by modulating the flux through central carbon metabolism. The biochemical capacity of carbon metabolism, in turn, depends on the abundance of the metabolic enzymes in conjunction with their regulation [6], [7]. Many of these proteins undergo metabolite allosteric binding or post-translational modifications (PTM) that control their activity, such as the phosphorylation of phosphofructokinase 2 (Pfk2p) [8] and pyruvate kinase (Pyk1p) [9]. Thus, protein abundance and the interactions among proteins strongly influence the cellular phenotype. Proteome measurements can reveal changes between conditions and facilitate the identification of cellular responses as well as elucidate regulatory mechanisms under diverse growth conditions and strains [10]–[13].

Protein kinase A (PKA) is known to play a key role in the transcriptional regulation of genes encoding ribosomal proteins and involved in ribosomes biogenesis, as well as in stress responsive genes, such as *RIM15*, *MSN2* and *MSN4*. Moreover, this kinase also regulates the activity of metabolic enzymes at the post-translational level during carbon source shifts [2], [14]. The latter includes regulation of

proteins for the synthesis and degradation of storage carbohydrates [15]–[17], for the glycolytic pathway [18], [19], and for gluconeogenesis [20]. Specifically for the glycolytic enzymes, PKA directly phosphorylates and activates the proteins Pfk2p and Pyk1p, which implies a positive correlation between PKA activity and the glycolytic flux. Both substrates, sucrose and glucose, induce the signaling cascade leading to PKA activation via the Gpr1p-Gpa2p protein coupled receptor [21]. This receptor has a higher affinity for the disaccharide [21], leading to the hypothesis that PKA is involved in enabling a higher growth rate on sucrose [22].

In this work, the proteomes of the *S. cerevisiae* strains CEN.PK113-7D, JP1, and UFMG-CM-Y259 during growth on sucrose were analyzed in comparison to the corresponding proteomes during growth on glucose. These three strains were chosen due to their different phenotypes on sucrose (Chapter 3). We performed comparative proteomic analysis using label-free quantification of protein abundance via mass spectrometry. Statistically significant changes in the proteome profiles related to glucose conditions were subjected to Gene Ontology (GO) overrepresentation and pathway enrichment analyses.

MATERIAL AND METHODS

Yeast strains and cultivation

Three *Saccharomyces cerevisiae* strains were used in this work: the laboratory strain CEN.PK113-7D, the wild isolate strain UFMG-CM-Y259, and the ethanol-industry strain, JP1. Yeast strains were pre-cultured in 500-ml shake flask (Certomat BS-1, Braun Biotech International) with 100 ml synthetic medium [23] containing urea (2.3 $g.l^{-1}$) as nitrogen source and supplemented with either 1% (w/w) glucose or the hexose equivalent amount of sucrose. After 24 h of growth at 30 °C and 200 rpm, a 1-ml aliquot of this pre-culture was transferred to another 500-ml flask containing 100 ml fresh pre-culture medium. The cells were again cultured for 24 h, then harvested by centrifugation (3500 *g*) and washed twice with fresh cultivation medium. The bioreactor cultivation medium was prepared according to [23] with 2% glucose or the hexose equivalent amount of sucrose as sole carbon and energy source. Enough cells to start a batch cultivation with an optical density of 0.2 at 600 nm were diluted in fresh cultivation medium and transferred to a 2-l bioreactor with a working volume of 1.2 l (Applikon Biotechnology B.V., Delft, The Netherlands). Aerobic bioreactor cultivations were carried out at 30 °C, 800 rpm, and with 0.5 $l.min^{-1}$ air flow rate. The culture pH was kept at 5.0 by automatic addition of 0.5 $mol.l^{-1}$ KOH.

Sample preparation, protein extraction and trypsin proteolytic digestion

Samples for proteomic analysis were collected during the exponential growth phase (EGP). Aliquots with approximately 2-mg protein content were withdrawn from the bioreactor cultivations and immediately centrifuged at 867 *g* for 5 minutes. The supernatant was discarded and the pellet frozen at

-80 °C until further use. Cell pellets were resuspended in 100 mM TEAB lysis buffer containing 1% SDS and phosphatase/protease inhibitors. Lysis occurred by glass bead milling through a 10-time shaking for 1 min with a bead beater alternated with 1 min rest on ice. Proteins were reduced by addition of 5 mM DTT and incubated for 1 h at 37 °C. Subsequently, the proteins were alkylated for 60 min at room temperature in the dark by addition of 15 mM iodoacetamide. Protein precipitation was performed adding four volumes of ice-cold acetone (-20 °C) and proceeded for 1 h at -20 °C. The proteins were solubilized using 100 mM ammonium bicarbonate. Proteolytic digestion was performed by Trypsin (Promega, Madison, WI), 1:100 enzyme to protein ratio, and incubated at 37° C overnight. Solid phase extraction was carried out with an Oasis HLB 96-well µElution plate (Waters, Milford, USA) to desalt the mixture. Eluates were dried using a SpeedVac vacuum concentrator at 45° C. Dried peptides were resuspended in 3% ACN/0.01% TFA prior to MS-analysis to give an approximate concentration of 250 ng per µl.

Large scale shot-gun proteomics

An aliquot corresponding to approx. 250 ng protein digest was analysed using an one dimensional shot-gun proteomics approach [24]. Briefly, the samples were analysed using a nano-liquid-chromatography system consisting of an ESAY nano LC 1200, equipped with an Acclaim PepMap RSLC RP C18 separation column (50 µm x 150 mm, 2µm), and an QE plus Orbitrap mass spectrometer (Thermo). The flow rate was maintained at 350 $nl.min^{-1}$ over a linear gradient from 6% to 26% solvent B over 65 minutes, followed by an increase to 50% solvent B over 20 min and a subsequent back equilibration to starting conditions. Data were acquired from 2.5 to 90 min. Solvent A was H_2O containing 0.1% formic acid, and solvent B consisted of 80% acetonitrile in H_2O and 0.1% formic acid. The Orbitrap was operated in data dependent acquisition mode acquiring peptide signals from 385-1250 m/z at 70K resolution. The top 10 signals were isolated at a window of 2.0 m/z and fragmented using a NCE of 28. Fragments were acquired at 17.5K resolution.

Data analysis

Raw data were mapped to the proteome database from *Saccharomyces cerevisiae* (Uniprot, strain ATCC 204508 / S288C, Tax ID: 559292, July 2018) using PEAKS Studio X (Bioinformatics Solutions Inc, Waterloo, Canada) [25] allowing for 20 ppm parent ion and 0.02 m/z fragment ion mass error, 2 missed cleavages, carbamidomethyl as fixed and methionine oxidation and N/Q deamidation as variable modifications. To limit false-positive peptide identification, 1% false discovery rate (FDR) was applied to peptide spectrum matches (PSM), and subsequently the identified peptides were filtered with ≥ 2 unique peptides in all three biological replicates. Label free quantitative analysis was carried out on the identified peptides by using such module in the PEAKS Q software tool (Bioinformatics Solutions Inc,

Waterloo, Canada) [25]. The peak areas were normalized to the total ion count (TIC) of the respective analysis run before performing pairwise comparison between the carbon sources for each strain. Protein abundance ratio between sucrose and glucose conditions was filtered with fold change ratios ≥ 1.2, and analysis of variance (ANOVA) was performed to test the statistical significance of the observed abundance changes, with *p* values below 0.05 considered to be statistically significant.

Gene Ontology (GO) term overrepresentation and pathway enrichment analyses of differentially expressed proteins

Differentially abundant proteins for each sucrose-glucose comparison were subjected to GO overrepresentation as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. GO overrepresentation analysis was carried out using PANTHER [26] directly through the GO webpage (http://geneontology.org). For this, GO biological processes were obtained with all identified proteins used as background reference. Fisher's exact test with FDR multiple test correction were set for the GO overrepresentation analysis. KEGG enrichment analysis was performed using the *clusterProfiler* [27] (version 3.16.0) package within the Bioconductor project [28] in the R environment (version 4.0.0).

RESULTS

Proteins identification and differential expression on sucrose relative to glucose conditions

Using label-free quantification (LFQ), we investigated how protein abundance altered when *S. cerevisiae* cells were cultivated in aerobic batch bioreactors with either sucrose or glucose as carbon source, using strains CEN.PK113-7D (laboratory), UFMG-CM-Y259 (wild isolate), and JP1 (industrial). Cells from early stage of the exponential growth phase (EGP) were collected and disrupted by bead beating, reduced and alkylated. Proteins were precipitated with ice-cold acetone, digested with trypsin and the resulting peptides were analyzed by MS, with triplicate injections. The acquired data were evaluated for putative systematic differences on the overall proteome distribution among experiments due to sample preparation bias, and no bias was detected. We identified a total of 237,916 peptides across all six conditions performed in triplicate injections, of which 30,197 were unique, resulting in 2,311 proteins identified (**Table 4.1).**

Changes in the untargeted proteome measurements between the two growth conditions were accessed for each strain by determining the protein abundance fold changes (FC) with respect to the glucose growth condition. The significance of each observed fold change was determined by calculating *p* values. A FC of 1.2 and an adjusted *p*-value < 0.05 were defined as the biological and statistical threshold values, respectively. The number of proteins with statistically significant differential abundance in

regard to glucose conditions diverged among strains, either for increased or decreased levels (**Figure 4.1A, B**). For the CEN.PK113-7D strain, the abundance of 418 proteins were found statistically significant different (272 up; 146 down), against 510 (242 up; 268 down) and 224 (183 up; 41 down) proteins for the UFMG-CM-Y259 and JP1 strains, respectively. Remarkably, a cross strain analysis showed only one up-regulated differentially expressed protein, named Mnp1p – a mitochondrial ribosomal protein of the large subunit. No down-regulated protein was shared by the three strains using the above defined criteria.

Common up- or down-regulated proteins were observed when the analysis concerned each pair of strains, with CEN.PK113-7D sharing 28 and 33 in the increased level, and 22 and 2 in the decreased level with UFMG-CM-Y259 and JP1 strains, respectively. Between the two latter strains, 35 proteins were mutually up-regulated, whereas 12 were jointly down-regulated.

Table 4.1. Summary of mass spectrometry-based proteome data of *S. cerevisiae* CEN.PK113-7D, UFMG-CM-Y259, and JP1 cultivated in aerobic batch bioreactors with either glucose or sucrose as sole carbon and energy source. Data represent the total number peptides and proteins identified across all six conditions.

	Number
Total peptides	237916
Unique peptides	30197
Identified proteins	2311
Proteins with significant altered abundance	1152

Sucrose metabolism related proteins

With no need for sucrose hydrolysis during growth on glucose, we especially expected different levels for the protein invertase between the two growth conditions. Furthermore, the transcription of the *SUC2* gene, which is the most common allele encoding for this enzyme, is subject to glucose repression. However, invertase abundance was not detected as significantly changed for UFMG-CM-Y259 and JP1 strains with the chosen criteria (**Figure 4.1C**). Moreover, this enzyme was not identified in samples originating from the CEN.PK113-7D strain, which could be due to low abundance (below detection limit). This is in agreement with the low invertase activity observed earlier for the CEN.PK113-7D strain cultivated on sucrose (Chapter 3).

Notably, for all strains, altered abundance in at least one of the proteins known to be involved in the regulation of *SUC2* expression [1], [29] was observed (**Figure 4.1C**). The general transcriptional co-repressor Cyc8p was significantly changed in both CEN.PK113-7D and UFMG-CM-Y259 strains, although to different extents: up-regulated in CEN.PK113-7D and down-regulated in UFMG-CM-Y259. For JP1, an increase of Tup1p, which is a general repressor of transcription that forms a complex with

Cyc8p, was found. This strain also exhibited increased levels of the protein phosphatase catalytic subunit Glc7p in the sucrose condition, contrary to the observations for UFMG-CM-Y259. Besides, Glc7p's regulatory subunit, Reg1p, was up-regulated only in CEN.PK113-7D. The increased expression levels of both regulatory proteins Glc7p and Tup1p during the cultivations with sucrose for the JP1 strain gives evidence for the repressed state of the *SUC2* gene in this condition. It has to be noted that, when samples were taken from the sucrose experiments with both UFMG-CM-Y259 and JP1, the glucose concentration in the media was above the threshold concentrations of 2-3.2 $g.l^{-1}$ [30], [31] reported for *SUC2* repression.

Furthermore, with glucose consumption throughout the sucrose cultivations, the invertase abundance is expected to increase due to the release of glucose repression. This could result in changes between the two growth conditions at a different time point. For example, Paulo and colleagues [13], reported significant differential invertase expression levels for *S. cerevisiae* BY4742 cells cultivated in batch mode with raffinose versus glucose-grown cells. Raffinose is a trisaccharide composed of galactose, fructose and glucose that relies on invertase to break down the α-D-glucose (1↔2) β-D-fructose glycosidic bonds. Though the trisaccharide is similar w.r.t. the glucose regulation, the difference in strain background and/or sampling time points could explain the different observation.

Carbon source shift related Protein kinase A

The protein kinase A activity is reported to influence protein expression in response to the available carbon sources [2], [14]. Especially, PKA influences the transcription of growth-related genes and the activity of key glycolytic enzymes. Therefore, correlation between growth rate and the activity of this kinase was expected.

Such correlation was observed for the UFMG-CM-Y259 strain with a higher growth rate on sucrose ($\mu_{MAX\_sucrose}$ = 0.37 ± 0.01 h^{-1}) compared to glucose ($\mu_{MAX\_glucose}$ = 0.29 ± 0.00 h^{-1}; Chapter 3). For CEN.PK113-7D, the PKA's regulatory subunit called Byc1p was up-regulated in sucrose-grown cells relative to glucose-grown cells (**Figure 4.1C**). The up-regulation could imply in reduced PKA activity as Byc1p inhibits the PKA's catalytic subunits. Byc1p was down-regulated in UFMG-CM-Y259, suggesting enhanced PKA activity for the sucrose condition. No significant differential expression of Bcy1p was detected in the JP1 strain. The redundant catalytic subunits Tpk1/2/3p of PKA could not be quantified in all strains, due to weak MS signals.

The PKA activity is mainly regulated by the binding of the cyclic adenosine monophosphate (cAMP) molecule to its regulatory subunit, through a signaling pathway mediated by the RAS proteins, and that is triggered by the presence of glucose or sucrose sensed by the G protein coupled receptor [32], [33]. CEN.PK113-7D cells do not carry the adenylate cyclase encoding gene *CYR1* in their genome, and therefore cAMP cannot be formed [34]. This could explain why sucrose did not positively affect PKA

activity in CEN.PK113-7D cells, despite the higher affinity of the *GPR1* receptor for this substrate. Alternatively to cAMP biding, Kelch-repeat proteins (Krh1/2p) can control PKA activation [35], [36]. However, CEN.PK113-7D cells are also unable to produce these proteins [34]. With the current literature knowledge, the mechanism by which this strain regulates PKA activity remains unknown.

Hexose transporters

As the expression of hexose transporters in *S. cerevisiae* is sensitive to the extracellular concentrations of hexoses [37], [38], we expected differential expression for these proteins between the glucose and sucrose conditions. A significant increase in abundance, for sucrose-glucose comparison, of the low-affinity hexose transporters Hxt2p and Hxt6p was observed for the JP1 strain. For UFMG-CM-Y259, Hxt3p increased (**Figure 4.1C**). For CEN.PK113-7D, no hexose transporter change could be quantified due to weak identification. This was also the case for other hexose transporters that were not quantified in JP1 or UFMG-CM-Y259.

The upregulation of Hxt3p in sucrose-grown UFMG-CM-Y259 cells is in agreement with a previous report claiming that this protein is involved in enhanced fructose utilization [39]. However, the unchanged expression of this protein in JP1suggests its regulation is strain-specific.

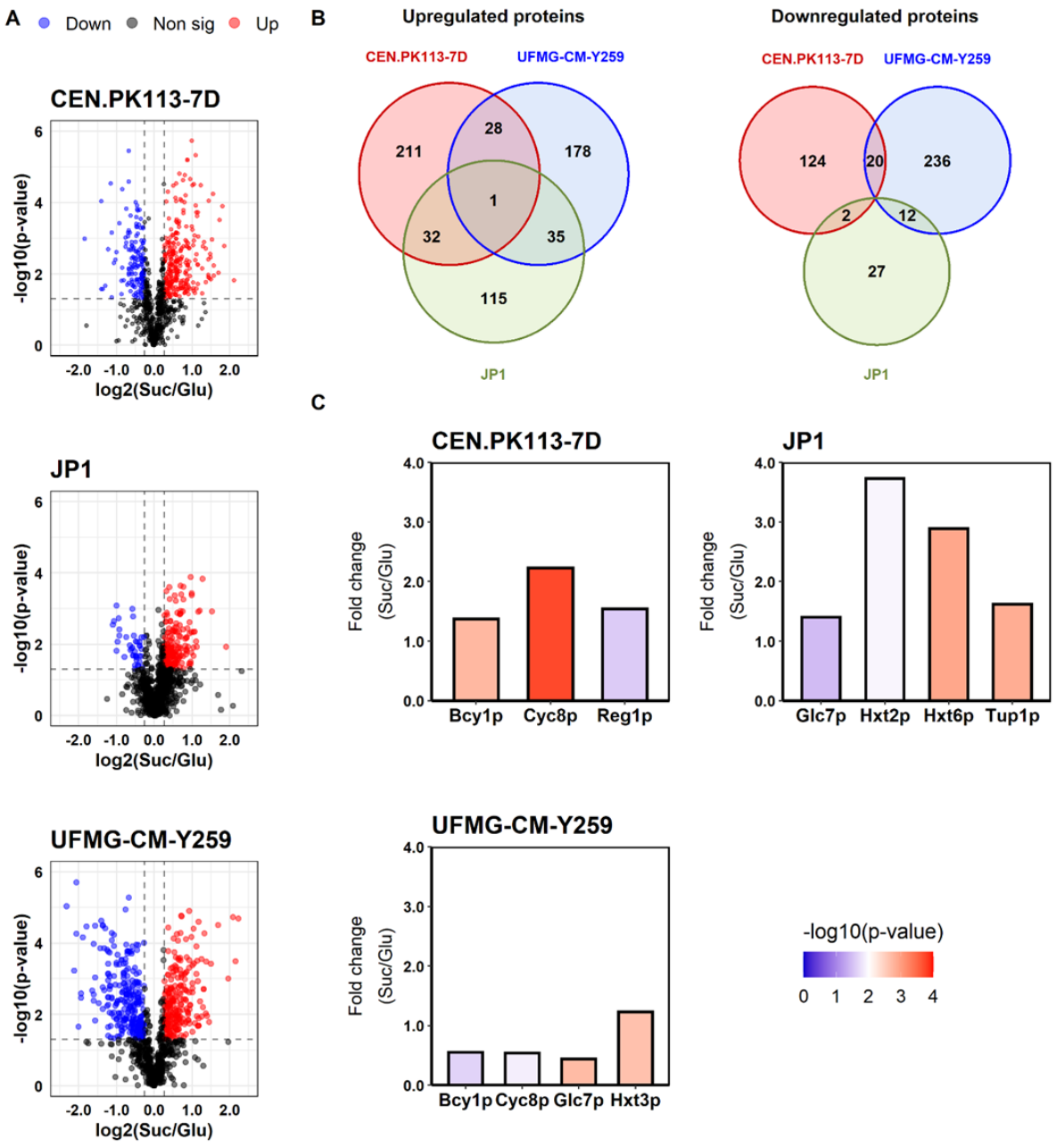

Figure 4.1 Comparative protein abundance between sucrose and glucose growth conditions for Saccharomyces cerevisiae CEN.PK113-7D, UFMG-CM-Y259, and JP1 strains. (A) Volcano plots highlight proteins with statistically significant differential abundance between growth on either substrate as sole carbon and energy source, considering a significance level of p value < 0.05. Upregulated proteins in sucrose over glucose condition are shown in red, downregulated proteins in blue, and proteins which abundance did not change significantly are represented in grey. (B) Venn diagrams illustrate the extent of differentially abundant proteins shared among the strains. (C) Differential expression of proteins known to play a role in sucrose metabolism and regulation, hexose transport, and in the protein kinase A (PKA) signaling cascade. Color coding indicates the statistical significance.

Gene Ontology term overrepresentation and pathway enrichment analyses of differently expressed proteins

Next to the targeted analysis of differentially expressed proteins, gene ontology (GO) term overrepresentation and pathway enrichment analyses were performed to identify changes in biological functions. These analyses were based on the pairwise comparisons, separately for up- and down-regulated proteins. GO biological processes as well as KEGG annotations were used to categorize the biological functions affected due to carbon source.

No significantly overrepresented biological process nor enriched pathways were identified for the proteins of JP1 cells grown on sucrose compared to glucose. This is consistent with the very similar physiology under the two conditions. This industrial strain presented a slightly higher maximum specific growth rate during the cultivations with the disaccharide, but comparable O_2 consumption rates, as well as yields of products (Chapter 3). In the following comparisons, JP1 will therefore not be included.

The GO analysis for the CEN.PK113-7D strain showed that the sucrose-induced up-regulated proteins were mainly associated with translation, aerobic respiration and energy metabolism. Down-regulated proteins were involved in secondary metabolites and amino acid metabolic processes (**Figure 4.2**). For the UFMG-CM-Y259 strain a reverse pattern of represented biological processes to that of CEN.PK113-7D was observed: A significant number of amino acid biosynthetic process proteins increased levels during sucrose growth, while proteins associated to translation and generation of precursor metabolites and energy were decreased.

KEGG pathway enrichment analysis showed similar results. For CEN.PK113-7D, an increase in ribosome and respiratory metabolism proteins under sucrose conditions (**Figure 4.3**). Down-regulated proteins play a role in biosynthesis of amino acids as well as of secondary metabolites pathways. As for GO biological processes terms, for the UFMG-CM-Y259 strain, the proteins which reduced in abundance can be found in the ribosome pathway. Moreover, KEGG annotation identifies proteins in both increased and decreased groups with function in the biosynthesis of secondary metabolites pathway, although with a higher relative proportion (GeneRatio) for the down level proteins.

Figure 4.2 GO biological process overrepresentation analysis for differentially expressed proteins for *Saccharomyces cerevisiae* strains CEN.PK113-7D and UFMG-CM-Y259 cultivated on sucrose as sole carbon and energy source relative to glucose grown cells. Count indicates the number of proteins in the dataset that falls in each overrepresented biological process.

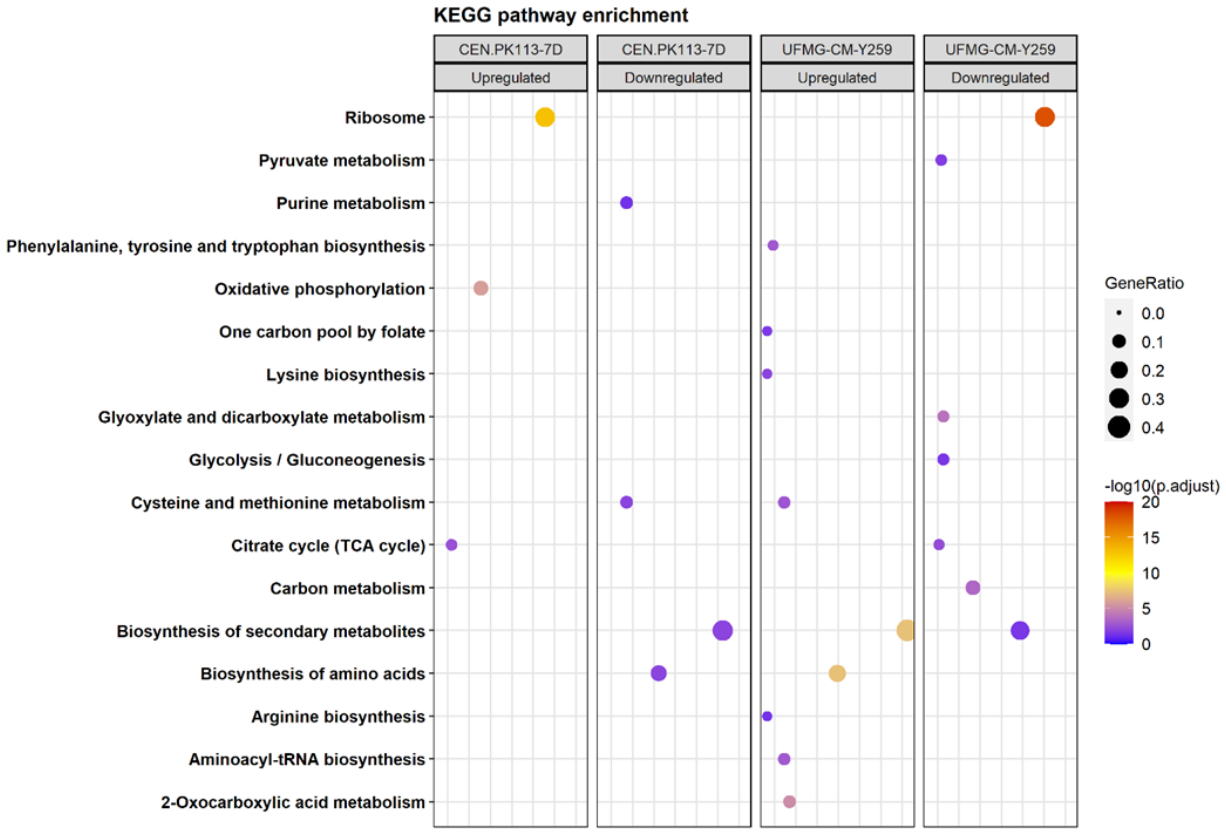

Figure 4.3 Pathway enrichment analysis for statistically significant proteins with an adjusted p-value < 0.05 for *Saccharomyces cerevisiae* strains CEN.PK113-7D and UFMG-CM-Y259 grown on sucrose as sole carbon and energy source relative to glucose grown cells. KEGG annotation was used for mapping the pathways. GeneRatio represents the proportion of proteins (gene products) in the correspondent abundance groups that is found in each category.

DISCUSSION

The regulatory network in yeast cells is very sensitive to glucose concentrations in the media. When *S. cerevisiae* is cultivated on sucrose alone, the glucose released due to the hydrolysis of this disaccharide reaches much lower levels relative to a growth on the hexose as sole carbon and energy source. Because of this, alterations are expected in carbon metabolism and related metabolic pathways, reflecting changes from the favored reference carbon source, glucose, to sucrose. Such alterations can be evidenced by physiology data. In our previous work (Chapter 3), the maximum specific growth rate of the *S. cerevisiae* laboratory strain CEN.PK113-7D was shown to be lower for sucrose-grown cells compared to glucose-grown cells. This is very consistent with the expectations in the view of the regulatory role of glucose on the use of alternative carbon sources. However, this trait did not repeat for the strains UFMG-CM-Y259 and JP1, for which, in that same work, we observed higher growth rates (although only slightly higher for the latter strain) on sucrose than on glucose. To further investigate the

mechanisms leading to *S. cerevisiae* strain-specific behavior on sucrose, in this work we used label free quantification to compare protein abundances in the three aforementioned strains under the sucrose and glucose growth conditions.

The comparative analysis revealed that there is not a pattern of sucrose-induced up- or down-regulated proteins amid all three studied strains, although some overlaps between a pair of strains were observed. This suggests that none of the proteins identified in this work, and thus the related cellular function, is under the exclusive control of sucrose. It is worth mentioning that there is no transcriptional factor (TF) among the common proteins to each pair of strains both in the up and down levels. However, individually, UFMG-CM-Y259 shows increased abundance of the TF Rtg2p, and decreased abundance of the TFs Cyc8p, Hmo1p, and Wtm1p. In CEN.PK113-7D, in turn, the TFs Cyc8p, Wtm1p, and Rap1p were up-regulated, whereas Hog1p was down-regulated in sucrose condition (**Supplementary Table S4.1**).

On the other hand, the extracellular glucose concentration in each sucrose cultivation, which varied depending on the strain, might have influenced the different responses to growth on the disaccharide due to the multiple regulatory effects triggered by this monosaccharide levels in the yeast *S. cerevisiae.* One of these effects is the repression of the transcription of several genes associated to the respiratory metabolism under high glucose concentrations [1]. For the CEN.PK113-7D strain, we observed the lowest hexose concentrations in the extracellular environment, compared to the values for UFMG-CM-Y259 and JP1 that were one order of magnitude higher (**Supplementary Figure S4.1**). In addition, the GO term and pathway enrichment analyses showed that cellular aerobic respiration proteins increased abundance only in sucrose-grown CEN.PK113-7D cells (**Figures 4.2 and 4.3**). Hence, it is reasonable to think that these three yeasts might have experienced glucose regulation to different extents during sucrose experiments, and that the low glucose concentrations in the sucrose cultivations of CEN.PK113-7D allowed for a more derepressed state of the respiratory genes. Consequently, a relative increased expression of the proteins encoded by these genes. This in turn resulted in a larger fraction of the substrate metabolized via the respiratory pathway by this strain (**Table 4.2**).

Through a systems biology perspective, being respiration catalytically less efficient than fermentation regarding adenosine triphosphate (ATP) production per protein mass, Crabtree-positive yeasts, such as *S. cerevisiae*, that rely more on oxidative phosphorylation to metabolize a specific carbon and energy source would grow at a lower rate on such substrate when compared to growth on another carbon source that is primarily metabolized via the fermentation pathway. This is because the use of respiratory metabolism implies a greater protein demand, consequently protein allocation to biomass production (biosynthesis of amino acids and secondary metabolites) is diminished in preferentially respiring cells in comparison to others with an enhanced fermentation rate. Thus, the slower growth on

sucrose relative to glucose observed for the CEN.PK113-7D strain could be a consequence of this trade-off between ATP yield and protein mass as proposed by Nilsson and Nielsen [40], since this strain showed enhanced oxidative phosphorylation on the sucrose-glucose pairwise comparison.

Concerning the JP1 strain, the lack of overrepresented biological processes or enhanced pathways at either regulation level suggests that glucose repression on the respiratory proteins was insensitive to the change in substrate. Indeed, the use of fermentative metabolism by this strain was comparable under both substrate conditions (**Table 4.2**). It is worth mentioning that hexose levels during sucrose cultivations with JP1 were the highest among the conditions we tested (**Supplementary Figure S4.1**).

Similar to JP1, the lack of enrichment for the oxidative phosphorylation pathway in the UFMG-CM-Y259 suggests that glucose repression on respiratory genes was also unaffected by the carbon source in this strain, which could as well be a reflection of the relatively higher extracellular hexose concentrations in sucrose-grown cells. Interestingly, a higher flux toward fermentation during sucrose cultivations with UFMG-CM-Y259 was observed compared to glucose cultivations (**Table 4.2**), and in analogy to the abovementioned interpretation for CEN.PK113-7D, this might explain the higher growth rate on sucrose achieved by this strain.

In sucrose-grown UFMG-CM-Y259 cells, the tricarboxylic acid (TCA) cycle pathway was down-regulated relative to glucose-grown cells (**Figure 4.3**), which upholds the observed enhanced fermentative metabolism. Because the expression of the TCA cycle proteins is glucose repressible under *HAP* control, when glucose-grown cells are cultivated on nitrogen sources that require α-ketoglutarate for assimilation, such as the ammonium used in this work, sufficient levels of this metabolite are assured by alternatively upregulating the transcription of genes encoding for proteins involved in its production pathway via the retrograde proteins Rtg1-3p [14], [41]–[43]. The products of the retrograde target genes *CIT2*, *ACO1*, *IDP2*, and *IDH1/2* were down-regulated in the sucrose-glucose pairwise comparison (**Supplementary Table S4.1**). Moreover, for the activation of the retrograde response, the phosphorylation of Rtg2p is required, as well as its binding to the protein Mks1p [42], [44]. As aforementioned, in UFMG-CM-Y259, the TF Rtg2p was up-regulated during growth on sucrose. The differential expression of this protein in its solo, non-phosphorylated version gives evidence for the influence of the retrograde pathway in controlling the respiratory metabolism capacity of this wild isolate strain, parallel to the control exerted by the glucose repression phenomenon. These observations corroborate the reduced use of respiration during sucrose cultivations in comparison to glucose cultivations with the UFMG-CM-Y259 strain, despite unchanged oxidative phosphorylation capacity (**Table 4.2**).

Correlation between specific consumption rates and protein abundance changes

Interesting observations can be drawn regarding the specific consumption rates achieved by these strains during growth on either carbon and energy source and this proteome study. Regardless of the minor use of respiration, UFMG-CM-Y259 showed a 1.2 fold change in the maximum oxygen specific uptake rate (qO_{2MAX}) for the sucrose-glucose pairwise comparison (**Table 4.2**). Although this higher qO_{2MAX} was not accompanied by increased abundance of cytochrome c oxidase, except for its subunit 13, several proteins involved in the ergosterol and heme biosynthesis were up-regulated (**Supplementary Table S4.1**). This suggests a higher demand for molecular oxygen by the UFMG-CM-Y259 strain to sustain non-respiratory pathways that require O_2 [45] during growth on sucrose. Likewise, the biological function oxoacid metabolic process was upregulated according to the GO overrepresentation analysis (**Figure 4.2; Table 4.2**).

Additionally, despite a reduction in the maximum substrate specific consumption rate for the sucrose-glucose pairwise comparison for CEN.PK113-7D cells (**Table 4.2**), the pathway enrichment analysis showed that proteins which function in glycolysis were not affected by the change in substrate for this strain (**Figure 4.3; Table 4.2**). This indicates that the glycolytic flux is mainly controlled at the post-translation level, either by metabolite allosteric regulation of the glycolytic enzymes or by PTM, which is consistent with previous findings using continuous culture of the strain CEN.PK113-7D [7], [46].

Table 4.2 Fold change of some physiological parameters, and proteins associated with corresponding biological process and pathways for *S. cerevisiae* CEN.PK113-7D, UFMG-CM-Y259 and JP1 during growth on sucrose relative to growth on glucose as sole carbon and energy source. Data are from previous work (Chapter 3).

	CEN.PK113-7D	**UFMG-CM-Y259**	**JP1**
Physiological parameters			
qO_{2MAX}	1.21	1.27	1.03
Proportion of substrate metabolized via the fermentative pathway	0.87	1.10	0.97
qS_{MAX}	0.62	1.07	1.05
Biological process			
Oxoacid metabolic process	Down	Up	N.S.
Pathway			
Oxidative phosphorylation	Up	N.S.	N.S.
Glycolysis/Gluconeogenesis	N.S.	Down*	N.S.

N.S. = no significant change.
* Protein which reduced abundance: Acs1p, Adc1p, Ald4p, Eno1p, Lat1p, Lpd1p, Pck1p, Pda1p, Pfk2p, Pgm2p, Tdh2p.

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SUPPLEMENTARY MATERIAL

Table S4.1 Summary of label free quantification and significance analyses for transcription factors, and proteins involved in retrograde response and ergosterol or heme biosynthesis for *S. cerevisiae* CEN.PK113-7D and UFMG-CM-Y259 cultivated in aerobic batch bioreactors with either sucrose or glucose as sole carbon source.

Accession	Gene code	Log2_(Suc/Glu)	Significance	Function
Transcription factor				
CEN.PK113-7D				
Q12363	WTM1	0.7312	27.22	Transcriptional modulator WTM1
P11938	RAP1	0.6229	20.47	DNA-binding protein RAP1
P14922	CYC8	1.1570	37.27	General transcriptional corepressor CYC8
P32485	HOG1	-0.4540	33.07	Mitogen-activated protein kinase HOG1
UFMG-CM-Y259				
P32608	RGT2	0.5059	14.01	Retrograde regulation protein 2
P14922	CYC8	-0.8890	18.61	General transcriptional corepressor CYC8
Q03973	HMO1	-0.4344	22.78	High mobility group protein 1
Q12363	WTM1	-0.6439	25.40	Transcriptional modulator WTM1
Retrograde target				
CEN.PK113-7D				
P41939	IDP2	-0.4941	14.57	Isocitrate dehydrogenase [NADP] cytoplasmic
P28834	IDH1	0.5059	44.72	Isocitrate dehydrogenase [NAD] subunit 1 mitochondrial
P28241	IDH2	0.3561	17.86	Isocitrate dehydrogenase [NAD] subunit 2 mitochondrial
UFMG-CM-Y259				
P08679	CIT2	-0.8890	24.20	Citrate synthase peroxisomal
P19414	ACO1	-0.4540	24.11	Aconitate hydratase mitochondrial
P41939	IDP2	-0.4540	28.86	Isocitrate dehydrogenase [NADP] cytoplasmic
P28834	IDH1	-0.4739	17.81	Isocitrate dehydrogenase [NAD] subunit 1 mitochondrial
P28241	IDH2	-0.6666	52.80	Isocitrate dehydrogenase [NAD] subunit 2 mitochondrial
Ergosterol or heme biosynthesis				
CEN.PK113-7D				
P54839	ERG13	0.7991	40.05	Hydroxymethylglutaryl-CoA synthase
P07143	CYT1	0.5656	25.95	Cytochrome c1 heme protein mitochondrial
P11353	HEM13	0.6229	21.05	Oxygen-dependent coproporphyrinogen-III oxidase
P21147	OLE1	1.2690	21.52	Acyl-CoA desaturase 1
UFMG-CM-Y259				
P32476	ERG1	0.4436	17.94	Squalene monooxygenase
P54781	ERG5	0.5460	28.03	Cytochrome P450 61
P10614	ERG11	0.2750	24.54	Lanosterol 14-alpha demethylase
P07277	ERG12	1.3951	19.63	Mevalonate kinase

Accession	Gene code	Log2_(Suc/Glu)	Significance	Function
P54839	ERG13	0.5850	18.04	Hydroxymethylglutaryl-CoA synthase
P53199	ERG26	0.7137	27.93	Sterol-4-alpha-carboxylate 3-dehydrogenase decarboxylating
P32352	ERG2	-0.2863	17.05	C-8 sterol isomerase
P32353	ERG3	-0.2863	15.41	Delta(7)-sterol 5(6)-desaturase
P53045	ERG25	-2.0000	16.54	Methylsterol monooxygenase
P09950	HEM1	0.4647	19.40	5-aminolevulinate synthase mitochondrial
P05373	HEM2	0.7398	17.85	Delta-aminolevulinic acid dehydratase
P28789	HEM3	1.0072	26.17	Porphobilinogen deaminase
P40075	SCS2	0.9411	21.38	Vesicle-associated membrane protein-associated protein

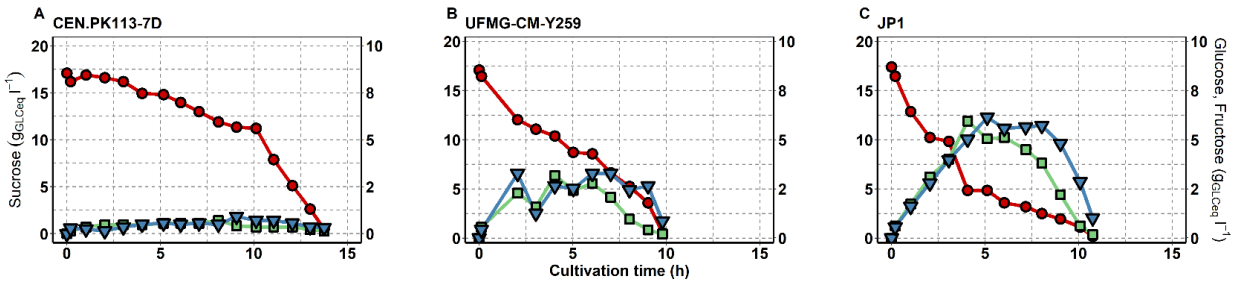

Figure S4.2 Sugar concentration during aerobic batch cultivations of *S. cerevisiae* CEN.PK113-7D, UFMG-CM-Y259, and JP1 in synthetic medium supplemented with sucrose as sole carbon and energy source.

5

Conclusions & Outlook

The shift to a bio-based economy will rely on a good combination of substrates and microorganisms and optimized process design [1]. The continuous improvement of the sucrose-based industry contributes to the broader strategy aimed at tackling the environmental issues triggered by fossil-based manufactures, while keeping the market competitiveness. The capacity of the yeast *Saccharomyces cerevisiae* to readily metabolize sucrose represents reduced costs with upstream processing as compared to the use of lignocellulosic feedstocks, for instance. In this view, unravelling the physiology and regulatory mechanisms involved in the growth of *S. cerevisiae* on sucrose is the starting point to promote the development of technologies to aid in the enhancement of existing sucrose-based bioprocesses and in the development of new ones. This thesis addressed this matter using well-controlled aerobic batch cultivations. The main focus was a quantitative physiological study using the *S. cerevisiae* CEN.PK113-7D, JP1, and UFMG-CM-Y259 strains, which display different behaviors on sucrose, with a subsequent comparative analysis of the untargeted global proteome.

During bioreactor cultivations with sucrose as sole carbon and energy source, the three studied strains displayed distinct growth capacity, hexose accumulation profiles, and periplasmic invertase activity. This is, to our knowledge, the first comprehensive report to reveal that the physiological traits of sucrose metabolism in *S. cerevisiae* are strain-specific. By performing a comparative analysis with growth on a mixture of glucose and fructose, we could speculate that the mechanism of sucrose utilization also differed amid the strains. Furthermore, a remark finding was the capability of the native strain, UFMG-CM-Y259, to achieve higher maximum specific growth rate on sucrose than on glucose under a well-controlled cultivation system. These interesting observations emphasize that *S. cerevisiae*'s physiology on sucrose has not been sufficiently researched.

Sucrose regulation at the proteome level in the three *S. cerevisiae* strains was studied by means of comparative label free quantification analyses with growth on glucose as the reference condition. The cellular responses to a change in carbon source were characterized by a lack of significant changes in abundance of transcription factors and invertase in all strains, reduced expression levels of the PKA's regulatory subunit Bcy1p in the UFMG-CM-Y259 strain, and a negative correlation between growth rate on sucrose and respiratory capacity in the UFMG-CM-Y259 and CEN.PK113-7D strains.

The knowledge acquired with the research described in this thesis can guide future studies aiming at optimizing sucrose-based industrial fermentations. Some of the interesting new avenues that can be pursued towards elucidating sucrose metabolism and regulation in *Saccharomyces cerevisiae* are summarized below:

Validation of hypotheses using mutant strains

We hypothesized that the *S. cerevisiae* JP1 strain utilizes sucrose via a combination of active transport and periplasmic hydrolysis with the subsequent uptake of the monosaccharides. The cultivation of the mutant JP1 *AGT1*Δ strain on a mixture of glucose and fructose could aid in the elucidation of the contribution of active transport to a phenotype of enhanced sucrose consumption in this industrial strain.

Moreover, our observations suggested that invertase activity might be a constraint in sucrose utilization by the laboratory strain CEN.PK113-7D. This could be verified, for instance, by overexpressing the *SUC2* gene, and comparing sucrose consumption in the mutant and in the parental strains.

In addition, we speculated that the *SUC2* gene of the UFMG-CM-Y259 strain might present mutations compared to this gene's sequence in the CEN.PK113-7D, and these mutations might confer the high performance of invertase in this wild isolate strain. If this hypothesis is confirmed by genome sequencing, whether such mutations are responsible for enhanced invertase activity in the wild isolate strain or not should also be verified. For instance, by expressing this mutant gene in the laboratory strain. In analogy, the role of Hxt3p in enhancing fructose utilization in the UFMG-CM-Y259 strain could be tested by expressing the mutant gene in the laboratory strain.

Increasing glycolytic rates by means of evolution and reverse engineering

Adaptive laboratory evolution has been widely applied to improve microbial performance [2]. The potential of an evolved CEN.PK113-7D strain to increase the maximum specific growth rate on sucrose could be exploited. Tracking the mechanism behind the expected improved phenotype of this evolved strain by means of genome sequencing, and confirming the modifications using reverse engineering could provide leads to strain engineering for increasing the glycolytic rates on sucrose in other *S. cerevisiae* strains.

Evaluation of the potential of the UFMG-CM-Y259 strain for industrial applications

Particularly in the fuel ethanol industry scenario in Brazil, the largely employed strains CAT-1 and PE-2, which once have had high performance, have been demonstrating loss of viability and lower persistence in the fermentation tanks [3]. This calls the attention for a continuous need of novel strains capable of withstanding the harsh conditions encountered in industrial fermentations. The assessment of UFMG-CM-Y259's robustness towards low pH, high temperature, slow growth, and high concentrations of inhibitory compounds [3]–[5] will deliver insights of its potential for industrial

applications, including, but not exclusively, in the fuel ethanol sector. In addition, the characterization of this strain in fed-batch cultivations as well as under rapid perturbation experiments [6]–[8], such as to mimic the oxygen and substrate gradient profiles typical of large-scale reactors, would complement the assessment.

Furthermore, the characterization under anaerobic conditions of a UFMG-CM-Y259 strain lacking the periplasmic invertase, which would generate the mutant UFMG-CM-Y259 *iSUC* [9] that mandatorily consumes sucrose via active transport, could be specially interesting for the fuel ethanol industry. In principle, a phenotype of enriched ethanol yield should be observed in the mutant compared to the parental strain.

At last, assessing the performance of the UFMG-CM-Y259 strain on industrial feedstocks, such as sugarcane and molasses, would provide further indications of its feasibility for industrial applications [10]. Eventually, this strategy could be combined with strain design to evaluate the titer, productivity, and yields of the manufacturing of primary and recombinant products of commercial interest.

Signaling pathway studies

In yeast, high glycolytic rates and cell proliferation are tightly linked but the molecular basis underlying this correlation is still poorly understood [11]. Cell proliferation is to a great extent regulated by the Ras proteins by means of the cAMP-PKA signaling cascade [12], [13]. Future studies should also focus on untangling signaling pathways to allow for a better understanding of the relationship between cellular growth and metabolism regulation. This could be achieved for example through phosphoproteomics since phosphorylation plays a significant role in posttranslational regulatory events.

Metabolic flux analysis for identification of differential pathway usage

We have demonstrated the strain-specific behavior of *Saccharomyces cerevisiae* during growth on sucrose. A comprehensive characterization of the dynamic compartmentalization of metabolites could aid in explaining the observed differences in cellular phenotype. Studies involving metabolic flux analysis using stable isotope tracers, such as ^{13}C and ^{15}N, will facilitate the identification of differential pathway usage.

Kinetic modelling

Kinetic modelling is a valuable tool to comprehend the temporal responses of a biological process or system to external stimuli, thus allowing for a better understanding, prediction and optimization of platform cell factories [14]. The research presented in this thesis has delivered a comprehensive data set

of different strains and cellular levels containing proteomics and physiology. These data sets are a solid basis for kinetic modelling approaches that could identify further targets for metabolic engineering.

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Carla Inês Soares Rodrigues

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About the Author

"Wisest is she who knows she does not know"

Jostein Gaarder

CURRICULUM VITAE

Carla Inês Soares Rodrigues was born on 5th January 1989 in Visconde do Rio Branco, Minas Gerais, Brazil. She was admitted in the Chemical Engineering undergraduate program at Federal University of Viçosa (UFV), in 2007. During her bachelor's degree, she worked as an undergraduate research scientist, being a beneficiary of a scientific initiation scholarship provided by the *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (FAPEMIG; Apr, 2008 – Jul, 2010). Under the supervision of prof.dr.ir. Juarez de Sousa e Silva, Carla performed projects on the techno-economic analysis of sustainable production of 1G fuel ethanol, liquor, and milk in small farms (pilot scale). In 2010, Carla was awarded the CAPES-FIPSE fellowship, which sponsored her studies during the fall semester at the University of Kentucky (UK) – the United States of America, where she also worked as an undergraduate research assistant in bioprocessing. Her academic performance as an international exchange student yielded her the Dean's List award. Back in Brazil, she performed a project on the hydrolysis of pretreated eucalyptus kraft pulp for fuel ethanol production, applying a cellulolytic enzymatic cocktail produced by thermophilic microbial communities, for her final diploma thesis, supervised by Dr.ir. Fábio Ávila Rodrigues.

Carla graduated with B.Sc. in July 2012. Soon after, she moved to Lexington, in Kentucky, to start the M.Sc. program in Biosystems and Agricultural Engineering, which she concluded in April 2015. During her masters' degree studies, she was supervised by Dr.ir. Michael D. Montross, and conducted research on characterization, pretreatment and hydrolysis of lignocellulosic raw materials.

Motivated by her passion for learning and willingness to further develop her knowledge of bioprocessing, Carla joint the Laboratory of Metabolic Engineering and Bioprocess (LEMeB) at University of Campinas (UNICAMP) in Brazil for her Ph.D. training in Bioenergy, in 2016. A year later, she was admitted in the dual degree Ph.D. program between UNICAMP and Delft University of Technology (TU Delft; The Netherlands). At TU Delft, she was part in the Cell Systems Engineering (CSE) group of the Biotechnology department. During her Ph.D. studies, Carla was jointly supervised by prof.dr.ir. Andreas K. Gombert and Dr. S.A. Wahl. Her doctoral thesis focused on the quantitative physiology of the yeast *Saccharomyces cerevisiae* during growth on sucrose, of which results are discussed in this dissertation.

PUBLICATIONS

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