BIANCA ELI DELLA BIANCA

Stress tolerance of *Saccharomyces cerevisiae* strains employed in the fuel ethanol production in Brazil

A thesis submitted to the Polytechnic School of the University of São Paulo for the degree of Doctor in Science

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Concentration Area: Chemical Engineering

Supervisor: Prof. Andreas Karoly Gombert

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To my mother, for always being there.

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I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie

RESUMO

DELLA-BIANCA, B. E. **Stress tolerance of *Saccharomyces cerevisiae* strains employed in the fuel ethanol production in Brazil**. 2013. 109 f. Tese (Doutorado) – Escola Politécnica, Universidade de São Paulo, São Paulo, 2013.

À medida que cresce a necessidade por biocombustíveis, devido a sua essência sustentável e aos altos preços do petróleo, também aumenta a importância da produção brasileira de etanol combustível no contexto global. No Brasil, etanol combustível é produzido via fermentação de substratos derivados da cana-de-açúcar por linhagens robustas de *Saccharomyces cerevisiae*. Entender a fisiologia dessas linhagens de levedura se tornou um passo necessário para aumentar o rendimento alcoólico, visto que outros processos da cadeia de produção de etanol já foram otimizados significativamente. O objetivo desta tese foi avaliar sistematicamente a fisiologia das linhagens brasileiras PE-2, CAT-1, BG-1 e JP-1, em resposta a condições de estresse relacionadas ao processo de produção de etanol. As linhagens de laboratório S288c e CEN.PK113-7D e a linhagem de panificação Fleischmann foram incluídas neste estudo como referência. Ensaios de diluição em placas contendo diversos fatores de estresse mostraram que as linhagens industriais toleram melhor altas concentrações de etanol e de ácido acético e substratos industriais (caldo de cana e melaço). Só foi observada diferença entre as linhagens de etanol e de panificação sob condições de estresse térmico e de pH baixo. Estas condições foram consideradas fatores-chave de pressão seletiva no ambiente industrial de produção de etanol. O estresse térmico foi estudado em cultivos em frasco agitado a 37 °C utilizando meio sintético. Nessa condição somente linhagens de laboratório apresentaram rendimentos em biomassa e etanol significativamente menores em relação a cultivos a 30 °C. Balanços de carbono mostraram que, naquela condição, essas linhagens direcionam mais carbono para a formação de outros metabólitos que não o etanol (como glicerol e ácidos orgânicos), provavelmente devido a uma maior ativação de mecanismos de resposta ao estresse. A resposta das linhagens PE-2 e CEN.PK113-7D a condições de estresse ácido foi estudada em anaerobiose em quimiostatos a pH 3.0 (em meio sintético ou complexo), em quimiostatos em meio sintético contendo ácido acético 105 mM e em cultivos contínuos dinâmicos com pH variável. Em todas essas

condições as duas linhagens apresentaram fisiologia semelhante, com exceção do metabolismo de acetato da linhagem PE-2. No entanto, em bateladas em meio complexo e pH 2.7, a linhagem PE-2 apresentou uma velocidade específica de crescimento 33 % maior e um rendimento em biomassa 86 % maior do que a linhagem CEN.PK113-7D. Essa distinção não foi observada em bateladas em meio complexo a pH 5.0 ou em bateladas em meio sintético a pH 5.0 ou 2.8. A resposta ao estresse ácido também foi analisada em ambiente não-proliferativo, através da determinação da viabilidade celular após tratamento com H_2SO_4 em pH 1.5. A linhagem PE-2 apresentou a maior viabilidade (64.7 %), seguida das linhagens Fleischmann (50.4 %) e CEN.PK113-7D (34.9 %). Em conjunto, os dados apresentados nesta tese sustentam a hipótese de que a linhagem PE-2 foi selecionada por sobreviver nas condições ácidas encontradas na etapa industrial de reciclo celular e por se propagar rapidamente neste ambiente estressante, utilizando as células mortas da linhagem de panificação como substrato. Essas características permitem que a linhagem PE-2 prospere e domine os fermentadores na produção industrial de etanol.

Palavras-chave: Etanol. Fermentação alcoólica. *Saccharomyces cerevisiae*. Tolerância a estresse. Estresse ácido.

ABSTRACT

DELLA-BIANCA, B. E. **Stress tolerance of *Saccharomyces cerevisiae* strains employed in the fuel ethanol production in Brazil**. 2013. 109 f. Tese (Doutorado) – Escola Politécnica, Universidade de São Paulo, São Paulo, 2013.

As the need for biofuels rise, given their sustainable nature and the high prices of oil, so does the importance of the Brazilian fuel ethanol production in a global context. In Brazil, fuel ethanol is produced via fermentation of sugarcane feedstocks using robust strains of *Saccharomyces cerevisiae*. Understanding the physiology of these yeast strains has become the next necessary step to increase ethanol yields, since other major industrial processes in the ethanol production chain have already been significantly optimized. The aim of this thesis was to systematically evaluate the physiological responses of Brazilian fuel ethanol strains PE-2, CAT-1, BG-1, and JP-1, towards stress conditions associated to the process in which they are employed. Laboratory strains S288c and CEN.PK113-7D and baker's strain Fleischmann were also studied and considered as reference strains. Spot dilution assays in plates with a range of stressors in varying concentrations showed that industrial strains perform better under ethanol and acetic acid stresses and on industrial media (sugarcane juice and molasses). A distinction between fuel ethanol and baker's strains was observed only during growth under heat and low pH stresses, conditions that may be considered major factors of selective pressure in the fuel ethanol production environment, hindering the replication of the starter baker's strain. Heat stress was further studied in synthetic medium shake-flask cultivations at 37 °C, in which only laboratory strains exhibited a significant decrease on biomass and ethanol yields in relation to 30 °C. Carbon balance analysis showed that these strains channel more carbon to metabolites other than ethanol (like glycerol and organic acids), probably due to a higher triggering of stress response mechanisms under heat stress. Response of strains PE-2 and CEN.PK113-7D towards acid-related stress conditions was analyzed in anaerobiosis in chemostats at pH 3.0 (on synthetic and rich media), in chemostats on synthetic medium added with 105 mM acetic acid and in dynamic continuous cultivations on synthetic medium with time-varying pH. In all these conditions both strains displayed similar physiology, with the exception of PE-2's particular

acetate metabolism. In batch cultivations in rich medium at pH 2.7, however, remarkable differences could be noticed—PE-2 strain exhibited a 33 % higher growth rate and an 86 % higher biomass yield. No differences between the strains were observed in batch cultivations in rich medium at pH 5.0 or in batch cultivations in synthetic medium at pH 5.0 or 2.8. Response to acid stress was also assessed in a non-proliferative environment, through measurements of cell viability after a 4-h H_2SO_4 treatment at pH 1.5. Strain PE-2 exhibited the highest viability (64.7 %), followed by strains Fleischmann (50.4 %) and CEN.PK113-7D (34.9 %). Analyzed together, the data presented in this thesis support the hypothesis that strain PE-2 was selected by surviving at low pH conditions found in the industrial cell recycle step, and by replicating fast in this stressful environment, most likely using dead baker's strain cells as a substrate. These features allow strain PE-2 to thrive in and dominate the fermentors in the fuel ethanol production process.

Keywords: *Saccharomyces cerevisiae*. Fuel ethanol. Stress tolerance. Growth physiology. Low acid stress.

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LIST OF ABBREVIATIONS

1G	First generation (ethanol production process)
2G	Second generation (ethanol production process)
AAD15	Aryl-alcohol dehydrogenase gene
ADH7	Alcohol dehydrogenase gene
AGP3	High-affinity glutamine permease gene
AGT1	High-affinity maltose transporter gene
ANCOVA	Analysis of covariance
APJ1	Anti-prion DnaJ gene
ARR1-ARR3	Arsenicals resistance genes 1-3
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BDS1	Bacterially derived sulfatase gene
BIOEN	Programa FAPESP de Pesquisa em Bioenergia
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCT	Coleção de Culturas Tropical
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CoA	Co-enzyme A
COX1	Cytochrome c oxidase gene
CUP1-1/CUP1-2	Metallothionein genes
DAK2	Dihydroxyacetone kinase gene
DNS	3,5-Dinitrosalicylic acid
DW	Dry weight
EC	Enzyme Comission number
ENA	Exitus natru genes (P-type ATPase sodium pump genes)
ERR3	Enolase-related repeat gene
ETH	Ethanol
EUROSCARF	European *Saccharomyces cerevisiae* archive for functional analysis
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FDC1	Ferulic acid decarboxylase gene

FIT2/FIT3	Facilitator of iron transport genes
FLO	Flocculation genes
FRE5	Ferric reductase gene
GEO	Gene Expression Omnibus
GLR1	Glutathione oxidoreductase gene
GM	Genetically modified
H_0	Null hypothesis
H_1	Alternative hypothesis
HAc	Acetic acid
HAP1	Heme activator protein gene
HO	Homothallic switching endonuclease gene
HOG	High osmolarity glycerol
HPLC	High performance liquid chromatography
Hsp	Heat shock protein
HXT15/HXT16	Hexose transporter genes (similar to other *HXT* family members)
HXT6/HXT7	Hexose transporter genes (high-affinity glucose transporter)
IMB	Industrial Microbiology Group (TUDelft)
kb	kilobase
MAL	Maltose fermentation genes
MAL11	High-affinity maltose transporter gene
ME	Metabolic engineering
MKT1	Maintenance of K2 killer toxin gene
MPH2/MPH3	Maltose permease homolog genes
N/G	No growth
PAD1	Phenylacrylic acid decarboxylase gene
PCR	Polymerase chain reaction
PDA1	Pyruvate dehydrogenase alpha gene
PDH	Pyruvate dehydrogenase
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrates
pH_c	Cytosolic pH
PHMB	Polyhexamethyl biguanide

PHR1	Photoreactivation repair deficient gene
PMA1/PMA2	Plasma membrane ATPase genes
Proálcool	Programa Nacional do Álcool
QTL	Quantitative trait loci
RDS1	Regulator of drug sensitivity gene
RI	Refractive index
ROS	Reactive oxygen species
RTM1	Resistance to toxic molasses gene
SAM3/SAM4	S-Adenosylmethionine metabolism genes
SC	Sugarcane
SEO1	Suppressor of sulfoxyde ethionine resistance gene
SGE1	Suppression of Gal11 expression gene
SM	Synthetic medium
SNO	SNZ proximal open reading frame gene (pyridoxine metabolism)
SNP	Single nucleotide polymorphism
SNZ	Snooze gene (pyridoxine metabolism)
SOR1	Sorbitol dehydrogenase gene
SOR2	Uncharacterized gene similar to *SOR1*
SRA	Sequence Read Archive
STL1	Sugar transporter-like protein gene
SUC	Sucrose fermentation (invertase) genes
TCA	Tricarboxylic acid
TRS	Total reducing sugars
TUDelft	Delft University of Technology
UFPE	Universidade Federal de Pernambuco
Usga	Usina Serra Grande Alagoas
USP	Universidade de São Paulo
UV/Vis	Ultraviolet-visible
v/v	Volume/volume
VHG	Very high gravity
vvm	Volume per volume per minute
w/v	Weight/volume

w/w	Weight/weight
YP	Yeast extract-peptone culture medium
YPD	Yeast extract-peptone-dextrose culture medium
YPE	Yeast extract-peptone-ethanol culture medium

LIST OF SYMBOLS

α	Significance level
a	Slope of a regression line
Abs_{600}	Absorbance at 600 nm
b	Intercept of a regression line
D	Dilution rate
DF	Degrees of freedom
E	Ethanol concentration in the fermentor/shake-flask
E_F	Ethanol concentration in the medium feed
i	i^{th} observation
k	Ethanol evaporation constant
μ	Specific growth rate
μ_{max}	Maximum specific growth rate
n	Number of observations
P	Product concentration in the fermentor/shake-flask
P_F	Product concentration in the medium feed
q	Specific rates of consumption or formation
q_{ACET}	Specific rate of acetate formation
q_E	Specific rate of ethanol formation
q_E^{max}	Maximum specific rate of ethanol formation
q_G	Specific rate of glycerol formation
q_G^{max}	Maximum specific rate of glycerol formation
q_{LACT}	Specific rate of lactate formation
q_P	Specific rate of product formation
q_P^{max}	Maximum specific rate of product formation
q_{PYR}	Specific rate of pyruvate formation
q_S	Specific rate of substrate consumption
q_S^{max}	Maximum specific rate of substrate consumption
q_{SUCC}	Specific rate of succinate formation
S	Substrate concentration in the fermentor/shake-flask

SE	Standard error
S_F	Substrate concentration in the medium feed
t	Time
X	Biomass concentration
x_i	Value of the independent variable for observation *i*
$\bar{x}$	Mean of the independent variable
$Y_{E/S}^{exp}$	Ethanol yield on substrate during exponential growth phase
$Y_{G/S}^{exp}$	Glycerol yield on substrate during exponential growth phase
$\hat{y}_i$	Estimated value of the dependent variable for observation i
y_i	Value of the dependent variable for observation *i*
$Y_{P/S}^{exp}$	Product yield on substrate during exponential growth phase
$Y_{X/ATP}$	Biomass yield from ATP (ATP yield)
$Y_{X/ATP}^{exp}$	Biomass yield from ATP (ATP yield) during exponential growth phase
$Y_{X/S}$	Biomass yield on substrate
$Y_{X/S}^{exp}$	Biomass yield on substrate during exponential growth phase

CONTENTS

INTRODUCTION

BACKGROUND AND MOTIVATION

Biofuels, which can be translated simply as fuels manufactured from biomass[1], are not new. Engine prototypes developed by Nikolaus Otto around 1860 were run on ethanol (ANTONI; ZVERLOV; SCHWARZ, 2007), and the World's Fair held in Paris in 1900 witnessed a Diesel engine running wholly on peanut oil (KNOTHE, 2001). After being set aside for decades, given the highly competitive low prices of gasoline, biofuels emerge once again as a cheaper alternative to fossil fuels. This time, however, it is not just a matter of market prices—biofuels hold the more appealing "sustainable" label, and this becomes particularly important as the consequences of unrestrained use of oil start to appear, in the form of air contamination by pollutants and changes in climate with unpredictable effects.

In Brazil, endeavors to use ethanol as a motor fuel date back to the 1900s. The first noteworthy attempt was a mixture of ethanol, diethyl ether and castor oil called Usga (from Usina Serra Grande Alagoas), which was made commercially available in the Northeast in 1927 (RODRIGUES, 2000). Since then, the Brazilian government has taken several measures that affected, positively in most cases, the production of ethanol and its use in gasoline blends. These measures were mostly prompted by historical events that pushed oil prices up, as the American Great Depression, the World Wars and the oil crises, and sugar prices down. Still, it was only in 1979, when Proálcool program's second phase was launched, that ethanol was considered not only an additive to gasoline, but again an exclusive motor fuel (ZANIN et al., 2000).

In addition to the great socio-economic impact that ethanol production has had on our country for decades, this industry was responsible for a number of technological breakthroughs, which include process engineering achievements and utilization of byproducts like bagasse (currently for electricity generation), vinasse (as a fertilizer) and dry yeast (a source of single cell protein) (LALUCE, 1991). The implementation of these and other

[1] Current definitions of biomass exclude fossil fuels, since even though they are formed from organic matter their renewal cycle takes millions of years (CENTRO NACIONAL DE REFERÊNCIA EM BIOMASSA, 2013).

innovations led to the classification of the Brazilian ethanol as an advanced biofuel, meaning that its greenhouse gas emissions are reduced by at least 50 % when compared to those from gasoline (US ENVIRONMENTAL PROTECTION AGENCY, 2010).

While some key unit operations used in ethanol production have worked for many years at high efficiencies—distillation efficiency was already 99 % more than a decade ago (ROSA; TOLMASQUIM; AROUCA, 1998)—, other ones have been improving in a much slower pace. Studies on the industrial yeast responsible for the fermentation step were intensified only in the early 1990s, following the development of molecular methods for strain characterization. Furthermore, although ethanol yields during fermentation have reached a plateau at around 90 % of the stoichiometric yield since that time, a minor but steady decrease in this parameter can be noticed in the past few years (DELLA-BIANCA et al., 2013). This clearly points out the lack of attention given to research on fermentation and yeast physiology in Brazil, when compared to another complex, biology-related area that is sugarcane breeding—while there are more than 500 new sugarcane varieties with high productivity and sugar content, adapted to several soil types and climate conditions (GOLDEMBERG; GUARDABASSI, 2010), only 6 yeast strains are available specifically to ethanol distilleries, besides baker's yeast (DELLA-BIANCA et al., 2013).

These strains, responsible for the conversion of sugarcane sucrose into ethanol, belong to the species *Saccharomyces cerevisiae*, a eukaryotic model organism extensively used in both research and industrial applications. In this species, firstly sucrose is hydrolyzed mainly extracellularly by invertase (β-fructofuranosidase; EC 3.2.1.26) to glucose and fructose, which are internalized by hexose transporters that mediate facilitated diffusion. These hexoses follow through the glycolytic pathway and are catabolized to pyruvate, which in turn proceeds through alcoholic fermentation and is converted to ethanol and CO_2 (DICKINSON; SCHWEIZER, 2004). *S. cerevisiae* is particularly suitable for large-scale ethanol production as it exhibits a "make-accumulate-consume" strategy: it consumes sugar and produces ethanol in fast rates, rendering a toxic environment for other microorganisms, and then later, when sugar is exhausted and if O_2 is present, it is able to oxidize the generated ethanol (PISKUR et al., 2006). This is only possible because *S. cerevisiae* displays a Crabtree-positive phenotype (DE DEKEN, 1966) under fully aerobic, high sugar environments. This phenotype is caused by a limited respiratory capacity or a glucose-repressed respiratory

metabolism (VEMURI et al., 2007), and leads to an overflow of pyruvate towards the alcoholic fermentation pathway in those environments.

In the industrial fermentation setting, yeast cells tackle a range of fluctuating conditions that are quite distinct from the defined, controlled laboratory setting. Cells are subjected to selection pressures caused simultaneously by the use of substrates with varying composition, the operation of open fermentors prone to bacterial contamination, the presence of cell stress factors, among others. Along the season, this affects cell population dynamics and enables indigenous, contaminating yeast to replace the starter baker's strain and dominate the fermentor (BASSO et al., 2008, BASSO et al., 1993). Some of these wild strains, selected for their good physiological and technological attributes (such as high ethanol yield, absence of flocculation/foaming and high cell viability), are the ones commercially available today.

Although the selection and the re-introduction of indigenous strains seem a rather straightforward concept, it does not happen so easily. Since the rationale for these strains' better performance is not yet fully explained, this selection process becomes a difficult trial-and-error exercise. Besides, the ethanol production process varies greatly from one distillery to another, and this compromises the implantation of only a few strains across the whole range of distilleries. To understand the mechanisms that make these strains so unique has been the target of a growing number of recent studies (ARGUESO et al., 2009, BABRZADEH et al., 2012, BASSO et al., 2008, BRAVIM et al., 2010, BROWN et al., 2013, DA SILVA-FILHO et al., 2005a, DE MELO et al., 2010, STAMBUK et al., 2009, SWINNEN et al., 2012), and higher tolerance to several stress factors has emerged as a potential explanation for their dominance in industrial fermentors. This topic is particularly important at the moment as second generation (2G) ethanol production processes, which are being implemented in large scale in Brazil using sugarcane bagasse hydrolysates as substrate, pose even more stress to yeast cells than the process used today (for first generation—1G—ethanol production).

SCOPE, OBJECTIVES AND STRUCTURE

This thesis aimed at systematically assessing the cell stress tolerance of industrial strains of *S. cerevisiae*—PE-2, CAT-1, BG-1 and JP1—used in the Brazilian fuel ethanol production process. The whole range of cell stress factors studied is specifically related to the industrial processes carried out in Brazil for 1G and 2G ethanol production. The distinct approach used in this study was based on comparative analysis of quantitative physiology data, obtained from cells under stress conditions, and included as references laboratory strains—S288c and CEN.PK113-7D—and the industrial baker's strain Fleischmann.

The specific objectives of this thesis were to:

1. Compare, in a qualitative and systematic manner, the growth of the above-mentioned strains on solid complex medium containing several stress factors, namely heat; high NaCl, sugar, ethanol and acetic acid concentrations; low pH; presence of industrial substrates and oxidative compounds. This provided a clear picture of the strains' stress tolerance in a fast and reproducible way, allowed the identification of key stress factors that distinguished industrial from laboratory strains, and guided the subsequent experiments.

2. Calculate physiological parameters and estimate carbon balance data from exponential growth of the above-mentioned strains in shake-flasks containing liquid synthetic medium, in standard and heat stress conditions.

3. Calculate physiological parameters from anaerobic chemostat cultivations of strains PE-2 and CEN.PK113-7D on synthetic medium, in standard conditions used for yeast physiology studies. This was performed for the first time for strain PE-2, which was chosen as the model fuel ethanol strain given its wide utilization by Brazilian distilleries.

4. Calculate physiological parameters from anaerobic chemostat cultivations of strains PE-2 and CEN.PK113-7D on synthetic medium, under the presence of acetic acid.

5. Calculate physiological parameters from anaerobic chemostats of strains PE-2 and CEN.PK113-7D on synthetic and complex media, under low pH stress.

6. Calculate physiological parameters from anaerobic dynamic continuous cultivations of strains PE-2 and CEN.PK113-7D on synthetic medium, under low pH stress.

7. Calculate physiological parameters from anaerobic batch cultivations of strains PE-2, CEN.PK113-7D and S288c on synthetic and complex media, under standard and low pH stress.

8. Construct cell viability curves of strains PE-2, CEN.PK113-7D and Fleischmann under extremely low pH.

To compare the transcriptome of industrial strain PE-2 to that of laboratory strain CEN.PK113-7D, both growing under stress conditions during chemostat cultivations, was also among the specific objectives of this thesis. This study was aborted due to the similarity between the values of physiological parameters obtained from the strains.

In Chapter 1 a literature review on the Brazilian industrial yeast strains is presented, corresponding to a paper published in Applied Microbiology and Biotechnology (DELLA-BIANCA et al., 2013). The objectives stated above are tackled in Chapters 2 and 3, which correspond to a manuscript already accepted for publication in Antonie van Leeuwenhoek Journal of Microbiology and another one that will soon be submitted. All the experiments from Chapter 2 and the cell viability curves detailed in Chapter 3 were performed at the Bioprocess Engineering Group (GEnBio, Grupo de Engenharia de Bioprocessos) from the Polytechnic School of the University of São Paulo (Brazil) and supervised by Prof. Andreas Karoly Gombert. The remaining experiments from Chapter 3 were performed at the Industrial Microbiology Section of the Delft University of Technology (the Netherlands) and supervised by Prof. Ton van Maris, Prof. Jean-Marc Daran, Prof. Jack Pronk and Prof. Andreas Karoly Gombert. With the aim of providing more details on the methods employed throughout this work, a supplementary section is presented in Appendix A. Finally, additional results obtained in this work are presented at the end of this thesis (Appendix B).

1 WHAT DO WE KNOW ABOUT THE YEAST STRAINS FROM THE BRAZILIAN FUEL ETHANOL INDUSTRY?[2]

1.1 ABSTRACT

The production of fuel ethanol from sugarcane-based raw materials in Brazil is a successful example of a large-scale bioprocess that delivers an advanced biofuel at competitive prices and low environmental impact. Two to three fed-batch fermentations per day, with acid treatment of the yeast cream between consecutive cycles, during 6–8 months of uninterrupted production in a non-aseptic environment are some of the features that make the Brazilian process quite peculiar. Along the past decades, some wild *Saccharomyces cerevisiae* strains were isolated, identified, characterized, and eventually, reintroduced into the process, enabling us to build up knowledge on these organisms. This information, combined with physiological studies in the laboratory and, more recently, genome sequencing data, has allowed us to start clarifying why and how these strains behave differently from the better known laboratory, wine, beer, and baker's strains. All these issues are covered in this minireview, which also presents a brief discussion on future directions in the field and on the perspectives of introducing genetically modified strains in this industrial process.

1.2 INTRODUCTION

Bioethanol and biodiesel are the main biofuels used worldwide today, with the first occupying an outstanding position in the Brazilian economy. In the 1970s, as oil became more expensive and sugar cheaper, the Brazilian government established a national program (Pro-Álcool), which aimed at the substitution of gasoline with fuel ethanol (GOLDEMBERG,

[2] DELLA-BIANCA, B. E.; BASSO, T. O.; STAMBUK, B. U.; BASSO, L. C.; GOMBERT, A. K. What do we know about the yeast strains from the Brazilian fuel ethanol industry? **Appl Microbiol Biot**, v. 97, n. 3, p. 979-991, 2013.

2008) produced by fermentation of sucrose from sugarcane. This initiative ensured Brazil about 30 years of expertise ahead of other countries in first-generation (1G) bioethanol production and a privileged position in the fuel sector. While nowadays sugarcane 1G bioethanol replaces ≈1 % of the gasoline used in the world, the potential of this technology is far from being exhausted. Gains in productivity and geographical expansion to larger areas may allow ethanol production from sugarcane to replace ≈10 % of the world's demand for gasoline before second-generation (2G) bioethanol production (from lignocellulosic biomass) reaches technological maturity and possible economical competitiveness (AMORIM et al., 2011, BUCKERIDGE et al., 2012, GOLDEMBERG, 2007, GOLDEMBERG; GUARDABASSI, 2010, SOCCOL et al., 2010, STAMBUK et al., 2008). Indeed, Brazilian sugarcane fuel ethanol is highly competitive when compared with production processes from other crops (e.g., corn and sugarbeet), as it shows the highest percentage of greenhouse gas emission reduction, the highest energy balance and yields per hectare, and lower production costs (GAROMA; BEN-KHALED; BEYENE, 2012, LEAL; WALTER, 2010, SÁNCHEZ; CARDONA, 2008).

In the 2011/2012 crop, Brazil produced approximately 23 billion liters of bioethanol, and almost 60 % of all Brazilian light vehicles can run on this fuel (BRAZIL, 2012a, BRAZILIAN SUGARCANE INDUSTRY ASSOCIATION, 2012). The energy content of sugarcane corresponds to 18 % of the country's energy matrix and represents a higher share than hydroelectricity (ALTIERI, 2012). This value includes the solid part, mainly bagasse, which could be used as a substrate for 2G bioethanol production but is currently burned in boilers to generate electricity to the mills and, if there is a surplus, to the national grid. This sugar/energy market employs 4.3 million people (in both direct and indirect jobs) and has a turnover of more than 80 billion dollars annually (SOUZA; MACEDO, 2010). Obviously, the achievement of such impressive numbers is only possible because Brazil takes advantage of its soil characteristics, climatic conditions, and vast land area. Sugarcane crops are concentrated in two regions: the Center-South, which produces approximately 90 % of all sugar and ethanol, and the North–Northeast, responsible for the other 10 % (BRAZIL, 2012a).

Since 1975, the overall yield of the Brazilian fuel ethanol industry has increased steadily by 3–4 % per year (Fig. 1a), reaching nowadays over 6,500 L ethanol per hectare of sugarcane. This increase was possible due to several agricultural improvements, including selection of new sugarcane varieties with increased amounts of sugarcane biomass per hectare (Fig. 1b). While the amount of total sugar per ton of sugarcane also increased

significantly during the first years (Fig. 1c), in the last 15 years, it seems to have reached a plateau around 140 kg of sugar per ton of sugarcane. Thus, there is a need for improvement in sucrose production and accumulation by the sugarcane plant, a feature that is under current research in Brazil (LAM et al., 2009, WACLAWOVSKY et al., 2010). This trend in the amount of sugar per ton of sugarcane biomass reflects directly into the amount of ethanol produced from each ton of sugarcane, as the fermentation and distillation processes have also reached high industrial efficiency with an apparent plateau in the last years of approximately 80 L ethanol per ton of sugarcane (Fig. 1d).

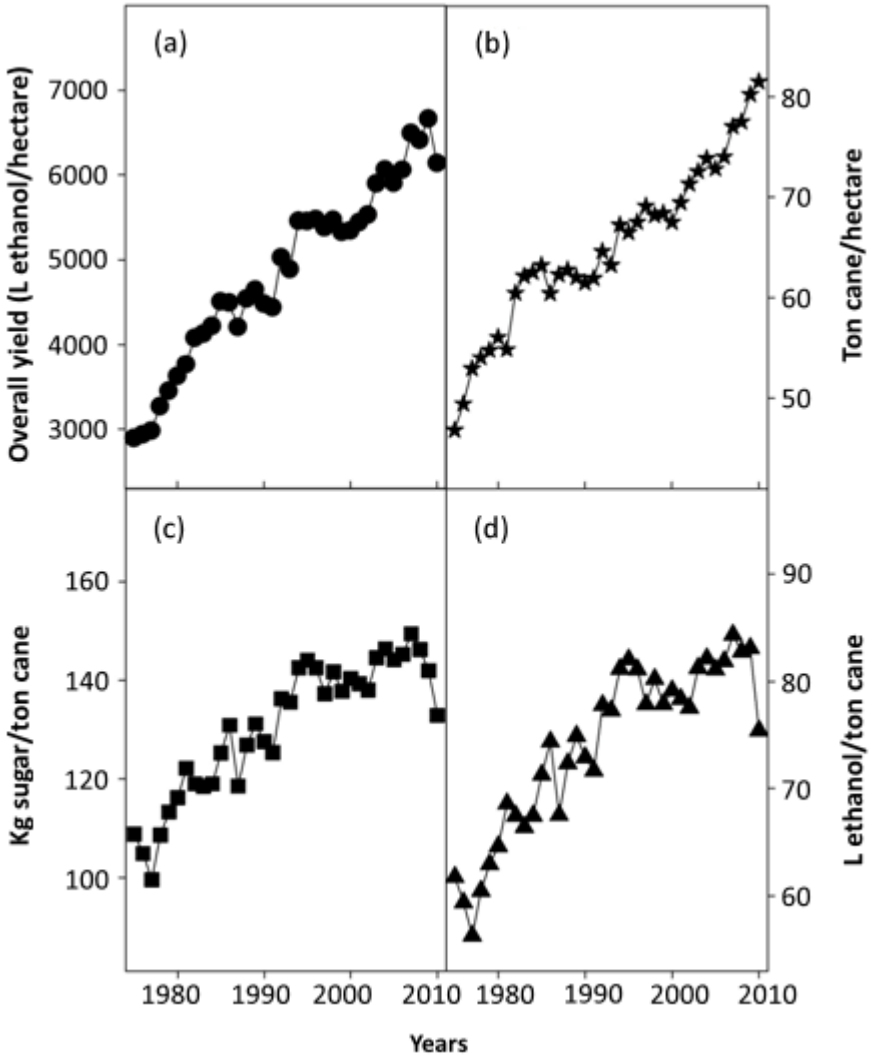

Figure 1 – Evolution of the Brazilian fuel ethanol production industry. The data show the growth in overall yield (industrial+agricultural) (a), due to improvements in the agricultural sector (b), including the selection of higher sucrose-producing sugarcane varieties (c) as well as the industrial fermentation performance (d) from 1975 to 2010; data from BRAZIL (2012b).

During fermentation, the key step in bioethanol production, sucrose (and glucose and fructose) is converted into ethanol by the yeast *Saccharomyces cerevisiae* in vats containing millions of liters. When there are no major operational issues (such as rain, contamination, and power failure), yields as high as 92 % of the stoichiometric conversion (0.511 g ethanol g hexose equivalent^{-1}) can be achieved, yet the average industrial yield in Brazil has slightly decreased during the last decade (Fig. 2). Since more than half of ethanol's final cost is due to the cost of sugarcane, any increment on the already high yield value would represent great economical gains. As an example, for an annual production of 30 billion liters of ethanol, a 1 % increase in fermentation yield would allow for the production of extra 300 million liters from the same amount of raw material or, in other words, from the same cultivated area, and this yield increase would represent an economical gain of more than R$ 368 million[3].

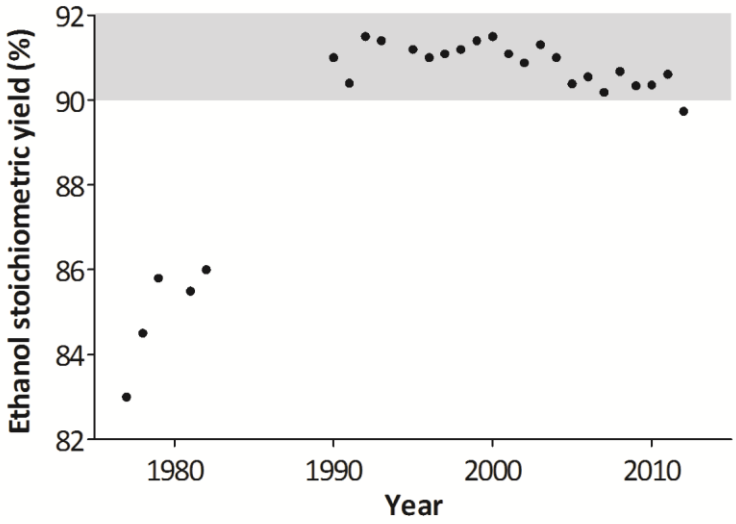

Figure 2 – Average ethanol yields (relative to 0.511 g ethanol g hexose^{-1}) from the Brazilian fuel ethanol production industry since the mid 1970s. The 90–92 % range is highlighted, with the aim of enabling a better observation of the recent drops in the fermentation yield. Each data point corresponds to the average of values obtained from at least 30 ethanol-producing plants, which were sampled every day (with a minimum of four samples per day) during at least 200 days in the season (information kindly provided by Jaime Fingerut, Centro de Tecnologia Canavieira, Piracicaba, SP, Brazil).

[3] Considering the producer price for anhydrous fuel ethanol of R$ 1.2273 per liter (price observed in São Paulo state in August 2013 (BRAZILIAN SUGARCANE ASSOCIATION, 2013)).

One of the ways to achieve this increase is through the use of adequate yeast strains, as has traditionally been done in wine and brewing industries. It has been proven that strains adapted to the harsh environment found in the industrial ethanolic fermentations are capable of delivering higher yields and productivities; studying these strains became an essential task for optimizing the process or having them genetically modified—not only for ethanol fermentation but also for the production of other biofuels, chemical precursors, or higher value compounds from sugarcane-based substrates (e.g., artemisinin).

1.3 THE PECULIARITIES OF THE BRAZILIAN FUEL ETHANOL INDUSTRIAL PROCESS

In Brazilian distilleries, sugarcane (*Saccharum* sp.) is the raw material of choice for sugar and bioethanol production. It provides readily fermentable sugars (11–18 % wet w/w, with 90 % of those being sucrose and 10 % glucose and fructose) that do not need any prior treatment to be metabolized by *S. cerevisiae* (WHEALS et al., 1999). Moreover, endophytic nitrogen-fixing bacteria can supply up to 60 % of sugarcane's nitrogen demand in low fertility soils, reducing the need for nitrogen fertilizers known to require large amounts of fossil energy for their production. These features contribute to a highly favorable energy balance (output:input ratio)—sugarcane ethanol's energy balance for Brazilian 2005/2006 crop season was estimated as 9.3:1 and is predicted to be higher than 11:1 in 2020 (MACEDO; SEABRA; SILVA, 2008), while for corn ethanol, it varies from less than 1:1 (PIMENTEL, 2003) to 2.8:1 (SHAPOURI et al., 2008).

Sugarcane juice and/or sugarcane molasses are used as substrates for bioethanol production. After washing and chopping, sugarcane is crushed for juice extraction. For sugar manufacturing, sucrose crystals are obtained after intense juice evaporation and centrifugation. The remaining viscous phase is called molasses, which still contains 45 to 60 % sucrose and 5 to 20 % glucose and fructose. Generally, the fermentation substrate is a mixture of varying proportions of sugarcane juice and molasses. This is because molasses is nutrient-rich and can even provide a buffering effect during fermentation, while sugarcane juice can be nutrient-deficient. On the other hand, molasses may also contain salts in high amounts and other inhibitors (mainly browning reaction compounds) (AMORIM; BASSO;

LOPES, 2009). The mineral composition of the substrate varies widely, depending on molasses proportion, sugarcane variety and maturity, soil, climate, and juice processing (Fig. 3).

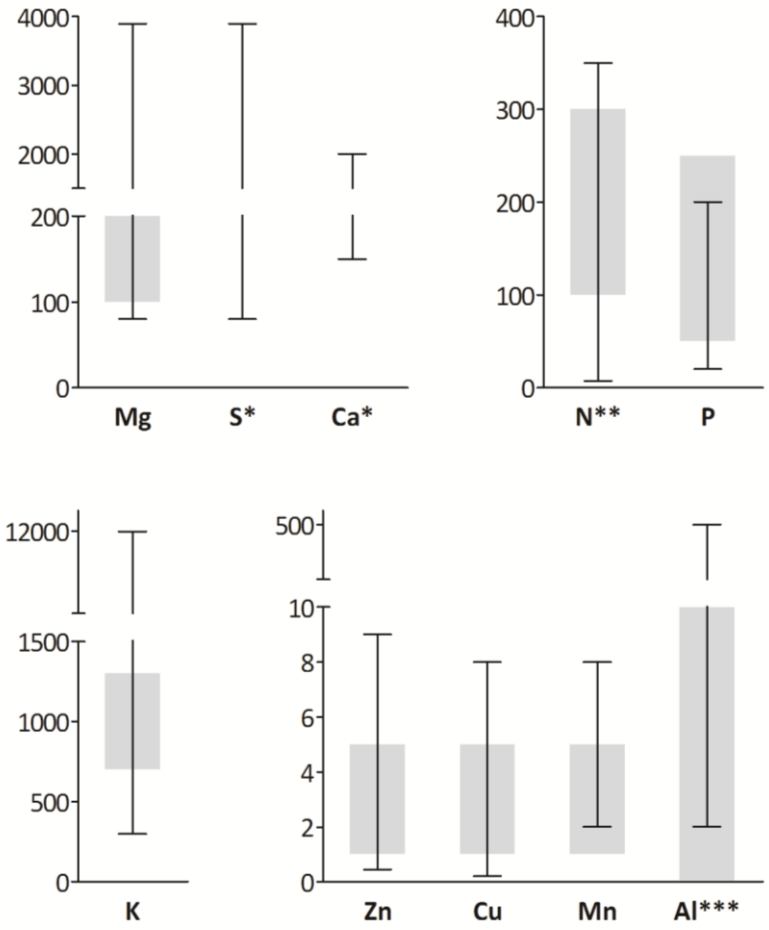

Figure 3 – Mineral composition of sugarcane-based substrates. Black lines represent the concentration range found in the substrates, and gray bars show the ideal concentration range for industrial production of ethanol. Data are given in mg L^{-1} of each element. *Ideal range is as low as possible. **In form of NH_4^+ and R-NH_2 nitrogen. ***A toxicant; ideal range for juice-only substrates. Data obtained from Amorim (2005) and Basso, Basso and Rocha (2011).

Introduced in Brazil in the 1960s (ZANIN et al., 2000), the Melle-Boinot process is the method of choice for ethanol fermentation and comprises three main features. First, fed-batch mode is used due to higher production stability, simpler flow adjustment, and lower equipment costs when compared to the continuous mode (AMORIM; BASSO; LOPES, 2009,

DÖRFLER; AMORIM, 2007, GODOY et al., 2008); however, according to Andrietta et al. (2007), the usual low-cost adaptations of plants from batch to continuous modes may hide the benefits from the latter option. Second, up to 90–95 % of the yeast cells are recycled by centrifugation and this allows high cell densities during fermentation (10 to 17 % (wet w/v)), so that no intensive yeast propagation is needed before (or during) each fermentation cycle. This, combined with the low nitrogen content of sugarcane-based substrates, allows for an increase of only 5 to 10 % of yeast biomass during one fermentation cycle, which is sufficient to replace the loss of cells during centrifugation. Accordingly, fermentation time becomes very short (6 to 10 h) in comparison to other fermentation processes (e.g., 40 to 50 h for corn ethanol), yet this benefit makes cooling the fermentors more difficult and temperatures go from 32 to 35 °C up to 40 °C in the summer season (AMORIM; BASSO; LOPES, 2004, ANDRIETTA et al., 2007, LALUCE, 1991, LIMA; AQUARONE; BORZANI, 1975, LIMA; BASSO; AMORIM, 2001, WHEALS et al., 1999). Lastly, recycled yeast goes through an acid treatment that decreases bacterial contamination (AMORIM, 2005, NEPOMUCENO; FERNANDES; BACCHI, 1997). This treatment lasts 1 to 3 h and consists of dilution with water (1:1) and addition of sulfuric acid (pH 1.8–2.5), after which the cells are reused. This particular step occurs at least twice a day during a season that may last up to 250 days (BASSO et al., 2008). In summary, fermentation starts by adding cane substrate, containing 18 to 22 % (w/w) total reducing sugars (TRS), to a 30 % (wet basis) yeast suspension that represents 25 to 30 % of the fermentation vat total volume. Feeding time normally lasts for 4 to 6 h and fermentation is finished within 6 to 10 h, resulting in ethanol titers of 8 to 12 % (v/v). Ethanol titers could be higher, but this would mean increasing fermentation time to unacceptable levels and it would also compromise yeast viability and, consequently, its fitness during the subsequent fermentation cycle.

The fuel ethanol production process provides an environment that is far from the optimal physiological condition for yeast. Several stress factors alternate during the process, among which the following are the most relevant ones (Fig. 4): high sugar and ethanol concentrations, elevated temperatures, pH variations, and presence of toxic compounds. In this unusual environment, yeast exhibits stress responses—a good industrial strain must be sufficiently robust to respond well to these environmental variations, without altering its fermentative characteristics over the cycles it is exposed to during the whole crop season (ATTFIELD, 1997). Sugarcane must is an environment that does not preclude the

proliferation of contaminants, mainly bacteria, given its high nutrient concentration, high water activity, and favorable pH for some species (AMORIM, 2005). Besides bacteria, wild yeasts (including *S. cerevisiae* strains) can contaminate and dominate the medium; after isolation and molecular and physiological characterizations (BASSO et al., 2008), some of these strains were found to be: (1) more adapted to the process than the starter cultures, (2) able to promote higher ethanol yields, and (3) not causative of technological issues, such as flocculation and/or foaming.

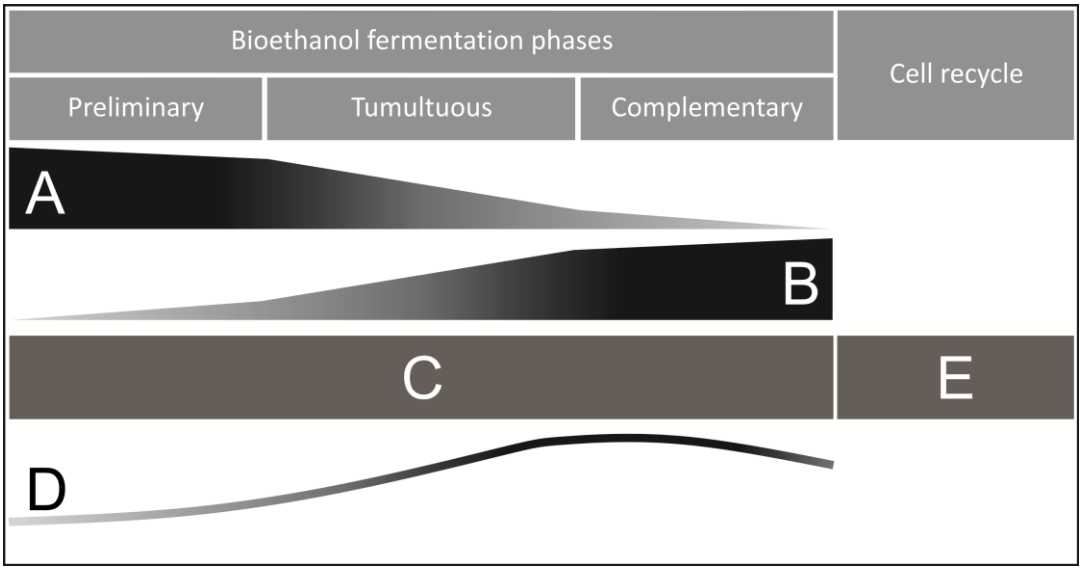

Figure 4 – Environmental stresses found by yeast during alcoholic fermentation phases in the Brazilian fuel ethanol production process; A high sugar concentration, B high ethanol concentration, C osmotic stress and bacterial contamination, D high temperature, E acid stress (adapted from Gibson et al. (2007)).

1.4 IDENTITY OF BRAZILIAN DOMINANT AND EFFICIENT FUEL ETHANOL YEAST STRAINS

One of the first attempts to determine which strains predominate in a group of five distilleries in Brazil was performed by Basso et al. (1993). Using a karyotyping protocol adapted from Vezinhet, Blondin and Hallet (1990), it was shown that starter strains (baker's and other two *S. cerevisiae* strains—TA and NF) were unable to compete with indigenous yeasts that contaminated the industrial process. This study also showed that a succession of different indigenous *S. cerevisiae* strains was detected throughout the fermentation season

and that only a wild strain (JA-1), previously isolated from one distillery, could survive the recycle step. Similar observations have been reported more recently—da Silva-Filho et al. (2005b) concluded that only strains that were previously isolated from ethanol plants (and then used as starter cultures) were detected during almost the whole crop season and Basso et al. (2008) noticed that baker's yeast, traditionally used as a starter culture, was rapidly replaced by wild strains in a short period (20 to 60 days) of cell recycle. Apart from karyotyping, other molecular techniques have also been employed to investigate yeast population dynamics during ethanol fermentation, such as a PCR fingerprinting method based on microsatellite $(GTG)_5$ primer used by da Silva-Filho et al. (2005b), which was used to identify dominant indigenous strains.

Wild yeast contaminants have also been monitored in the ethanol industry by molecular techniques during the fermentation step (BASÍLIO et al., 2008, BASSO et al., 2008, DA SILVA-FILHO et al., 2005b, DE SOUZA LIBERAL et al., 2007) as well as during the production of cachaça (a spirit made from sugarcane juice) in several regions of Brazil (BADOTTI et al., 2010, BERNARDI et al., 2008, GOMES et al., 2007, MARINI et al., 2009). da Silva-Filho et al. (2005b) reported on the presence of a significant proportion of non-*Saccharomyces* contaminant strains (12–30 %) during a particular fermentation season. In distilleries in Northeastern Brazil, the most frequent contaminants were identified as *Dekkera bruxellensis* (DE SOUZA LIBERAL et al., 2007, LEITE et al., 2012), responsible for severe impacts on ethanol productivity. It is known that polyhexamethyl biguanide (PHMB), a fungicide that affects the cell membrane, kills *D. bruxellensis* strains (ELSZTEIN; DE MENEZES; DE MORAIS JR, 2008) but not the industrial fuel ethanol strain JP1 (although strain PE-2 is sensitive to this compound). Strain JP1 tolerance to PHMB was later found to be related to the induction of genes involved in cell wall integrity and in general and oxidative stress responses (ELSZTEIN; DE LUCENA; DE MORAIS JR, 2011).

In the same region, *Candida tropicalis* and *Pichia galeiformis* were also detected as major yeast contaminants in acute contamination episodes, being responsible for decreased ethanol yields (BASÍLIO et al., 2008). However, it should be stressed that in a wider study covering Central-Southeastern Brazil, in only a few distilleries could non-*Saccharomyces* contaminants be identified (mainly *Schizosaccharomyces pombe*, *D. bruxellensis*, and *C. krusei*), representing less than 5 % of the yeast population (BASSO et al., 2008). It was demonstrated that such contaminant non-*Saccharomyces* strains were more frequently

found in distilleries operating with relatively lower final ethanol concentrations (6 % v/v) and would not pose a problem for regularly operating ethanol plants that work with higher ethanol titers.

It is generally accepted by researchers and industrial personnel involved in large-scale ethanol production that starting the fermentation season with adapted (selected) strain(s) is of crucial importance in order to keep yeast population stability, which, in turn, leads to reproducible (constant) ethanol yield and productivity. Thus, the large inoculum required at the beginning of the season (starter) is commonly prepared by mixing 2 to 12 t of baker's yeast with 10 to 300 kg of selected strains in active dry yeast form. One example is the study performed by da Silva-Filho et al. (2005a), which describes the use of both PCR fingerprinting and physiological assays in the selection of an indigenous strain (JP1) as a proposed starter culture. This strain showed stress tolerance (towards acidic pH, ethanol, and high temperature conditions) and fermentative capacity similar to those of other commercial industrial strains. After reintroduction as the starter strain in the distillery it had been isolated from, it was found that JP1 strain was able to dominate the population while conferring high ethanol yields (>90 %) for two consecutive production seasons.

Although these results might suggest that selecting well-fitted indigenous strains is an attractive strategy to guarantee high product yields and population homogeneity during industrial fermentations, one should be aware that such a task is quite laborious and not always successful. First, the selected strain must have appropriate physiological characteristics, such as good fermentation performance, high dominance, and high persistence. In this context, dominance is the proportion of a particular strain inside the fermentor at a given point of the season and is related to competitiveness among strains and to their specific growth rate. In contrast, persistence means the strain's ability to withstand the multiple stress conditions found in the bioethanol fermentation process and, therefore, survive throughout the season and can be expressed as the proportion of distilleries in which a particular strain was able to be implanted, or implantation capability. Second, the strain must possess appropriate technological properties, like absence of flocculation and of stuck fermentations, and low foam formation. In the ethanol production process adopted in Brazil, flocculating yeast can increase fermentation time because they cannot reach the substrate as efficiently as non-flocculating cells as well as cause clogging and lower centrifugation yield. Although Universidade Estadual de Campinas (2000) and Gomes et al. (2012)

constructed flocculating ethanol strains, suggesting the use of an approach more similar to the repitching performed by the brewing industry and a decrease in cell recycle costs, this would require equipment adaptation in current Brazilian ethanol plants.

The 12-year yeast selection study performed by Basso et al. (2008) also made use of karyotyping as a tool to spot out dominant indigenous strains at various ethanol producing plants. Surprisingly, among more than 300 dominant isolates, only 20 % showed appropriate technological characteristics and were further evaluated at laboratory conditions that simulated the industrial process. Of these strains, only 14 presented good physiological properties. These strains were then reintroduced in the distilleries they had been isolated from and also in other ethanol plants in order to evaluate their implantation capability throughout successive production seasons. This program selected, among others, the strains PE-2 and CAT-1, which showed the highest dominance during the research time frame. These two strains currently represent 70 % of the Brazilian market for fuel ethanol yeast strains (Henrique Amorim, personal communication). Besides these, four other strains (SA-1, BG-1, FT858, and JP1) are commercially available (LNF-Latino Americana Ltda. and Marcos Morais Jr., personal communication). According to Basso et al. (2008), it was not possible to establish a relationship between strain adaptability and particular process features, such as mode of operation and type of substrate. On the other hand, da Silva-Filho et al. (2005a) showed a relationship between the prevalence of a particular strain and the type of substrate used in the industrial plant, in this case, juice- or molasses-based medium.

1.5 PHYSIOLOGY OF BRAZILIAN INDUSTRIAL FUEL ETHANOL STRAINS

Until the mid 1990s, IZ-1904, TA and baker's yeast were the most used starter strains for industrial fuel ethanol fermentation. However, in laboratory studies, their physiological parameters were obtained after a single fermentation round (without cell recycle). When the same strains were evaluated under cell recycle conditions, valuable physiological data arose. Indeed, when compared with baker's yeast, IZ-1904 presented higher ethanol yield (and titer) and also lower biomass gain, but higher glycerol formation, lower viability, and very low levels of intracellular trehalose. Such data suggested that IZ-1904 would not be able

to endure cell recycle, since the higher ethanol yield observed was at the expense of biomass formation and trehalose accumulation (ALVES, 1994, BASSO; AMORIM; GUTIERREZ, 1988). These data were the starting point for studies on the implantation capability of these starter strains in distilleries, and further results demonstrated that the only strain able to persist in industrial fermentation conditions was JA-1, a strain previously isolated from an ethanol plant (BASSO et al., 1993). When compared with TA, JA-1 showed superior fermentation capabilities, such as higher ethanol yield, lower glycerol formation, higher cell viability, and higher trehalose content (BASSO; AMORIM, 1994).

PE-2 and VR-1 strains were first introduced in the 1995/1996 season in 24 distilleries, showing a remarkable implantation capability. These strains represented 80 to 100 % of the total yeast biomass in the fermentors of 12 distilleries and were implanted in 63 % of the plants, accounting for 42 % of the biomass at the end of the season (BASSO; AMORIM; OLIVEIRA, 1996). Since then, PE-2 strain has been used as a reference industrial strain and, therefore, compared with baker's yeast to disclose physiological traits that could be related to its superior fermentation performance. It also proved to be suitable for microvinification of raspberry juice (DUARTE et al., 2010) and sweet potato hydrolysate (PAVLAK et al., 2011). Its high ethanol tolerance made PE-2 appropriate for very high gravity (VHG) fermentation, attaining final ethanol contents of >19 % (v/v) with a productivity of >2.5 g L^{-1} h^{-1}, while the laboratory CEN.PK113-7D strain reached 17.5 % (v/v) ethanol with a productivity of 1.7 g L^{-1} h^{-1} (PEREIRA et al., 2010a, PEREIRA et al., 2011). This strain was also studied under VHG fermentations with cell recycle, attaining high ethanol titer and productivity during 15 cycles (PEREIRA et al., 2012). Also, a recombinant derivative of PE-2 bearing the *FLO1* gene, responsible for a flocculation protein, performed successfully under VHG and flocculation–sedimentation cell recycle conditions (GOMES et al., 2012).

These industrial strains usually accumulate high levels of storage carbohydrates (trehalose and glycogen, which can account for 20 % w/w of yeast dry weight) towards the end of fermentation, as demonstrated by Paulillo, Yokoya and Basso (2003). The degradation kinetics of these storage carbohydrates in strains SA-1 and PE-2 was analyzed and, under high temperature and high biomass concentration, these compounds could be further metabolized. This, in turn, resulted in extra ethanol formation and a consequent increase in protein levels, which went from 35 to 42 % (w/w) and from 42 to 52 % (w/w) in PE-2 and SA-1 strains, respectively. This strategy is outlined as an important way to add economical value

to the excess yeast slurry commercialized by the ethanol industry as animal feed, in which the protein levels are expected to be at least 40 % (AMORIM; BASSO, 1991).

Regarding the patterns of sugar utilization by industrial fuel ethanol yeasts, their ability to ferment sucrose (MIRANDA JR et al., 2009) and maltose and maltotriose (AMORIM NETO et al., 2009, DUVAL et al., 2010) has been evaluated. The results indicated that strain CAT-1 ferments maltose but not maltotriose, and it showed similar fermentation performance and higher thermotolerance when compared to standard commercial whisky-distilling yeast (AMORIM NETO et al., 2009). These industrial yeast strains can also easily ferment sugar (glucose) in high concentrations, up to 330 g L^{-1} for strain PE-2 (PEREIRA et al., 2010b).

Alves (2000) highlighted the high rate of sucrose hydrolysis by baker's yeast in relation to PE-2 strain during fermentation, in both cases exceeding glucose and fructose uptake capacity. Accordingly, a greater accumulation of hexoses (nearly double) was observed for baker's strain in relation to PE-2 strain. It was proposed that this condition would be imposing a higher osmotic stress and thus, accounting for the higher glycerol production observed in cultivations with baker's yeast; internal glycerol was also much higher during fermentation with this strain. It was also demonstrated that both strains were deprived of a succinic acid transporter. Nevertheless, when they were placed in ^{14}C-succinate-containing medium, intracellular radioactivity was twice higher in baker's yeast, suggesting a less selective membrane than PE-2's. During cell recycle, PE-2 presented higher performance than baker's yeast, as indicated by physiological and technological parameters (BASSO et al., 2008) such as higher ethanol yield, lower glycerol formation, vigorous growth, and high cell viability, presumably promoted by its higher levels of intracellular trehalose and glycogen. Compared to the laboratory strain CEN.PK113-7D, the PE-2 strain could indeed accumulate higher contents not only of trehalose and glycogen (1.1- and 2.6-fold, respectively) but also of sterols (threefold), which may be another form of adaptation to the stressful VHG conditions that the strains have been exposed to (PEREIRA et al., 2011).

PE-2 strain indeed showed higher viability and trehalose levels in cell recycle conditions when compared to another Brazilian industrial strain, M26, but few differences regarding ethanol yield (DORTA et al., 2006). On the other hand, M26 presented higher production of succinic acid, what can, at least in part, explain the higher antibacterial effect previously observed against *Lactobacillus fermentum* CCT 1396 (DE OLIVA-NETO; FERREIRA;

YOKOYA, 2004) and *L. fermentum* CCT 1407 (CHERUBIN, 2003). Dorta et al. (2006) also showed that low pH (3.6) and high ethanol titers (9.5 % w/v), acting synergistically, were the major stress factors to decrease industrial yeast cell viability and that low pH (3.6) was the main factor to decrease ethanol yield.

Apart from presenting the population dynamics that resulted in the isolation of strain JP1 (described in the previous section), da Silva-Filho et al. (2005a) also studied several of its physiological parameters. They showed that under laboratory conditions, JP1 displayed ethanol yields similar to those observed for industrial fuel strains BG-1, VR-1, PE-2, and SA-1 (ranging from 0.46 to 0.50 g ethanol g sucrose^{-1}). It was also verified that JP1's tolerance towards acid, heat, and ethanol stresses are comparable to PE-2's response and that a temperature increase from 37 to 42 °C magnified cell viability decrease caused by low pH or high ethanol concentration. In a molasses-based medium, JP1 grew slower than PE-2 (0.16 vs. 0.23 h^{-1}), whereas they exhibited similar growth rates on diluted cane juice medium (around 0.3 h^{-1}); these observations probably reflect the distinct environments these strains were isolated from.

The majority of the (few) papers published on the physiology of industrial strains employed in Brazil investigated the response of these strains towards stress conditions associated with the industrial scenario, such as high ethanol titers, elevated temperature, high osmotic stress due to sugar and salts, low pH, possible inhibitors, and bacterial contamination. These conditions have been investigated in more detail for other fuel ethanol-related strains, such as the ones involved in corn-based ethanol industry (ZHAO; BAI, 2009). For baker's strain, temperature, pH, sugar concentration, nitrogen (ALVES, 1994, GUTIERREZ, 1989, 1991), potassium, sulfite (ALVES, 1994), nitrite (GUTIERREZ; ORELLI, 1991), 2,4-dinitrophenol (GUTIERREZ, 1989, 1991), benzoic acid (GUTIERREZ et al., 1991a), acetic acid (GUTIERREZ et al., 1991b), and octanoic acid (GUTIERREZ, 1993) had their effects investigated. Noteworthy, sulfite up to 300 mg L^{-1} slightly increased glycerol formation but did not affect ethanol yield, yeast viability, or cellular trehalose levels, possibly because it reduced bacterial growth and, consequently, lactic and acetic acid concentrations in the culture medium (ALVES, 1994).

Some metals, such as aluminum and cadmium, can also affect industrial strains. The toxic form of aluminum (Al^{3+}) is favored by the acidic environment of fermentation and, although very low levels of cadmium were detected in sugarcane from crops fertilized with

municipal sewage sludge, toxic levels of this element were found during fermentations, since yeast has the ability to accumulate cadmium throughout the intensive cell recycle. Among the toxic effects of both metals are lower cell viability, decreased intracellular trehalose content, and reduced ethanol yield. While the presence of magnesium only minimizes aluminum toxicity, the use of a molasses-rich medium eradicates it. The use of vinasse/stillage (effluent from ethanol distillation) is efficient to alleviate both aluminum and cadmium toxic effects. When compared to baker's yeast, strain PE-2 presents higher tolerance and lower intracellular accumulation of aluminum during cell recycle; concerning cadmium toxicity, it is more tolerant than strain IZ-1904 (BASSO et al., 2004, DE SOUZA OLIVEIRA et al., 2009, MARIANO-DA-SILVA; BASSO, 2004, SILVA et al., 2010).

Excessive yeast growth observed in some distilleries compromised ethanol yield; for this reason, increasing ethanol yield by lowering biomass and/or by-product formation (mainly glycerol) was performed by addition of 2,4-dinitrophenol (GUTIERREZ, 1989), acetic acid (GUTIERREZ et al., 1991b), or benzoic acid (GUTIERREZ et al., 1991a). The latter treatment was more efficient and kept high yeast cell viability. During the 1992/1994 crop seasons, it was used in three different distilleries and evaluated in laboratory scale, simulating cell recycle process and using baker's yeast (BASSO; AMORIM; OLIVEIRA, 1996). In all distilleries, a significant increase in ethanol yield and a reduction in glycerol formation were observed during the initial cycles, but later on, bacterial growth was high enough to make the use of benzoic acid in ethanol plants impracticable. In laboratory conditions, not only the same trend was observed but also a drastic reduction of succinic acid formation by yeast was noticed. Additionally, it was demonstrated that succinic acid produced by yeast exerts an antibacterial effect during fermentation, being an important inhibitor of lactic acid bacteria (BASSO; ALVES; AMORIM, 1997). An ecological reason for succinic acid production was then suggested, since it renders yeast more competitive in an industrial fermentation environment, especially in fuel ethanol distilleries where bacterial contamination is very frequent.

Bacterial contamination is often regarded as a major drawback during industrial ethanol fermentation. Besides deviating feedstock sugars from ethanol formation, there are also detrimental effects of bacterial metabolites (such as lactic and acetic acids) upon yeast fermentative performance, resulting in reduced ethanol yields, yeast cell flocculation, and low yeast viability. Most of the bacterial contaminants of the fermentative step of ethanol

production are lactic acid bacteria, probably because they are more able to cope with low pH values and high ethanol concentrations when compared to other microorganisms; indeed, *Lactobacillus* was the most abundant genera isolated from Brazilian ethanol plants, from both homo- and heterofermentative types (BASSO; BASSO; ROCHA, 2011).

1.6 FIRST GENOMIC INFORMATION OF BRAZILIAN FUEL ETHANOL YEAST STRAINS

Industrial strains are a rich source of unexplored genetic diversity, mainly for their alleles and/or mutations controlling traits relevant to the industrial fermentation. Identifying and characterizing these mutations could provide the basis for a reverse metabolic engineering (ME) approach in order to develop even better fermenting strains. The detailed genomic structure of some of these industrial strains has started to be determined (ARGUESO et al., 2009, STAMBUK et al., 2009), revealing significant structural and sequence variations when compared to laboratory strains or others isolated from nature. For example, while many brewing and wine industrial yeast strains are interspecies hybrids between two or even three yeast species (*S. cerevisiae*, *S. bayanus*, and/or *S. kudriavzezii*), generating polyploid, aneuploid, or even allopolyploid genomes with sometimes mosaic chromosomes (BORNEMAN et al., 2011, NOVO et al., 2009, QUEROL; BOND, 2009), the industrial fuel ethanol yeasts selected in Brazil are bona fide heterothallic diploid *S. cerevisiae* strains, exceptions being strains JP1, which is a homothallic diploid (REIS et al., 2012), and FT-858, a new polyploid strain probably derived from PE-2 that exhibited a high implantation rate during the 2011/2012 crop season (Henrique Amorim, personal communication). The genome from strain BG-1 contains two *S. paradoxus* introgression events; however, this strain is not an interspecific hybrid. These introgression events encompass regions containing *MAL* (maltose utilizing) genes and *STL1* (glycerol transport) and *PAD1* and *FDC1* (both phenolic acid decarboxylases) genes, all of them relevant to the industrial conditions (DUNN et al., 2012). A list of all Brazilian industrial strains which genomic data have already been published is shown in Table 1.

Table 1 – Brazilian fuel ethanol strains and their genomic details.

Strain	Ploidy	Details[a]	References	Databases[b]
PE-2	Diploid	Heterothallic; presents efficient sporulation and ≈2.6 SNPs/kb between allelic regions in homologous chromosomes; carries amplified *SAM3/SAM4* and *SNO/SNZ*	Argueso et al. (2009) Stambuk et al. (2009)	GEO accession nos. GSE14601, GSE17578, GSE13875
JAY291	Haploid	Presents 5.4 SNPs/kb in comparison to S288c *ho FLO8 HAP1 SUC2* Carries amplified *SEO1* and *ADH7;* does not carry *RTM1*	Argueso et al. (2009) Babrzadeh et al. (2012)	GenBank accession no. ACFL01000000
CAT-1	Diploid	Heterothallic; presents high heterozygozity and large-scale loss of heterozygozity but no sequences corresponding to the 2-µm plasmid Carries amplified *SEO1*, *ADH7*, *RDS1*, *SGE1*, *ARR1-ARR3*, *SAM3/SAM4*, *SNO/SNZ; c*arries lower copy number of *MPH2*, *SOR2*, *HXT15*, *MPH3*, *SOR1*, *HXT16*, *DAK2*, *AGP3*, *SNO4*, *ERR3*, *AAD15*, *BDS1*, *FIT2*, *FIT3*, *FRE5*, *PHR1*, *MAL11*(*AGT1*), *HXT6*, *HXT7*, *ENA1*, *ENA2*, *ENA5*, *CUP1-1*, *CUP1-2;* does not carry *RTM1*, *FLO1* or *FLO9* Missing regions in *FLO5*, *FLO10* and *FLO11*	Stambuk et al. (2009) Babrzadeh et al. (2012)	GEO accession no. GSE13875 SRA accession no. SRA012578
BG-1	Diploid	Heterothallic; carries a smaller version of the 2-µm plasmid and amplified *SNO/SNZ* Presents *S. paradoxus* introgression regions containing *MAL*, *STL1*, *PDA1* and *FDC1* Missing regions in mitochondrial *COX1*, *ENA* and *HXT6/7*	Stambuk et al. (2009) Dunn et al. (2012)	GEO accession no. GSE13875 and GSE26689 SRA accession no. SRA049752
JP1	Diploid	Homothallic	Da Silva-Filho et al. (2005a) Reis et al. (2012)	
VR-1	Diploid	Amplified *SNO/SNZ*	Stambuk et al. (2009) Swinnen et al. (2012)	GEO accession no. GSE13875 SRA accession no. SRA049724
SA-1	Diploid	Amplified *SNO/SNZ*	Stambuk et al. (2009)	GEO accession no. GSE13875

[a] Gene amplifications, altered copy number, and missing regions are described in comparison to S288c genome sequence

[b] Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/), GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra)

The complete genome sequence of a haploid derivative of strain PE-2 (JAY291) is published (ARGUESO et al., 2009), and the diploid PE-2 strain is already sequenced (Gonçalo A. Pereira, personal communication). More recently, the complete genome of the diploid strain CAT-1 was also determined (BABRZADEH et al., 2012). The genomes of these fuel ethanol strains were found to be highly heterozygous (around 2–3 SNPs/kb), affecting over half of the predicted protein-coding genes present in the *S. cerevisiae* reference genome (from laboratory strain S288c). These industrial yeast strains also display significantly less transposable elements in their genomes, several structural polymorphisms between homologous chromosomes, and large regions of loss of heterozygosity, when compared to the S288c strain (ARGUESO et al., 2009, BABRZADEH et al., 2012, BORNEMAN et al., 2011). In fact, chromosomal rearrangements were identified earlier in PE-2 strain (LOPES; BASSO; AMORIM, 2002) and in JP1 strain (LUCENA et al., 2007), both in the industrial environment and when cultivated for long periods in laboratory conditions. It was observed, however, that since these chromosomal rearrangements occur near chromosome ends, i.e., in regions that do not contain essential genes, they are unlikely to impair meiosis (ARGUESO et al., 2009); instead, they contain amplifications that improve fitness for industrial fermentations (ARGUESO et al., 2009, STAMBUK et al., 2009). It was proposed that these polymorphisms could play a role in the higher adaptability of these variants throughout the fermentation process (BASSO et al., 2008), contributing to their higher productivity. Although not observed by Lopes, Basso and Amorim (2002), this was further investigated by Argueso et al. (2009), who showed that such characteristics contributed to high ethanol and cell mass production as well as to high temperature and oxidative stress tolerance, probably accounting for their high adaptation to the industrial environment.

Another important feature is the amplification of telomeric *SNO* and *SNZ* genes, shared by five industrial strains (PE-2, CAT-1, BG-1, SA-1, and VR-1) (STAMBUK et al., 2009). These genes are involved in thiamine (vitamin B1) and pyridoxine (vitamin B6) biosyntheses, required for sugar catabolism by yeast. An increased copy number of these genes contributed for efficient growth under thiamine repression, when in medium lacking pyridoxine and with high sugar concentration. Surprisingly, these five strains did not show amplification of the *SUC2* gene (STAMBUK et al., 2009), as seen for telomeric *SUC* genes in baker's and distillers' strains adapted to sucrose-rich broths (BENÍTEZ et al., 1996, CODÓN; BENÍTEZ; KORHOLA, 1998). As *SUC2* encodes for an extracellular invertase, this indicates that

sucrose conversion is not a limiting step for these strains during industrial fermentation. *RTM1*, another gene usually found in association with telomeric *SUC* genes and implicated in "Resistance To Molasses" of baker's and distillers' yeasts by an unknown mechanism (DENAYROLLES et al., 1997, NESS; AIGLE, 1995), is also absent from the JAY291 genome (ARGUESO et al., 2009, BORNEMAN et al., 2011). In the BG-1 strain genome, there are presumed deletions in regions containing *ENA* genes (as corroborated by unpublished results from our laboratory, which show a lower osmotic tolerance of this strain) and in the *HXT6/7* locus (DUNN et al., 2012).

Swinnen et al. (2012) applied pooled segregant whole-genome sequence analysis to map all the quantitative trait loci (QTL) that determine the high ethanol tolerance (up to 17 % ethanol) presented by a segregant of strain VR-1. The main genes associated with this trait were *MKT1*, which has a regulatory role in global gene expression, and *APJ1*, which encodes for an Hsp40 family protein with a negative effect on ethanol tolerance.

1.7 CONCLUSIONS AND PERSPECTIVES

The Brazilian ethanol production process is quite peculiar, given its features like yeast cell recycle and acid treatment. Analysis of strains isolated from this process shows us that successfully implementing a modified laboratory strain for use in this industry can be very difficult, if not impossible, since even some highly dominant isolates, as PE-2, are now disappearing from the distilleries and giving space to more adapted ones.

In this sense, applying evolutionary engineering techniques to these industrial strains can be challenging, as they have been evolving since decades in the process itself. Probably just as challenging is the implementation of metabolically engineered strains. ME could be used not only to increase their ethanol productivity and/or yield but also to change substrate utilization or the desired end product while keeping their highly desired tolerance traits. Examples of ME targets would be heterologous enzyme expression for production of 2G ethanol, biopolymers (e.g., polyhydroxyalkanoates (PHA) and polyhydroxybutyrates (PHB)), or other biofuels (e.g., synthetic diesel and butanol), consequently upgrading sugar/ethanol distilleries to biorefineries. However, one should consider the regulatory issues involved in

the industrial use of genetically modified (GM) strains—recently, one process for farnesene production from sugarcane-based media by GM yeast was approved by the Brazilian biosafety commission (BRAZIL, 2010).

Presently, there are many aspects about the Brazilian ethanol strains that remain unknown, such as why they dominate the fermentors so quickly in some distilleries, but in 40 % of the plants, none of them can be implanted. At the same time, these questions should be the object of more research, and there is the need of isolating or producing new strains, since the number of commercially available strains for this industry is rather small. One alternative to the challenge of finding a strain versatile enough to be implanted in multiple distilleries is the isolation of customized strains in an in-house manner, selecting the ones that are already at the plants and probably more fitted to the distinct environment they encounter every day. It is important to keep in mind that, among the more than 400 mills currently in operation in Brazil, there are no two identical industrial processes.

Isolating new suitable strains is also challenging because the sugar and ethanol production processes are constantly changing, mainly due to: new sugarcane crops, different proportions of sugarcane juice to molasses used in substrate formulation, different molasses composition (due to improved sucrose extraction during edible sugar production), and increased mechanical harvesting that might bring other plant materials to the crushing step, such as tops and leaves, among others.

The possibility of using GM strains for industrial fuel ethanol production is highly dependent on the perspectives of implementing asepsis in the different stages of the process, since wild yeasts with higher specific growth rate and viability will most probably outcompete the GM strains. Currently, the price of ethanol does not justify such practices, but what would happen if we had GM strains with improved performance in a scenario of high gasoline prices? A laboratory strain was already engineered and evolved for an 11 % higher ethanol yield on sucrose (BASSO et al., 2011), and this strategy was recently implanted in an industrial strain and is currently being tested under industrial conditions, with promising results (Boris Stambuk's unpublished data).

To conclude, we can affirm that we are still at the beginning of understanding what makes these industrial strains dominate and persist (or not) inside the industrial vats. More genome sequencing and annotation, targeted physiological studies, and also field trials are required to fill this gap. Last but not least, we do not even know where these wild yeast

strains come from: do they have an origin in the sugarcane fields, are they brought by insects that visit the production plant or, since they are more related to baker's strains than to wine or brewing yeast (STAMBUK et al., 2009), did they simply evolve from commercial baker's yeast?

1.8 ACKNOWLEDGEMENTS

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2 STRESS TOLERANCE AND GROWTH PHYSIOLOGY OF YEAST STRAINS FROM THE BRAZILIAN FUEL ETHANOL INDUSTRY[4]

2.1 ABSTRACT

Improved biofuel production requires a better understanding of industrial yeast strains. Some wild *Saccharomyces cerevisiae* strains, isolated from the fuel ethanol industry in Brazil, present exceptional fermentation performance in the harsh industrial environment. Nevertheless, they have not yet been systematically compared to other well-known strains, in terms of their physiology. With this purpose, we evaluated the tolerance of the widely used industrial strains PE-2, CAT-1, BG-1 and JP1 towards process-related stressors and analyzed their growth physiology under heat stress, in comparison with laboratory and baker's strains. Whereas industrial strains performed better than laboratory strains under ethanol or acetic acid stresses and on industrial media, high sugar stress was tolerated equally by all strains investigated. Heat and acidic stresses distinguished fuel ethanol strains from the others, indicating that these conditions may exert selective pressure on cells in the industrial environment. During synthetic medium shake-flask cultivations at 37 °C, industrial strains presented the highest ethanol yields on glucose, indicating that they could have been selected for this trait—a response to energy-demanding fermentation conditions. These results may guide future improvements of large-scale ethanol production using strain engineering, and eventually their use in different industrial bioprocesses.

2.2 INTRODUCTION

As one of the largest ethanol producers in the world, Brazil has played a key role in the bioenergy scenario during recent years. The Brazilian 1st generation (1G) ethanol,

[4] DELLA-BIANCA, B. E..; GOMBERT, A. K. Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. **A van Leeuw J Microb**, Sept 2013. In press.

recently classified as an advanced biofuel (US ENVIRONMENTAL PROTECTION AGENCY, 2010) and the most ecoefficient 1G ethanol (MACEDO, 2007), is produced primarily by fed-batch fermentation of sugarcane juice and/or molasses. Cell recycling is used to achieve high cell concentrations in the fermentors, which decreases fermentation times and increases ethanol yields (BASSO; BASSO; ROCHA, 2011, DELLA-BIANCA et al., 2013).

Baker's strains of the yeast *Saccharomyces cerevisiae* are utilized as starter cultures in the fuel ethanol industry, but contamination by indigenous yeast strains is normally detected already some weeks after start of the sugar cane crushing season. This results from the lack of sterilization of the culture medium before fermentation and the non-aseptic manner in which the process is conducted. Over the past few decades, it has been revealed that some indigenous isolates are able to persist and even dominate the fermentors, thus being more suited to the process than the starter yeast. Some of these wild yeast strains promote higher ethanol yields than the latter ones and are now commercially available to compose the inoculum at the distilleries (BASSO et al., 2008, DA SILVA-FILHO et al., 2005a, WHEALS et al., 1999).

The environment found by yeast in the distilleries is very distinct from controlled laboratorial conditions. The substrate itself possesses some compounds in toxic levels (e.g. potassium, aluminum, cadmium, iron, and phenols), as well as high sugar concentrations, which can promote osmotic stress (BASSO; BASSO; ROCHA, 2011). High sugar concentrations also lead to high ethanol concentrations (8-11 % v/v (WHEALS et al., 1999)), which also trigger stress responses in yeast. There are also great variations in temperature, due to the exothermic nature of cell metabolism associated with very high volume vats and inefficient cooling systems—in the hottest regions of Brazil temperatures can go up to 40 °C inside the fermentors (BASSO; BASSO; ROCHA, 2011). Even though it is not a condition found in the fermentation vessel itself, low pH acts as a stress factor during the cell recycling stage, when sulfuric acid is added to the cell suspension and pH values can drop down to 1.8 (BASSO; BASSO; ROCHA, 2011). This operation decreases bacterial contamination but also affects yeast cell viability (DORTA et al., 2006). On the other hand, it is well known that in 2nd generation (2G) ethanol production, which is close to being a commercial enterprise, a number of yeast inhibitors are released during the pre-treatment of lignocellulosic raw materials. Acetic acid is one of the most prominent inhibitors, since it is usually present in higher proportions, when compared to other inhibitors, and negatively affects yeast

performance (ALMEIDA et al., 2007). All the stress factors mentioned above exert a continuous selective pressure on yeast through the fermentation cycles, allowing only the most resistant strains to survive in the process during the 8-month sugarcane crop season in Brazil (BASSO et al., 2008).

Systematic analyses of stress tolerance have been performed on *Saccharomyces cerevisiae* strains isolated from and/or used in distinct fermentation environments, e.g., wine (CARRASCO; QUEROL; DEL OLMO, 2001, MASSERA et al., 2012, ZUZUARREGUI; DEL OLMO, 2004), mezcal (PÁEZ et al., 2011), hydrolysates (ALMEIDA et al., 2009) and baker's strains (LEWIS et al., 1997). Similarly, *Saccharomyces* beer hybrids (BELLOCH et al., 2008) and clinical isolates (LLANOS; FERNÁNDEZ-ESPINAR; QUEROL, 2006) have also been investigated. These analyses are a valuable tool in strain characterization and may provide a foundation for optimized strain choice in an industrial setting. This type of study has not been hitherto carried out on strains isolated from the Brazilian fuel ethanol industry, so we analyzed for the first time these strains' responses towards stress conditions, both qualitatively and quantitatively (the latter for heat stress alone). Recently, there has been growing interest in elucidating the mechanisms that confer advantages to these fuel ethanol strains (ARGUESO et al., 2009, BABRZADEH et al., 2012, BASSO et al., 2008, BORNEMAN et al., 2011, DA SILVA-FILHO et al., 2005a, DE MELO et al., 2010, DUNN et al., 2012, ELSZTEIN; DE LUCENA; DE MORAIS JR, 2011, STAMBUK et al., 2009, SWINNEN et al., 2012). Despite these efforts, much of the physiology of these wild strains is not yet known, and this type of information is required to pave future attempts to improve them and, by consequence, the industrial processes in which they are involved. Comparative physiological data on the main fuel ethanol strains used in Brazil and on well-known laboratory and other industrial strains are almost absent in the scientific literature, to our knowledge. Thus, the aims of the present work were to evaluate the Brazilian fuel ethanol strains' tolerances towards stress factors; to characterize their growth physiology; and to compare them with well-known laboratory and baker's strains.

2.3 MATERIALS AND METHODS

2.3.1 Yeast strains, preservation and pre-culture preparation

The yeast strains used in this study are listed in Table 2. Stocks were kept at -80 °C in YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) with 20 % v/v glycerol. All experiments were started by first transferring some cells directly from the frozen stocks onto the surface of solid YPD plates (YPD containing 2 % w/v agar) and incubating them at 30 °C; after 48 h, a loop containing cells from one isolated colony was transferred to pre-culture flasks.

Table 2 – *Saccharomyces cerevisiae* strains used in this study.

Strain designation	Group	Ploidy	Precedence	References
S288c	Laboratory	Haploid	Dr. E. J. Vicente (USP, Brazil)	Mortimer and Johnston (1986)
CEN.PK113-7D	Laboratory	Haploid	Dr. P. Kötter (EUROSCARF, Germany)	van Dijken et al. (2000)
PE-2	Industrial (fuel ethanol)	Diploid	Dr. L. C. Basso (USP, Brazil)	Argueso et al. (2009) Basso et al. (2008)
CAT-1	Industrial (fuel ethanol)	Diploid	Dr. L. C. Basso (USP, Brazil)	Babrzadeh et al. (2012) Basso et al. (2008)
BG-1	Industrial (fuel ethanol)	Diploid	Dr. L. C. Basso (USP, Brazil)	Basso et al. (2008)
JP1	Industrial (fuel ethanol)	Diploid	Dr. M. A. de Morais Jr (UFPE, Brazil)	da Silva-Filho et al. (2005a)
Fleischmann	Industrial (baking)	Diploid	Dr. L. C. Basso (USP, Brazil)	

2.3.2 Serial dilution spotting on stress plates

The dilution spot assays were based on the protocol of Netto (2006), with modifications. Strains were transferred from YPD plates to pre-culture 250-mL shake-flasks containing 100 mL of YPD medium and grown overnight at 30 °C, 200 rpm. Baffled 500-mL shake-flasks with 100 mL of YPD medium were then inoculated with an initial absorbance at 600 nm (Abs_{600}) of 0.2. Strains were incubated at 30 °C, 200 rpm and grown for 3 h (until early exponential phase). At this point, cells were harvested, diluted in sterile water to Abs_{600} 0.1 and four successive dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were prepared. Spots of 3 or 5 µL of each dilution were placed onto stress plates, which were incubated for at least 48 h at 30 °C, with the exception of heat stress plates (37, 40 or 42 °C).

Control and heat stress plates were composed solely of YPD medium. Plates with high glucose or sucrose concentration were prepared by adjusting the sugar concentration of the YPD medium to 200 g L^{-1} hexose-equivalent; the sugar being added by sterile filtration. Industrial substrates were also tested by preparing plates with sugarcane juice (80 % v/v sterile-filtered juice, 1 % w/v agar, resulting in 162 g L^{-1} total reducing sugars (TRS)), sugarcane molasses (41 % w/v autoclaved molasses, 2 % w/v agar, resulting in 200 g L^{-1} TRS), and with sugarcane juice and molasses in a TRS proportion of 1:1 (49 % v/v juice, 20.5 % w/v molasses, 2 % w/v agar, also resulting in a TRS concentration of 200 g L^{-1}). Sugarcane juice was sterilized as follows: centrifugation at 10,000 rpm for 10 minutes followed by serial filtration (20, 14, 8, 6.5, 3, 1.2, 0.45 and 0.22 µm-pore membranes), being kept on ice at all times (SCHWAB; TEIXEIRA; BALDANI, 2008), and later stored at -20 °C. Fresh sugarcane juice (200 g L^{-1} TRS) and molasses (490 g kg^{-1} TRS) were obtained from a local market and from Prof. Luiz Carlos Basso, respectively, and their sugar concentrations were measured by the DNS method (MILLER, 1959) following acid hydrolysis.

Saline stress plates were composed of YPD medium supplemented with 0.2-2.0 M NaCl; oxidative stress plates of YPD with 1.0-10.0 mM H_2O_2 (added by sterile filtration); ethanol stress plates of YPD with 2.5-20.0 % v/v ethanol (added aseptically); and acetic acid plates of YPD with 2.5-5.0 g L^{-1} acetic acid (added aseptically). Acidic stress plates were composed of YPD in the pH range 2.0 to 5.5 (the latter corresponds to YPD without any

adjustment); pH adjustments were made with sterile 1 M H_2SO_4. Ethanol was also tested as a carbon source, by using YP plates supplied with 8.0-20.0 % v/v ethanol (added aseptically).

2.3.3 Shake-flask cultivations in synthetic medium

Strains were transferred from YPD plates to pre-culture baffled 500-mL shake-flasks containing 100 mL of synthetic medium with 20 g L^{-1} glucose (VERDUYN et al., 1992) and grown overnight at 30 °C, 200 rpm. pH variation was kept to a minimum by using urea instead of ammonium sulfate as a nitrogen source, as in Luttik et al. (2000) and van Leeuwen et al. (2009). After centrifuging cells and washing them twice with fresh synthetic medium, baffled 500-mL shake-flasks with 100 mL of the same synthetic medium were then inoculated with an initial Abs_{600} of 0.2 and incubated at 30 °C (*control*), 37 °C (*mild heat stress*) or 40 °C (*severe heat stress*), at 200 rpm. Samples were taken every hour until glucose exhaustion, which was detected by an increase in pH and confirmed by a Roche Accu-Chek glucose test strip. All experiments were carried out in duplicate, in random order.

2.3.4 Sample treatment and analysis

Cell growth, metabolite formation and substrate consumption were followed along the shake-flask cultivations with synthetic medium, which allowed for the calculation of the main physiological parameters during exponential growth, namely specific rates and yields. Cell concentration was indirectly determined by spectrophotometry (Abs_{600}) and the data were converted into cell concentration in terms of *grams dry cell weight (DW) per volume.* For this purpose, in each cultivation the final sample had both its Abs_{600} and its dry cell weight determined (the latter using the gravimetric method as described by Olsson and Nielsen (1997)). After sample filtration through 0.22 µm-pore membranes, the concentrations of extracellular metabolites were determined by HPLC, on an Aminex HPX-87H column at 60 °C eluted with 0.5 mM H_2SO_4 at 0.6 mL min^{-1}, coupled with refractive index

and UV detectors (Waters IR 2414 at 50 °C for glucose, ethanol, glycerol, acetate, succinate and lactate and Waters UV/Vis 2489 at 214 nm for pyruvate, respectively).

2.3.5 Calculation of physiological parameters and statistical comparisons

All calculations and statistical comparisons were performed with GraphPad Prism 5 (La Jolla, USA). Exponential phase data from two independent replicates of each experiment were analyzed together and produced only one curve. The maximum specific growth rate (μ_{max}) was obtained by plotting the natural logarithm of Abs_{600} values against time and then calculating the slope of the straight line corresponding to the exponential growth phase (by linear regression). Yields were calculated as the absolute value of the slope of a straight line (by linear regression) using solely data points from the exponential growth phase: the biomass yield on substrate ($Y_{X/S}^{exp}$) from a plot of cell concentration data against substrate concentration data (for this purpose the Abs_{600} values were first converted into *grams dry cell weight per volume*, as described above), and the product (ethanol or glycerol) yield on substrate ($Y_{P/S}^{exp}$) from a plot of product concentration data against substrate concentration data.

These physiological parameters were represented by the slope of the curves ± the standard errors reported by the software, and further compared statistically using an ANCOVA test between different conditions (ZAR, 2010) with a significance level of 0.05. This test yielded the same results as t-tests conducted in parallel on Microsoft Excel. Specific rates of substrate consumption (q_S^{max}) and product (ethanol or glycerol) formation (q_P^{max}) were calculated through the combination of growth rate and yield values, and are shown in eq.(1) and eq.(2), respectively.

$$q_S^{max} = -\mu_{max} * \left(\frac{1}{Y_{X/S}^{exp}}\right) \quad (1)$$

$$q_P^{max} = \mu_{max} * \left(\frac{Y_{P/S}^{exp}}{Y_{X/S}^{exp}}\right) \quad (2)$$

2.3.6 Carbon balance estimation

In order to estimate the carbon fraction from the substrate incorporated into the main products of cell metabolism (biomass, ethanol, CO_2 and glycerol), a carbon balance was performed, using yield values in C-mol C-mol^{-1} units. The biomass composition was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ and 5 % ash (NIELSEN; VILLADSEN; LIDÉN, 2003), resulting in 25.89 g DW biomass C-mol^{-1}, in all cases.

2.4 RESULTS AND DISCUSSION

2.4.1 Serial dilution spotting on stress plates

In order to improve our understanding on the physiology of the most important fuel ethanol strains isolated from Brazilian mills, we started by evaluating their tolerance towards classical stress factors and also particular ones faced by yeast in the industrial fuel ethanol production. Although these conditions are not the exact representation of what takes place inside the industrial fermentors, this approach based on individual stress factors was pursued in order to obtain clearer results, as suggested by Ivorra et al. (1999). Since no difference in stress response was observed between haploid and diploid strains in previous studies (see Albers and Larsson (2009) for laboratory and Bravim et al. (2010) for industrial strains), the laboratory strains used in our experiments were chosen based on the public availability of genomic and quantitative physiological data, even though they are of a distinct ploidy than the industrial strains we were interested in investigating.

To evaluate heat tolerance, the strains were grown on solid YPD medium under a control temperature (30 °C) or under different levels of heat stress (37, 40 or 42 °C) for at least 48 h. Growth at 30 °C (Figure 5a) and 37 °C (data not shown) was very similar for all strains after 48 h, indicating that lab and industrial strains are alike in their tolerance to mild heat stress, as observed by Argueso et al. (2009) for strains S288c and JAY270 (a PE-2 diploid

derivative) and by da Silva-Filho et al. (2005a) for strains PE-2 and JP1 at the same temperature. No growth of any strain could be detected at 42 °C, even after 7 days. At 40 °C (Figure 5b), strains JP1, CAT-1 and BG-1 showed a noticeable higher thermotolerance than the other strains. The robustness towards heat stress shown by the JP1 strain relates to the region it was isolated from (DA SILVA-FILHO et al., 2005a)—the Northeast of Brazil, where higher temperatures are reached throughout the year, when compared to the Southeast of the country, where the other industrial strains used in this work were isolated from (Table 2).

Tolerance to osmotic stress was analyzed on solid YPD medium containing NaCl, as in Posas et al. (2000), or by raising the sugar concentration of the medium from 2 % to 20 % hexose-equivalent, in the form of glucose or sucrose. Addition of 0.5 M NaCl (Figure 5c) reduced colony size for all strains in comparison to the control plate, and strain JP1 presented again a slightly higher tolerance than the others did. Furthermore, two strains were hypersensitive to this condition, laboratorial CEN.PK113-7D and industrial BG-1. As observed by Garay-Arroyo et al. (2004) and later explained by Daran-Lapujade et al. (2009a), CEN.PK113-7D is hypersensitive to sodium ions since it contains a single *ENA* allele, which encodes a Na^+-ATPase (in contrast, the tolerant S288c strain possess 5 alleles). More recently, it was shown by Dunn and colleagues (2012) that BG-1 presents deletions in the same genomic region, and this fact probably accounts for its sodium hypersensitivity. Under 1 M NaCl, these two strains did not show any growth and the others showed little growth after 48 h (data not shown), which is in good agreement with the threshold of about 1 M NaCl verified by Blomberg (1997). No growth was observed on 1.5 and 2 M NaCl plates after 48 h, for any of the strains investigated. Regarding high sugar concentration (Figures 5d and 5e), all strains presented growth patterns similar to the control condition—this is in accordance to studies on other strains (ALBERS; LARSSON, 2009, BELLOCH et al., 2008, JIMÉNEZ-MARTÍ et al., 2011, PÁEZ et al., 2011). No detectable growth difference was found between high glucose and high sucrose plates, most likely due to the action of the extracellular invertase, which hydrolyzes sucrose to glucose and fructose (DICKINSON; SCHWEIZER, 2004). It is worth emphasizing that 200 g L^{-1} hexose-equivalent is the typical sugar concentration used in the feeding stream of sugar cane juice and/or molasses that enters the fermentation vats in the Brazilian mills. Since the process is carried out in fed-batch mode with a high amount of yeast in the fermentor right at the beginning of each

fermentation cycle, this means that the residual sugar concentration to which cells are exposed during fermentation never surpasses this value. Thus, osmotic stress by sugar alone does not exert an important selective pressure on the cells in the process, unless this effect is potentiated by the concomitant effect of other stress factors.

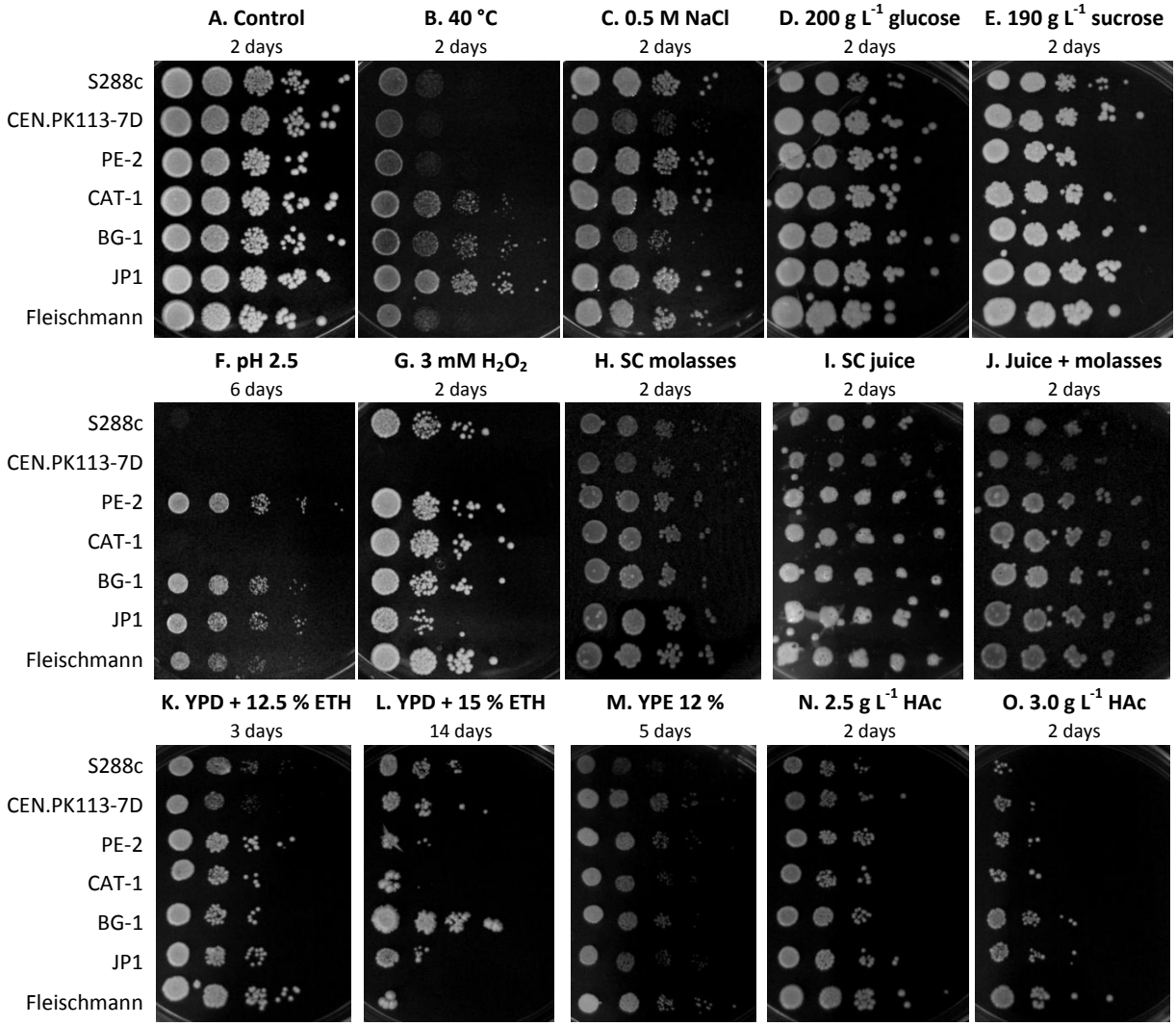

Figure 5 – Cells growing exponentially in liquid YPD medium were transferred onto plates with solid YPD medium and different stress factors, as indicated. Control condition (A) is stress-free. Lines represent different strains, and columns, dilutions ranging from Abs_{600} 10^{-1} to 10^{-5}. Pictures were taken after the number of days described for each plate. SC: sugarcane; ETH: ethanol; HAc: acetic acid.

Analysis of low pH tolerance was done by correcting the medium pH with sulfuric acid, the same compound used in Brazilian mills during the cell recycling step (between two consecutive fermentations), with the aim of decreasing bacterial contamination. For the

plates with pH 4.0 and 3.0 (data not shown), there was a decrease in colony size, but not in colony number, after 48 hours for all strains in a similar manner. At pH 2.0 (data not shown), no growth was detected after 144 h for any of the strains (data not shown). At pH 2.5 (Figure 5f), the most remarkable distinction between the fuel ethanol strains and the other strains (both the laboratorial ones and the baker's strain) could be observed. Only industrial strains exhibited growth after 144 h and the Fleischmann strain (baker's yeast), utilized as starter culture in the fuel ethanol industry, showed poor growth under this condition. This indicates that the acid treatment of cells used industrially during the cell recycling step could be a key selective pressure that has allowed these wild strains to dominate the fermentors already during the first month of the sugar cane crushing season in Brazil. An exception was the CAT-1 fuel ethanol strain, which did not grow under this condition of pH 2.5. Further experiments would be necessary to clarify this difference between CAT-1 and the remaining fuel ethanol strains.

Although reactive oxygen species (ROS) production is usually associated with cellular respiration, there is evidence that ROS is formed even during fermentative metabolism (LANDOLFO et al., 2008), which means that oxidative stress may take place during industrial fuel ethanol production. Thus, growth under this condition was assessed with the use of hydrogen peroxide. On plates containing 1 mM and 2 mM H_2O_2, all the strains showed a decrease in colony number, but not in colony size, and no growth was detected for any strain on plates containing 5 mM and 10 mM H_2O_2, after 48 h (data not shown). In a general fashion, industrial strains presented higher tolerance in comparison with laboratorial strains, as can be seen on the plate containing 3 mM H_2O_2 (Figure 5g). The Fleischmann strain, commonly used in baker’s yeast production, was the most tolerant under this condition. This was already expected, since baker’s yeast strains are highly tolerant towards oxidative stress (ATTFIELD, 1997).

One can also notice from Figure 5g that laboratory strain CEN.PK113-7D is more sensitive towards H_2O_2 stress than the remaining ones, as it did not present any growth at 3 mM H_2O_2, whereas the others did. JP1 is the least tolerant industrial strain towards H_2O_2 stress, among those tested, despite the fact that it was the most tolerant strain towards heat and NaCl stresses. This may be related to the fact that this strain was isolated from mills in the Northeast of Brazil, which employ sugarcane juice, but not molasses, as a substrate for fermentation (DA SILVA-FILHO et al., 2005a). On the other hand, the PE-2 strain, which

presented higher tolerance to oxidative stress than JP1, was isolated from mills that use a mixture of sugarcane juice and molasses (DA SILVA-FILHO et al., 2005a). It is known that sugarcane molasses may contain SO_2 in detrimental levels for yeast cells (BASSO; BASSO; ROCHA, 2011), and that SO_2 exposure induces the expression of *GLR1*, which encodes a glutathione reductase involved in the sulfitolysis of glutathione (PARK; HWANG, 2008), a compound with a role in the cellular defense against oxidants (DICKINSON; SCHWEIZER, 2004). An explanation for JP1's sensitivity towards H_2O_2 could be a failure in stress response mechanisms activated more specifically under oxidative conditions, such as those related to glutathione; however, this is only speculative. Even though the genomic data from JP1 are not yet available, its sensitivity towards H_2O_2 is unlikely related to the absence of the *RTM1* gene, linked to resistance to sugar beet molasses toxicity (NESS; AIGLE, 1995), since this gene is absent from JAY291's genome, which is a haploid descendant of the PE-2 strain (ARGUESO et al., 2009), and also from CAT-1's genome (BABRZADEH et al., 2012).

To further analyze this issue, we inoculated all strains on a medium composed of only sugarcane molasses (Figure 5h), on which the industrial strains, including JP1, performed better than the lab strains. This contradicts previous results published by da Silva-Filho et al. (2005a), who observed a lower growth rate for JP1, in comparison to the PE-2 strain, in a molasses-based liquid medium. Nevertheless, it should be emphasized that industrial media are hardly, if at all, reproducible. Growth on sugarcane juice (Figure 5i) and on a mixture of juice and molasses (Figure 5j) was also investigated, since these are all common situations in the Brazilian fuel ethanol industry. The results obtained were similar, i.e. industrial strains grew better than laboratory strains on these media.

The concentration range used to analyze ethanol tolerance was based on the alcoholic levels achieved in the Brazilian industrial process. Based on their growth on YPD containing 12.5 % ethanol (Figure 5k), it is possible to distinguish less tolerant laboratory strains from more tolerant industrial ones. However, on plates containing 15 % ethanol (Figure 5l), different results were obtained after 14 days of incubation. However, we believe that this result is an artifact, since ethanol probably evaporates during such a long incubation time. No growth of any strain could be observed after 48 h under 17.5 or 20 % ethanol. Using different experimental conditions, Carrasco, Querol and del Olmo (2001) found only one wine strain capable of growing in 15 % ethanol after 5 days of incubation. Pereira et al. (2011), on the other hand, reported a 19.1 % v/v ethanol titer in very high

gravity (VHG) fermentation using the PE-2 strain and corn steep liquor in the medium, showing that industrial strains are actually capable of producing ethanol concentrations of almost 20 %, although accompanied by a sharp decrease in viability.

Ethanol tolerance was also assessed on plates containing ethanol as the main carbon source (no glucose added, 12 % ethanol, Figure 5m). Since this condition activates fully respiratory metabolism, the oxidative-stress tolerant Fleischmann strain displayed a slightly better growth than the other ones. In general, the different growth behaviors of all strains on similar ethanol concentrations in the presence and in the absence of glucose (Figures 5k and 5m, respectively) is probably due to the lower specific growth rate of *S. cerevisiae* on ethanol than on glucose (VAN DIJKEN et al., 2000).

Addition of acetic acid was used to evaluate the strains' tolerance to one of the main yeast inhibitors in sugarcane bagasse hydrolysates, now strongly considered for 2G ethanol production (BUCKERIDGE et al., 2012). Once again, industrial strains grew slightly better than laboratory strains, presenting increased colony size if compared to the latter ones under 2.5 g L^{-1} acetic acid (Figure 5n). This trend was also observed under 3.0 g L^{-1} acetic acid (Figure 5o), a condition that also evidences that colony numbers are higher for the industrial strains, when compared to the others. Acetic acid in concentrations of 3.5, 4.0 and 4.5 g L^{-1} allowed only residual growth while 5.0 g L^{-1} was completely inhibitory for all strains (data not shown).

In summary, it is possible to state that none of the investigated strains is the most tolerant to all stress conditions tested in this study. This is probably related to the fact that the fuel ethanol strains were selected and isolated from different mills, which employ different process conditions. Nevertheless, it is possible to observe that, in general, the industrial fuel ethanol strains are more tolerant than laboratorial strains, and the stress conditions that mostly distinguished fuel ethanol strains from lab and baker's strains were heat and low pH. This result indicates that these stressors could be the most relevant sources of selective pressure on yeast cells used in the Brazilian ethanol production process, and so they were further considered for quantitative experiments, as described below.

2.4.2 Growth physiology in synthetic liquid media

In order to study the physiology of the Brazilian fuel ethanol strains in a more quantitative manner, we chose a reproducible condition and analyzed growth and metabolite formation in shake-flask cultivations, using a synthetic medium, glucose as the sole carbon and energy source and 30 °C, which can be considered standard conditions for studies on yeast physiology. Besides this reference condition, we wished to investigate the physiology of the different yeast strains under the most relevant conditions detected in the plate assays, as detailed above, i.e. low pH and heat stress. Since shake-flask cultivations do not allow for an appropriate pH control, we set out to study heat stress first, starting with the temperature of 40 °C, based on the heat stress plate results. However, at this temperature, there was a large difference between replicates (Figure 6b), which is not observed at 30 °C (Figure 6a). It is known that under sublethal conditions (such as growth at 40 °C), even genetically identical cells can exhibit phenotypic heterogeneity (SUMNER; AVERY, 2002) and this therefore may account for the large difference observed between our experimental replicates. This result led us to choose a milder heat stress condition, namely 37 °C, for the quantitative comparison among strains under heat stress.

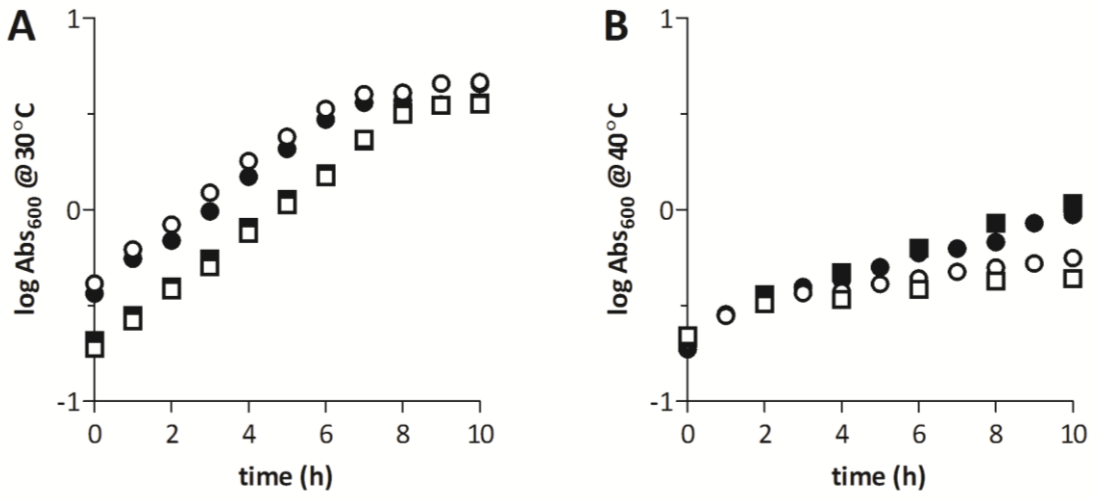

Figure 6 – Time profile of the logarithm (base 10) of cell concentration (indirectly assessed by absorbance measurements at 600 nm) for strains CEN.PK113-7D (circles) and PE-2 (squares) at 30 °C (A) and 40 °C (B) over 10 hours of shake-flask cultivation in liquid synthetic medium. Filled and open symbols correspond to duplicate experiments.

At 30 °C, both strain groups (industrial and laboratorial) showed similar values for the maximum specific growth rate, namely 0.345 ± 0.038 h^{-1} (Figure 7a), except for strain Fleischmann whose μ_{max} was around 0.42 h^{-1}. Strains CAT-1 and JP1, which were observed to be the most thermotolerant ones during the plate assays (Figure 5b), presented higher growth rates at 37 °C than at 30 °C (+8.32 and +19.7 %, respectively). The other thermotolerant strain, BG-1, exhibited a slight decrease in μ_{max} at 37 °C, which was not statistically significant.

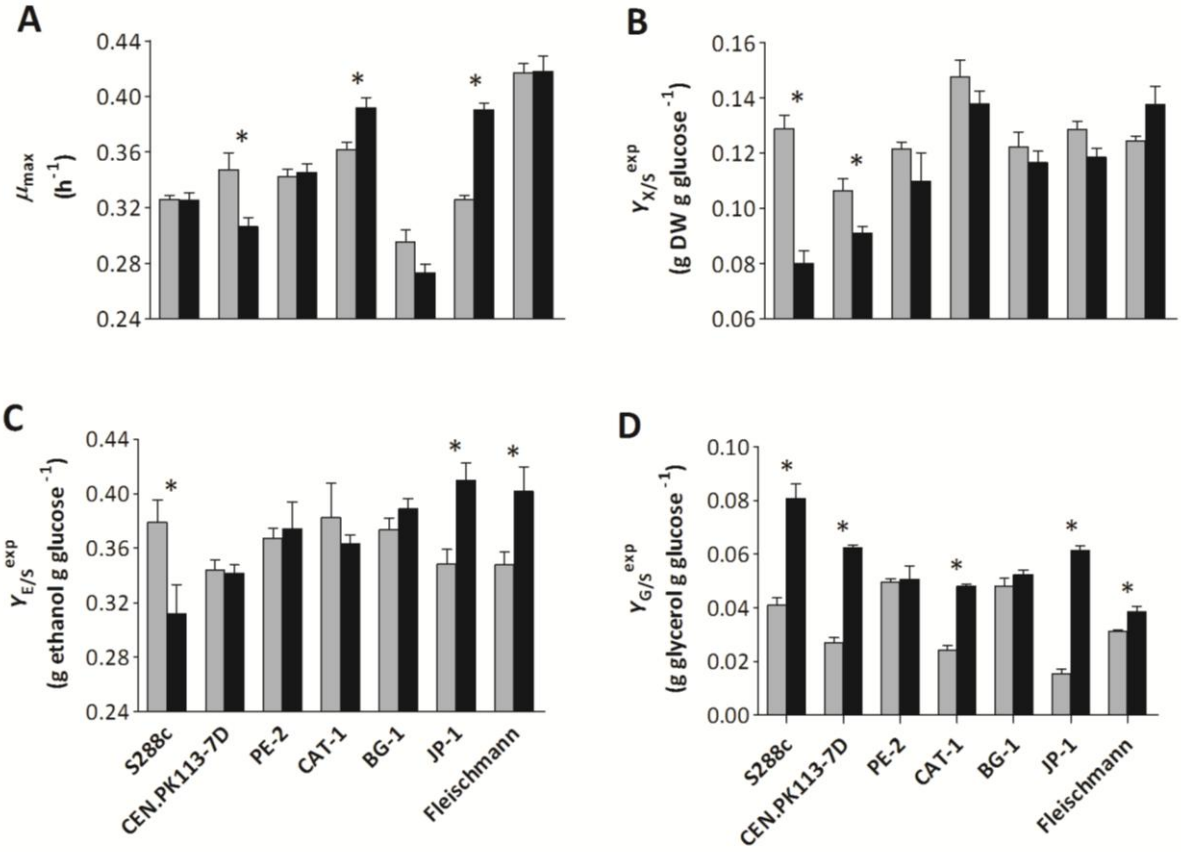

Figure 7 – Cells grown in shake-flasks containing synthetic medium had their physiological parameters calculated during the exponential growth phase. Maximum specific growth rate (μ_{max}), biomass yield on glucose ($Y_{X/S}^{exp}$), ethanol yield on glucose ($Y_{E/S}^{exp}$) and glycerol yield on glucose ($Y_{G/S}^{exp}$) are represented for each strain cultivated at 30 °C (gray) and 37 °C (black). Error bars indicate standard errors obtained from two replicates (as described in Materials and Methods). * indicates statistical difference between values at 30 °C and 37 °C for the same strain; significance level is 0.05.

As observed with the μ_{max} values at the reference condition, the biomass yield on glucose was similar among strains cultivated at 30 °C, namely 0.126 ± 0.012 g g^{-1} (Figure 7b).

At 37 °C, the laboratorial strains showed the lowest values for $Y_{X/S}^{exp}$. A decrease trend in biomass yield can be observed at 37 °C for all strains (again exception being Fleischmann), but only for the laboratorial strains this decrease was statistically significant. This reveals that a mild heat stress condition is enough to affect biomass generation—cell growth becomes energetically more costly (MENSONIDES et al., 2002) since more carbon is required to counteract stress effects. Accordingly, most strains showed higher specific rates of glucose consumption at 37 °C, when compared to 30 °C (data not shown).

At 30 °C, the average and standard deviation for the ethanol yield on glucose ($Y_{E/S}^{exp}$), considering all strains cultivated, was 0.363 ± 0.016 g g^{-1}. At 37 °C, the laboratorial strains showed lower values for this parameter, when compared to the industrial ones (Figure 7c). This could be the consequence of the fact that, in industrial practice, although highly desired, it has been impossible to keep the temperature inside the fermentors around 30 °C. Insufficient heat-exchange capacity, high costs of both heat-exchange equipment and water supply, besides daily temperature variations are among the reasons for this. Thus, it is logical to expect that the industrial process has selected cells for the trait of producing enough ethanol (which implies enough energy in an anaerobic environment) in order to survive under heat stress, even if that means producing less cells. By comparing ethanol yields at 30 °C and 37 °C for each individual strain, no general tendency of decrease or increase could be observed. Still, all strains showed higher specific rates of ethanol formation (q_E^{max}) under heat stress (data not shown), indicating a higher rate of ATP formation, by each cell, even when the ethanol yield decreased, since q_E^{max} calculations depend also on $Y_{X/S}^{exp}$ and μ_{max}. This observation was expected, as higher temperatures push cells to produce energy faster, once again to counteract effects caused by stress.

By comparing glycerol formation at 30 °C for all strains investigated, it is possible to conclude that this trait is clearly strain-dependent (Figure 7d). A clear trend of increase in glycerol production, both in terms of yield ($Y_{G/S}^{exp}$) and specific rate (q_G^{max}, data not shown), can be observed at 37 °C for all strains, when compared to the corresponding situation at 30 °C. Besides being the compatible solute synthesized by yeast during osmotic stress, glycerol is also produced as a response to elevated temperatures through the activation of the HOG pathway (HOHMANN, 2002, OUGH; FONG; AMERINE, 1972, WINKLER et al., 2002).

2.4.3 Carbon balance

Carbon balances were set up for all shake-flask cultivations (Figure 8). Since CO_2 formation was not measured, we calculated it to be equimolar to ethanol production, i.e., a purely fermentative metabolism was assumed. This assumption is obviously hypothetical and corresponds to the minimum CO_2 produced, since any oxidative utilization of glucose would contribute to increase CO_2 production, with respect to purely fermentative metabolism.

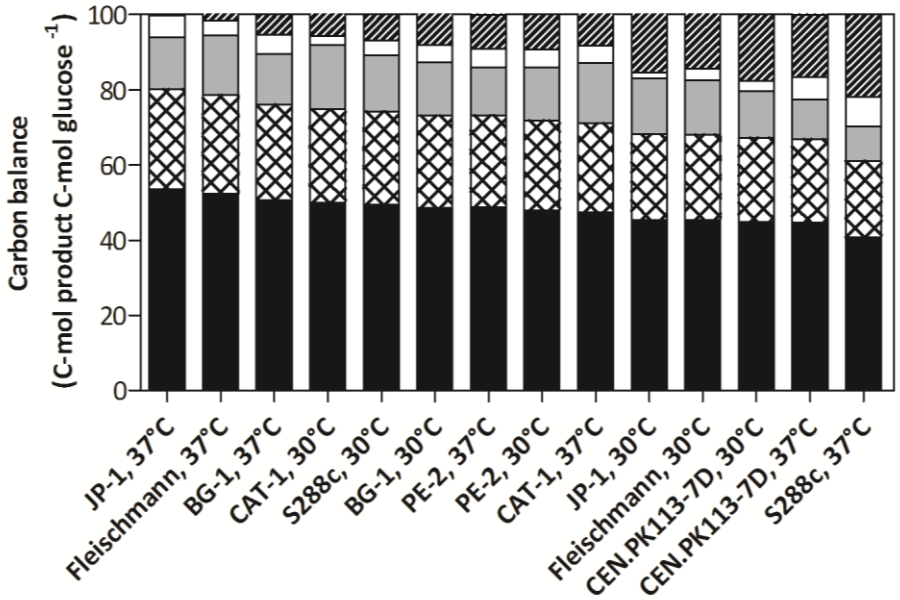

Figure 8 – Percentage of carbon (from glucose) incorporated into the following products: ethanol (black), CO_2 (diamond), biomass (gray), glycerol (white) and others (acetate, succinate, lactate and pyruvate plus nonquantified compounds) (diagonal), during the exponential growth phase of all strains in shake-flask cultivations in synthetic medium, at 30 °C or 37 °C. Data were sorted in decreasing order of percentage of carbon incorporated into ethanol.

As Locher et al. (1993) showed in a highly quantitative manner, 10-15 % of the total carbon consumed during the exponential phase of a *S. cerevisiae* batch cultivation on glucose cannot be traced in the main products of cell metabolism under these conditions, namely biomass, ethanol and CO_2, meaning that this carbon must be incorporated into other compounds, including glycerol and some organic acids (acetate, lactate, pyruvate and

succinate, among others), as well as other compounds these authors did not measure. In our cultivations, this value (white plus diagonal fractions in Figure 8) ranged from 5.5 % (Fleischmann, 37 °C) to 29.7 % (S288c strain, 37 °C).

From Figure 8, it is possible to notice that the three cultivations with the lowest percentages of carbon incorporated into ethanol were performed with laboratorial strains. Two of the latter cultivations (CEN.PK113-7D and S288c strains under heat stress) also led to high percentages of carbon incorporated into glycerol, organic acids and nonquantified compounds (as discussed above), pointing to a probable higher triggering of stress response mechanisms. In contrast, the strains that produced more ethanol and less from other metabolites from glucose, are industrial (exception being strain S288c cultivated at 30 °C) (left part of Figure 8). This is another indication that the fuel ethanol strains used in Brazil might indeed have been selected along decades of industrial practice for the trait of high ethanol yields, maybe as a response to stressful, energy-demanding fermentation conditions.

To conclude, we believe that the data presented here will aid researchers in considering the use of the Brazilian fuel ethanol strains not only for fuel ethanol production, but also for different applications that require high tolerance to a certain stress factor. These data may also guide future studies on the improvement of large-scale fuel ethanol production using e.g. metabolic and evolutionary engineering strategies.

2.5 ACKNOWLEDGEMENTS

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Natalia Perrella and Lirian Aranda, for their help in the experiments. The authors confirm that there are no Conflicts of Interest involved in this work.

3 TOLERANCE OF LABORATORY AND FUEL ETHANOL *SACCHAROMYCES CEREVISIAE* STRAINS TOWARDS ACIDIC CONDITIONS[5]

3.1 ABSTRACT

There has been growing interest worldwide in the Brazilian fuel ethanol yeast strains, currently responsible for the production of the most energetically efficient first-generation ethanol. Although some genomic and transcriptomic information is already available for some of these strains, quantitative physiological data have not yet been made available in the scientific literature. Our aim was to study *Saccharomyces cerevisiae* strain PE-2, one of the most widely used strains in Brazil for fuel ethanol production, in comparison to the popular lab strain CEN.PK113-7D, regarding their physiology under standard and stressful conditions, focusing on low pH and acetic acid stresses. For this purpose, we used highly-quantitative anaerobic fermentation set-ups including batch, chemostat and dynamic continuous cultivations. Surprisingly, in spite of their different origins, both strains exhibited similar physiological traits under standard conditions used for yeast cultivations in bioreactors (synthetic medium with glucose as the sole carbon and energy source, pH 5 or 3). Our data show that under such conditions the lab strain presented slightly higher ethanol and CO_2 yields on glucose than the industrial one. The only condition under which undoubtable superior growth and fermentation performances were observed for the PE-2 strain, in comparison with CEN.PK113-7D, was during batch cultivations on a complex medium (yeast extract, peptone and glucose) at low pH (2.8). Survival curves of post-diauxic cells at non-proliferating conditions (pH 1.5) also indicate a superior performance of PE-2, with respect to the lab strain. These data indicate that the industrial practice of cell recycling with extremely low pH using sulfuric acid might be crucial for the natural capability of PE-2 to survive during long periods of fuel ethanol production, outcompeting other *S. cerevisiae* strains in the non-aseptic vats.

[5] DELLA-BIANCA, B. E.; DE HULSTER, E.; PRONK, J.; VAN MARIS, T.; GOMBERT, A.K. Tolerance of laboratory and fuel ethanol *Saccharomyces cerevisiae* strains towards acidic conditions. Manuscript in preparation.

3.2 INTRODUCTION

While a number of biofuels, e.g. biodiesel and biobutanol, are currently under research and/or in the market, fuel ethanol produced from renewable resources is already a global commodity (GOLDEMBERG, 2007). In Brazil, where first generation (1G) bioethanol is produced in large scale by fermentation of sugarcane juice and/or molasses, indigenous strains of *Saccharomyces cerevisiae* have been isolated and reintroduced in the process (BASSO et al., 2008, DA SILVA-FILHO et al., 2005a), displaying remarkably better performances than baker's strains, used industrially as starters at the beginning of the sugarcane crushing season, and finally outcompeting them inside the fermentors.

Since yeast cells endure several stress conditions in the fuel ethanol production process, these wild strains must possess distinctive stress-related attributes in order to thrive in this environment. While a range of physiological traits from these strains have been described in the past few years (and reviewed recently by our group (DELLA-BIANCA et al., 2013)), there is a lack of accurate knowledge regarding their basic physiology under laboratory, well-defined conditions. In this context, chemostats come in handy, as a reliable tool for strain characterization. This mode of cultivation allows a constant physicochemical state to be kept inside the fermentors and the manipulation of cell growth rates (DARAN-LAPUJADE et al., 2009b, MONOD, 1950, NOVICK; SZILARD, 1950), facilitating data comparison between strains.

In a previous work, we screened several Brazilian fuel ethanol strains for tolerance towards a number of stress factors (Chapter 2 of this thesis). Low pH was the condition towards which these strains in general presented higher tolerance, when compared not only to laboratorial strains, but also to baker's strains. After the industrial fermentation is complete, cells undergo a washing treatment in which pH is lowered to 1.8-2.5 for 1-2 hours by addition of H_2SO_4, in order to reduce bacterial contamination (BASSO; BASSO; ROCHA, 2011). This may happen up to 3 times a day and also affects the yeast cells being recycled (DE MELO et al., 2010, KAPTEYN et al., 2001). In view of this, we thought it would be interesting to investigate this stress factor in more detail, since acidic pH could be the environmental factor that mostly contributed to the phenotypes we encounter today in the Brazilian fuel ethanol strains.

Moreover, high acetic acid concentrations can be found in sugarcane bagasse hydrolysates (ALMEIDA et al., 2007), the main substrate for second generation (2G) bioethanol production in Brazil. Acetic acid is known to affect yeast metabolism in many ways, primarily by causing intracellular acidification and anion accumulation (PIPER et al., 2001), and so the response towards this compound was also studied by us. The aim of this study was to investigate the physiological responses of *S. cerevisiae* strains PE-2, widely used in the Brazilian fuel ethanol production (BASSO et al., 2008), and CEN.PK113-7D, one of the most popular laboratory strains used in physiological studies (VAN DIJKEN et al., 2000), towards low pH and the presence of acetic acid. Since our previous results were obtained using spot assays in plates (see Chapter 2), in this work we used both chemostat and batch cultivations in fermentors to compare these strains in a highly quantitative manner.

3.3 MATERIALS AND METHODS

3.3.1 Yeast strains

Four *Saccharomyces cerevisiae* strains were used in this study: the laboratory reference strains S288c and CEN.PK113-7D (obtained from the Yeast Genetic Stock Center, Berkeley, USA and Dr. P. Kötter, Frankfurt, Germany, respectively), the Brazilian fuel ethanol strain PE-2 (BASSO et al., 2008), and the baker's strain Fleischmann (the latter two obtained from Dr. L. C. Basso, Piracicaba, Brazil).

3.3.2 Growth conditions

The following two media were used in shake-flasks: synthetic medium corrected to pH 6.0 by addition of KOH (VERDUYN et al., 1992), and YPD (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose). Inocula were prepared by adding 1 mL of stock cultures to 500 mL

shake-flasks containing 100 mL of medium. After overnight cultivations at 30 °C and 200 rpm, the cultures were transferred to fermentors that contained the same medium used for inoculum preparation.

Strains were grown at 30 °C in fermentors (Applikon, Schiedam, The Netherlands) with a working volume of 1 L, which was kept constant during continuous cultivations by means of an electric level sensor. Stirrer speed was controlled at 800 rpm and pH was controlled at the desired value (or using a desired pre-established pH profile) with the automatic addition of 2 M KOH or 2 M H_2SO_4. Anaerobic conditions were maintained by equipping the fermentors with Norprene tubing and Viton O-rings, sparging them with N_2 gas at 0.5 vvm, and also flushing the medium vessel with the same gas. Anaerobic growth factors (10 $mg.L^{-1}$ ergosterol and 420 $mg.L^{-1}$ Tween 80) were added to both synthetic (VERDUYN et al., 1992) and YPD media (REINER et al., 2006), as well as 0.15 $g.L^{-1}$ antifoam C emulsion (Sigma, St. Louis, USA). Acetic acid 105 mM was added to the medium in some of the experiments. All fermentations were carried out at least in duplicate.

During batch cultivations, samples were drawn at selected time intervals. For chemostats, the dilution rate *D* was set to 0.10 h^{-1} and steady state was assumed when, after at least five volume changes, the culture dry weight and specific CO_2 production rate changed by less than 2 % over 2 volume changes. At this moment, sampling proceeded. Dynamic continuous cultivations were started as chemostats and, after steady state was reached, a controlled pH decrease was applied with a linear rate of -0.01 pH units h^{-1}, during which samples were taken every 8 hours.

3.3.3 Analytical methods

Culture dry weights were determined in duplicate via filtration onto dry, pre-weighted nitrocellulose membranes with a pore size of 0.45 µm, which were then washed with demineralized water, dried in a microwave oven for 20 minutes at 360 W and weighted again (POSTMA et al., 1989). Residual glucose concentrations were analyzed enzymatically using EnzyPlus EZS781+ kit (BioControl, Bellevue, USA) after rapid sampling using pre-cooled steel beads (MASHEGO et al., 2003). Fresh media and supernatants, the latter obtained after

culture broth centrifugation, were analyzed via HPLC using an AMINEX HPX-87H ion exchange column (BioRad, Richmond, USA) at 60 °C with 5 mM H_2SO_4 as the mobile phase. Glucose, ethanol, glycerol, succinate and lactate were detected by a Waters 2410 refraction index detector at 50 °C, and pyruvate and acetate were detected by a Waters 2487 UV detector at 214 nm. Ethanol concentrations were corrected for evaporation as in Medina et al. (2010). Off-gas was first cooled with a condenser (2 °C) and then dried with a PD-625-12P dryer (PermaPure, Toms River, USA), and had its flow rate measured by a Saga digital flowmeter (Ion Science, Cambridge, UK). CO_2 and O_2 concentrations in the off-gas were measured with an NGA 2000 gas analyzer (Rosemount Analytical, Orrville, USA).

3.3.4 Physiological data determination

The biomass yield from ATP (ATP yield; $Y_{X/ATP}$) was determined taking into account biomass, ethanol and glycerol concentrations (PAMPULHA; LOUREIRO-DIAS, 2000, VERDUYN et al., 1990a). For batch cultivations, only points from exponential growth phase were considered. Maximum specific growth rates (μ_{max}) were calculated based on continuous off-gas CO_2 measurements, whereas product yields on substrate ($Y_{P/S}^{exp}$) were determined from plots of product concentration vs. substrate concentration. For chemostats, only data from steady state were considered; yields were calculated using specific rates of consumption and/or formation (q); C recovery was calculated as the percentage of carbon from substrates recovered in biomass and metabolites, using a biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$ and 5 % ash (NIELSEN; VILLADSEN; LIDÉN, 2003) that corresponds to 25.89 g DW biomass C-mol^{-1}. During dynamic continuous cultivations, instantaneous specific growth rates (μ) and specific rates of consumption and/or formation were obtained using mass balances and the geometrical differentiation method proposed by Leduy and Zajic (1973), implemented in Microsoft Excel by Gombert (2001). Maximum specific growth rates during washout were determined using the slope of the logarithmic plot of biomass against time, which equals to ($\mu_{max} - D$), as in Pirt and Callow (1960).

3.3.5 Viability curves

Cells were grown overnight in YPD medium as described previously for inoculum flasks and harvested by centrifugation at 4000 rpm for 5 minutes. These post-diauxic[6] cells were either resuspended in 50 mL water or transferred to new flasks and cultivated until exponential growth phase was reached (3 h). These exponentially growing cells were also centrifuged and resuspended. Cells were kept in suspension with minor agitation on a magnetic stirrer plate at 30 °C. After adjusting cell suspension pH to 1.5 using H_2SO_4 2 M (BASSI et al., 2013), samples were taken every hour for 4 h and incubated 1:1 (v/v) with 0.4 % Sigma trypan blue solution for 5 minutes. Cells were then diluted appropriately and counted microscopically on a Neubauer chamber using visible light, and viability was determined by the percentage of non-stained cells.

3.4 RESULTS AND DISCUSSION

3.4.1 Chemostat data of the *S. cerevisiae* PE-2 strain

For the first time to our knowledge, strain PE-2's physiology was explored in a glucose-limited anaerobic chemostat, a classical set-up for studying yeast physiology. Under the reference condition (D = 0.1 h^{-1}, pH 5), residual glucose concentration was higher for strain PE-2, as well as biomass, glycerol and lactate yields (and these metabolites' specific production rates) (Table 3). On the other hand, strain CEN.PK113-7D presented higher ethanol and CO_2 yields and specific production rates than PE-2 did. Previous results obtained using the same medium, but in shake-flask cultivations, differ from the present ones, since strain PE-2 exhibited a higher ethanol yield than CEN.PK113-7D (Chapter 2 of this thesis). Pyruvate and succinate but no acetate could be detected in the effluent for strain PE-2. This

[6] The post-diauxic growth phase is characterized by exhaustion of glucose and slow growth on ethanol (HERMAN, 2002).

may be due to a distinct regulation of the pyruvate dehydrogenase (PDH)-bypass, such as a lower acetaldehyde dehydrogenase activity and/or a higher acetyl-CoA synthetase activity (POSTMA et al., 1989), which all reduce the intracellular acetate pool available for excretion.

Growth with a very low extracellular acetate concentration is an advantageous and highly desirable phenotype, especially in acidic environments, as is the industrial fermentation process for fuel ethanol production. Low external pH favors the undissociated form of the acid, which will diffuse back across the membrane and dissociate in the near-neutral cytosolic pH (PAMPULHA; LOUREIRO-DIAS, 1989), generating an ATP-spending, futile cycle of pumping out both H^+ and acetate anion. This could in part explain why the PE-2 strain exhibited higher biomass (+22.2 %) and ATP (+28.7 %) yields, in comparison to CEN.PK113-7D.

Table 3 – Physiology of *Saccharomyces cerevisiae* strains CEN.PK113-7D and PE-2 in glucose-limited, anaerobic chemostats in synthetic medium at a dilution rate of 0.1 h^{-1}. Specific rates *q* are given in mmol g^{-1} h^{-1}. Results are given as average values from triplicate experiments ± standard deviation, unless otherwise stated. *X*: biomass concentration.

	pH 5		pH 5 + 105 mM HAc		pH 3		
	CEN.PK113-7D[a]	**PE-2**	**CEN.PK113-7D**[a]	**PE-2**	**CEN.PK113-7D**[b]	**CEN.PK113-7D**[c]	**PE-2**[c]
***q* glucose**	-6.03 ± 0.10	-5.06 ± 0.15	-12.17 ± 0.20	-10.17 ± 0.94	-6.96 ± 0.39	-6.74 ± 0.14	-5.95 ± 0.02
***q* CO_2**	10.40 ± 0.45	8.51 ± 0.28	22.97 ± 0.50	21.03 ± 1.53	11.76 ± 0.29	12.32 ± 0.02	10.37 ± 0.00
***q* ethanol**	9.52 ± 0.16	7.70 ± 0.26	21.45 ± 0.35	19.32 ± 1.32	10.99 ± 0.43	10.99 ± 0.17	9.40 ± 0.04
***q* glycerol**	0.79 ± 0.02	0.89 ± 0.04	0.54 ± 0.01	0.68 ± 0.04	0.83 ± 0.04	0.69 ± 0.01	0.95 ± 0.01
***q* lactate**	0.05 ± 0.01	0.052 ± 0.003	0.09 ± 0.00	0.093 ± 0.008	0.09 ± 0.01	0.079 ± 0.001	0.039 ± 0.002
***q* acetate**	0.02 ± 0.00	0.000 ± 0.000	-0.57 ± 0.02	-0.28 ± 0.06	0.02 ± 0.00	0.007 ± 0.000	0.002 ± 0.000
***X* (g DW L^{-1})**	2.25 ± 0.02	2.63 ± 0.01	1.13 ± 0.02	1.27 ± 0.04	2.03 ± 0.04	1.97 ± 0.01	2.26 ± 0.04
$Y_{X/S}$ (g DW g glucose^{-1})	0.09 ± 0.00	0.11 ± 0.00	0.05 ± 0.01	0.054 ± 0.002	0.09 ± 0.00	0.080 ± 0.001	0.093 ± 0.002
C recovery (%)	99.4 ± 0.8	100.9 ± 0.7	95.1 ± 0.6	101.5 ± 2.1	96.5 ± 3.1	100.2 ± 0.9	100.9 ± 0.3
Residual glucose (mM)	0.2 ± 0.0	0.60 ± 0.06	2.1 ± 0.1	1.69 ± 0.23	0.4 ± 0.1	0.55 ± 0.06	0.77 ± 0.08
$Y_{X/ATP}$ (g DW mol ATP^{-1})	11.19 ± 0.28[d]	14.41 ± 0.10	5.24 ± 1.05[d]	5.53 ± 0.13	11.10 ± 0.78[d]	9.40 ± 0.01	11.76 ± 0.33

[a] Data from Abbott et al. (2007)
[b] Data from Abbott et al. (2008)
[c] Average values from duplicate experiments ± average deviation
[d] Calculated by us using data from reference papers

3.4.2 Response to acetic acid stress

PE-2's tolerance towards acetic acid was evaluated under the same experimental set-up described above, with the addition of 105 mM acetic acid in the feed, a concentration known to decrease in 50 % CEN.PK113-7D's biomass yield under such cultivation conditions (ABBOTT et al., 2007). A similar drop in the biomass yield, in relation to the standard condition (no acetic acid added), was obtained for PE-2 strain (Table 3). The residual glucose concentration, on the other hand, was lower for PE-2 than for CEN.PK113-7D in this condition; the opposite of what we observed during the cultivations without acetic acid added. The specific rates of ethanol and CO_2 formation during the cultivations with acetic acid added were the same for both strains (considering the standard deviations), in contrast to what was observed for the cultivations without acid added.

Acetate in the medium was internalized at different rates by the two strains—PE-2's acetate consumption rate was almost 51 % lower than the one shown by CEN.PK113-7D. Given this observation, we calculated the acetate accumulation factor for both strains under the condition tested—13.3 for PE-2 and 28.9 for CEN.PK113-7D. These values are much lower than the ones calculated by Verduyn et al. (1990b) for strains CBS8066 and H1022 (84 and 65, respectively, for acetate concentrations ranging from 3.5 to 35.5 mM). This low accumulation phenotype is energetically favorable since less acetate inside the cell means less energy spent to pump it (and protons) to the extracellular environment. Nijkamp et al. (2012) showed that CEN.PK113-7D's genome possesses features from both laboratory and industrial strains, and so this advantageous feature may originate from the strains' industrial backgrounds. Besides, Garay-Arroyo et al. (2004) demonstrated that CEN.PK113-7D was one of the most tolerant towards the addition of 166 mM acetic acid in YPD batch cultivations, compared to a number of industrial strains.

The results above may also be an indication that PE-2's membrane is less permeable to acetate. Alves (2000) suggested that PE-2 has a more selective membrane than baker's yeast, based on its lower intracellular radioactivity when incubated with ^{14}C-succinate. Taken together, these findings point to a decreased diffusion of these organic acids through PE-2's membrane. Still, the ATP yield exhibited by PE-2 was comparable to CEN.PK113-7D's in this condition.

3.4.3 Response towards low pH in continuous cultivations using a synthetic medium

Tolerance towards low pH conditions was evaluated using chemostats at pH 3.0 as in Abbott et al. (2008) and also by a dynamic continuous cultivation approach. For this purpose, after a chemostat at pH 3.0 had reached steady state, the pH inside the fermentor was decreased linearly in time until the CO_2 concentration in the off-gas was equal to zero, meaning that the cultures had been completely washed out; this happened around pH 2.15 for both strains.

Analyzing the steady state data at pH 3.0 for both strains (Table 3), it is possible to observe a similar behavior to the one observed at pH 5 (as discussed above): a higher residual glucose concentration, lower ethanol and CO_2 specific production rates and higher glycerol specific production rate for PE-2, when compared to CEN.PK113-7D. Nevertheless, the differences between the two strains are relatively smaller at pH 3 than at pH 5. The specific production of lactate, however, was 50 % lower for strain PE-2 and once again no acetate was detected in the effluent. The biomass yield was the same for both strains and ATP yield for strain PE-2, although higher than the one observed for strain CEN.PK113-7D in this experiment, was comparable to the one calculated using CEN.PK113-7D's data from Abbott et al. (2007).

For the dynamic part of this experiment, we calculated the instantaneous and maximum specific growth rates and the specific production/consumption rates for glucose, ethanol, glycerol, pyruvate, acetate, succinate and lactate (Figures 9 and 10). It is possible to separate the analyses into two time frames: in the first interval (pH > 2.68), there is a slow washout since the instantaneous and maximum growth rates are smaller but close to the dilution rate applied to the system. On the other hand, during the second interval (pH < 2.68) a faster washout could be noticed, in which the instantaneous and maximum growth rates drop sharply and get close to zero. This "no growth" phase was triggered for both strains around the same critical pH (2.68), although the maximum growth rate in pH values lower than the critical pH was higher for PE-2 strain.

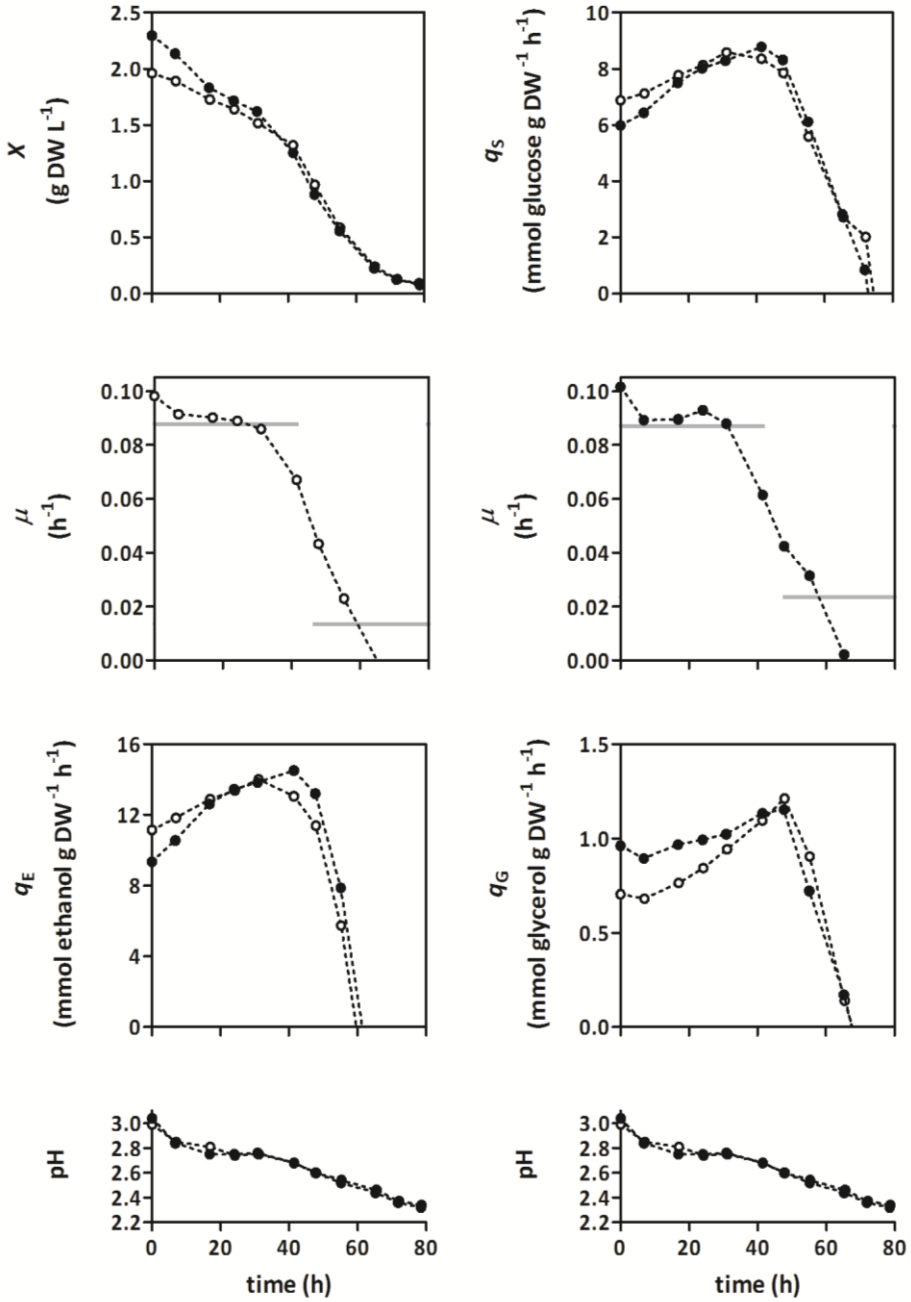

Figure 9 – Physiology of *Saccharomyces cerevisiae* strains CEN.PK113-7D (open circles) and PE-2 (solid circles) in anaerobic, glucose-limited continuous cultivations in synthetic medium and under a controlled pH decrease. Gray lines on growth rate graphs represent maximum specific growth rates (h^{-1}) for different time intervals. Time-zero data are from prior steady states reached at pH 3.0, just before the beginning of the pH decrease. Data shown are from one experiment out of two conducted with similar results for each strain. DW: biomass dry weight.

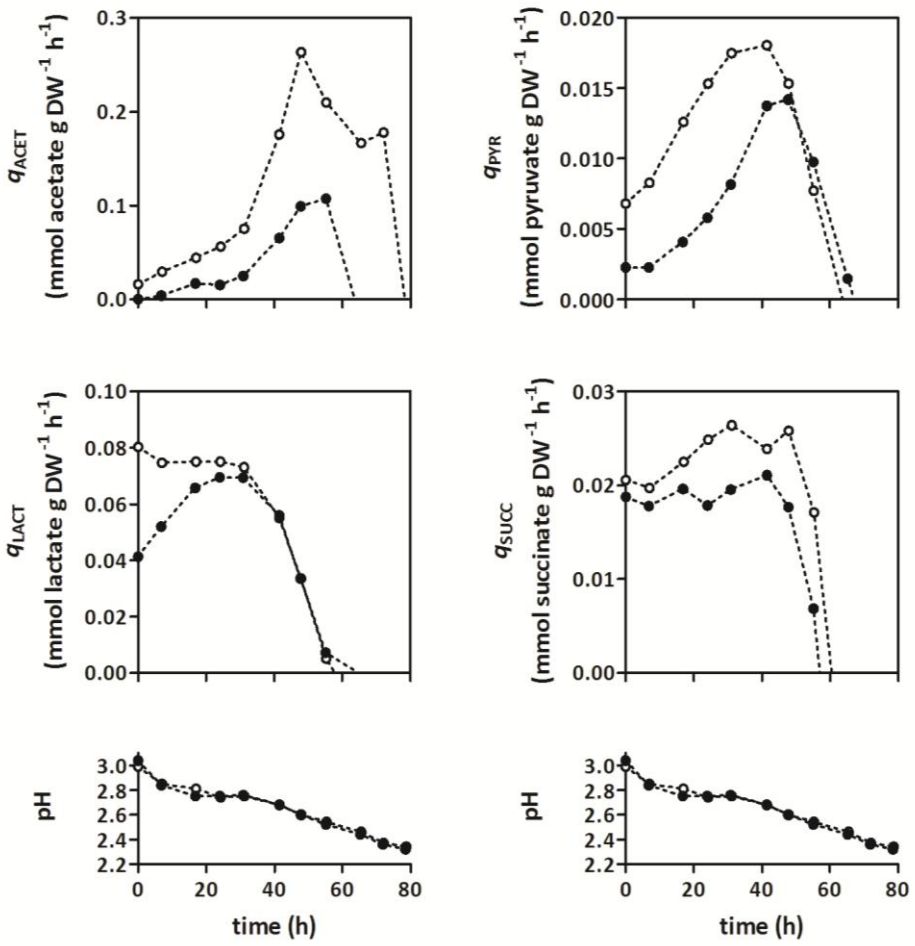

Figure 10 – Physiology of *Saccharomyces cerevisiae* strains CEN.PK113-7D (open circles) and PE-2 (solid circles) in anaerobic, glucose-limited continuous cultivations in synthetic medium and under a controlled pH decrease. Time-zero data are from prior steady states reached at pH 3.0, just before the beginning of the pH decrease. Data shown are from one experiment of two performed with similar results for each strain. DW: biomass dry weight.

Residual glucose profiles during the washout were very similar for both strains, as well as glucose specific consumption rates and glycerol and ethanol specific formation rates. Specific formation rates of the four organic acids analyzed (acetate, pyruvate, lactate and succinate) varied between the two strains along the washout, with acetate the metabolite which exhibited the most distinct profile when the two strains are compared. Pyruvate production rates exhibit a peak around the critical pH value, which can be explained by a faster flux through the glycolytic pathway, driven by greater cell maintenance requirements under highly acidic conditions. Acetate and lactate production rates followed the same trend, the first formed from pyruvate via the PDH-bypass pathway (POSTMA et al., 1989, REMIZE; ANDRIEU; DEQUIN, 2000) and the second formed from dihydroxiacetone-

phosphate via the methylglyoxal bypass (MARTINS; CORDEIRO; FREIRE, 2001, PRONK; YDE STEENSMA; VAN DIJKEN, 1996). Although succinate formation depends on pyruvate, which under anaerobiosis can go through the reductive branch of the TCA pathway (CAMARASA; GRIVET; DEQUIN, 2003), a succinate production peak was not evident in our data.

Although strains CEN.PK113-7D and PE-2 showed some distinct physiological aspects in the conditions discussed above, their tolerance to acetic acid and low pH can be considered, from the results above, highly similar. This observation was surprising to us, since we did not expect a laboratorial strain (CEN.PK113-7D) to be as tolerant to a typical stress found in the fuel ethanol industry as a strain that was isolated from this environment. Besides this, the results presented and discussed above apparently differ from the result obtained earlier by our group, in which strain PE-2 exhibited growth in YPD plates in pH 2.5 while strain CEN.PK1137-D did not (in Chapter 2 of this thesis). To further investigate this apparent contradiction, we tried to reproduce the condition from the YPD plates in a more quantitative and controlled way, taking two parallel approaches: 1) anaerobic bioreactor cultivations on YPD medium at low pH; and 2) assessment of cell viability under non-proliferative, highly acidic conditions (survival curve at low pH). The results are discussed next.

3.4.4 Response towards low pH during bioreactor batch cultivations using YPD medium

In batch cultivations using YPD medium at pH 2.7, carried out in bioreactors, strain PE-2 showed a 33 % higher maximum growth rate and an 86 % higher biomass yield than strain CEN.PK113-7D (Figure 11). This result supports our previous observation in YPD plates at pH 2.5. Furthermore, the ethanol yield was 7 % higher and the glycerol yield 37 % lower for the PE-2 strain, when compared to CEN.PK113-7D. These yields' values were analyzed together in the form of ATP yield, which was also 46 % higher for strain PE-2. In contrast, during a control experiment using YPD medium at pH 5.0 the strains showed comparable physiological data. Another control experiment was tried using synthetic medium at pH 2.7, but since neither of the two strains showed any growth (data not shown), the cultivation was repeated at the slightly higher pH of 2.8. Under this condition, the strains' performances

were once again very similar to each other. It is clear from these data that these two strains behave distinctly under specific acidic conditions—YPD medium and low pH. Strain PE-2 shows a higher stress tolerance under these conditions, by means of utilizing energy more efficiently towards cell generation.

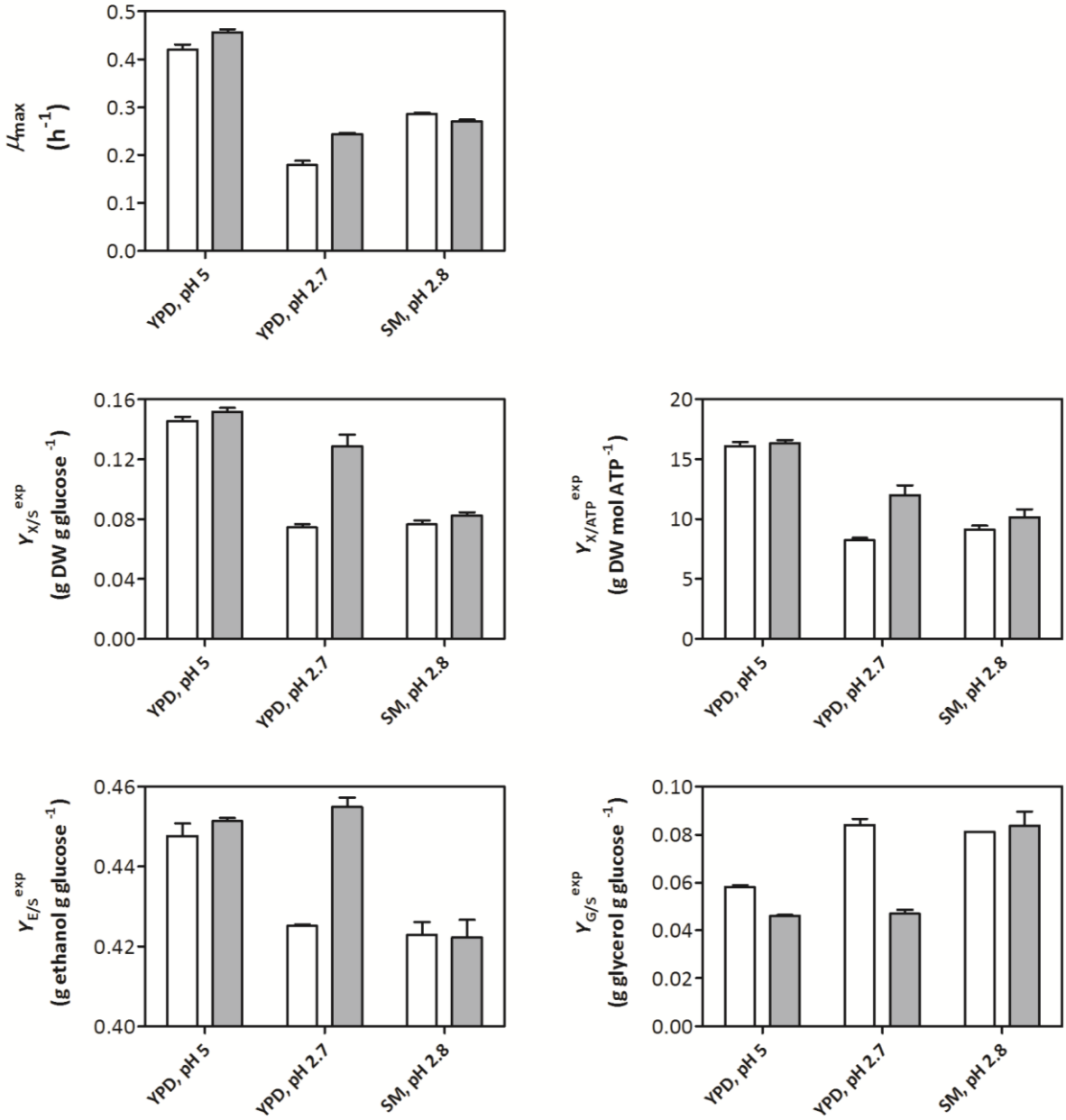

Figure 11 – Maximum specific growth rate (μ_{max}), biomass yield ($Y_{X/S}^{exp}$), ethanol yield ($Y_{E/S}^{exp}$), glycerol yield on substrate ($Y_{G/S}^{exp}$) and biomass yield on ATP ($Y_{X/ATP}^{exp}$) from exponential growth phase of *Saccharomyces cerevisiae* strains CEN.PK113-7D (white bars) and PE-2 (gray bars), in anaerobic batch cultivations on YPD and synthetic (SM) media. Bars indicate average values from duplicate experiments and error bars, the average deviation. DW: biomass dry weight.

3.4.5 Cell viability under very low pH

Strains PE-2 and CEN.PK113-7D cell viabilities under low pH were assessed in a non-proliferative and lethal environment: an H_2SO_4 solution at pH 1.5 and 30 °C, in an attempt to mimic industrial cell recycling conditions used in Brazilian 1G ethanol production (DELLA-BIANCA et al., 2013). In this experiment, the baker's strain Fleischmann was used as an industrial reference strain, since it is used as a starter strain in the Brazilian fuel ethanol industry, disappearing during the first month of fermentation cycles (BASSO et al., 2008). Exponentially growing cells from all three strains, when placed at pH 1.5, showed equivalent low viabilities after 4 hours (Figure 12). On the other hand, post-diauxic cells exhibited viabilities that were strain-dependent and overall higher than those of exponentially growing cells, as expected since post-diauxic cells are more stress resistant (DICKINSON; SCHWEIZER, 2004). Strain PE-2 presented the highest viability after 4 hours at pH 1.5 (64.7 % ± 5.4 %), followed by Fleischmann strain (50.4 % ± 2.9 %) and CEN.PK113-7D (34.9 % ± 1.2 %). These values, although not drastically different, may help explain why PE-2 outcompetes Fleischmann in the industrial environment, since a relatively higher number of Fleischmann cells are eliminated at each post-fermentation cycle of cell washing.

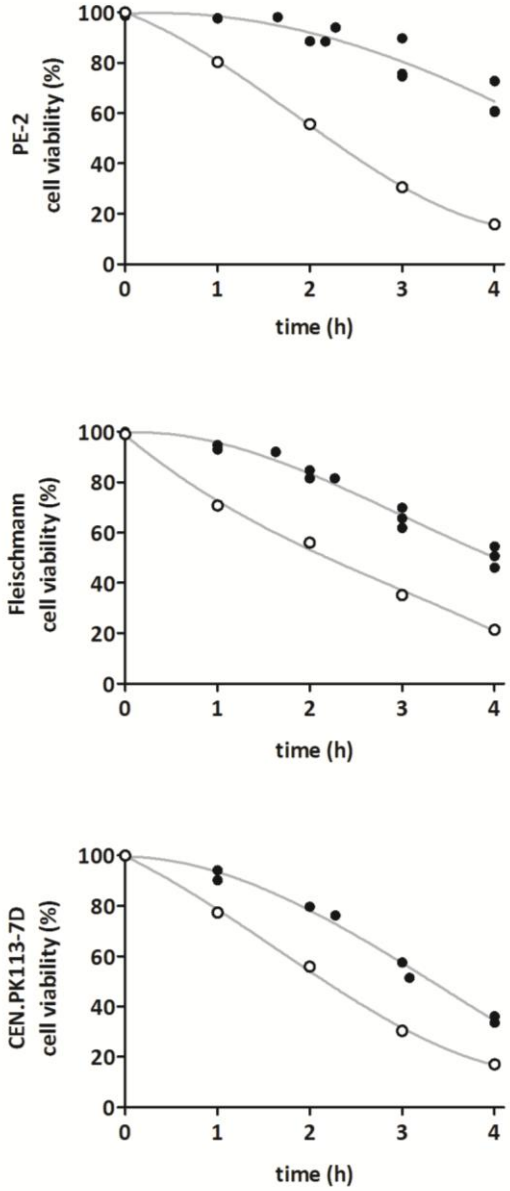

Figure 12 – Viability curves of exponentially growing (open circles) or post-diauxic cells (solid circles) from *Saccharomyces cerevisiae* strains PE-2, Fleischmann and CEN.PK113-7D, following a 4-hour treatment with sulfuric acid at pH 1.5 in a non-proliferative condition. Gray lines represent third order polynomials fitted for viewing purposes only.

3.4.6 Response towards low pH in chemostats using YPD medium

Based on the results obtained during low pH batch cultivations and cell viability curves, we performed carbon-limited anaerobic chemostats on YPD medium (2 % glucose) and low pH, even though certain limitations arise from the use of a complex medium in this highly quantitative experiment, i.e. quantification of certain compounds. At this point, we included strain S288c as a second reference laboratory strain.

From the data presented in Table 4 it is possible to see that all three strains exhibited the same physiological data in YPD chemostats, at least in what concerns the formation of cells and main products (ethanol, CO_2 and glycerol). These results did not confirm the higher tolerance of strain PE-2 towards low pH revealed in YPD batch cultivations and viability measurements, and so a possible influence of the set-up must be considered, in terms of a distinct accumulation of toxic metabolites during batch cultivations or the distinct control of specific growth rate in batch (lack of control; growth at μ_{max} under this particular condition) and chemostat (μ_{max} equal to 0.1 h^{-1}) set-ups. It was proposed that the cytosolic pH (pH_c) determines the specific growth rate (ORIJ et al., 2012), and that pH_c, albeit neutral in normal conditions, is dynamic and responds to environmental conditions (ORIJ; BRUL; SMITS, 2011), e.g. extracellular pH. An adequate response to a decrease in extracellular pH depends on a number of pH_c regulation players, e.g. the H^+-ATPase-coding genes *PMA1* (ORIJ et al., 2012) and *PMA2* (CARMELO; BOGAERTS; SÁ-CORREIA, 1996), and so mutations in these players can affect pH_c homeostasis. In chemostats, where the growth rate is not strain-dependent but is controlled by the dilution rate, any variations in pH_c homeostasis that would lead to different growth rates could be overridden and go unnoticed. Given that similar results were obtained from chemostats with the addition of acetic acid, and cytosolic acidification was shown to be the main cause of growth inhibition by acetate (ULLAH et al., 2012), it is still possible that PE-2 possesses a higher, but still overlooked, tolerance to acetic acid, as seen previously in cultivations in YPD plates supplemented with this compound (see Chapter 2 of this thesis).

Another hypothesis for the differences observed between batch and chemostat experiments regards the cell population dynamics in the industrial acid washing step. In the beginning of the cane crushing season, tons of baker's yeast cells are used as starters, with the purpose of achieving very high biomass concentrations in the fermentors and prioritize

ethanol production over biomass propagation. In the first rounds of fermentation, however, the fuel ethanol yeasts are able to contaminate the process. Since baker's yeast cells possess lower viability under very low pH, at each acid washing cycle they are killed relatively faster, and become a rich and abundant substrate for fuel ethanol strains. Therefore, these strains may have evolved to multiply fast in a rich environment under low pH and this condition resembles more the batch cultivation than the chemostat (both performed on YPD medium and low pH), due to the substrate and growth rate limitations that are inherent to chemostats.

Table 4 – Physiology of *Saccharomyces cerevisiae* strains CEN.PK113-7D, PE-2 and S288c in anaerobic, glucose-limited chemostats in YPD medium at pH 3.0 and a dilution rate of 0.1 h^{-1}. Specific rates *q* are given in mmol g^{-1} h^{-1}. Results are given as average values from duplicate experiments ± average deviation. *X*: biomass concentration.

	CEN.PK113-7D	**PE-2**	**S288c**
***q* glucose**	-5.41 ± 0.24	-5.40 ± 0.07	-5.66 ± 0.04
***q* CO_2**	10.48 ± 0.31	10.74 ± 0.04	11.05 ± 0.03
***q* ethanol**	9.71 ± 0.27	10.15 ± 0.14	10.29 ± 0.14
***q* glycerol**	0.20 ± 0.05	0.42 ± 0.03	0.36 ± 0.02
***X* (g DW L^{-1})**	2.12 ± 0.13	1.98 ± 0.02	1.97 ± 0.02
$Y_{X/S}$ (g DW g glucose^{-1})	0.10 ± 0.01	0.10 ± 0.00	0.10 ± 0.00
C recovery (%)	105.7 ± 2.3	111.0 ± 0.1	107.4 ± 0.1
Residual glucose (mM)	0.77 ± 0.14	1.28 ± 0.06	1.72 ± 0.03
$Y_{X/ATP}$ (g DW mol ATP^{-1})	10.65 ± 0.68	10.43 ± 0.19	10.35 ± 0.01

3.5 CONCLUSIONS

In our study, we made available, for the first time, highly-quantitative physiological data for the *S. cerevisiae* strain PE-2, obtained from anaerobic glucose-limited chemostats. We have also shown that this strain, used in the Brazilian fuel ethanol industry, exhibits similar physiology to the laboratory strain CEN.PK113-7D, under a range of conditions tested in chemostat mode. However, strain PE-2 seems to be able to grow with lower excretion of acetate, when compared to CEN.PK113-7D, which may reflect a different regulation of the PDH bypass and/or a lower permeability of its membrane to extracellular acetic acid. The distinct acetate metabolism presented by strain PE-2 is a highly desirable phenotype in acidic

environments such as the industrial fuel ethanol fermentation, since less acetate inside the cell means less ATP used to extrude this anion and the corresponding proton.

During anaerobic YPD batch cultivations under low pH, the two strains presented remarkable differences—strain PE-2 exhibited a 33 % higher growth rate and an 86 % higher biomass yield. This strain also showed the highest cell viability during a 4-hour H_2SO_2 treatment at pH 1.5, even higher than baker's strain Fleischmann, also used in the fuel ethanol production in Brazil. The higher cell viability of PE-2, when compared to CEN.PK113-7D, after an acid treatment and its better performance during anaerobic YPD batches at low pH form a possible explanation to why cells of the starter baker's strain are outcompeted by PE-2 cells in the fuel ethanol industry, since yeast cells are subjected to a sulfuric acid washing step between consecutive fermentation cycles.

Taken together, the data presented in this paper provide new insights into *S. cerevisiae* PE-2's physiology, which have certainly been acquired by evolution over the years in the dynamic industrial process it came from. These data might aid us in starting to understand why it is capable of surviving during months of operation in the non-aseptic industrial vats.

3.6 ACKNOWLEDGEMENTS

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4 CONCLUSIONS AND FUTURE WORK

In this thesis, the aim was to evaluate the physiology and the stress tolerance of *S. cerevisiae* strains used in the Brazilian fuel ethanol industry, through the analysis of physiological responses in a variety of cultivation conditions.

Firstly, from the literature review presented in Chapter 1, it becomes clear that our knowledge on the Brazilian yeast strains has grown considerably in the last 5 years, mainly due to genome sequencing. However, since we are not yet capable of predicting cell behavior under different conditions from genome sequence alone, further research on these strains is still needed to improve their performances in the biorefinery. Within this context, physiological studies are of crucial importance.

From the data presented in Chapter 2, we concluded that none of the strains (laboratorial or industrial) is the most tolerant to all stress conditions tested, and this reflects the diverse environments in which the strains evolved, even when the fuel ethanol strains are compared to each other. In solid YPD growth assays, high sugar concentrations were not able to distinguish strains, although the use of sugarcane juice and molasses, which also resulted in high sugar concentrations, favored the growth of industrial strains over that of lab strains. Under ethanol and acetic acid stresses, the industrial strains also performed better. Oxidative stress enabled the detection of two hypersensitive strains, CEN.PK113-7D and JP1. In contrast, strain JP1 exhibited the highest tolerance towards heat and NaCl stresses, and PE-2 strain towards low pH stress. A distinction between fuel ethanol and baker's strains was only observed during growth under heat and low pH stresses. Therefore, these two conditions may be considered major factors of selective pressure in the fuel ethanol production environment, hindering the replication of the starter baker's strain. Regarding growth in shake-flasks with synthetic medium, the physiology of all strains was similar under the standard temperature of 30 °C, exception being the glycerol yield, which varied in a strain-dependent manner. Relative to 30 °C, only laboratorial strains exhibited a significant decrease on biomass and ethanol yields under heat stress (37 °C). Carbon balance analysis indicated that under heat stress laboratorial strains incorporate more carbon into other compounds (as glycerol and organic acids) instead of ethanol. This may result from a

higher triggering of stress response mechanisms, when compared to the response of industrial strains under the same condition.

In Chapter 3, the focus was on the physiological responses of strains CEN.PK113-7D and PE-2 to acid-related stress conditions (low pH and presence of acetic acid). For the first time the physiology of strain PE-2 was explored in a chemostat cultivation. In standard and low pH chemostats (pH 5.0 and 3.0, respectively), strain PE-2 did not excrete acetate. This phenotype can be energetically advantageous in acidic environments as the industrial fuel ethanol process, although the ATP yield of strain PE-2 at pH 3.0 was similar to that of CEN.PK11-7D. In a dynamic continuous cultivation with decreasing pH over time, there was a critical pH value (2.68) below which the instantaneous growth rate of strain PE-2 was higher than that of CEN.PK113-7D. In chemostats with the addition of 105 mM acetic acid at pH 5.0, both strains exhibited the same biomass yield but different acetate consumption rates and accumulation factors (51 % and 54 % lower for strain PE-2, respectively). Although certain variations exist, the tolerance of these strains to acetic acid and low pH appeared somewhat similar in chemostats.

A completely different picture emerged when the strains were analyzed in low pH batches using YPD medium (2.7), a condition that resembles more the plate assays from Chapter 2. Strain PE-2 presented higher specific growth rate (33 %), biomass yield (86 %) and ethanol yield (7 %), and lower glycerol yield (37 %) than strain CEN.PK113-7D did. However, the strains exhibited comparable physiology in YPD batches at pH 5.0 or in synthetic medium batches at pH 5.0 and 2.8, and even during YPD chemostats at pH 3.0. It becomes clear that these two strains behave distinctly only under specific acidic conditions—anaerobic YPD batches carried out at low pH. Furthermore, after 4 hours in a non-proliferative condition at extremely low pH (1.5), the viability of post-diauxic cells of strain PE-2 (64.7 %) was higher than of CEN.PK113-7D (34.9 %) and Fleischmann (50.4 %) strains. Taken together, these results may support the hypothesis that strain PE-2 has adapted not only to survive at the low pH values encountered during the industrial acid washing step, but also to replicate fast in this stressful condition, using dead starter strain cells as a substrate.

This work also evidenced that the fermentation set-up can exert great influence on the outcome of stress response experiments. In addition to the adequate intensity of the stress factor assayed, one must consider diverse fermentation set-ups (plates, shake-flasks,

batches, continuous cultivations) in order to compare thoroughly the physiology of yeast strains under stress conditions.

Finally, some questions regarding the topic of this thesis remain open and can be addressed in future projects. For example, it would be interesting to compare gene expression and enzyme activity data from strain PE-2 to those from strain CEN.PK113-7D, under chemostat conditions, with the aim of clarifying the differences found in acetate metabolism between these strains. Further studies may also elucidate the factors responsible for the hypersensitivity of strain JP1 towards oxidative stress. Competition assays with strains PE-2 and Fleischmann, coupled to the collection of genome data (for tracking strain population) and transcriptome data (for tracking gene expression), could expand the knowledge on cell population dynamics in the industrial fermentors. Finally, transcriptome and enzyme activity data from rich medium cultivations at low pH might also explain the large differences in physiology exhibited by strains PE-2 and CEN.PK113-7D in this stressful condition.

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APPENDIX A – SUPPLEMENTARY MATERIALS AND METHODS

A.1 TOTAL REDUCING SUGAR (TRS) DETERMINATION

Since sucrose, a non-reducing sugar, is the major sugar found in sugarcane juice and molasses, an acid hydrolysis was conducted in order to obtain quantifiable reducing sugars, as in Bassi (2011). First, sterilized sugarcane juice and molasses were diluted from 1:100 to 1:5000 and 1-mL aliquots of each solution (sample) were placed in 100-mL volumetric flasks, together with 30 mL of distilled water and 2.5 mL of concentrated HCl. The solutions were homogenized and placed in a water bath at 65 °C for 15 minutes, and then cooled down under running water. Samples were neutralized with the addition of 2.8 mL of 12 N NaOH and distilled water to the flask mark.

Before TRS determination, a DNS solution (MILLER, 1959) was prepared by slowly dissolving 1 g of 3,5-dinitrosalicylic acid in 20 mL of 2 N NaOH and 50 mL of distilled water. The solution was heated up to 40 °C to facilitate DNS dissolution. After complete dissolution, 30 g of potassium sodium tartrate (Rochelle salt; $KNaC_4H_4O_6.4H_2O$) were added and the volume was completed to 100 mL with distilled water. The solution was kept in an amber flask wrapped in aluminum foil.

TRS determination (BASSI, 2011) was conducted in test tubes containing 1 mL of sample, 1 mL of DNS solution and 1 mL of distilled water, which were placed in a boiling water bath for 5 minutes and cooled down under running water. Then, 8 mL of distilled water were added and the solution was homogenized for 5 s on a vortex-mixer. Sample absorbance was measured at 540 nm. A blank was prepared by replacing the sample with distilled water, and a standard calibration curve was prepared using glucose solutions with concentrations varying from 0.25 to 2 g L^{-1}; this yielded a linear curve used to correlate absorbance to TRS concentration.

A.2 PREPARATION OF STRESS PLATES AND CELL SUSPENSION DILUTIONS

Stress plates used in dilution spot assays contained 25 mL of solid medium and were prepared as follows. Saline stress plates were prepared by using 25 mL of solid YPD medium supplemented with NaCl before autoclaving. Oxidative stress plates were prepared by autoclaving concentrated solid YPD medium and then adding aliquots of a diluted 1 M H_2O_2 solution (in sterile distilled water) in a way that the final volume was 25 mL. This was done with the aid of a 0.22 µm-pore syringe filter when the medium temperature was low enough that the medium could be poured. High glucose or sucrose concentration plates were prepared the same way as oxidative stress plates, but using concentrated glucose or sucrose solutions instead. Ethanol stress plates were prepared similarly, except that ethanol was added after autoclaving with the aid of sterile pipettes.

Low pH stress plates and plates containing acetic acid required adjustments of medium pH. Test plates were done by autoclaving solid YPD medium and measuring its pH using a pHmeter and a temperature probe when the medium was ready to be poured. Then, the required volumes of a 1 M H_2SO_4 solution (for low pH stress plates) or of a combination of glacial acetic acid and a 4 M KOH solution (for acetic acid stress plates) were added to the medium in order to reach the desired pH values. Once these volumes were known, the actual plates were prepared by autoclaving solid YPD medium and then adding the same volumes of autoclaved H_2SO_4 and KOH solutions and/or glacial acetic acid (using a sterile pipette).

For preparing stress plates containing industrial media, minor adjustments were made. To prepare one sugarcane juice plate (with 162 g L^{-1} TRS), 0.3 g of agar was dissolved in 6 mL of distilled water, autoclaved, and supplemented with 24 mL of sterile sugarcane juice, resulting in 30 mL of solid medium (see item 2.3.2 in Chapter 2 for sugarcane juice sterilization procedures). A lower agar concentration was required in order to limit the sugarcane juice dilution caused by the addition of dissolved agar, since sugarcane juice could not be autoclaved. To prepare one sugarcane molasses plate (with 200 g L^{-1} TRS), 0.5 g of agar was dissolved in 17.36 mL of distilled water, autoclaved, and supplemented with 10.18 g of autoclaved molasses, resulting in 25 mL of solid medium. For the preparation of one plate containing both sugarcane juice and molasses (with 200 g L^{-1} TRS in a 1:1 sugar proportion), 0.5 g of agar was dissolved in 8.66 mL of distilled water, autoclaved, and

supplemented with 12.18 mL of sterilized sugarcane juice and 5.09 g of autoclaved sugarcane molasses, resulting also in 25 mL of solid medium.

Diluted cell suspensions were prepared using cells from exponential growth phase. First, the culture had its absorbance measured at 600 nm. The required culture volume to produce a 1-mL suspension with absorbance of 0.1 was placed in an Eppendorf tube, centrifuged, washed in sterile distilled water twice and resuspended in 1 mL. This was considered the first dilution (10^{-1}), from which four more dilutions were prepared (10^{-2} to 10^{-5}) using sterile distilled water.

A.3 SHAKE-FLASK SAMPLING DEVICE

Sampling from shake-flasks was optimized by the use of the device pictured in Figure 13. Two needles were connected by a piece of silicon tubing, which was kept closed by a clamp to avoid contamination. During sampling, a syringe (not shown) was connected to the outside needle and the clamp was held open, allowing the culture to be quickly collected by the syringe. After discarding a small volume of culture, approximately 3 mL were used as the real sample. This device allowed the shake-flasks to be sampled without removing them from the rotary shaker.

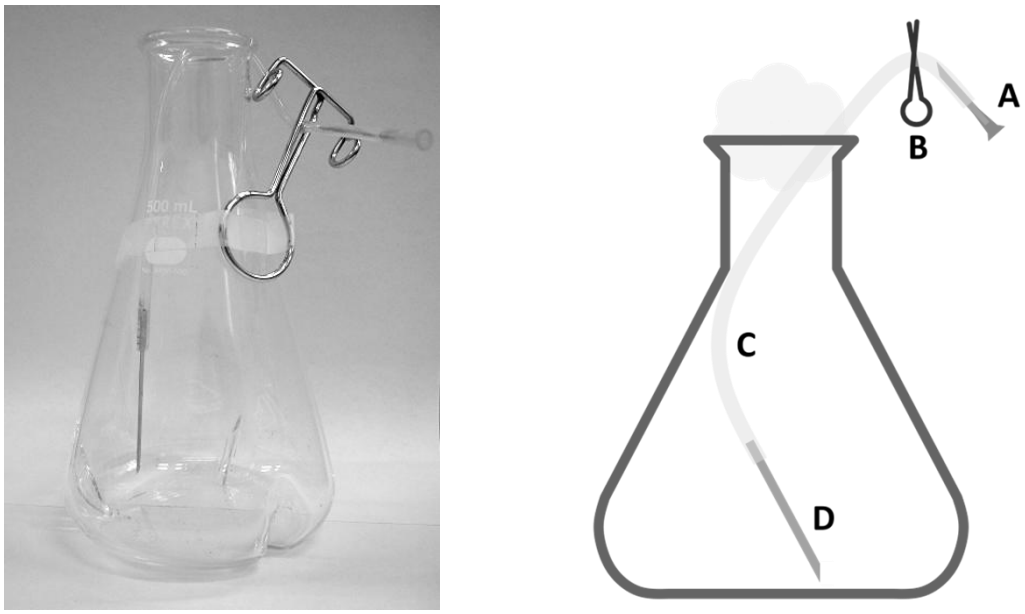

Figure 13 – Device used to sample shake-flasks. A: outside needle; B: tubing clamp; C: silicon tubing; D: inside needle.

A.4 STATISTICAL COMPARISONS

Statistical comparisons of physiological parameters were performed between different cultivation conditions and between different strains using GraphPad software. In this thesis, linear regression slopes were compared first by adjusting linear regression models and choosing the Compare option in the software, which evaluates whether slopes and intercepts are significantly different. This option performs an F-test and yields a P-value, which is compared to the significance level used in this thesis (0.05). Another method used to compare slopes statistically is to perform a t-test that can be implemented in Microsoft Excel software, as described next.

First, one linear regression ($y = ax + b$) is fitted to all the data obtained from replicates of the same experiment using the least-squares method. After this, the slope a and the intercept b of the regression line are known. Then the standard error of the slope SE can be calculated using eq.(3):

$$SE = \frac{\sqrt{\frac{\sum(y_i - \hat{y}_i)^2}{n-2}}}{\sqrt{\sum(x_i - \bar{x})^2}} \quad (3)$$

where y_i is the value of the dependent variable for observation *i*, $\hat{y}_i$ is the estimated value of the dependent variable for observation *i*, *n* is the number of observations, x_i is the value of the independent variable for observation *i* and $\bar{x}$ is the mean of the independent variable (STATTREK, 2013).

After the slopes and the standard errors of the slopes from conditions (or yeast strains) 1 and 2 are known, two hypotheses can be stated: the null hypothesis (H_0: $a_1 = a_2$) and the alternative hypothesis (H_1: $a_1 \neq a_2$), which will be tested with a significant level α of 0.05 using a two-tailed t-test. The t-score statistic is defined by eq.(4) and the degrees of freedom *DF* by eq.(5):

$$t = \frac{|a_1 - a_2|}{\sqrt{SE_1^2 + SE_2^2}} \quad (4)$$

$$DF = n_1 + n_2 - 4 \quad (5)$$

Using these values above, the P-value can be determined using a t-distribution calculator (or TDIST function in Microsoft Excel). If the P-value is less than the significance level, the null hypothesis can be rejected and the slopes are significantly different.

A.5 DESCRIPTION OF THE BIOREACTOR CULTIVATION SET-UP

The bioreactor set-up used in this thesis is shown in Figures 14 to 16 and described in detail next. Medium vessels (A) consisted of 20-L glass carboys (Schott Duran, Germany), covered with black plastic in order to protect light-sensitive compounds present in the

medium. These vessels were located near the floor level and placed on magnetic stirrer plates. Plastic carboys were used as effluent reservoirs (B) and located behind the medium vessels. Medium was pumped from the vessels to the fermentor by a Masterflex (Cole-Parmer, Vernon Hills, USA) peristaltic pump connected to a Masterflex console drive 77521-57 (C), and pumped out of the fermentor to the effluent reservoirs by a similar pumping system (D). A sampling flask (E) was placed in the effluent line before the effluent pump.

The fermentor (F) consisted of a single-wall glass vessel with an upper stainless steel lid. It was equipped with 2 Rushton 6-blade impellers attached to the agitation shaft, an L-type gas sparger and 4 metal baffles. The agitation shaft was powered by an Applikon P100 motor and controlled by an Applikon stirrer controller ADI 1032 (G). Gas inflow was controlled by a Brooks Instruments (Hatfield, USA) Thermal mass flow controller 5850S and a Brooks Read Out & Control unit 0154 (H). Outflowing gas left the fermentor through a condenser (I), which was kept at low temperatures by a Lauda circulation chiller WK300 (J).

The upper lid contained ports for one pH electrode (Mettler Toledo, Columbus, USA), one dissolved O_2 electrode (Mettler Toledo), both connected to an Applikon Biocontroller unit ADI1030 (K), and a thermowell filled with water. The sensor placed in the thermowell was connected to a Lauda (Lauda-Königshofen, Germany) cooling thermostat RE307 (L), which in turn was connected to the fermentor's internal heat exchanger. Attached to the lid there was also supply ports and an electric level sensor, which was coupled to the effluent pump drive. Acid and base (M) were added to the fermentor by Masterflex peristaltic pumps (N), which were controlled by the Applikon Biocontroller unit.

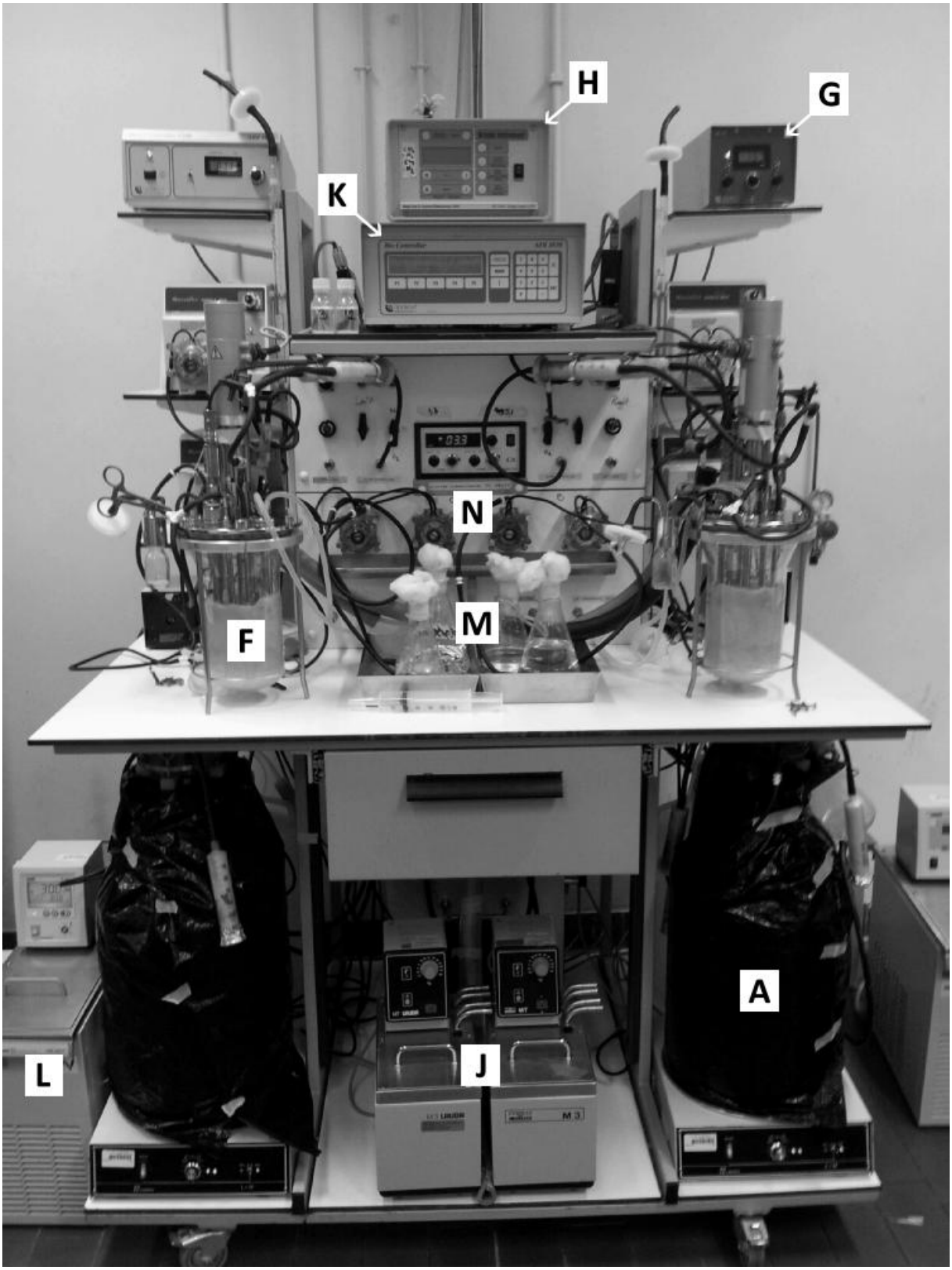

Figure 14 – General view of two running fermentors.

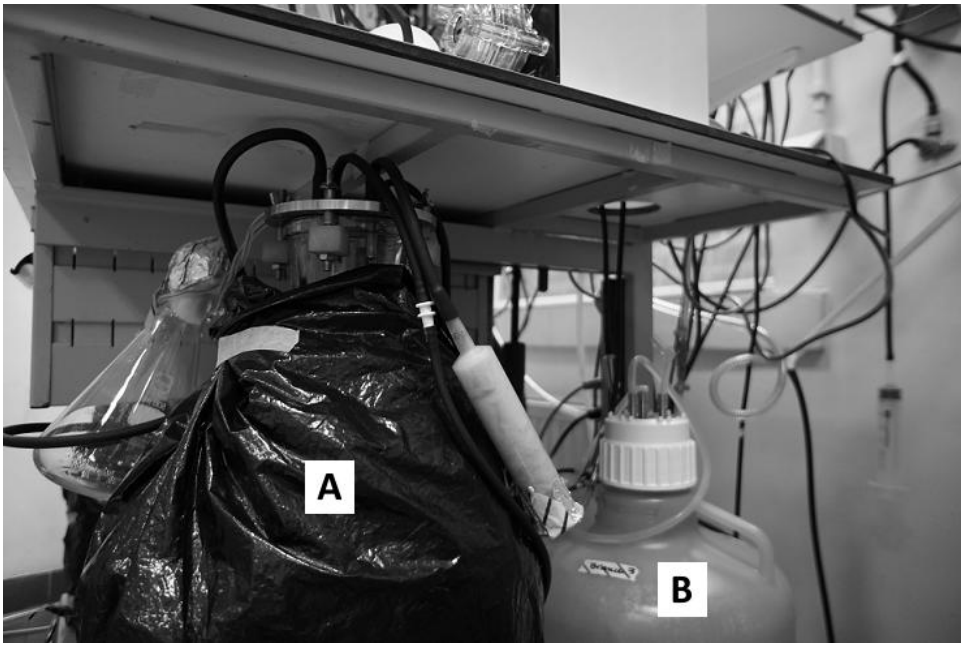

Figure 15 – Medium vessel and effluent reservoir placed close to the floor level.

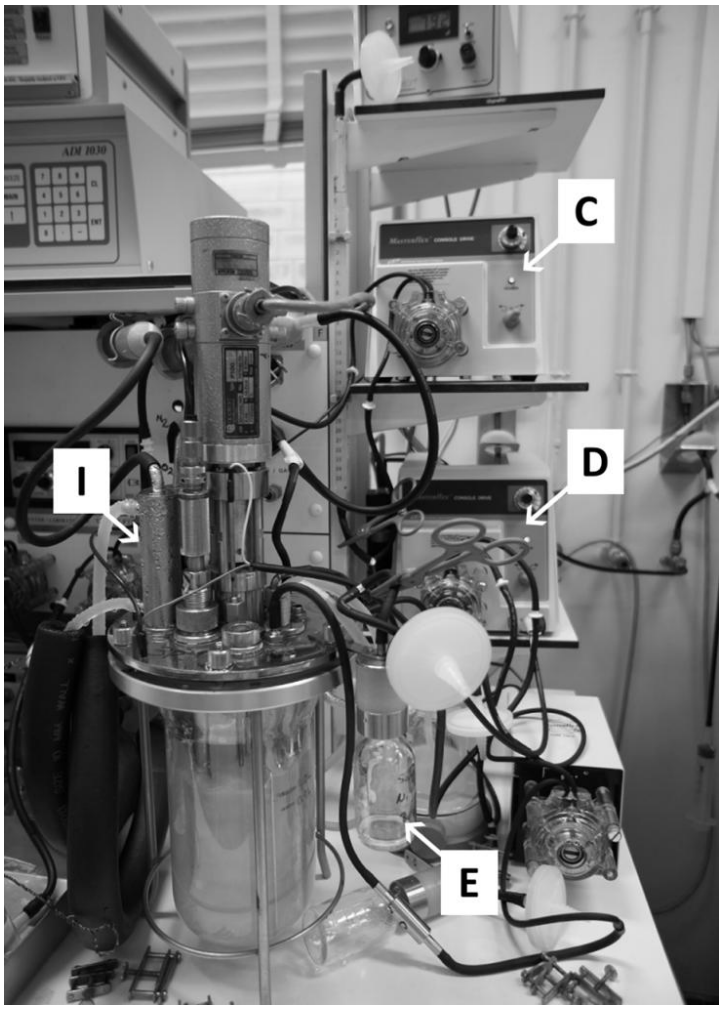

Figure 16 – Detail of one fermentor and pumps.

A.6 MASS BALANCE EQUATIONS

The following equations (eq.(6) to eq.(8)) were used to calculate instantaneous specific growth rates (μ) and specific rates of substrate consumption (q_S) and of product formation (q_P) during dynamic continuous cultivations:

$$\mu = \frac{1}{X} * \left(\frac{dX}{dt} + D * X\right) \quad (6)$$

$$q_S = \frac{1}{X} * \left[\frac{dS}{dt} - (S_F - S) * D\right] \quad (7)$$

$$q_P = \frac{1}{X} * \left[\frac{dP}{dt} - (P_F - P) * D\right] \quad (8)$$

where X, S and P are biomass, substrate and product concentrations in the fermentor, respectively, S_F and P_F are substrate and product concentrations in the medium feed, respectively, and D is the dilution rate. The derivatives dX/dt, dS/dt and dP/dt were calculated by the analytical geometry-based method described by Leduy and Zajic (1973).

A.7 ETHANOL EVAPORATION CORRECTION

Since there is ethanol evaporation with the off-gas in sparged bioreactors, the ethanol concentration values obtained from culture supernatants were corrected by an ethanol evaporation constant k equal to 0.0080 ± 0.0002 h^{-1}. This constant was determined by Bianca van Leeuwen and Eline Huisjes of the IMB group/TU Delft, under standard conditions of temperature (30 °C), culture volume (1 L), gas flow rate (0.5 vvm) and agitation frequency (800 rpm), and its value can be used for dilution rates ranging from 0.03 to 0.2 h^{-1}. In a chemostat, the specific ethanol formation rate q_E can then be given by eq.(9):

$$q_{\mathrm{E}} = -\frac{D}{X} * (E_F - E) + \frac{k}{X} * E \ (9)$$

where E_F and E are the ethanol concentrations in the medium feed and in the fermentor, respectively.

A.8 CELL VIABILITY CURVES

For viability measurements of post-diauxic cells, 1 mL of stock culture was added to a 500-mL shake-flask containing 100 mL of YPD medium and cultivated overnight at 30 °C and 200 rpm. Next 80 mL of culture were centrifuged in two tubes containing 40 mL each, at 4000 rpm for 5 minutes. The supernatant was discarded and cells were resuspended by adding 25 mL of sterile distilled water in each tube and gently using a vortex-mixer. Contents from both tubes were placed in a small beaker, which was previously cleaned using 70 % alcohol. For viability measurements of exponentially growing cells, the same procedure was used until the centrifugation step. After centrifugation, cells were resuspended in 5 mL instead of 25 mL. Contents from both tubes were then inoculated in another 500-mL shake-flask containing 100 mL of YPD medium and cultivated at 30 °C and 200 rpm for 3 h. After the cultivation, cells were collected and resuspended as described above for post-diauxic cells.

The beaker containing the cell suspension was placed in a magnetic stirring plate and stirred at a slow speed. The heat generated from the stirrer plate was enough to maintain the suspension temperature around 30 °C during the experiment. Before pH adjustment, the pH of the cell suspension was close to 5.3 and a time-zero sample (1 mL) was taken. Next, pH was adjusted to 1.5 using a 2 M H_2SO_4 solution and samples were taken every hour for 4 h. A pHmeter was kept in contact with the suspension during all the experiment and additional pH correction was seldom required.

Samples were immediately stained using a 0.4 % trypan blue solution (Sigma) in a 1:1 (v/v) proportion for 5 minutes and counted using an improved Neubauer chamber (Figure 17). For this, the stained cell suspension was diluted so that every counted square contained from 20 to 50 cells. Cell viability was calculated by the average viable (non-stained) cell count per square divided by the average number of cells (stained plus non-stained) per square. The cell concentration in the sample (eq.(10)) is calculated using the average cell count per square, the square volume and the dilution factor.

*cells/mL = average cell count per square * square volume * dilution factor* (10)

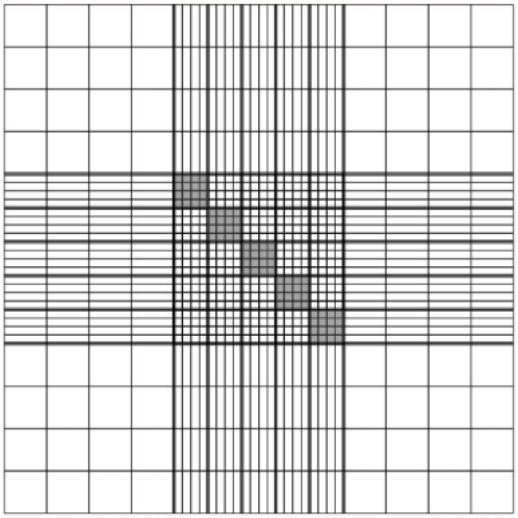

Figure 17 – Improved Neubauer chamber. The squares shown in gray (side length of 0.2 mm and depth of 0.1 mm) were counted on both sides of the chamber.

APPENDIX B – SUPPLEMENTARY RESULTS

B.1 SERIAL DILUTION SPOTTING ON STRESS PLATES

All serial dilution spotting experiments performed on stress plates are shown in Figures 18 to 24.

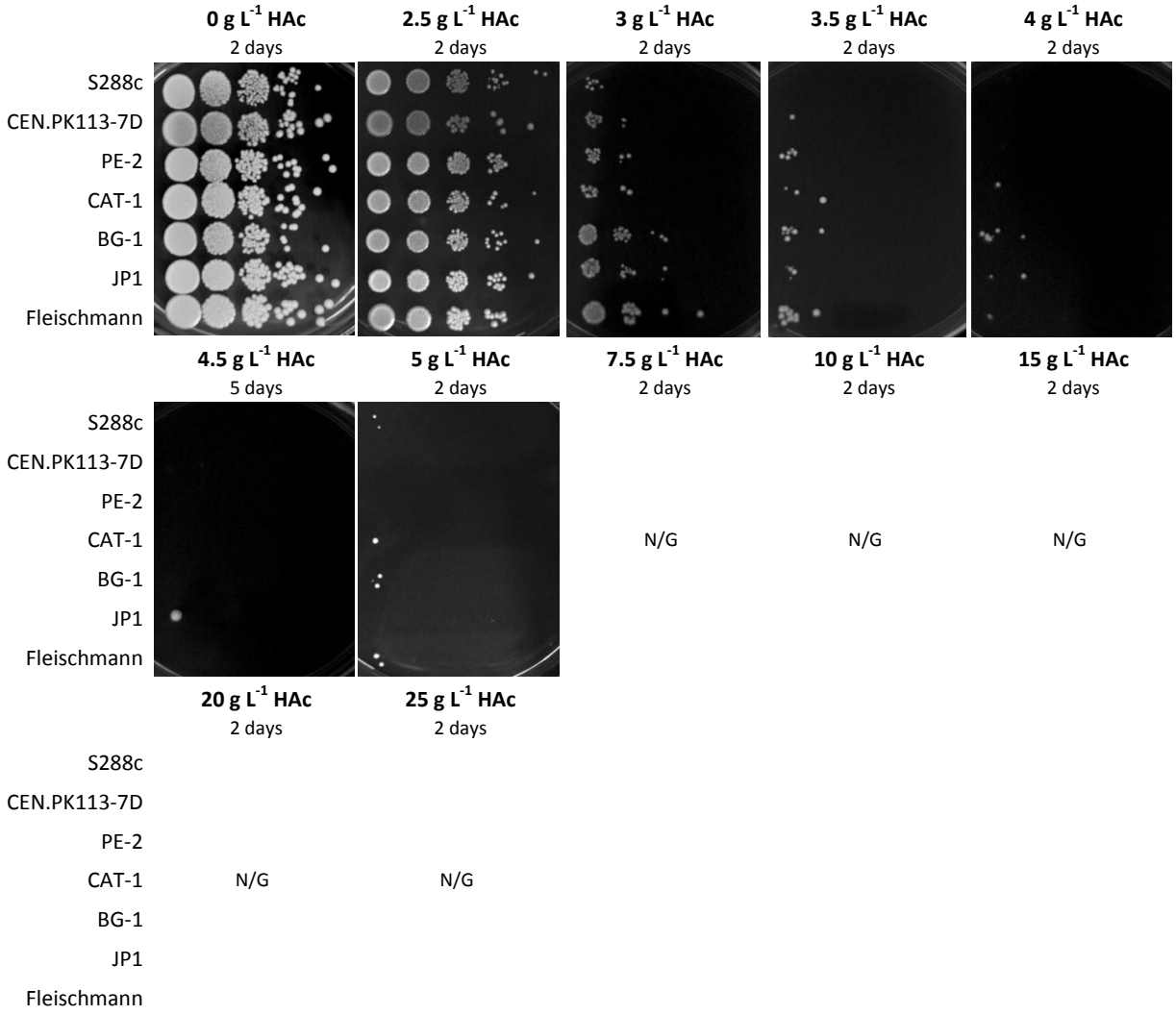

Figure 18 – Cells growing exponentially in liquid YPD medium were transferred onto plates with solid YPD medium and different acetic acid (HAc) concentrations, as indicated. Lines represent different strains, and columns, dilutions ranging from Abs_{600} 10^{-1} to 10^{-5}. Pictures were taken after the number of days described for each plate. N/G: no growth.

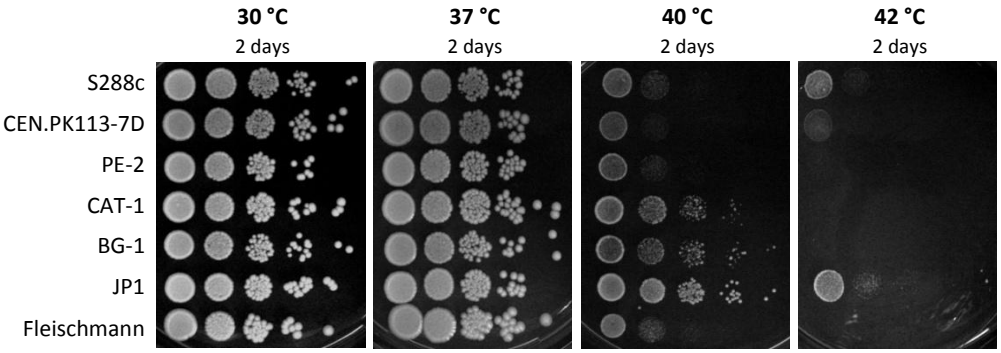

Figure 19 – Cells growing exponentially in liquid YPD medium were transferred onto plates with solid YPD medium and incubated at different temperatures. Lines represent different strains, and columns, dilutions ranging from Abs_{600} 10^{-1} to 10^{-5}. Pictures were taken after the number of days described for each plate.

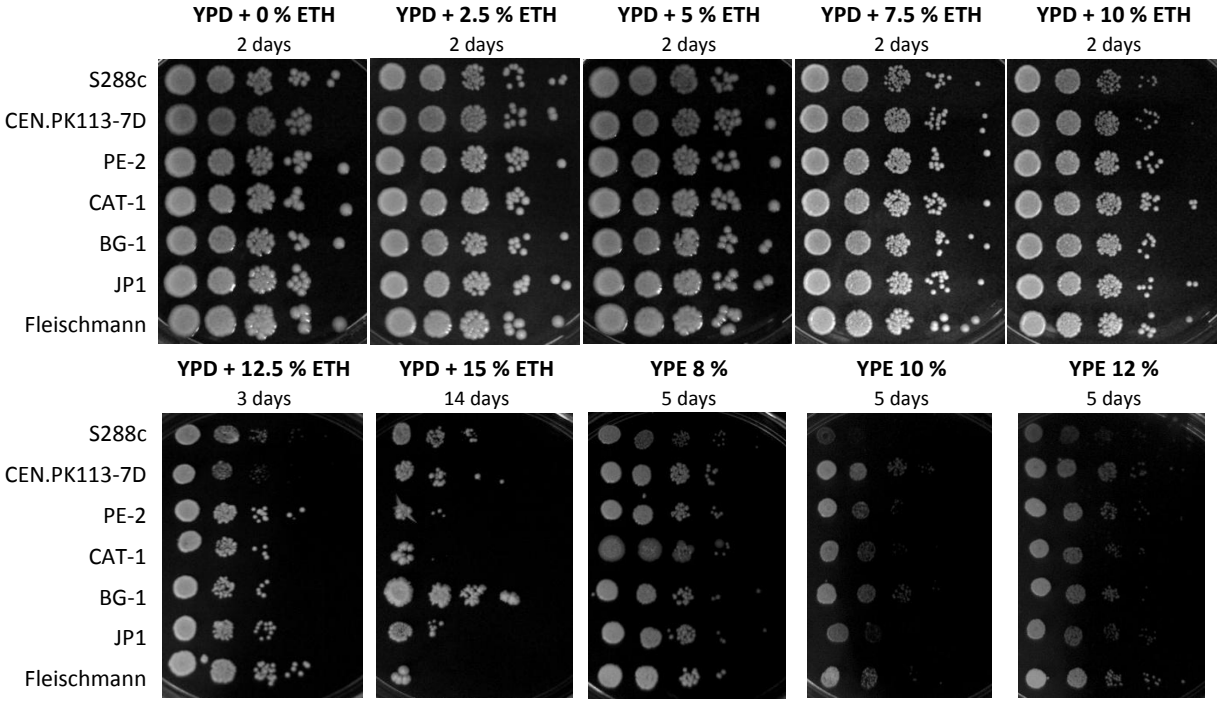

Figure 20 – Cells growing exponentially in liquid YPD medium were transferred onto plates with solid YPD or YP medium and different ethanol (ETH; E) concentrations, as indicated. Lines represent different strains, and columns, dilutions ranging from Abs_{600} 10^{-1} to 10^{-5}. Pictures were taken after the number of days described for each plate.

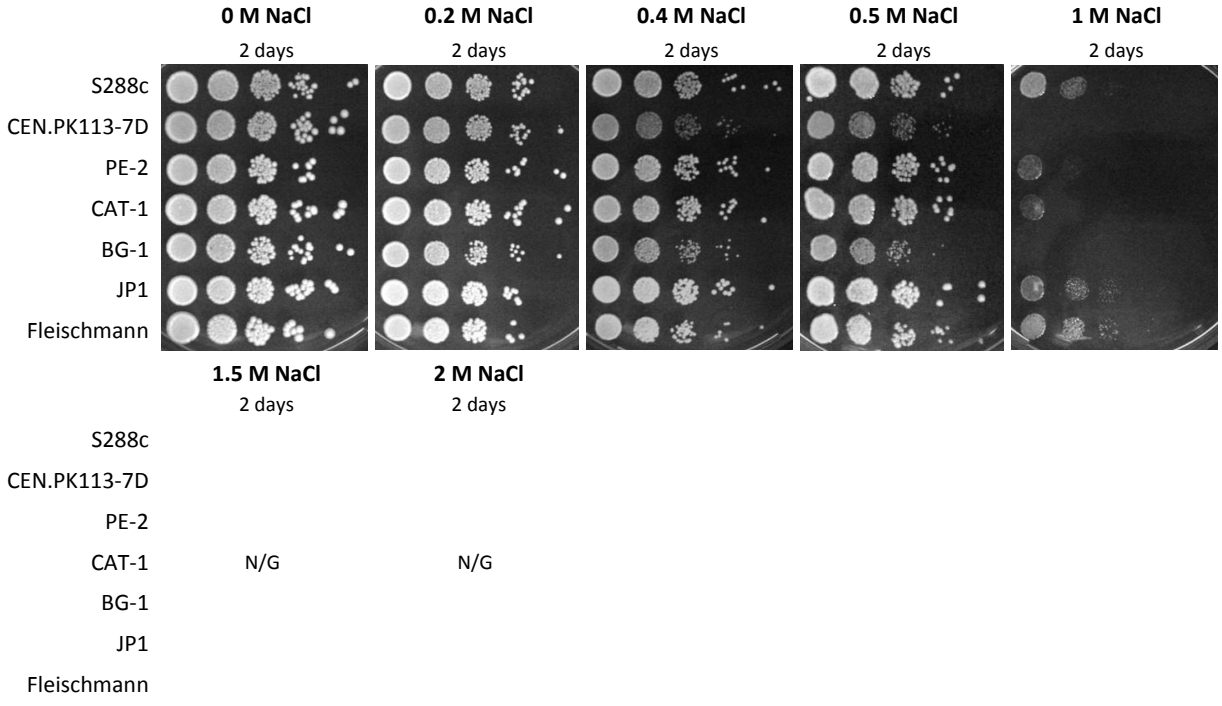

Figure 21 – Cells growing exponentially in liquid YPD medium were transferred onto plates with solid YPD medium and different NaCl concentrations, as indicated. Lines represent different strains, and columns, dilutions ranging from Abs_{600} 10^{-1} to 10^{-5}. Pictures were taken after the number of days described for each plate. N/G: no growth.

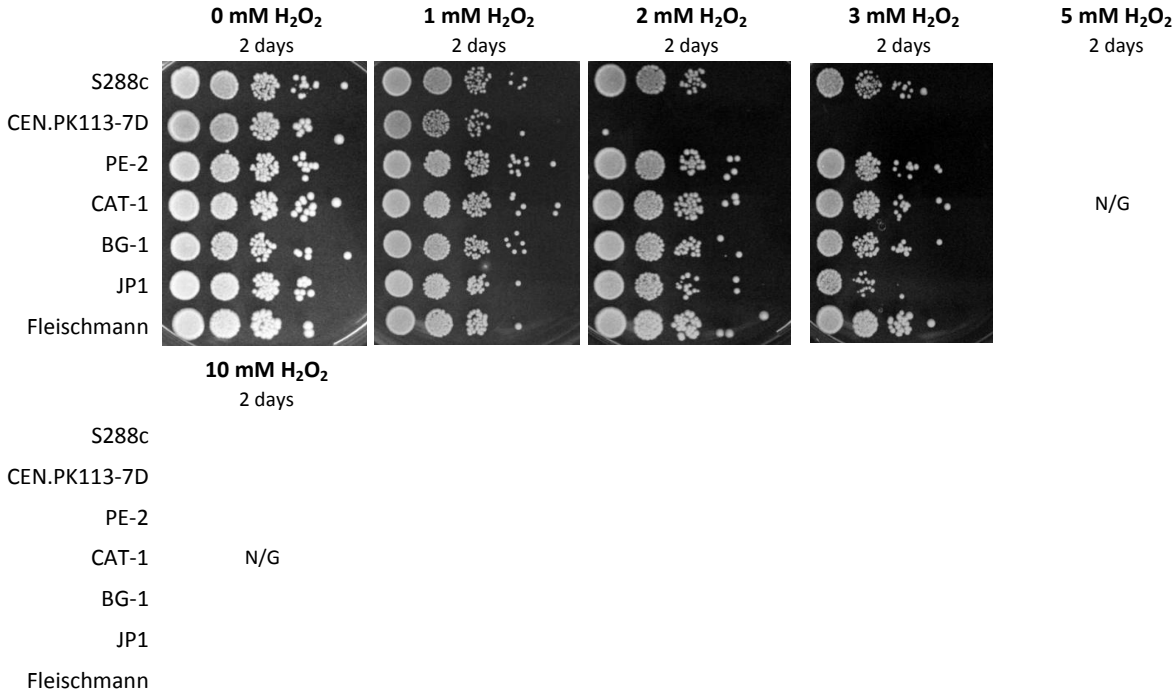

Figure 22 – Cells growing exponentially in liquid YPD medium were transferred onto plates with solid YPD medium and different H_2O_2 concentrations, as indicated. Lines represent different strains, and columns, dilutions ranging from Abs_{600} 10^{-1} to 10^{-5}. Pictures were taken after the number of days described for each plate. N/G: no growth.

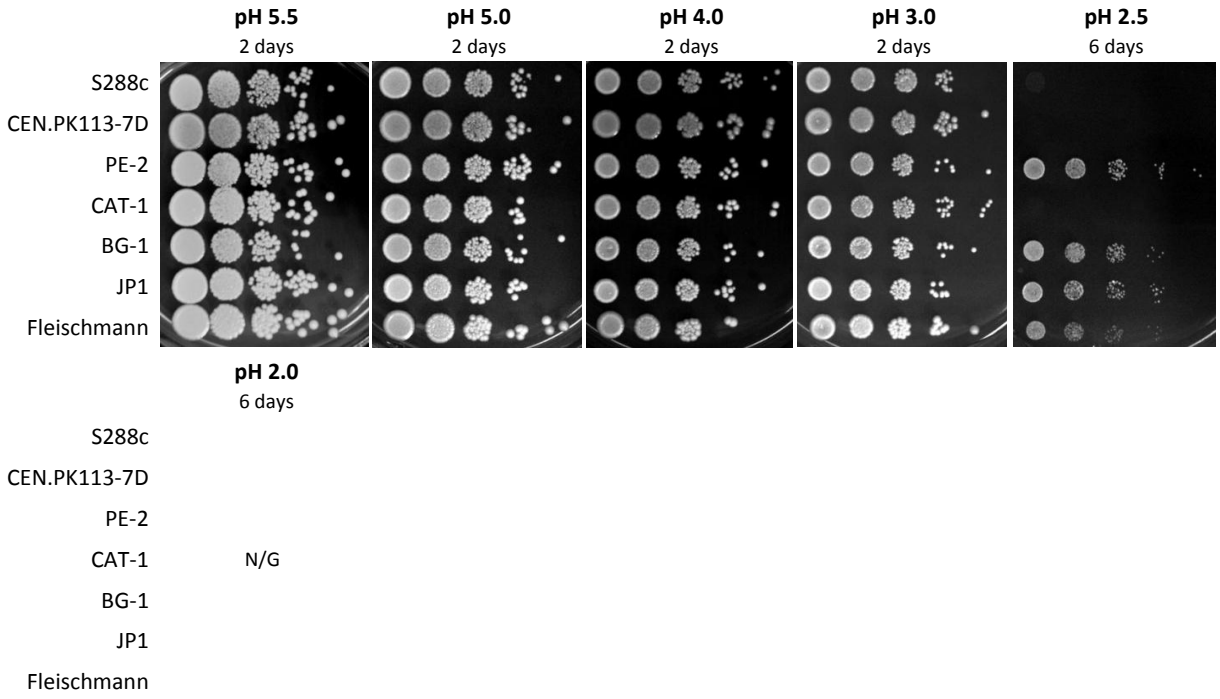

Figure 23 – Cells growing exponentially in liquid YPD medium were transferred onto plates with solid YPD medium at different pH values, as indicated. Lines represent different strains, and columns, dilutions ranging from Abs_{600} 10^{-1} to 10^{-5}. Pictures were taken after the number of days described for each plate. N/G: no growth.

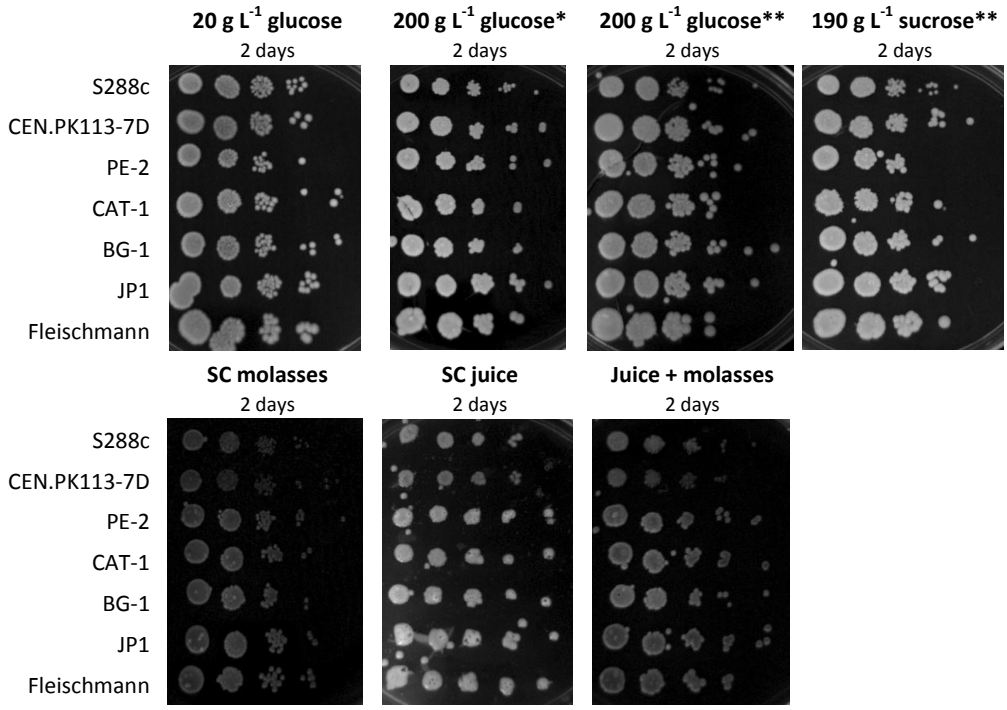

Figure 24 – Cells growing exponentially in liquid YPD medium were transferred onto plates with solid YPD medium and different sugar concentrations, or with solid industrial medium, as indicated. Lines represent different strains, and columns, dilutions ranging from Abs_{600} 10^{-1} to 10^{-5}. Pictures were taken after the number of days described for each plate. Sugars were either autoclaved (*) or filter-sterilized (**).

B.2 GROWTH PROFILES OF INDUSTRIAL STRAINS DURING SHAKE-FLASK CULTIVATIONS ON SYNTHETIC MEDIUM

During shake-flask cultivations on synthetic medium at 30 °C and at 37 °C, all strains tested (S288c, CEN.PK113-7D, PE-2, CAT-1, BG-1, JP1 and Fleischmann) presented similar profiles of cell growth, metabolite formation and substrate consumption; representative profiles are shown in Fig. 25.

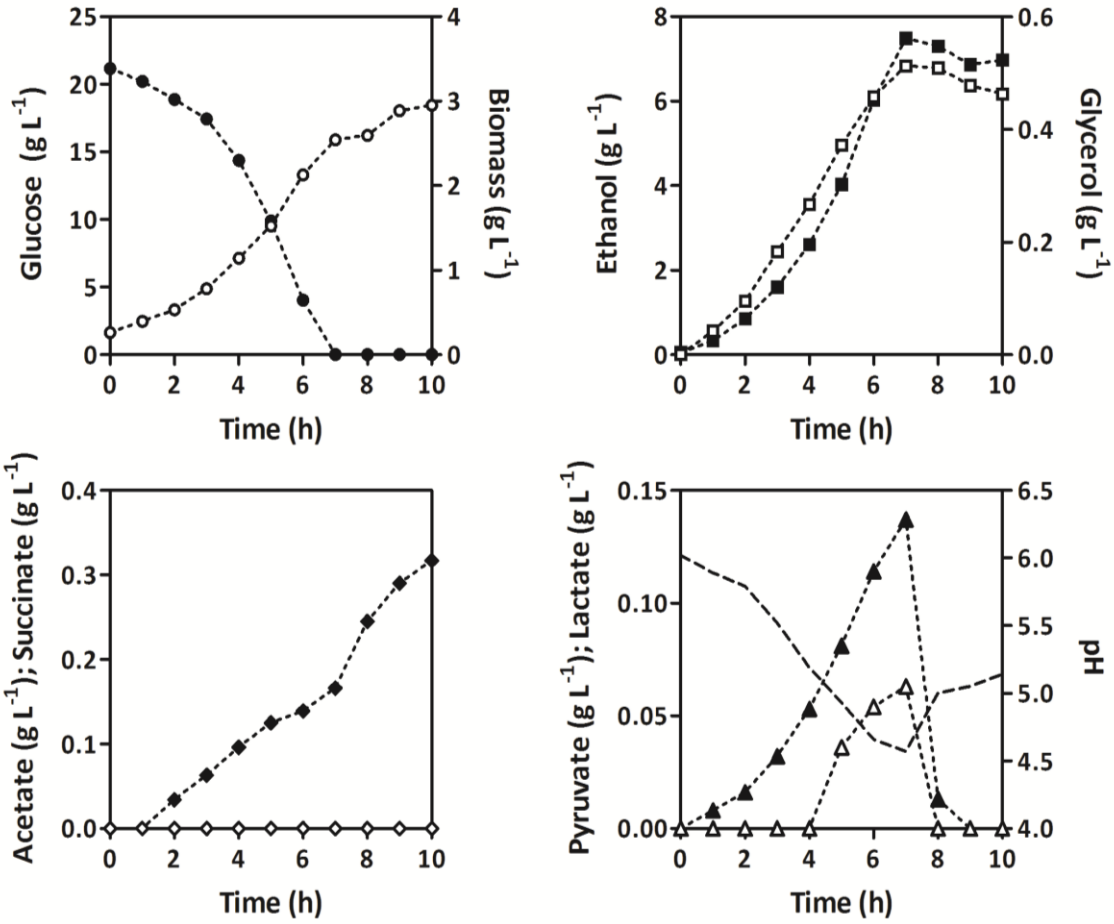

Figure 25 – Profiles of glucose consumption (●), biomass generation (○), formation of ethanol (■), glycerol (□), acetate (◆), succinate (◇), pyruvate (▲) and lactate (△), and pH values (--) from a shake-flask cultivation using strain CEN.PK113-7D at 30 °C (data from one replicate).