THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Tools and applications to assess yeast physiology and robustness in bioprocesses

Lab-scale methods from single cells to populations

LUCA TORELLO PIANALE

Department of Life Sciences
CHALMERS UNIVERSITY OF TECHNOLOGY

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LUCA TORELLO PIANALE
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Division of Industrial Biotechnology
Department of Life Sciences
Chalmers University of Technology
SE-412 96 Gothenburg
Sweden
Telephone + 46 (0)31-772 1000

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Artwork by: Luca Torello Pianale

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** THWOORP **

– Katya

Preface

This dissertation partially fulfils the requirements for obtaining the degree of Doctor of Philosophy at the Department of Life Sciences at Chalmers University of Technology. The work was supported by the Novo Nordisk Foundation grant Distinguished Investigator 2019 - Research within biotechnology-based synthesis & production (grant # 0055044) awarded to Prof. Lisbeth Olsson. The PhD studies were carried out between November 2019 and February 2024 under the supervision of Prof. Lisbeth Olsson and the co-supervision of PhD Peter Rugbjerg. The thesis was examined by Prof. Carl Johan Franzén.

Most of the work in this thesis was carried out at the Division of Industrial Biotechnology at the Department of Life Sciences at Chalmers University of Technology. Microfluidics experiments were conducted at Bielefeld University (Germany) by Luisa Blöbaum (Paper VI) or in collaboration with Luisa Blöbaum during a research visit (unpublished data).

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Luca Torello Pianale
Division of Industrial Biotechnology – Department of Life Sciences
Chalmers University of Technology

Abstract

Bioprocesses enable the efficient production of valuable chemicals by microorganisms such as the yeast *Saccharomyces cerevisiae*. Predictable and stochastic perturbations affect microbial performance in an industrial-scale bioreactor. Because some of these complex and dynamic perturbations are difficult to mimic at a small scale, strains selected and developed in the lab might underperform in industrial settings, creating challenges during scale-up. Moreover, the ability of a system to maintain a stable performance, defined as microbial robustness, has been overlooked owing to a scarcity of suitable quantification methods.

This thesis describes novel approaches for characterising industrially relevant microorganisms at laboratory scale. The developed methods and techniques were applied to one laboratory and two industrial yeast strains predominantly in the context of second-generation biofuel production. Yeast physiology was explored by both canonical methods and real-time monitoring of eight intracellular parameters using the *Sc*EnSor Kit. To complement physiology, the concept of robustness was explained and elaborated. A recently formulated method for quantifying robustness was applied to physiological data to determine the stability of cell performance and expand the concept of robustness itself. Lastly, the physiology and robustness of yeast cells exposed to rapid feast-starvation oscillations were investigated using dynamic microfluidics single-cell cultivation. This technique proved instrumental in mimicking, at a laboratory scale, the fast dynamics encountered within large-scale bioreactors.

In summary, the tools presented in this thesis address some of the challenges associated with the scaling up of bioprocesses. Owing to the multilevel resolution, ranging from populations to single cells, the developed techniques have the potential to advance our understanding of microbial performance and robustness, ensuring more efficient and reliable industrial applications of engineered microorganisms.

Keywords: Scale-up, scale-down, bioproduction, *Saccharomyces cerevisiae*, fluorescence, biosensors, microfluidics

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Torello Pianale, L.**, Rugbjerg, P., & Olsson, L. (2022). Real-Time Monitoring of the Yeast Intracellular State During Bioprocesses With a Toolbox of Biosensors. *Frontiers in microbiology*, *12*, 802169. doi.org/10.3389/fmicb.2021.802169

II. **Torello Pianale, L.**, & Olsson, L. (2023). *Sc*EnSor Kit for *Saccharomyces cerevisiae* Engineering and Biosensor-Driven Investigation of the Intracellular Environment. *ACS Synth. Biol.*, *12*(8), 2493–2497. doi.org/10.1021/acssynbio.3c00124

III. Olsson*, L., Rugbjerg*, P., **Torello Pianale*, L.**, & Trivellin*, C. (2022). Robustness: linking strain design to viable bioprocesses. *Trends in biotechnology*, *40*(8), 918–931. doi.org/10.1016/j.tibtech.2022.01.004

IV. **Torello Pianale, L.**, Caputo, F., & Olsson, L. (2023). Four ways of implementing robustness quantification in strain characterisation. *Biotechnol Biofuels Bioprod.*, *16*, 195. doi.org/10.1186/s13068-023-02445-6

V. Trivellin, C., **Torello Pianale, L.**, & Olsson, L. (2023). Robustness quantification of a mutant library screen revealed key genetic markers in yeast. *Submitted*.

VI. Blöbaum*, L., **Torello Pianale*, L.**, Olsson, L., & Grünberger, A. (2023). Microfluidic assessment of microbial robustness in dynamic environments from single-cell to population level. *Submitted to Microbial Cell Factories*. Pre-print DOI: doi.org/10.21203/rs.3.rs-3644873/v1

*The authors contributed equally

Contribution Summary

I. I planned and performed the experimental work. I analysed the data, interpreted the results, and wrote the manuscript. I edited the manuscript together with other authors.

II. I planned and performed the experimental work. I analysed the data, interpreted the results, and wrote the manuscript. I edited the manuscript together with other authors.

III. I contributed to group discussions to conceive concepts. All authors shared the writing and editing of the manuscript.

IV. I planned and performed the experimental work, except the material analysis, which was carried by FC. I analysed the data, interpreted the results, and wrote the manuscript. I edited the manuscript together with other authors.

V. I designed and performed the construction of the deletion strains. I helped CT with data interpretation after she performed the screenings. Along with the other authors, I provided feedback while CT was writing the manuscript.

VI. I conceived the study together with LB. I planned the experiments with LB. I focused on the data processing pipeline, while the experimental work was performed by LB. I performed the data analysis, interpretation of the results and manuscript writing together with LB. I edited the manuscript together with other authors.

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Abbreviations

1G	First generation
2G	Second generation
ART-FCM	Automated real-time flow cytometry
BiH	Birch hydrolysate
cpFP	Circularly permuted fluorescent protein
CSH	Corn stover hydrolysate
dMSCC	Dynamic microfluidics single-cell cultivation
EthPro	Ethanol consumption probe (biosensor)
FBP	Fructose-bis-phosphate
FP	Fluorescent protein
GlyOx	Glycolytic flux and oxidative stress biosensor
GlyRNA	Aptameric glycolytic flux biosensor
HGSH	High gravity spruce hydrolysate
HMF	Hydroxymethylfurfural
LH	Lignocellulosic hydrolysate
LPB	Lignocellulosic plant biomass
OHH	Oat hulls hydrolysate
OxPro	Oxidative stress response probe (biosensor)
OxSR	Oxidative stress response
PyruPro	Pyruvate metabolism probe (biosensor)
PyruEth	Pyruvate metabolism and ethanol consumption probe (biosensor)
R(c)	Robustness across conditions
R(p)	Robustness across populations
R(s)	Robustness across systems
R(t)	Robustness over time
RibPro	Ribosome abundance probe (biosensor)
RibUPR	Ribosome abundance and UPR probe (biosensor)
SBH	Sugarcane bagasse hydrolysate

*Sc*EnSor	*Saccharomyces cerevisiae* engineering + biosensor
SCH	Synthetic corn hydrolysate
SLRH	Softwood logging residues hydrolysate
SSH	Synthetic spruce hydrolysate
SWSH	Synthetic wheat straw hydrolysate
TRY	Titres, rates, yields
TU	Transcriptional unit
UPR	Unfolded protein response
UPRpro	Unfolded protein response probe (biosensor)
WSH	Wheat straw hydrolysate

Chapter 1. Introduction

The introductory chapter gives an overview of bioprocesses and the challenges associated with them, as well as a description of *Saccharomyces cerevisiae* as a production host. It ends with a statement and description of the research questions and aims of the thesis.

1.1. Bioprocesses and *Saccharomyces cerevisiae* as a cell factory

Bioprocesses are a cornerstone of modern biotechnology, in which substrates, such as rich media or industrial side streams, are converted into valuable products using living organisms, including bacteria, yeast, filamentous fungi or mammalian cells. The products encompass primary and secondary metabolites, biomass, and macromolecules. The production of primary metabolites is coupled with generation of energy (ATP), such as with ethanol production during yeast fermentation. Instead, the production of secondary metabolites, biomass, and macromolecules requires energy. Bacteria, yeast, and filamentous fungi are the most used production hosts. However, the production host choice depends on the desired product and/or conditions required by the bioprocess (Table 1) [1–3].

One of the most versatile production hosts is the yeast *Saccharomyces cerevisiae* [4]. *S. cerevisiae* has been domesticated and used for human purposes for centuries, making it also a well-characterised model organism [5]. Historically, bioprocesses have focused on yeast fermentation for food and beverage production [6]. However, they have branched out to address contemporary challenges and opportunities. These include biofuels as renewable and environmentally friendly energy sources [7] or bio-based production of cutting-edge monoclonal antibodies, proteins, and vaccines for the pharmaceutical industry [8]. Moreover, synthetic biology contributed to tackling sustainability and environmental conservation issues by stimulating advances in bio-based plastics and materials [9]. Bioprocesses are generally performed in either batch (system with constant volume, neither inlet nor outlet), fed-batch (semi-open system, inlet only) or continuous cultures (open system, constant volume, both inlet and outlet). While the first two are more common as they limit the risk of contamination, the latter is preferred as it maximises production, while

shortening times and lowering costs between runs [10,11]. Industrial-scale bioprocesses typically use bioreactors of 1 to 100 m^3 [12]. Large-scale settings inevitably lead to phenomena and dynamics that are difficult to mimic in the laboratory. The sum of predictable and stochastic perturbations will eventually affect microbial performance, making it a challenge to optimise strains for industrial purposes [13].

Table 1. Comparison among different production hosts in bioprocesses.

	Bacteria	**Yeasts**	**Filamentous fungi**	**Mammalian cells**
Growth (μ_{max})	< 2 h^{-1}	< 0.5 h^{-1}	< 0.25 h^{-1}	< 0.04 h^{-1}
Spores	Yes	No	Yes	No
GRAS[1]	Some	Most	Most	No
O_2 demand	Wide variety	Obligate aerobes, facultative anaerobes	Primarily obligate aerobes	Obligate aerobes
Nutrient requirements	Low	Medium	Medium	High
Extremophiles	Common	Uncommon	Uncommon	No
pH range	4.5 – 6.5	3 – 8	2 – 9	7.2 – 7.4
Temperature range (°C)	20 – 45 / 45 – 80	25 – 35	20 – 40 / 40 – 60	35 – 40
Protein PTM[2]	No	Yes, some	Yes, various	Yes, extensive
Downstream process	Challenging (small cells, toxins)	Easy (large cells)	Easy (large cells, secreted proteins)	Challenging (pharmaceutical grade)
Main Industrial applications	Dairy, antibiotics, bioplastics, bioenergy, pollution control	Baking, brewing, biofuels, enzymes, hormones, biomass	Enzymes, organic acids, biomass, food industry, antibiotics, secondary metabolites	Pharmaceuticals, vaccines, therapeutic proteins

[1]Generally recognized as safe; [2]Post-traslational modifications.

1.2. Challenges in bioprocesses

Cell factories are exposed to numerous and complex perturbations, which affect their titres, rates, and yields (TRY metrics) (**Paper III**) [13]. Some perturbations are predictable, such as batch variability [14], the presence of inhibitory compounds in the substrate [15], product inhibition towards the end of the process [16], and suboptimal physicochemical variables (e.g. pH or temperature) [17,18]. Instead, other perturbations are more stochastic, including viability of seed cultures [19], contaminations [20], mutations, ageing [21,22], and even a malfunction of bioreactor control systems (e.g. a sudden stop of aeration). While some predictable perturbations are used for selecting and improving cell factories (e.g. inhibitory compounds or product inhibition), stochastic ones are rarely investigated (Fig. 1.1). Moreover, microorganisms are often selected based on overall performance rather than their ability to withstand different challenges or exhibit a stable performance (a feature often referred to as "robustness"). During the "scale-up process", a microorganism is transferred from the laboratory, where it was developed, selected, or improved, to a much larger industrial environment [23]. By ignoring the stability of microbial performance in the face of key perturbations, the scale-up process is likely to fail because of poor growth at the industrial scale or suboptimal TRYs. Therefore, mimicking industrial conditions at a smaller scale would provide information on microbial physiology in response to large-scale perturbations at an earlier stage of strain development, thereby increasing the chances of a subsequent successful scale-up. However, downscaling the conditions found in bioreactors, especially stochastic perturbations, is challenging. For example, a sudden stop of aeration in the reactor is easily reproducible in a small-scale reactor, but more difficult in high-throughput systems such as 96-well plates or flasks. To simulate ageing and mutations, one would have to ensure that the seed culture is grown for many generations or pre-expose it to mutagenic compounds/conditions, which is difficult to reproduce or compare over time. However, setups, which reveal the effects of stochastic perturbations on strains during the early stages of development, could improve the reliability and reproducibility of bioprocesses when challenged with such perturbations. This approach would prevent and reduce the risk of failed or suboptimal cultivations.

Another important consideration is the insufficient mixing in large-scale cultivations, which leads to gradients within the bioreactor [24,25]. Gas [26], substrates [27], temperature [28], and pH [29] gradients expose cells to different and changing environments, resulting in increased metabolic costs for cells [30], decreased productivity [31], and population heterogeneity [32]. The small time-constant dynamics cells are subjected to are in the order of minutes or even seconds [24]. Attempts to simulate such rapid dynamics *in silico* included a model for the prediction and

estimation of how much time a cell spends in each condition within a 22 m^3 bioreactor, thus giving the different metabolic regimes cells might be subjected to [33]. Nevertheless, it is extremely challenging to simulate rapidly changing dynamics at a laboratory scale and obtain reliable experimental data. One way to simulate large-scale gradients is with scale-down reactors, whereby the cell culture is exposed to different conditions or circulated through reactor compartments with varying conditions [34]. However, scale-down reactors yield mainly population-averaged results and do not reveal the behaviour of individual cells over time. Thus, finding a way to simulate large-scale dynamics at single-cell resolution over time, and allowing for simultaneous investigation of physiology and production, would improve our understanding and selection of microorganisms for bioprocesses (Fig. 1.1).

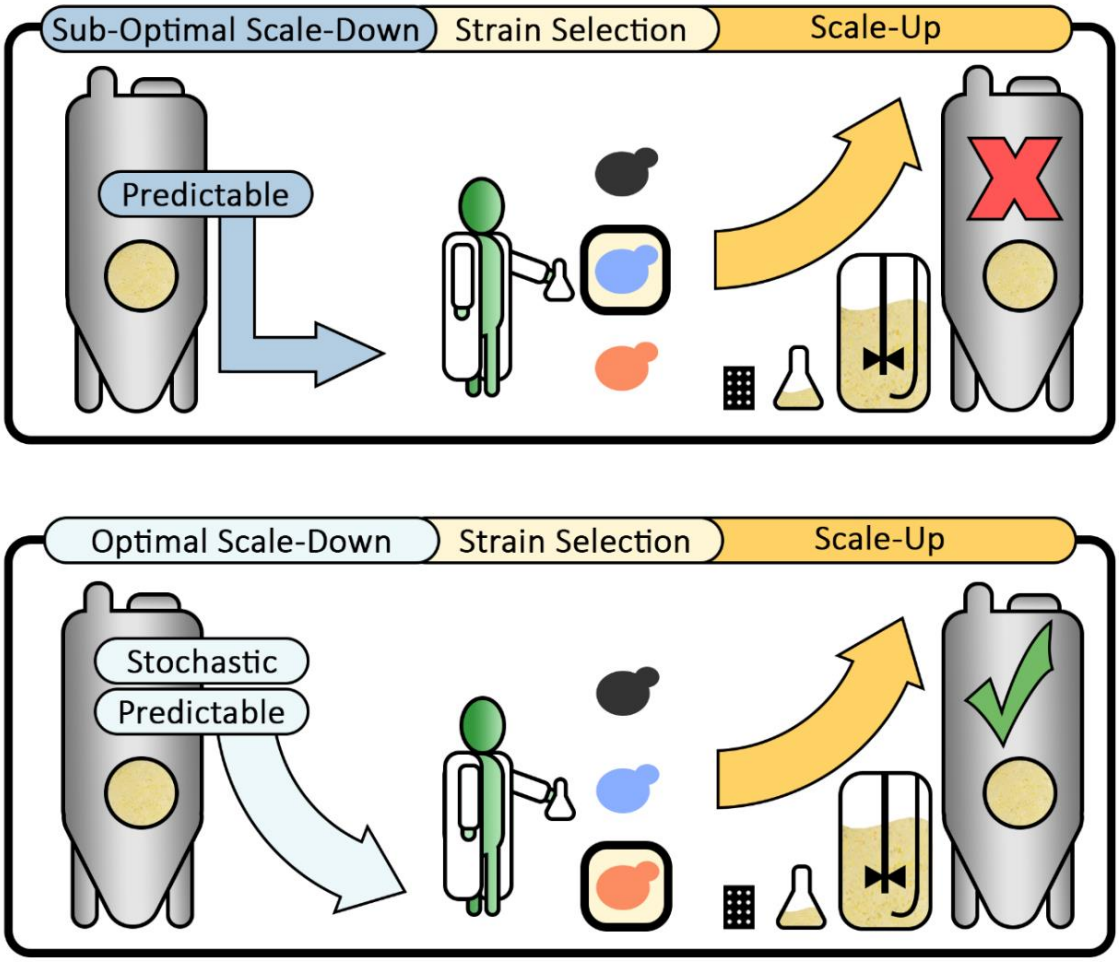


Figure 1.1. Steps in the selection of production hosts. *Strain selection/screening prior to scaling up is generally performed by considering predictable perturbations only (top panel). However, many microorganisms and strains selected or developed in the laboratory often fail to perform optimally in a large-scale reactor. A more holistic approach that incorporates stochastic perturbations and tools to better mimic industrial-scale dynamics might instead increase the chances of succeeding in the scaling up (bottom panel).*

1.3. Aim of the thesis

The growing demand for bio-based products calls for microorganisms with increasingly elevated TRY metrics. Microorganisms are often selected and improved in the laboratory, where they might perform optimally. However, they might fail the scale-up process to industrial production. Moreover, industries often strive for improved "robustness" by their production hosts, but no clear and common definition or way to quantify it exists. Being able to assess the ability to withstand the dynamic (both small time and large time-constants), heterogeneous, and harsh industrial conditions at early stages can be crucial for optimising cell factories. Therefore, my **thesis** work set out to address the following research questions:

- How do microorganisms respond to the complex and dynamic environments found in large-scale bioreactors?
- How can predictable and stochastic large-scale perturbations be studied at small scale?
- How can microbial robustness be defined and applied in academic research or in industry?

While reflecting on the above research questions, it became clear that there were insufficient tools enabling the investigation at a laboratory (small) scale. Most screening methods available for such settings focus on large time-constants (in the order of hours). Examples include measurements of product formation (causing product inhibition) [35] or detoxification of inhibitory compounds in a substrate [36]. Instead, dynamics with small time-constants (seconds to minutes), such as the ones caused by substrate, pH, or gas gradients [24], are often overlooked. Moreover, the intracellular environment dynamics of cells exposed to these industrial settings has been poorly studied. Thus, the following aims were set to address the above-mentioned questions:

- Develop a toolbox to easily investigate the intracellular environment in yeast cells and better understand cell physiology under industrial settings (**Papers I** and **II**).
- Give a clear definition of microbial robustness (**Paper III**) and showcase how to implement different aspects of robustness quantification in multiple setups (**Papers IV, V**, and **VI**).
- Devise a setup to investigate the small time-constant dynamics found in large-scale bioreactors and mimic the conditions cells are exposed to during production (**Paper VI**).

Chapter 2. Yeast Physiology in Bioprocesses

The second chapter focuses on *S. cerevisiae* metabolism and physiology, using predominantly fermentation of lignocellulosic hydrolysates (LHs) for second-generation (2G) biofuel production. Analysis of growth functions was complemented by high-throughput characterisation of the intracellular environment using fluorescent biosensors. To achieve the latter, the fluorescent *Sc*EnSor Kit was developed and is described hereafter.

2.1. Lignocellulosic hydrolysates as industrial substrate

Biofuels offer a promising and sustainable alternative to fossil fuels. They are produced via microbial fermentation of plant biomass, such as crops (first-generation, 1G) or lignocellulosic substrates (2G) [37]. The main advantages of 2G over 1G bioethanol are the reduced competition with food production and the valorisation of underutilised biomass. Its main disadvantages are related to cost-effectiveness and scalability [38]. Moreover, the presence of inhibitory compounds originating from pretreatment of LHs tends to hinder microbial fermentation performance [39]. Variations in lignocellulosic plant biomass (LPB) and severity of pretreatment cause the composition and concentration of inhibitory compounds in LHs to vary widely. Therefore, each LH might exert different effects on yeast metabolism.

LPB is composed of 35%–50% cellulose, 20%–30% hemicellulose, and 10%–25% lignin, along with extractives [40,41]. Cellulose provides mechanical strength and structure [42]; whereas hemicellulose has a protective function and cross-links cellulose fibres [43]. Several hemicellulose types exist. While the primary cell wall of terrestrial plants is dominated by xyloglucan [43,44]; hemicellulose in the secondary cell wall varies with respect to plant species. For hardwood trees (e.g. birch) and grasses (e.g. wheat and sugarcane), xylan is the main hemicellulose and is composed primarily of pentose units [43]. Side substitutions are also species-specific: glucuronoxylan in hardwood, arabinoxylan in cereal grain and grasses, and glucuronoarabinoxylan in some agricultural crops and softwood [43]. Acetyl groups and uronic acid residues are found in the xylan chain [45]; whereas ferulic acid is common in the arabinose substitutions of arabinoxylan and

glucuronoarabinoxylan [46]. Ferulic acid is crucial for cross-linking, which ensures a strong hemicellulose network less accessible to degrading enzymes [46]. For softwood trees (e.g. pine and spruce), galactoglucomannan is the main hemicellulose component of the secondary cell wall [47]. These chains encompass a backbone formed by glucose and mannose residues, acetyl and galactosyl sidechains, as well as acetyl groups [48]. Lignin is a complex amorphous polymer formed by three phenyl-propane alcohols (p-coumaryl, coniferyl and sinapyl) as the main building blocks [49]. Its main function is to provide hydrophobicity and structural rigidity, it is highly recalcitrant to enzymatic attack, and, upon pretreatment, it releases compounds which have a strong inhibitory effect on both enzymes and microbes [50].

Due to its complex and strong structure, LPB is generally pretreated to open its crystalline structure and improve access to hydrolysing enzymes [51]. However, pretreatments may release inhibitory compounds from the plant biomass itself or from the degradation of hexoses, pentoses, and lignin (Fig. 2.1) [52]. Monosaccharides from cellulose and hemicellulose are degraded into aldehydes, such as hydroxymethylfurfural (HMF) from hexoses or furfural from pentoses [52]. Microbial exposure to furans leads to reduced biological activity, DNA damage, oxidative stress, and redox/energy imbalance. In fact, ATP is used by active transporters to pump the aldehydes out, while NAD(P)H+H^+ is used for their conversion into less inhibitory alcohols [53,54].

Weak acids are released from the hemicellulose fraction (e.g. acetic acid) or are derived from further degradation of aldehydes (e.g. formic or levulinic acids) [55]. When the pH of LHs is above the pKa of the weak acid (generally below 5), protonated (uncharged) weak acids can diffuse into the cell [56]. Once in the cytosol at neutral pH, weak acids release a proton and remain trapped, lowering the intracellular pH [56]. Consequently, yeast cells must use ATP to actively pump out the protons and acids, causing ATP depletion, metabolic stress, and interference with the glycolytic pathway [57,58]. Moreover, weak acids induce endoplasmic reticulum stress and the unfolded protein response (UPR) [59].

A mixture of aldehydes, ketones, acids, and alcohols, with varying degrees of growth inhibition are released from the lignin fraction [60]. Phenolic compounds, in particular, exert a high inhibitory effect already at low concentrations such as 0.2 g/L for ferulic acid [61,62]. Akin to aldehydes, phenolic compounds trigger oxidative stress and redox imbalance, while also increasing ergosterol production, mitochondrial activity, and membrane transport [62,63]. In summary, LHs are challenging substrates for fermenting microorganisms.

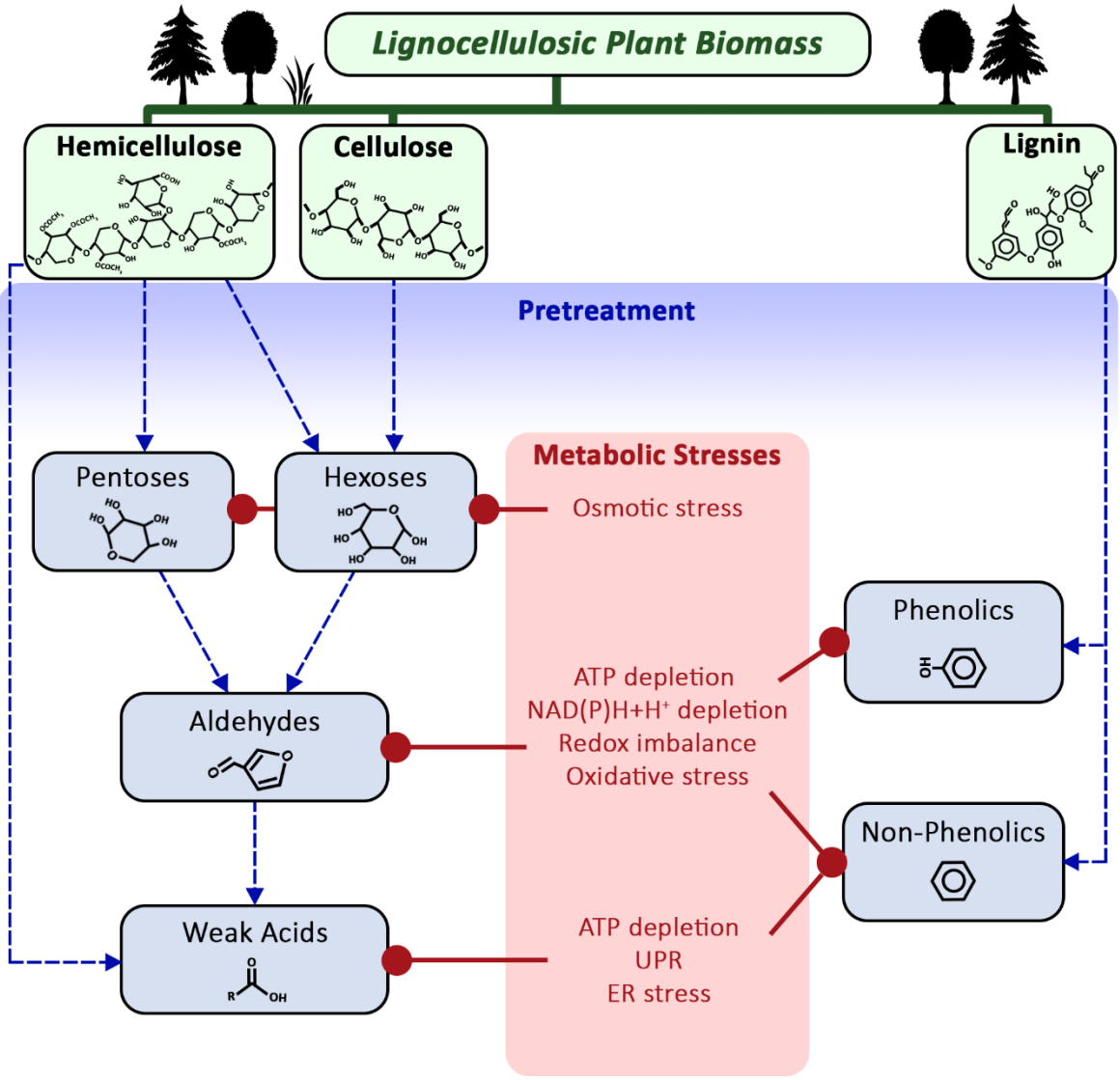


Figure 2.1. Degradation of plant biomass and inhibitory compounds. *Overview summarising the generation of inhibitory compounds from cellulose, hemicellulose, and lignin following pretreatment of lignocellulosic plant biomass. While cellulose and hemicellulose are present in all types of plant biomass (both woody and non-woody plants), lignin is found in higher amounts in woody plants (softwood and hardwood). The higher the severity of pretreatment, the further cellulose and hemicellulose are degraded. Some of the main metabolic forms of stress experienced by cell factories are listed in red for each compound type.*

2.2. Growth comparison of three *S. cerevisiae* strains

Numerous *S. cerevisiae* strains are available for bioproduction, each specialised for a certain target, such as ethanol, alcoholic beverages, biomass, and proteins [4]. Therefore, when it came to selecting the strains to be screened, some choices had to be made. First, at least one laboratory strain had to be included. Some of the most widely used *S. cerevisiae* laboratory strains are S288C and its derivatives BY4741 and BY4742 [5,64]. However, these strains have an impaired respiratory metabolism [65] and perform poorly under most stress conditions [66,67]. In contrast, CEN.PK113-7D is a laboratory strain with a wild-type and industrial background [68], good tolerance, and well-suited for cell-factory research [66,69]. As strain comparison would have been carried out in industrial-relevant conditions, CEN.PK113-7D was selected. Second, given that the screenings would entail perturbations commonly encountered during 2G biofuel production, osmotic stress-tolerant strains capable of growing in the presence of abundant sugars [35], and ethanol-tolerant strains resistant to product inhibition [70] were preferred as industrial candidates. Therefore, Ethanol Red and PE2, two strains used for 1G bioethanol production in Europe and Brazil, respectively [71], were chosen. In fact, both are often used as industrial strain proxies. The three selected strains were characterised in a 2G bioethanol production setting, using real and synthetic LHs, as well as de-constructed perturbation spaces. The screenings were carried out aerobically in 96-well plates and oxygen-limited flasks.

2.2.1. Aerobic and oxygen-limited cultures in lignocellulosic hydrolysates

When characterising strains to identify good 2G bioethanol producers, often only one or two LHs are analysed. However, owing to differences in LPB composition (**Chapter 2.1**), it would be better to investigate performance in multiple LHs. Therefore, the three strains mentioned above (CENPK.113-7D, Ethanol Red, and PE2) were screened in seven different LHs. These originated from non-woody plant biomass, such as wheat straw (WSH), corn stover (CSH), oat hulls (OHH), and sugarcane bagasse (SBH); as well as woody plant biomass, such as spruce (HGSH), softwood logging residues (SLRH), and birch (BiH) (**Paper IV**) [72]. In line with published reports, pentose concentrations were higher in non-woody and hardwood materials; whereas the inhibitors furfural and HMF were more abundant in non-woody and softwood materials, respectively (Table 2). For bioethanol production, batch fermentations are the preferred due to ease of control, flexibility, and lower risk of contamination [73]. Owing to the Crabtree effect, *S. cerevisiae* can exhibit

fermentative (absence of oxygen and abundance of sugars), respiro-fermentative (presence of oxygen and abundance of sugars) or respiratory (presence of oxygen and low levels of sugars) growth [74]. Although fermentation under anaerobic conditions would lead to the highest ethanol yield possible (0.51 $g/g_{substrate}$), oxygen is required for the biosynthesis of sterols and unsaturated fatty acids [75,76]. Therefore, yeast batch cultures aimed at bioethanol production begin under aerobic conditions and a respiro-fermentative metabolism, which is replaced by a fermentative metabolism once oxygen is exhausted [77].

Table 2. Composition of lignocellulosic hydrolysates (from Paper IV).

Compound	WSH[1]	SBH[1]	CSH[1]	OHH[1]	BiH[2]	HGSH[2]	SLRH[2]
Glucose	82.3	95.6	62.5	37	124.4	84.9	26.7
Mannose	--	--	--	--	3.2	31.6	21.6
Galactose	1.3	0.7	1.3	2.6	1.7	6.2	9.4
Arabinose	3.1	2	1.9	5.3	1.1	4	4.3
Xylose	38.1	47.1	26.3	87.7	63.8	15	16.5
Formic acid	0.2	--	--	3.1	1.5	1.4	0.1
Acetic acid	5	4.7	2.5	5.1	13.4	7.8	2.7
Levulinic acid	2.1	--	--	--	--	3.6	0.7
Furfural	3.2	1.1	2.7	3.4	0.6	0.4	0.5
Hydroxymethylfurfural	--	--	0.4	--	--	0.9	0.7

Concentrations are expressed in g/L and refer to the undiluted hydrolysate.

[1]Non-woody hydrolysates; [2]Woody hydrolysates.

BiH, birch hydrolysate; CSH, corn stover hydrolysate; HGSH, high gravity spruce hydrolysate; OHH, oat hulls hydrolysate; SBH, sugarcane bagasse hydrolysate; SLRH, softwood logging residue hydrolysate; WSH, wheat straw hydrolysate.

To achieve oxygen-limited conditions while maintaining a throughput high enough to compare three strains and seven LHs, the selected yeast strains were grown in flasks sealed with glycerol loop traps to prevent the diffusion of oxygen (**Paper IV: Fig. 1a**) [72,78]. All substrates used for the screening contained 50% (vol/vol) LH and multiple growth functions were assessed (Fig. 2.2A and **Paper IV: Fig. 1**) [72]. Ethanol Red was the best-performing strain and the one able to grow in most LHs. Interestingly, its performance varied less than for other strains, revealing greater stability across multiple conditions. CEN.PK113-7D and Ethanol Red were the most similar in terms of average ethanol output, as well as biomass and glycerol yields. PE2 was the least-performing strain of the three. Previous studies have compared CEN.PK113-7D and PE2 in very high gravity (i.e. highly concentrated sugar substrate) oxygen-limited fermentation using corn steep liquor, a by-product

of corn wet-milling, rich in amino acids, vitamins, and minerals [79–81]. Accordingly, PE2 attained a higher ethanol production rate and titre than CEN.PK113-7D. However, corn steep liquor composition resembled that of substrates used for 1G biofuels (rich in sugars, few-to-no inhibitors), which are the preferred substrates for PE2. Interestingly, in a follow-up study under the same growth conditions, CENPK.113-7D attained higher glycerol yields than PE2 [82], akin to the present results in LHs (Fig. 2.2A). Moreover, in the same study, the overall sterol accumulation was higher in PE2 than in CEN.PK113-7D when grown on very high gravity substrates, but the laboratory strain started accumulating sterols immediately after inoculation [82]. Sterols and unsaturated fatty acids play a pivotal role in cell tolerance towards stress [83–85]. The rapid accumulation of sterols might offer an advantage, as it could make a strain more tolerant to a stressful environment such as LHs. Therefore, a higher tolerance to stress might be due to strain-specific differences in lipid and sterol synthesis. Ergosterol has been shown to act as a cell protectant against inhibitory compounds [62] and Ethanol Red boosts ergosterol biosynthesis when exposed to lignocellulosic inhibitors [86]. This could explain the strong performance and good tolerance of Ethanol Red compared to other strains.

The synthesis of sterols is very energy-consuming: one molecule of ergosterol requires 24 molecules of ATP and 16 of $NADPH+H^+$ [76]. Therefore, elevated ATP production is required. Respiration of one glucose molecule in *Saccharomyces cerevisiae* yields around 18 ATP molecules, while fermentation yields only 2 ATP molecules. Due to overflow metabolism and the Crabtree effect, *S. cerevisiae* would consume most of the sugars via fermentation rather than respiration even in the presence of oxygen [74]. However, as high amounts of ATP and $NAD(P)H+H^+$ are used in response to LH inhibitors [87], sugars might be directed towards respiration rather than the fermentative pathway. A more favourable energy and redox metabolism might explain why the three strains tolerated the LH better under aerobic conditions than in oxygen-limited flasks (Fig. 2.2B and **Paper IV: Fig. 4**) [72]. While PE2 and CEN.PK113-7D exhibited similar performance, Ethanol Red presented the highest specific growth rate and shortest lag phase. These results are in line with previous studies performed in WSH [88]. Ethanol Red was shown to have high and stable specific growth rates in many conditions and robust (i.e. stable) ethanol yields, albeit still lower than for PE2 [69,89]. Based on its high tolerance and good performance, Ethanol Red was chosen as a target strain to engineer xylose consumption in its metabolism [90] and to further improve its tolerance towards spruce hydrolysates and high temperatures to make it a better candidate for simultaneous saccharification and fermentation [91]. Moreover, CEN.PK113-7D and PE2 have previously shown similar performance in batch, chemostat or dynamic continuous cultures run in the presence of acetic acid or low pH [92].

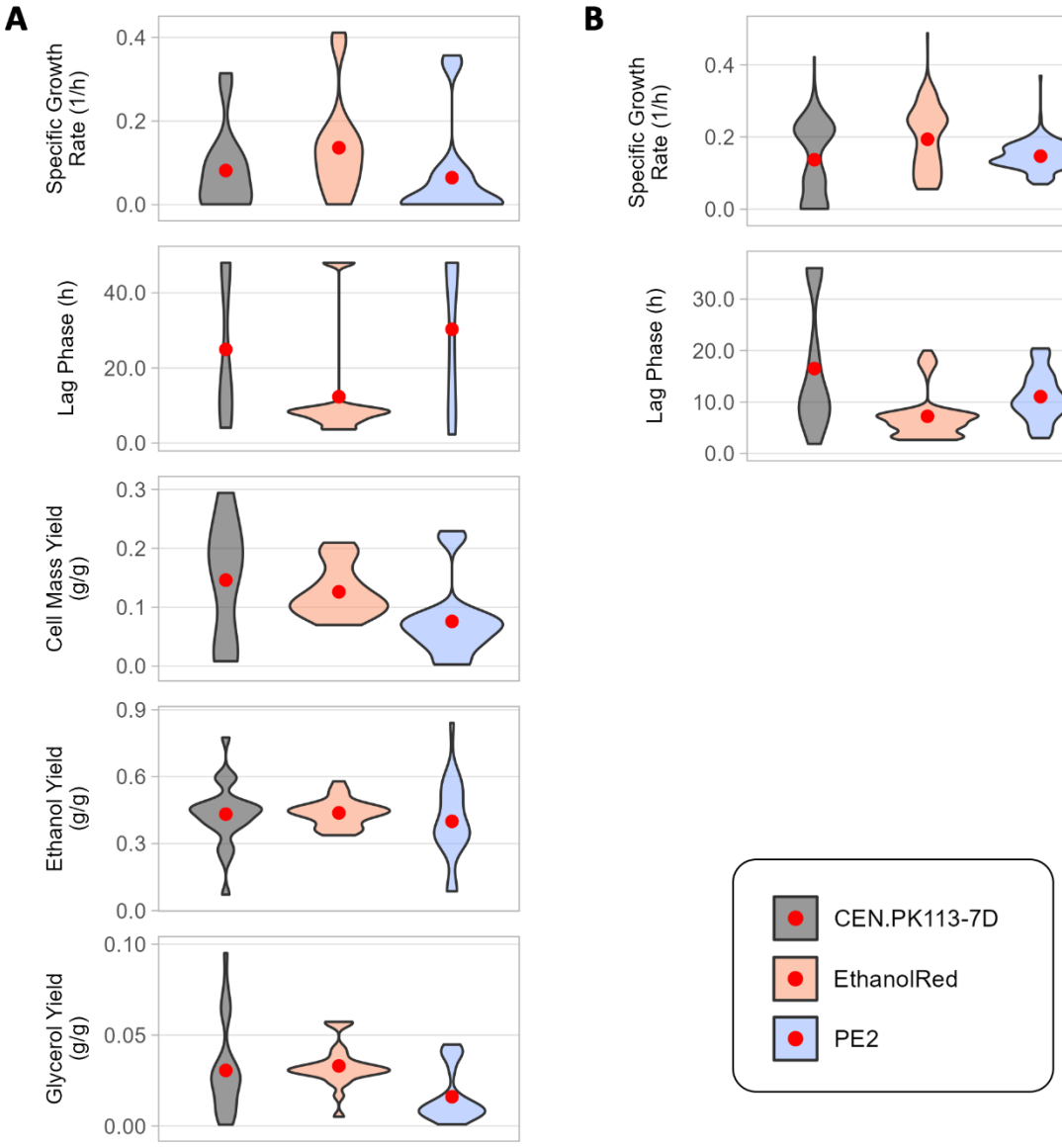


Figure 2.2. Performance of selected yeast strains grown in LHs. *Violin plots for selected growth functions (maximum specific growth rate, lag phase, cell mass/ethanol/glycerol yields) for the three selected yeast strains grown in seven LHs and a control condition (synthetic minimal medium) under oxygen-limited (A) or aerobic (B) conditions. Violin plots represent the distribution of the growth function considered for all media tested (each in triplicates). Red dots represent the mean across all media and replicates for each strain and function. (A, B) Data adapted from Paper IV.*

2.2.2. Standardising bioprocesses for the laboratory

Growing strains in LHs would take into account the complexity of these media and the synergistic effect of inhibitors. However, LHs vary substantially with respect to LPB source and across batches due to seasonal changes or the plant parts being used. As batch variation is very common in bioprocesses [14], testing how strains are influenced by variability in substrate composition should be carried out at an early stage. Even like that, all this variability makes it difficult to compare different studies. To ensure substrate composition is reproducible and comparable across studies, synthetic LHs containing inhibitor cocktails have been used to mimic actual LHs [93–96]. For this **thesis**, Ethanol Red and CEN.PK113-7D were grown in a synthetic wheat straw hydrolysate (SWSH) (**Paper I**) [96], along with other relevant plant-derived sources, such as synthetic corn hydrolysate (SCH) and synthetic spruce hydrolysate (SSH) (Table 3). Screenings to compare CEN.PK113-7D and Ethanol Red were carried out in synthetic LHs at 50% and 80% (vol/vol) under aerobic conditions (Fig. 2.3A-B). Not only did the industrial strain outperform the laboratory one, but it also grew under all conditions, whereas the latter failed to grow in SSH80 (Fig. 2.3A-B). The low specific growth rate and biomass formed during growth in SCH50 and SCH80 were due to the low concentration of hexoses and abundance of pentoses, which were not metabolised by these two strains. Although this might be a step forward in trying to standardise performance assays in LHs, synthetic LH should contain a wider range of inhibitors to better represent the real environment.

Table 3. Composition of synthetic lignocellulosic hydrolysates.

Compound	SSH	SWSH	SCH
Glucose	18	68.8	7.4
Mannose	16	1	0.8
Galactose	4.5	0.6	3.34
Xylose	9	36.4	40.2
Arabinose	4	4	7
Formic acid	1.2	1.2	0.7
Acetic acid	6.3	4.7	2
Levulinic acid	2.4	--	0.3
Furfural	1.1	3	--
HMF	3.4	0.6	0.4
Vanillin	0.11	0.03	0.05

Concentrations refer to undiluted media and are expressed in g/L.

SCH, synthetic corn hydrolysate; SSH, synthetic spruce hydrolysate; SWSH, synthetic wheat straw hydrolysate.

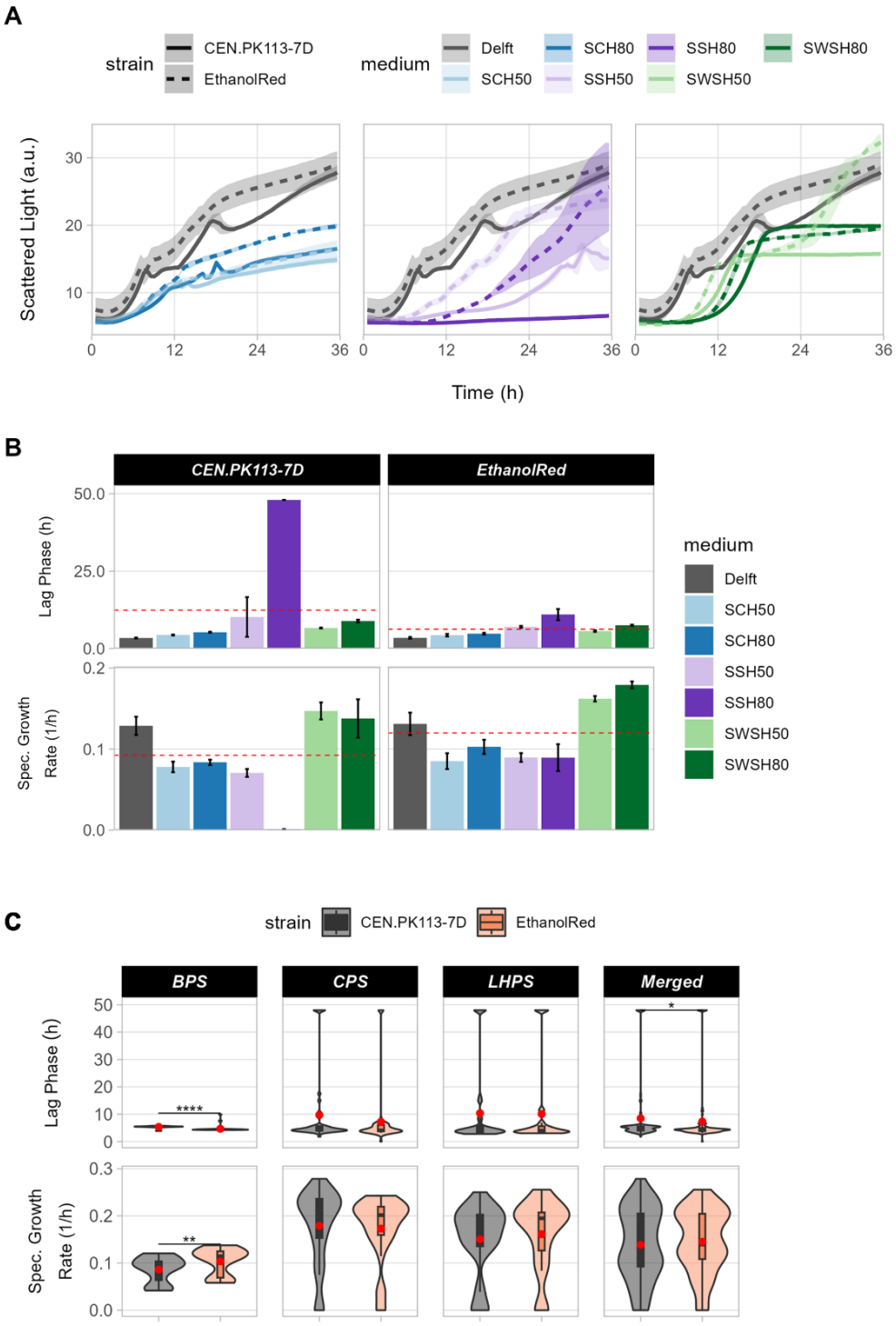


Figure 2.3. Yeast performance under laboratory conditions mimicking industrial setups. *Growth curves (A) and performance (B) of two yeast strains grown aerobically in "Delft" synthetic minimal medium and synthetic LHs at two different concentrations. (B) Red dashed horizontal lines represent the mean across all conditions for each strain and function. (C) Violin plots of growth functions for two yeast strains grown in different perturbation spaces mimicking beer production (BPS), antibiotics (CPS), LHs (LHPS), and all together (Merged). Red dots represent the mean across all conditions in each perturbation space. Wilcox test was performed to assess statistical differences between the two strains; *p ≤ 0.05, **p ≤ 0.01, and ****p ≤ 0.0001. (A, B) Partial data adapted from Paper I and unpublished data (Torello et al. 2022); (C) Data adapted from Paper V.*

Synthetic LHs represent “complex” media, from which it is still hard to pinpoint to the contribution of single components to overall strain physiology. To overcome this limitation, one could screen the strains using synthetic minimal media with only one inhibitor or condition at a time. Such an approach would create a set of conditions that together formed a perturbation space (PS). This concept has been used to compare three strains (S288C, CEN.PK113-7D, and Ethanol Red) in a perturbation space resembling conditions found in LHs [69]. Thus, CEN.PK113-7D and Ethanol Red were tested in three different perturbation spaces to assess their performance in a wider range of stressful conditions and inhibitors (**Paper V**). The perturbation spaces will be described in greater detail in a later chapter (**Chapter 3.3**). Briefly, one mimicked the LHs, one mimicked stress encountered during beer production, and one mimicked a set of antibiotics. Each perturbation space included 16 different conditions. When summing up all perturbations, Ethanol Red displayed a shorter lag phase and higher maximum specific growth rate compared to CEN.PK113-7D (Fig. 2.3C); only in the one containing antibiotics CEN.PK113-7D exhibited a higher averaged specific growth rate, although data were more dispersed.

Growth curves and growth-related functions (specific growth rates, production/consumption rates, and yields of individual compounds) are generally the starting point for most analyses and screening assays aimed at subsequent strain selection. However, having more information on the cells’ metabolic responses and states would be valuable to compare more aspects of cell physiology prior to selection. Omics data, such as transcriptomics, proteomics or metabolomics, can be reasonably easily collected and provide insightful information on cell physiology; however, these techniques are time-consuming, costly, and *at line*/*offline*. For a quicker, cheaper, and real-time/*online* investigation of physiological responses, fluorescent biosensors might be a better alternative.

2.3. Making intracellular environment monitoring more accessible

Biosensors are capable of detecting molecules or conditions within a sample or system using a reporter, such as a fluorescent protein (FP) [97]. While fluorescent biosensors are widely used to study basic cell physiology, their role in bioprocesses is geared primarily towards strain selection and improvement. Transcription-factor-based biosensors are commonly used to screen libraries and identify the best candidates for the desired goals [98]. One can screen for strains with higher production, as in the case of octanoic acid [99], or for better tolerance, as has been done for acetic acid [100]. Similarly, fluorescent biosensors can be used to improve either production itself, as shown for muconic acid [101], or tolerance to industrially-relevant stress [102]. Additionally, biosensors have been suggested to monitor the intracellular state during bioproduction [103]. There are several laboratory-scale examples of *online* monitoring via automated real-time flow cytometry (ART-FCM), which allows for operator-free long-term monitoring of bioreactors at variable sampling frequencies [104]. *In situ* microscopy has been used for *online* monitoring of cell size, morphology, and budding events to track growth over time [105]. However, these techniques have not found practical application in large-scale production due to instrumentation and software limitations [103,104]. Effective and practical real-time monitoring of the cells' intracellular state would allow us to better understand the microbial response in large reactors.

2.3.1. Fluorescent biosensors: a general overview

To grasp the potential and limitations of fluorescent biosensors, a general overview of their different types and mechanisms of action is needed. All fluorescent biosensors are based on some form of regulation (e.g. transcriptional or allosteric) of a FP. FPs contain a typical β-barrel structure, in which three key amino acids are rearranged in response to oxygen to form the chromophore, which emits fluorescence when excited by light [106]. The amino acids involved in chromophore formation vary depending on the FP [106]. Choosing which FP to use in an experiment depends on the FP's characteristics, as no FP is "good for all occasions" [107].

Although several biosensor categories and subcategories exist [97], only a few key ones will be considered and described hereafter to better understand the content of this **thesis**. First, biosensors can be divided into transcription-based and transcription-independent. Transcription-based biosensors regulate FP transcription levels to achieve differences in fluorescence output [97]. Expression of these FPs is driven by a promoter regulated by a transcriptional factor (TF). TFs bind

specific DNA sequences in the promoter region and regulate the expression of target genes [108]. Generally, to guarantee a specific response, TF-based biosensors are heterologous circuits, such as the bacterial xylose-sensing/regulation gene circuits introduced in yeast [109]. Other biosensors use native regulatory circuits instead, such as the native yeast promoters employed to monitor metabolic states [110,111]. Synthetic promoters can be developed to respond to specific conditions [112,113] or drive evolution to improve product yields [114]. Alternatively, one can also tag a native protein in the genome and then follow directly the amounts being synthesised [115].

In the case of transcription-independent biosensors, fluorescence output does not rely on the transcriptional activity of the FP gene [97]. This category encompasses nucleic-acid and protein-based biosensors. Nucleic-acid biosensors use nucleic acids (DNA or RNA) and their secondary structures to bind to desired molecules and change the fluorescent output [97]. Riboswitches are single-stranded RNA sequences capable of binding desired ligands, thereby causing a change in conformation then translated into a response [116]. Protein-based biosensors include FPs, whose fluorescence intensity, spectrum or response changes upon the applied condition or following allosteric regulation [97,117]. The biosensor pHluorin is a pH sensor with varying spectra based on the protonation state of the FP [118]. Circularly-permuted FPs (cpFPs) contain an extra ligand-sensitive domain, whose conformational rearrangements modulate the fluorescent intensity of the cpFP [117], as in the ATP biosensor QUEEN-2m [119]. Finally, fluorescent resonance energy transfer (FRET) biosensors rely on a set of two FPs with overlapping spectra connected by a ligand-sensitive allosteric linker, which can modify the distance between the two FPs and their fluorescent output [117].

FPs and biosensors come with some limitations. Oxygen must be present in the medium for proper chromophore formation [106]. Different FPs have varying pH sensitivity, affecting the overall intensity, which might be a problem for specific applications [107]. When using multiple FPs in the same cell, spectral overlap and cross-excitation must be avoided or minimised [107]. Last but not least, FPs are heterologous proteins and, therefore, their stability over time and effect on metabolic processes of the host need to be considered.

Many biosensors exist; choosing the right candidate to monitor the intracellular environment during bioprocesses requires extensive work. First, one should scan the literature for desired biosensors, then adapt them to the given system and setup, and finally start the screening. In this context, having a versatile and ready-to-use kit with trusted biosensors would be helpful as a starting point.

2.3.2. The *Sc*EnSor Kit

Several toolkits enable cloning, gene regulation, and genome integration in *S. cerevisiae* [120–122], but not monitoring of the intracellular environment. To overcome this limitation, the *Sc*EnSor (*Saccharomyces cerevisiae* engineering + biosensor) Kit was developed (**Papers I-II**) [96,111]. The kit is based on the three-step build-transform-assess workflow and consists of Genome-Integration and Biosensor Modules (Fig. 2.4).

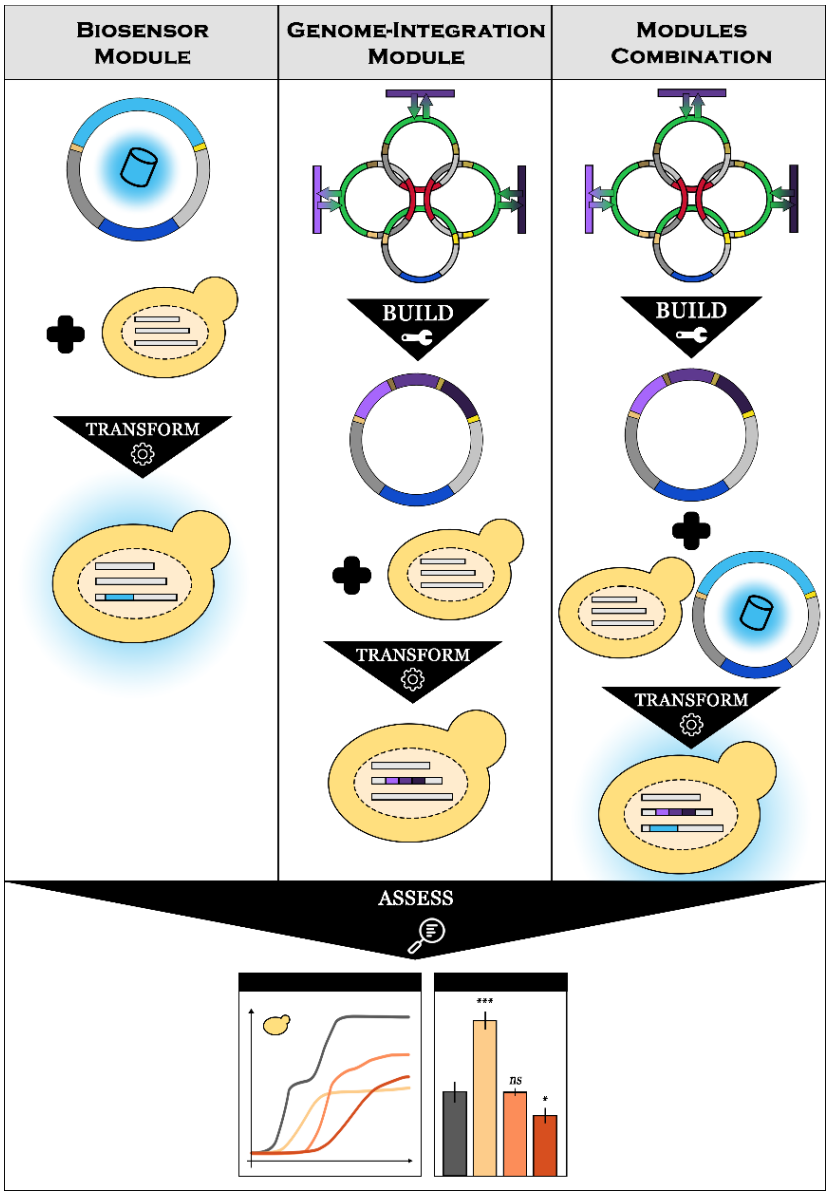


Figure 2.4. Overview of the toolkit. *The* Sc*EnSor (*Saccharomyces cerevisiae *Engineering + Biosensor) Kit is based on a three-step (build-transform-assess) workflow and includes two modules that can be used independently or in combination. The Genome-Integration module enables the integration of desired heterologous constructs into the cell. The Biosensor Module includes eight fluorescent reporters to investigate the intracellular environment of* Saccharomyces cerevisiae. *Image from the kit page on Addgene.*

Toolkits for efficient multi-locus genome integration of heterologous constructs into the *S. cerevisiae* genome are widely available [122,123]. However, single-locus integration would allow for fewer transformation rounds, higher efficiency, and equal chromatin accessibility to all constructs simultaneously. The HO locus in the *S. cerevisiae* genome is the most used site for genome integration as it does not affect the growth of transformants [124]. Over the years, more loci offering stable expression of the integrated constructs have been identified [125]. One of the preferred is the X2 locus on chromosome X. This locus is present in laboratory, industrial, and wild-type *S. cerevisiae* strains, as well as in a *Saccharomyces boulardii* commercial strain (**Paper I: Fig. 2A**) [96]. Marker-free CRISPR-Cas9-driven genome integration in this locus achieved >80% integration efficiency in all laboratory and industrial strains, although it varied somewhat between wild-type strains (**Paper I: Fig. 2B**) [96]. Therefore, both the X2 site and HO locus were chosen as targets for routine genome integration. To speed up the marker-free genome integration of transcriptional units (TUs, each consisting of promoter, coding sequence, and terminator), a set of backbone plasmids was developed and grouped into the Genome-Integration Module of the *Sc*EnSor Kit (**Paper II**) [111]. These backbone plasmids carry a GFP-dropout cassette that can be replaced by a desired TU. Moreover, thanks to the connectors from the yeast Molecular Cloning Kit [120], single TU plasmids can be assembled into Multi TU plasmids with 2–6 TUs. This module has been successfully applied to engineer a *S. cerevisiae* strain to consume a wider carbon pool (unpublished data, Ravn *et al.* 2023).

Aiming to create a starter-pack collection of key sensors to investigate the intracellular environment of *S. cerevisiae*, well-established biosensors or biosensors developed in yeast for the most relevant parameters were selected from the literature. Eventually, six biosensors were chosen from the literature and two were developed *de novo* to create the Biosensor Module in the *Sc*EnSor Kit (**Papers I-II**) [96,111]. The eight biosensors have very different modes of action and can detect intracellular pH, ATP, glycolytic flux, oxidative stress (OxSR), UPR, ribosome abundance, pyruvate metabolism, and ethanol consumption (Table 4 and Fig. 2.5). ATP levels are detected by the QUEEN-2m biosensor, a green cpFP whose fluorescent intensity is regulated by the binding of ATP to the FP [119]. To monitor intracellular pH, the more stable version of pHluorin was selected, sfpHluorin [126]. Glycolytic flux monitoring was achieved through an aptameric biosensor, here referred to as GlyRNA [127]. The aptamer is between the coding sequence and the terminator of the FP mRNA and can bind fructose-bis-phosphate (FBP). On the one hand, during high glycolytic flux, FBP is abundant and binds to the aptamer, which changes conformation and destabilises the mRNA, leading to decreased FP synthesis. On the other hand, with low glycolytic flux, FBP is scarce, and the FP mRNA is sufficiently stable to allow translation. To monitor the metabolic activity of cells,

two biosensors were developed *de novo*, PyruPro and EthPro [111]. While the first one senses pyruvate metabolism (predominantly fermentative) thanks to the PDC1 promoter [128], the second one senses ethanol consumption thanks to the ADH2 promoter [129]. Two synthetic promoter-based biosensors were selected to monitor oxidative stress (OxPro) [130] and UPR (UPRpro) [112]. The synthetic promoter in OxPro is responsive to the TF YAP1, the main oxidative stress response mediator in yeast [131]. Instead, the promoter in UPRpro is controlled by the TF HAC1, which regulates the UPR in yeast [132]. Lastly, the native RPL13A protein, one of the components of the 60S ribosomal subunit, was tagged with a FP to assess ribosome abundance [96]. Notably, by tagging the native protein in this biosensor (i.e. RibPro) rather than using the RPL13A promoter, it was possible to include post-transcriptional regulation of the corresponding mRNA, as RPL13A possess an intron. All the biosensors in the kit are ready to be integrated into the X2 locus using marker-free genome integration. Depending on the FP spectra, it is possible to combine some of the biosensors for simultaneous assessment of multiple intracellular parameters in the same cells. Indeed, this was the case of GlyOx for simultaneous detection of glycolytic flux and oxidative stress, RibUPR for the simultaneous detection of ribosome abundance and UPR, and PyruEth for the simultaneous detection of pyruvate metabolism and ethanol consumption (**Papers I-II** and **IV**) [72,96,111]. Moreover, none of the strains bearing single or multiple biosensors from the kit showed any defects in maximum specific growth rate or length of lag phase (at least) with respect to the parental strain (with no biosensors) in multiple conditions (e.g. synthetic media, synthetic media with single stressors, and LHs) (**Papers I-II** and **IV**) [72,96,111].

Table 4. Biosensors included in the* Sc*EnSor Kit.

Biosensor name	Intracellular parameter sensed	REF(s)
sfpHluorin	Intracellular pH	[96,126]
QUEEN-2m	Intracellular ATP	[96,119]
PyruPro	Pyruvate metabolism	[111]
EthPro	Ethanol consumption	[111]
GlyRNA	Glycolytic flux	[96,127]
OxPro	Oxidative stress	[96,130]
UPRpro	Unfolded protein response (UPR)	[111,112]
RibPro	Ribosome abundance	[96]
GlyOx	Glycolytic flux & oxidative stress	[96,127,130]
RibOx	Glycolytic flux & ribosome abundance	[96,127]
RibUPR	UPR & ribosome abundance	[96,111]
PyruEth	Pyruvate metabolism & ethanol consumption	[111]

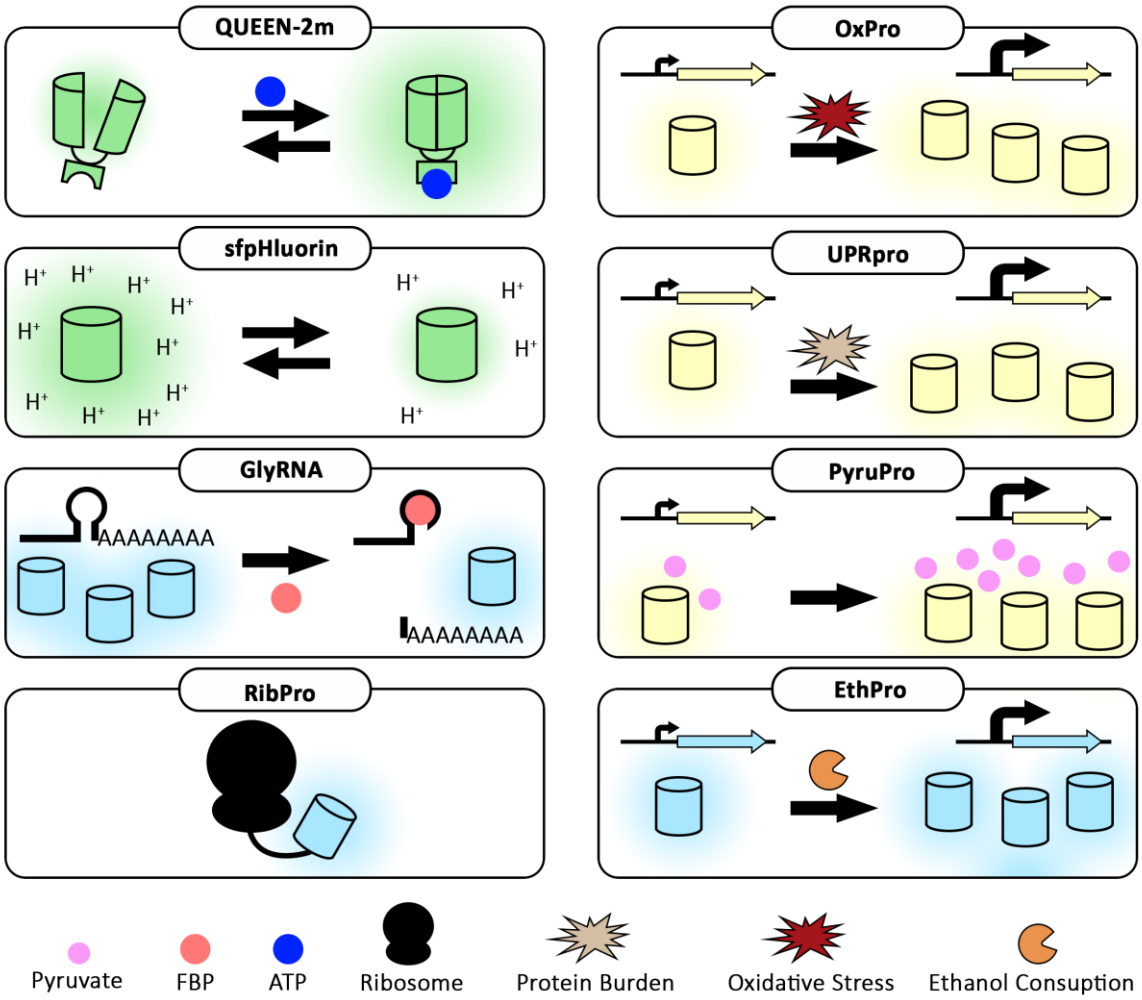


Figure 2.5. Overview of the biosensors included in the kit. *The ScEnSor kit includes eight biosensors that can detect the following intracellular parameters: ATP (QUEEN-2m), pH (sfpHluorin), glycolytic flux (GlyRNA), ribosome abundance (RibPro), oxidative stress (OxPro), unfolded protein response (UPRpro), pyruvate metabolism (PyruPro) and ethanol consumption (EthPro). The mechanism of action of each biosensor is illustrated.*

2.4. Evaluation of yeast intracellular environment in bioprocesses

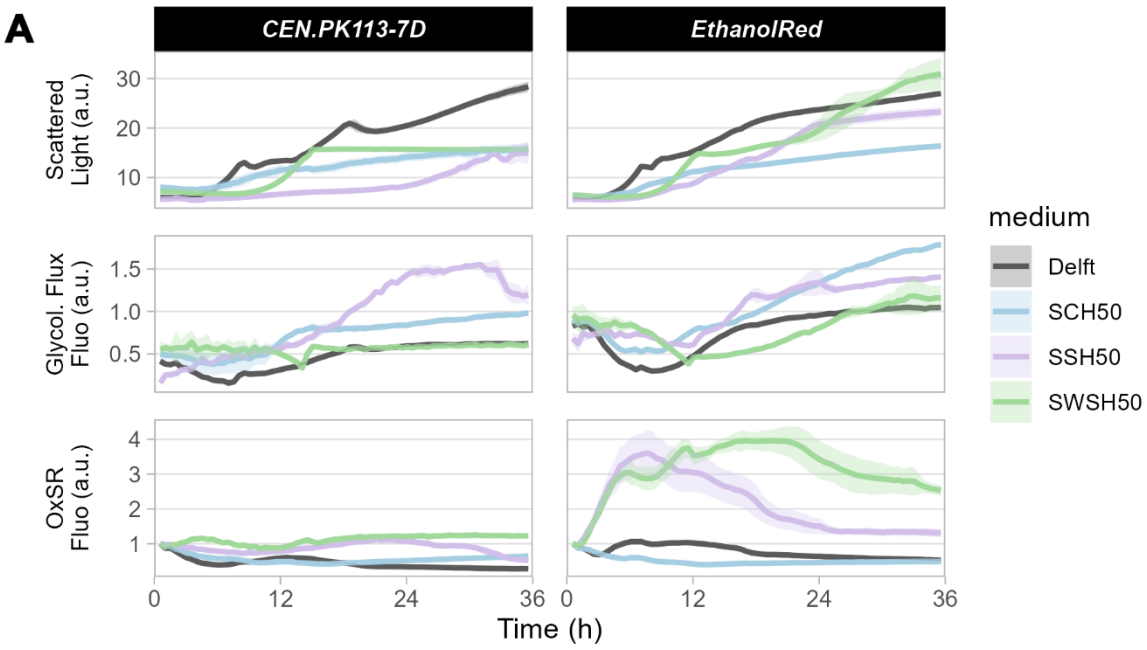
Assessing microbial TRY metrics is generally the first step in strain characterisation. The next step is to better understand the cellular and molecular reasons for the differences among strains using transcriptomics or proteomics. A multi-omics approach revealed the physiological response of the Brazilian bioethanol industrial strain SA-1 to *p*-coumaric acid [133]. High-throughput phenotyping of a strain library identified a strain with improved growth and fermentation performance in the presence of acetic acid and furfural, and subsequent multi-omics analyses elucidated the underlying mechanisms [134]. However, omics are time-consuming, expensive, and with a limited throughput. Moreover, when investigating LHs, one of the challenges is their dark colour, which limits the detection of cells. The BioLector system from m2p-labs, which allows for the detection of both biomass and fluorescence, can measure growth in LHs [88], but it was never shown to be reliable in monitoring fluorescence coming from biosensors. To overcome these limitations, the three yeast strains (CEN.PK113-7D, Ethanol Red, and PE2) analysed in this **thesis** were transformed with the biosensors in the *Sc*EnSor Kit and their intracellular responses to both synthetic and real LHs were explored (**Papers I** and **IV**) [72,96].

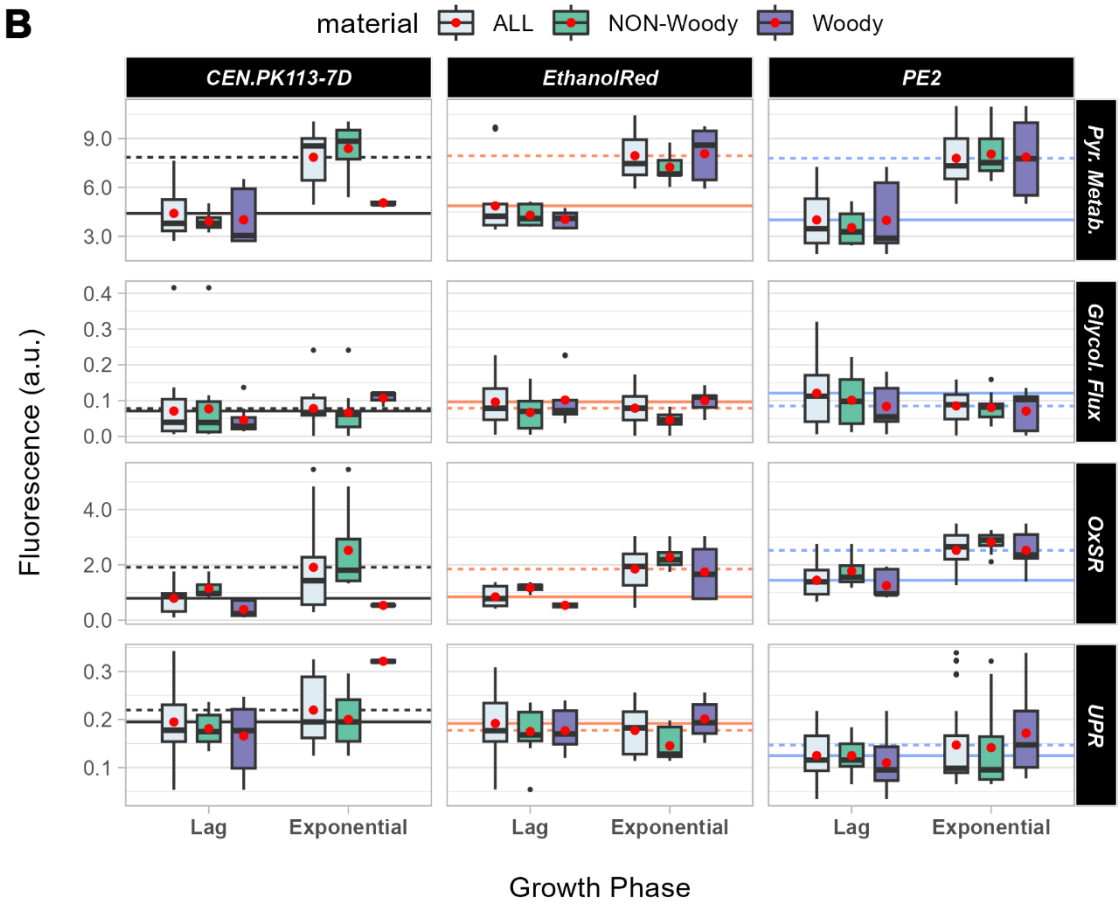
The OxSR and UPR play a pivotal role in the response towards LH inhibitors. The importance of these stress responses has been supported by transcriptomics [93,134,135], proteomics [95,136], and meta-data analysis of 7971 studies [137]. When grown aerobically in synthetic hydrolysates, Ethanol Red showed an overall higher oxidative stress response with respect to CEN.PK113-7D, pointing to a likely reason for its superior performance (Fig. 2.6A). During aerobic screening in LHs, PE2 and Ethanol Red displayed the most active OxSR and UPR, respectively (**Paper IV: Fig. 5**) [72]. The OxSR was higher in non-woody LHs, whereas the UPR was higher in woody LHs (Fig. 2.6B), in line with higher amounts of aldehydes and weak acids, respectively (Table 2). For all strains, the OxSR and UPR were more active during the exponential phase compared to the lag phase (Fig. 2.6B). The only exception was the UPR of Ethanol Red, which was higher during the lag phase, but only when considering all LHs. As cells were inoculated from a stress-free medium into LHs, no active stress response was present. The lag phase represents the cells' adaptation to a new environment [138]. When inoculated into a stressful environment such as the one encountered in LHs, cells need to activate a new set of genes to cope with the stress. One of the main forms of stress caused by LH inhibitors is ATP depletion and redox imbalance [131]. Therefore, cells might try to maximise ATP and NAD(P)H+H^+ during lag phase, probably by favouring respiration over fermentation, in spite of abundant fermentable sugars. Actively respiring cells present a shorter lag phase with respect to fermenting ones [138]. This could explain why the three strains exhibited a

comparable glycolytic flux in both lag and exponential phase, but a different pyruvate metabolism (Fig. 2.6B). The GlyRNA biosensor detected the glycolytic flux via cytosolic FBP levels. Accordingly, it provided information only on the metabolic activity of cells, but not on whether sugars were respired or fermented [127]. Generally, the glycolytic flux increased from the beginning of lag phase until the end of exponential phase (Fig. 2.6A). In contrast, PyruPro was based on the promoter of PDC1, whose protein is involved in the conversion of pyruvate to acetaldehyde during fermentation [128]. During respiration, pyruvate is converted to acetyl-CoA by the PDH complex [139]. Therefore, PyruPro activity indicated greater fermentation during exponential phase than lag phase. Redirecting energy production towards survival might eventually affect biosynthesis of the desired compound in an industrial setup. This may be particularly severe in the case of secondary metabolites, macromolecules or biomass. Pre-adapting the cells to LH inhibitors prior to the production phase could help avoid the above scenario [63,135,140]. When the transcriptome of adapted and non-adapted cells was compared, the former was characterised by a more active oxidative stress response, as well as biosynthesis of vitamins, oxidoreductases, and antiporters than the latter [135]. Therefore, pre-adapting cells to stresses might allow cell factories to optimise energy usage during production and possibly increase productivity.

Fluorescent biosensors are powerful tools to address a wide range of physiological questions. Besides monitoring the intracellular environment, they can be employed to follow the output of desired metabolites, such as branched-chain amino acids [141], natural products [142], and short- or medium-chain fatty acids [143]. Either way, having a tool to assess the stability of functions across different conditions or over time would not only enable a quicker comparison, but would also provide an additional evaluation tool when selecting, developing, or improving strains.

Figure 2.6. Intracellular parameters in synthetic and real LHs (Next Page ▶). *All data come from aerobic screenings. (A) Line plots for biomass, glycolytic flux, and oxidative stress response in Delft medium and synthetic LHs at 50% (vol/vol). (B) Barplots representing levels of selected intracellular parameters divided by growth phase, strain, and LH material. Eight conditions (control + seven LHs) are included. Red dots represent the mean value for each group. Horizontal lines represent the mean intracellular parameter considering all materials during lag (continuous) and exponential (dashed) phases. (A) Partial data adapted from Paper I and unpublished data (Torello et al. 2022); (B) Data adapted from Paper IV.*


A
CEN.PK113-7D
EthanolRed
Scattered Light (a.u.)
Glycol. Flux Fluo (a.u.)
OxSR Fluo (a.u.)
Time (h)
medium
Delft
SCH50
SSH50
SWSH50


B
material
ALL
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Woody
CEN.PK113-7D
EthanolRed
PE2
Pyr. Metab.
Glycol. Flux
OxSR
UPR
Fluorescence (a.u.)
Lag
Exponential
Growth Phase

Chapter 3. Microbial Robustness in Bioprocesses

This chapter describes the concept of robustness and explains possible quantification methods. The chosen robustness equation is described and applied to the results from **Chapter 2**, to investigate different aspects of function stability, as well as to analyse a pre-existing dataset and identify potential robustness genetic markers.

3.1. What is microbial robustness?

3.1.1. Defining robustness and its difference from tolerance

"Robustness" and "robust" are often used as positive descriptors of a system (i.e. a process or a microorganism). However, the concept of robustness is abstract, and its definition has varied based on the context. Often, "robustness" is used as a synonym for "tolerance", although they are entirely different phenomena (**Paper III**) [13]. Tolerance refers to the ability of a microorganism or a cell to grow (measured as viability or specific growth rate) in the presence of a given stress. Instead, robustness describes the stability of a function (e.g. specific growth rate) when confronted with different perturbations [13,144,145]. For example, assume three microorganisms are being screened in different concentrations of a weak acid (Fig. 3.1). Those capable of growing at the highest acid concentration or with the overall highest specific growth rate are defined as tolerant (e.g. microorganisms #1 or #2 in Fig. 3.1), whereas the one with the most stable specific growth rate across all tested conditions is considered more robust (e.g. microorganism #2 with respect to microorganism #1 in Fig. 3.1). Robustness per se does not give any information on the performance of a system. One microorganism might have a very low but constant specific growth rate across all the conditions tested, therefore making it more robust than another strain with higher but varying specific growth rates (e.g. microorganism #2 with respect to microorganism #1 in Fig. 3.1). Often, strains with high performances in very specific conditions have lower tolerance if tested in a wider set of variables, while low-performing strains might display higher tolerance (**Paper III: Fig. 2C**) [13]. For example, slow-growing subpopulations exhibit higher tolerance towards stresses or antibiotics

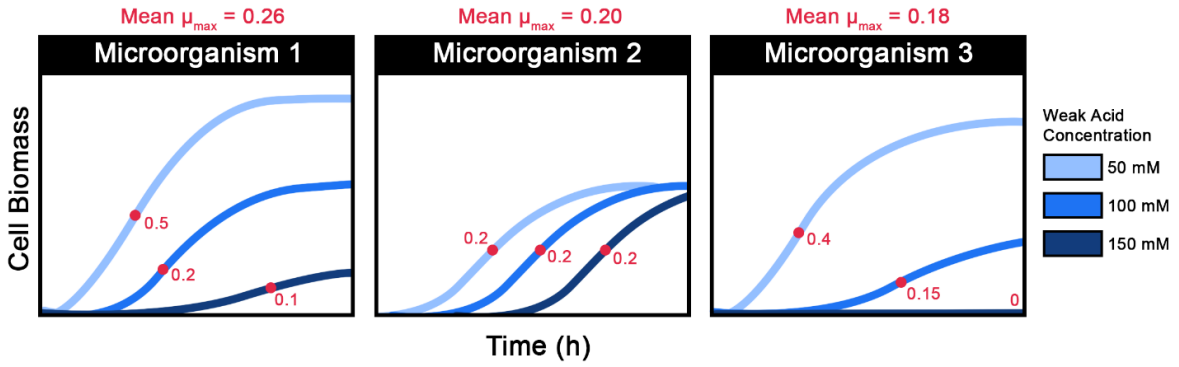


Figure 3.1. Difference between tolerance and robustness. *Visual representation of the difference between tolerance and robustness. Graphs summarise the growth of three microorganisms cultivated in three different concentrations of a weak acid. Values for the maximum specific growth rate are given in red close to each curve. The mean maximum specific growth rate across all conditions is displayed above each graph. Among the three microorganisms, #1 and #2 tolerate the two highest concentrations of weak acid, whereas #3 does not. Microorganism #2 has a more robust specific growth rate than microorganism #1, as it is more stable across all concentrations of weak acid; however, microorganism #1 has an overall higher performance than the other two.*

[146,147]. Similarly, there can be an inversely proportional trade-off between performance and robustness, as shown for ethanol yield, biomass yield, and cell dry weight in *S. cerevisiae* [89]. Owing to limited resources, as well as physical and thermodynamic limitations [148], a microorganism cannot maximize both performance and robustness. Hence, one or the other is favoured based on the environment. Not surprisingly, analysis of a large phenotypic dataset revealed poor overlap between strains with high performance and those with high robustness (**Paper V: Fig. 2A**).

Robustness is therefore a relatively abstract concept, and its analysis is often neglected or overlooked because no easy and versatile quantification methods exist. However, robustness quantification, especially at a small scale, might facilitate the physiological characterisation of microorganisms and their potential selection for industrial purposes.

3.1.2. Quantification of robustness

Robustness analysis during strain characterisation has been often neglected because no suitable quantification methods has been in practise. As robustness represents the stability of a function, the ideal robustness equation would require quantifying the distribution of a set of data with respect to its mean. The proposed quantification methods have come with some limitations. The coefficient of variance, defined as the ratio between standard deviation and mean, was suggested as a candidate [149]. However, this method poorly describes the dispersion of data with means between 0 and 1 [150]. Another strategy assessed the influence of a set of frequency-dependent perturbations (defined as perturbation space) on a function with respect to a control condition [144]. However, defining a universal control condition is challenging and makes the quantification method arbitrary. A common way to assess the dispersion of data is via the Fano factor. The Fano factor, defined as the variance-to-mean ratio of a series of data points [151], has been already suggested as a candidate for robustness quantification [145,150]. Therefore, it has been adapted by Trivellin *et al.* to formulate a frequency-independent and dimensionless robustness equation free from arbitrary control conditions (equation 1) [69].

$$\boldsymbol{R = -\frac{\sigma^2}{\bar{x}} \cdot \frac{1}{m}} \qquad (\boldsymbol{Eq.1})$$

The factors $\bar{x}$ and σ represent the mean and standard deviation of a function in a system across different conditions, while m represents the mean across all conditions and systems (Fig. 3.2). The normalisation factor m in equation 1 was introduced to obtain a dimensionless value for R, thus allowing comparison of different functions; however, it is not strictly necessary if no comparisons among different functions are performed. The minus sign in the formula ensures that high R values are associated with high robustness. A practical example can be the computation of robustness for the specific growth rate (function) of two strains (systems) across four conditions (Fig. 3.2).

Often, strains are deemed "robust" just because they can withstand harsh conditions. However, the use of such phrasing fits neither with the definition of robustness nor with its quantification method, regardless of which one is used. In fact, robustness is function-specific and relative, as it is applied to a specific perturbation space, rather than being a feature of the system or a universal variable. Therefore, rather than stating "Strain X is robust", it would be better to say "Robustness of the specific growth rate is higher for strain X than strain Y in perturbation space Z" or, at least, "Strain X has more robust functions than strain Y in perturbation space Z". As robustness refers to the stability of performance, one should specify which function is considered. Moreover,

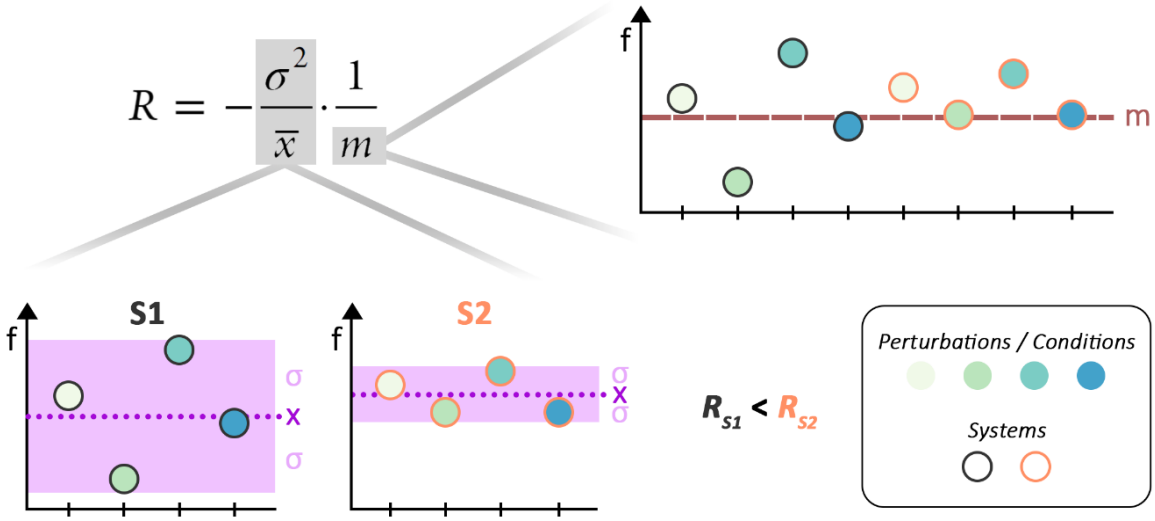


Figure 3.2. Representation of Trivellin's robustness quantification formula. *Visual representation of the robustness equation formula (Eq. 1). Robustness (R) of a function (f) can be computed for two systems (S1 and S2, such as two microorganisms) across a set of conditions/perturbations (four, in this case). After computing the mean ($\bar{x}$) and standard deviation (σ) for each system across all four conditions, the normalisation factor (m) can be computed as the mean across all systems and conditions to obtain a dimensionless value.*

robustness values and comparison among strains are heavily influenced by the perturbation space considered, making it essential to contextualize which data R was computed from.

The versatility of Trivellin's robustness quantification method allowed to expand the concept of robustness in **Paper IV**. Being based on the Fano factor, equation 1 can inherently grasp and condense in a single number the dispersion of a dataset, allowing for a wide variety of analyses. Therefore, equation 1 was applied to investigate different aspects of function stability, such as across different conditions, time, systems, and populations (**Papers IV-VI**). All these concepts will be explained and exemplified throughout **Chapter 3.2**.

3.2. Implementing robustness quantification in bioprocesses

3.2.1. Function stability across conditions

A valuable 2G bioethanol yeast cell factory should achieve stable (i.e. robust) production not only when exposed to different batches of the same LH, but also across different LHs. Because LPB might vary seasonally, the performance of the cell factory should not be affected by different substrates. When the performance of the three selected strains was analysed in real LHs (**Chapter 2.2**), Ethanol Red corresponded to the most condensed violin plots (Fig. 2.2). This suggested a relatively constant performance across all substrates. For a quantitative comparison of the strains, robustness of the functions was computed across different conditions and is referred to hereafter as R(c).

Ethanol Red exhibited always the best or nearly top function performances (specific growth rate and lag phase) across all strains in both real hydrolysates under aerobic and oxygen-limited conditions, as well as in synthetic hydrolysates (Fig. 2.2 and Fig. 3.3). In addition, it exhibited the highest R(c) for most functions under oxygen-limited conditions compared to CEN.PK113-7D and PE2 (Fig. 3.3A) and in synthetic LHs with respect to CEN.PK113-7D (Fig. 3.3C). When grown in LHs under aerobic conditions, PE2 was instead the strain with the most robust functions, though it had a longer lag phase and lower specific growth rate than Ethanol Red (Fig. 3.3B). A similar trend for these three strains was observed when R(c) was computed in a perturbation space composed of media containing individual stresses chosen to resemble WSH [69,89].

To underline the fact that high robustness does not necessarily mean best performance, one should use the example of CEN.PK113-7D grown under oxygen-limited conditions in woody LHs (**Paper IV**) [72]. Here, CEN.PK113-7D showed the highest R(c) for both specific growth rate and lag phase, even though it was the worst-performing strain (**Paper IV: Supplementary Fig. S1b, Additional File 1**) [72]. In this case, the high function robustness was due to the strain not growing in most conditions. When investigating a library of strains isolated from cachaça production (a spirit), two strains stood out as low-performing but with robust functions [89]. In that case, there might have been a trade-off between performance and robustness, as the strains grew in all conditions, even if they performed poorly. Probably, these two strain isolates deployed a set of responses that allowed them to withstand a wide range of conditions/perturbations at the cost of performance.

Therefore, robustness alone is not sufficient to describe and select a strain for bioproduction, because a strain with robust functions might not necessarily represent the best choice for a cell factory. Moreover, as substrates in other bioprocesses might not always vary, other aspects of function stability may become more relevant and will be outlined in **Chapter 3.2.2**.

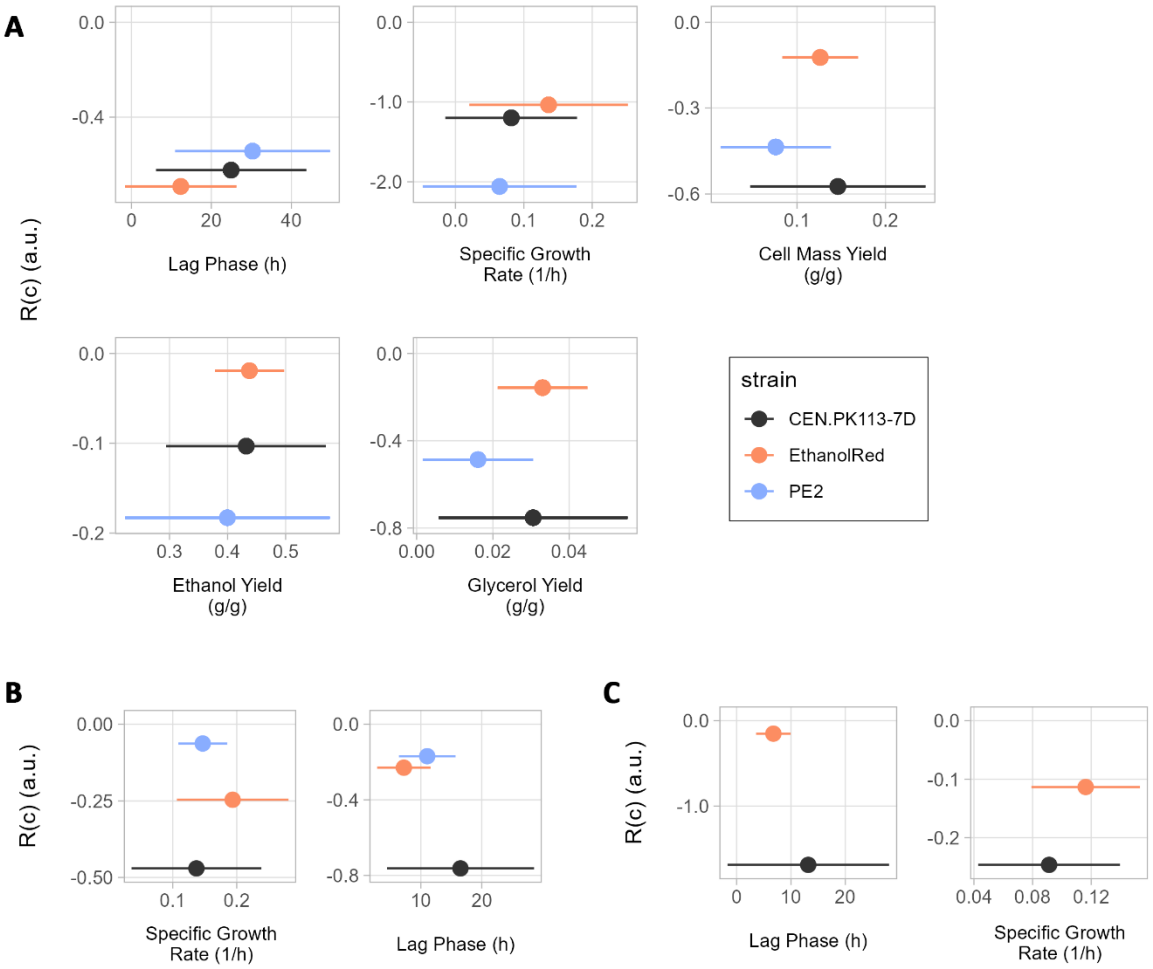


Figure 3.3. Robustness across conditions. *Correlation between R(c) and performance for various functions. Comparison of strains grown in LHs under oxygen-limited conditions (A), aerobic conditions (B), and in synthetic hydrolysates (C). (A–C) Horizontal lines represent the standard deviation of function performance across all tested conditions. (A, B) Data adapted from Paper IV. (C) Data from Paper I and unpublished dataset (Torello et al. 2022) with synthetic LH described in* ***Chapter 2.2.2****.*

3.2.2. Different aspects of function stability

By assessing the stability of functions across different conditions/perturbations, the robustness quantification formula offers a versatile tool with potentially infinite applications. The Fano factor has been used to investigate the variability of neuronal spike trains [151] and as a measure of transcriptional noise and bursting [152,153], thus describing the degree of diversity within a group and the dispersion of data. Thus, equation 1 can be applied to investigate other aspects of function stability other than across conditions, namely over time, across systems and across populations (Fig. 3.4) (**Papers IV** and **VI**) [72].

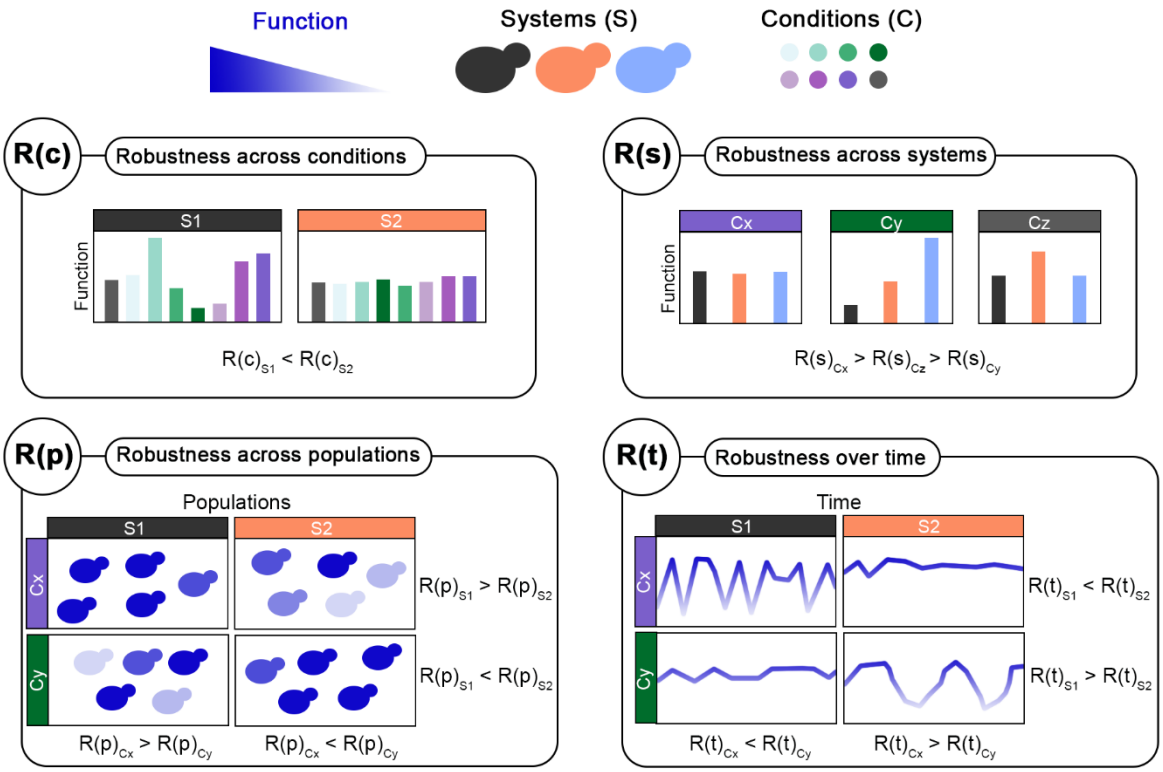


Figure 3.4. Possible applications of the robustness quantification method. *For a desired function (specific growth rate, ATP levels, ethanol yield, and cell size), systems "S" (such as different yeast strains) and set of conditions "C" (such as a set of media containing different growth inhibitors), it is possible to use the robustness quantification method to measure the stability of a function across conditions, systems, populations, and over time. Figure from Paper IV.*

3.2.3. Quantification of population heterogeneity

Population heterogeneity refers to the existence of phenotypically diverse subpopulations within an isogenic bulk population. For instance, PE2 showed two subpopulations with distinctive OxSR and glycolytic flux when grown in Delft and HGSH50 under aerobic conditions (**Paper IV: Fig. 7d, Supplementary Fig. S9, Additional File 1**) [72]. Phenotypic heterogeneity might be due to intrinsic (e.g. stochastic gene expression/noise) or extrinsic (e.g. gradients) factors [154]. Phenotypic heterogeneity from intrinsic factors might be a natural survival strategy to unexpected environmental challenges, a concept referred to as bet-hedging [155]. For example, a slow-growing yeast subpopulation was found to be more tolerant of sudden heat stress because of overexpression of TSL1, a gene involved in the synthesis of trehalose [147]. The variability in cell performance within a population can be noted not only in terms of physiological responses, but also in product titres and yields (**Paper III: Fig. 3**) [13]. In fact, population heterogeneity is a common problem in bioprocesses [156]. For instance, only a part of the bulk population might be involved in the formation of the desired product, as shown in L-valine production by *Corynebacterium glutamicum* [157] or insulin production by recombinant *S. cerevisiae* [158]. However, quantifying population heterogeneity remains a challenge. Although several tools can detect subpopulation formation [159], there are no mathematical formulae to quantify it. Computation of entropy in a cell population has been used to describe population heterogeneity, although it is actually an estimate of how likely a different phenotype may emerge within a bulk population [160]. In this context, Trivellin's robustness equation (eq. 1) [69] enables simple quantification of performance dispersion for a function in a population.

Robustness across populations, referred to as R(p), indicates how homogeneous a function is in a cell population (Fig. 3.4). High levels of R(p) indicate low levels of population heterogeneity, while low R(p) denotes elevated population heterogeneity. In line with what was seen from a qualitative point of view, PE2 appeared as the strain with the highest population heterogeneity among the three strains tested (**Paper IV: Fig. 8**) [72]. Moreover, CEN.PK113-7D exposed to feast-starvation oscillations revealed elevated heterogeneity for cell morphology in 6-min and 12-min oscillations and for ATP levels in 48-min oscillations (**Paper VI: Fig. 7, Supplementary Fig. S12, Additional File 2**). Equation 1 used for computing R(p) is easily adaptable to different setups, such as flow cytometry, which has been widely used to better understand population heterogeneity [161,162]. Being able to quickly assess and compare the levels of heterogeneity in a population might be pivotal in some cases. A method to quantify heterogeneity would have been useful when developing a light-responsive inducer to achieve homogeneous regulation of *lac*-promoter-

regulated genes [163] or when improving *Escherichia coli* eGFP production over time by decreasing the levels of population heterogeneity [164]. In bioproduction, population heterogeneity is often correlated with production TRY over a certain period, further supporting the need to quantify function fluctuation in time.

3.2.4. Assessment of function stability over time

Production half-life (number of generations to have a strain producing half of the initial amount) and production load (difference in growth with respect to a non/low-producing strain over generations) are two ways to assess industrial production stability over time [165]. However, the assessment of performance alone might not be sufficient to fully evaluate strains, as it does not provide any information on the stability of a function over time. Equation 1 can be used to compute the robustness of a function over time, referred to as R(t). When analysing two functions, the one with smaller data dispersion with respect to its mean will have a higher R(t) (Fig. 3.4). R(t) was used to assess the stability of multiple intracellular parameters of the three *S. cerevisiae* strains grown in LHs (**Paper IV: Fig. 6**) [72]. Given that the lag phase reflects an adaptation period with various metabolic rearrangements (**Chapter 2.4**), R(t) of intracellular parameters was generally lower in lag phase than in exponential phase (**Paper IV: Supplementary Fig. S6**). Moreover, R(t) was used to compare the difference between single-cell and population-averaged measurements over time for several functions (ATP levels, morphology, and growth) in CEN.PK113-7D exposed to feast-starvation oscillations (**Paper VI: Fig. 6**). Two observations stood out. First, population-averaged measurements were more stable than single-cell ones, highlighting how part of the information is lost when considering bulk populations compared to individual cells. Second, trends in the stability of functions over time for different oscillation frequencies were similar between population-averaged and single-cell measurements, but not identical. For instance, population-averaged measurements were less stable with 48-min oscillations; whereas single-cell growth was most affected by 12-min oscillations (**Paper VI: Fig. 6**).

On the one hand, the application of equation 1 might be useful for industrial processes requiring stable production over time, such as continuous or repeated batch (re-use of microorganisms multiple times in different batches) cultivations to produce lipid or single-cell protein [166,167]. On the other hand, equation 1 might not be useful to evaluate functions which are not meant to be stable over time. For instance, secondary metabolite production might be induced after a non-producing phase in which only biomass is formed [168]. Therefore, computing

how stable enzyme production is throughout the whole process would be misleading. Similarly, other setups might not have a stable function over time. For example, alternating growth and production phases in *Synechocystis* increased overall lactate yield [169]. In this case, it would not make sense to compute R(t) for either growth or production, as the setup aims for higher yields rather than stable functions. From a physiological point of view, establishing the stability of functions would be instead interesting as some responses, such as ATP level and cytotoxic protein aggregation, have been associated with unstable functions [170].

3.2.5. Relationship among R(c), R(t), and R(p)

Besides the relationship between the robustness of a function and its performance, it is possible to also assess the relationship between different types of robustness, whenever the experimental setup allows for the collection of relevant data. Such analysis provides a deeper and more complete insight into the differences between the tested systems. For example, in a set of strains with similar performance, analysis of different types of robustness would point to the best candidate based on the desired goal. As a case-in-point scenario, assume three strains have been selected from an original pool of 10 to upscale a process. In this scenario, the final goal is to have a strain that can grow in multiple versions of the same substrate with minimal population heterogeneity. Besides performance, one could decide to include a strain based on an R(c) vs R(p) plot. To facilitate the assessment of the relationship between two robustness types, each graph can be divided into four subpanels denoting the different relative relationships among systems (Fig. 3.5 and Fig. 3.6A).

Whether high or low robustness values are the goal, they will change based on the function and scenario considered. Therefore, to explain the concepts behind the different graphs in Fig. 3.5, the target function will be the product yield for a set of strains. Irrespective of whether R(c), R(p) or R(t) is considered, a strain falling in section A would probably be a good candidate for subsequent screenings, as all its cells exhibit stable production over time and across populations (low heterogeneity) no matter the conditions they face. In contrast, a strain in section C might be among the worst candidates as its production over time is unstable, presents a heterogeneous population, and its performance is highly affected by the different conditions. Strains falling in sections B and D require further evaluation, as they will have high robustness for only one of the two robustness types considered.

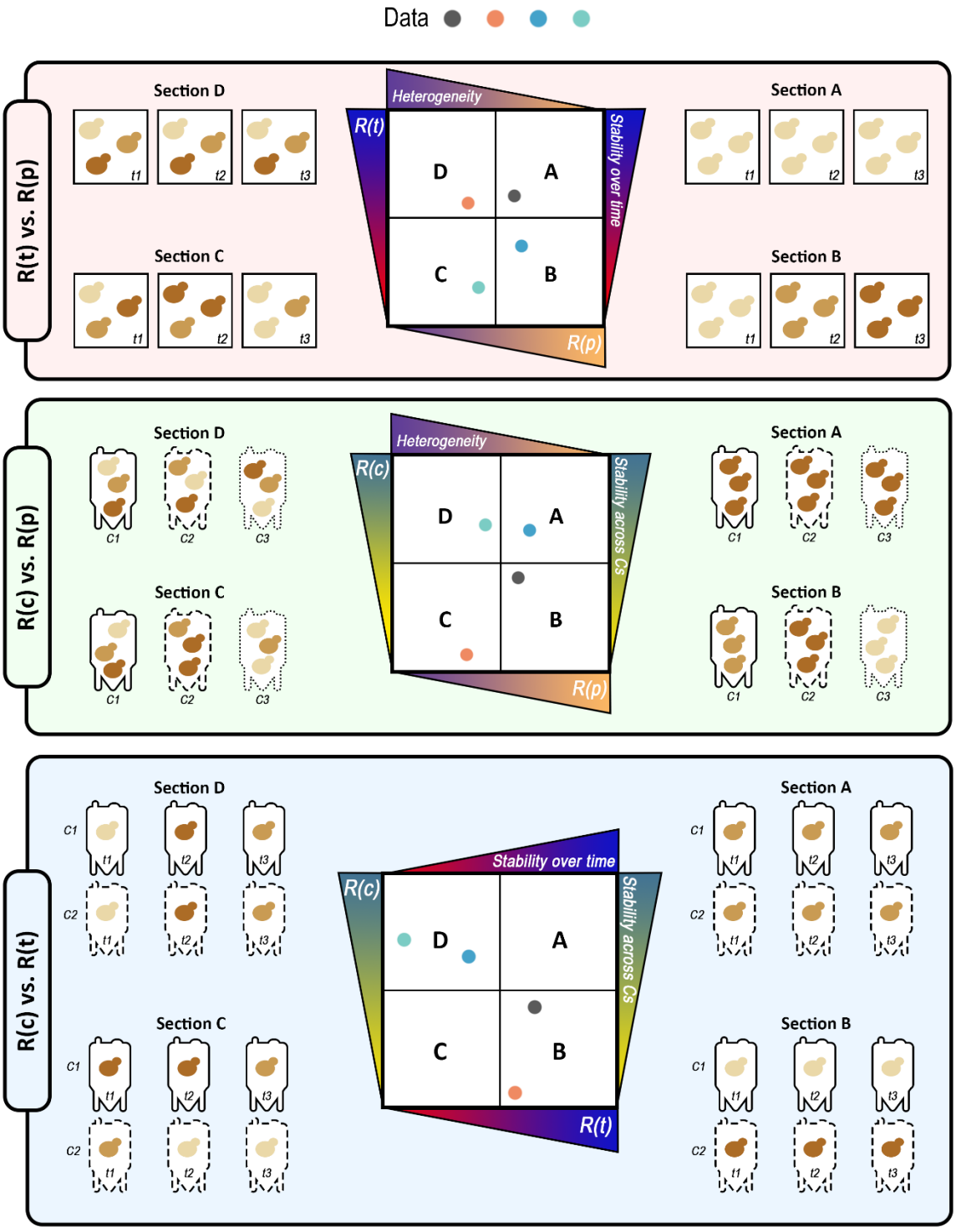


Figure 3.5. Relationships between different robustness types. *Visual representation of graphs plotting two robustness types together into a scatter plot, specifically R(t) vs R(p) (top), R(c) vs R(p) (middle), and R(c) vs R(t) (bottom). Four sections (A–D) can be identified in each graph. As an example, the graphs show data for four strains grown in different conditions (C1, C2, and C3, represented with continuous, dashed, and dotted lines, respectively) and timepoints (t1, t2, and t3). Shades of brown refer to the differences in function performance (e.g. ATP levels). Top panel adapted from Paper VI and here expanded.*

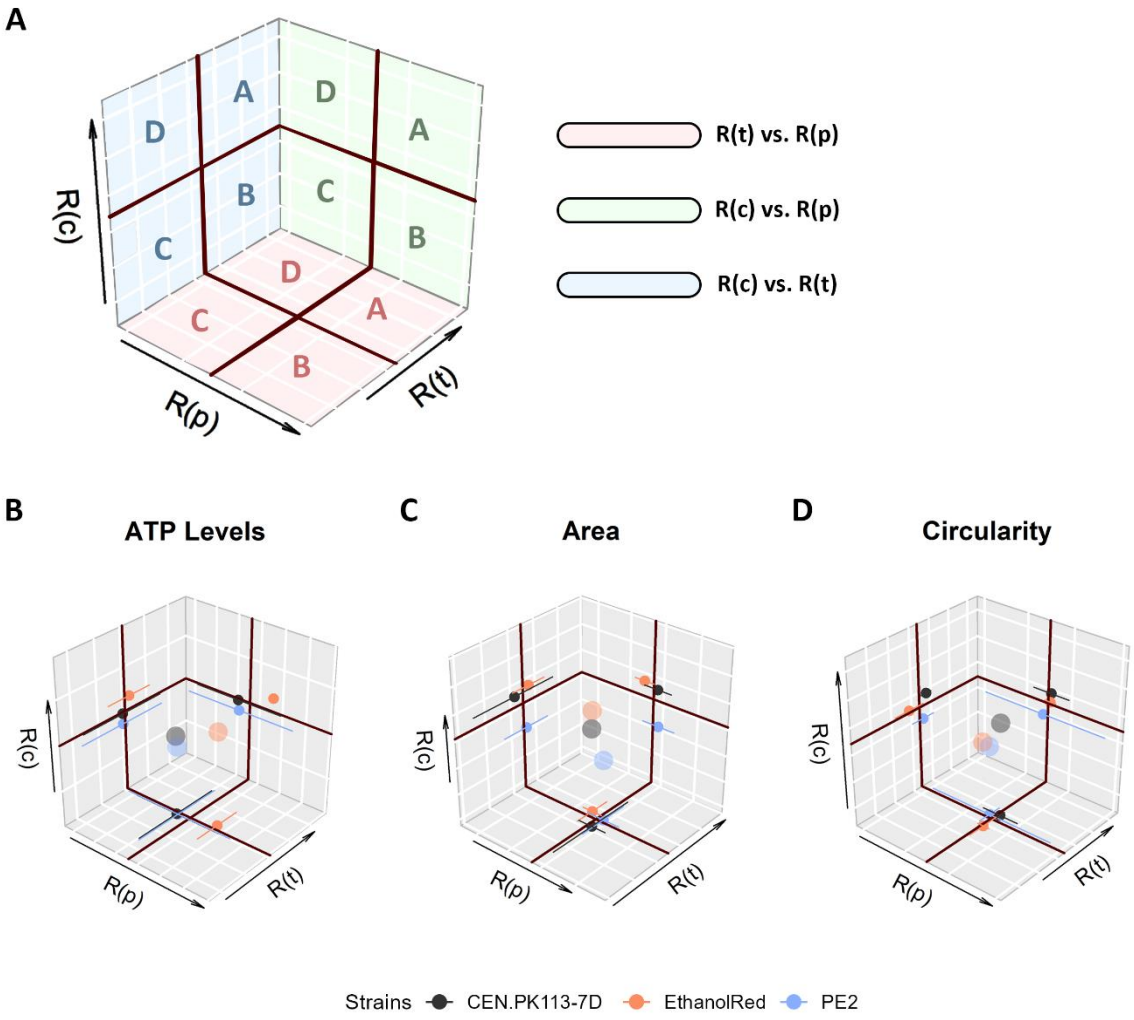


Figure 3.6. 3D robustness plots for relationships among R(c), R(p), and R(t). *(A) Visual representation of 3D robustness plots. The three 2D plots with the respective subdivisions in four sections described in Figure 3.5 were here detailed using the same colour palette as in Figure 3.5. (B–D) 3D plots for ATP levels (B) cellular area (C), and cell circularity (D). The unpublished dataset (Torello et al., 2024) includes three yeast strains grown in feast-starvation oscillation (feast = 50 g/L, starvation = 1 mg/L) of 0.75, 1.5, 6, 24, and 48 min. Each oscillation was performed in 5 replicates. 3D data (large, faded circles in the middle of the graphs) were projected onto 2D scatterplots for easier comparison. Standard deviations were computed based on 30 different chambers, 5 for each condition (5 oscillations + 1 control).*

When studying the relationship between R(t) and R(p), strains falling in sections B or D will have either a stable function over time or low population heterogeneity (Fig. 3.5 top). For example, yeast cells exposed to 1.5-min feast-starvation oscillations were homogeneous but displayed high instability for ATP over time, thereby falling in section B (**Paper VI: Fig. 9**).

When investigating the relationship between R(c) and R(p), systems will be characterised by either a stable function across different conditions or have low population heterogeneity in sections B and

D (Fig. 3.5 middle). In this case, section B will include homogeneous populations but unstable performance when different conditions are applied. Therefore, it should be determined which conditions affect the performance the most and, based on that, the choice of strain should be confirmed or not. Instead, section D would be the opposite (stable production across different conditions, but higher levels of population heterogeneity). Therefore, production can potentially be improved by decreasing the degree of heterogeneity so that all cells contribute to production.
From a bioprocess point of view, R(c) vs R(t) graphs might be more relevant for continuous cultures or repeated batch processes. Akin to the previous scenario, cells in section B exhibit stable production over time; however, unlike for section D, production varies greatly based on the condition tested (Fig. 3.5 bottom).

Regardless of the relationship considered, two points should always be taken into account. First, the relationships between strains are always relative: if a strain falls in section A when compared to a set of strains, it might instead fall in section C when compared to another set of strains. Second, the fact that a strain falls in section A does not necessarily mean that its performance is inferior to that of strains from other sections. Robustness itself simply offers an additional point of view from which to look at the data, but the desired strain must be chosen based on the intended goal.

The relationship between R(t), R(c), and R(p) can be further exemplified by considering three yeast strains exposed to different feast-starvation oscillations (50 and 1 mg/L of glucose, respectively). For each strain, R(c), R(t), and R(p) of three functions (ATP levels, cellular area, and circularity) were computed and compared to observe the relationships among them (Fig. 3.6). Ethanol Red presented the highest R(c), R(t), and R(p) with respect to ATP levels, indicating the most stable ATP content across populations, time, and conditions (Fig. 3.6B). Ethanol Red was previously shown to be better at maintaining a stable glycolytic flux compared to CEN.PK113-7D, ensuring stable ATP levels over time [30,171,172]. Ethanol Red's low population heterogeneity for multiple functions was observed also when the three strains were grown in different LHs (**Paper IV: Supplementary Fig. S9, Additional File 1**) [72]. In contrast, while Ethanol Red had the highest R(c) and R(t) with respect to cell area, it suffered from the highest heterogeneity. Instead, PE2 was strongly affected by the different conditions, but the cells maintained a stable size and circularity over time and across populations (Fig. 3.6C). Finally, CEN.PK113-7D was the one with the highest R(t), R(c), and R(p) (Fig. 3.6D). More details and considerations on strain comparison in feast-starvation experiments will be provided in **Chapter 4.3**.

3.2.6. Common strain response to stress conditions

When multiple strains are investigated in a large set of conditions, one can assess how stable the functions are in each condition across all strains, thus computing the robustness across systems, R(s) (Fig. 3.4). This type of robustness is particularly useful to determine the impact of each condition on different microorganisms and identify the most destabilising ones (**Paper IV**) [72]. Computing this value adds a level of consideration to the strain characterisation rather than just assessing the performance. For example, consider the glycerol yields of three yeast strains grown in Delft, OHH50, and SLRH50 media under aerobic conditions (**Paper IV: Fig. 3b**) [72]. In terms of performance, similar high glycerol yields were found for strains grown in Delft and SLRH50; while low yields were found in OHH50. However, a large difference in R(s) for glycerol yields was detected between Delft and SLRH50, with the former presenting higher R(s) values than the latter. This discrepancy suggested that, while strain performance was similar in these two conditions, the strains performed more uniformly in Delft than in SLRH50. Specifically, in SLRH50, some strains might have been overperforming while others underperforming. Even though glycerol yields in Delft and OHH50 were different, they had similar R(s) values. This suggested that all strains were equally affected by the condition, which caused yields to drop.

Such analysis offers a new point of view for understanding how different conditions affect the performance and physiology of strains, especially when using large datasets. Large datasets have already revealed differences in the performance of various strains in response to acids, alcohols, aldehydes, NaCl, and sugars [89]. Overall, large datasets are becoming increasingly important as a source of data to mine, as well as to grasp features that might have been hitherto overlooked.

3.3. Genetic markers of microbial robustness

Robustness is characterised by redundancy (different components or pathways perform the same task), dynamic control (regulation of a process by its components), and modularity (compartmentalisation of processes in modules) (**Paper III: Fig. 1**) [13,173]. Each of these principles is regulated by intricate pathways and multiple genes, which makes robustness a complex feature and complicates the identification of specific genes related to it. Nevertheless, the manipulation of individual genes may affect the performance and robustness of specific functions, thus pointing to the underlying pathways and/or metabolic processes. To determine the phenotypic outcome of each gene, high-throughput setups are necessary. Ever more sophisticated instruments and easier genome engineering techniques have facilitated the development and screening of libraries. Collections of strains such as gene-deletion libraries have been widely used to observe phenotypic changes caused by mutations and, consequently, discern the role of specific genes [174–176]. Phenotypic datasets have been crucial to understanding gene expression patterns [177], genotype-to-phenotype relationships [178] or trait correlations [179]. However, robustness analysis in these datasets has rarely been performed, making them a vast, yet underutilised source of knowledge.

A library containing single- and double-gene deletions as well as temperature-sensitive allele mutants (deriving from BY4741 or BY7092) has been used to better understand gene-gene, gene-environment, and gene-gene-environment interactions [180]. By growing the mutants (>4000) under 14 conditions, a phenotypic dataset measuring growth on agar plates was generated. This dataset was a potential source to mine and identify genetic markers related to robustness (**Paper V: Fig. 1**). Fourteen deletion mutants were selected for further characterisation based on whether the mutant had high robustness only, high robustness and high fitness (i.e. performance) or low robustness (**Paper V**). The 14 gene deletions were replicated in CEN.PK113-7D, and then investigated together with the parental strain and Ethanol Red (**Paper V**). As robustness is a very ample concept and is strictly connected to the perturbation space investigated, the 16 strains were screened in three different perturbation spaces. One was similar to the original one, mainly composed of antifungal agents and sugars. Another one included different combinations of malts, hops, aromas, and fining agents to resemble the beer fermentation processes. The last one mimicked 2G bioethanol production and contained acids, phenolics, aldehydes, salts, and sugars (**Paper V**). Following testing, two deletion mutants stood out, namely *met28* and *wwm1*. The WWM1 deletion mutant exhibited some of the lowest robustness values in each perturbation space, but presented a comparable specific growth rate as the parental strain and Ethanol Red, along with a shorter lag phase (Fig. 3.7 and **Paper V: Figs. 3-5**). On the contrary, the MET28 deletion

mutant had among the highest robustness values, but performed worse (longer lag phase and lower specific growth rate) than the parental strain and Ethanol Red (Fig. 3.7 and **Paper V: Figs. 3-5**).

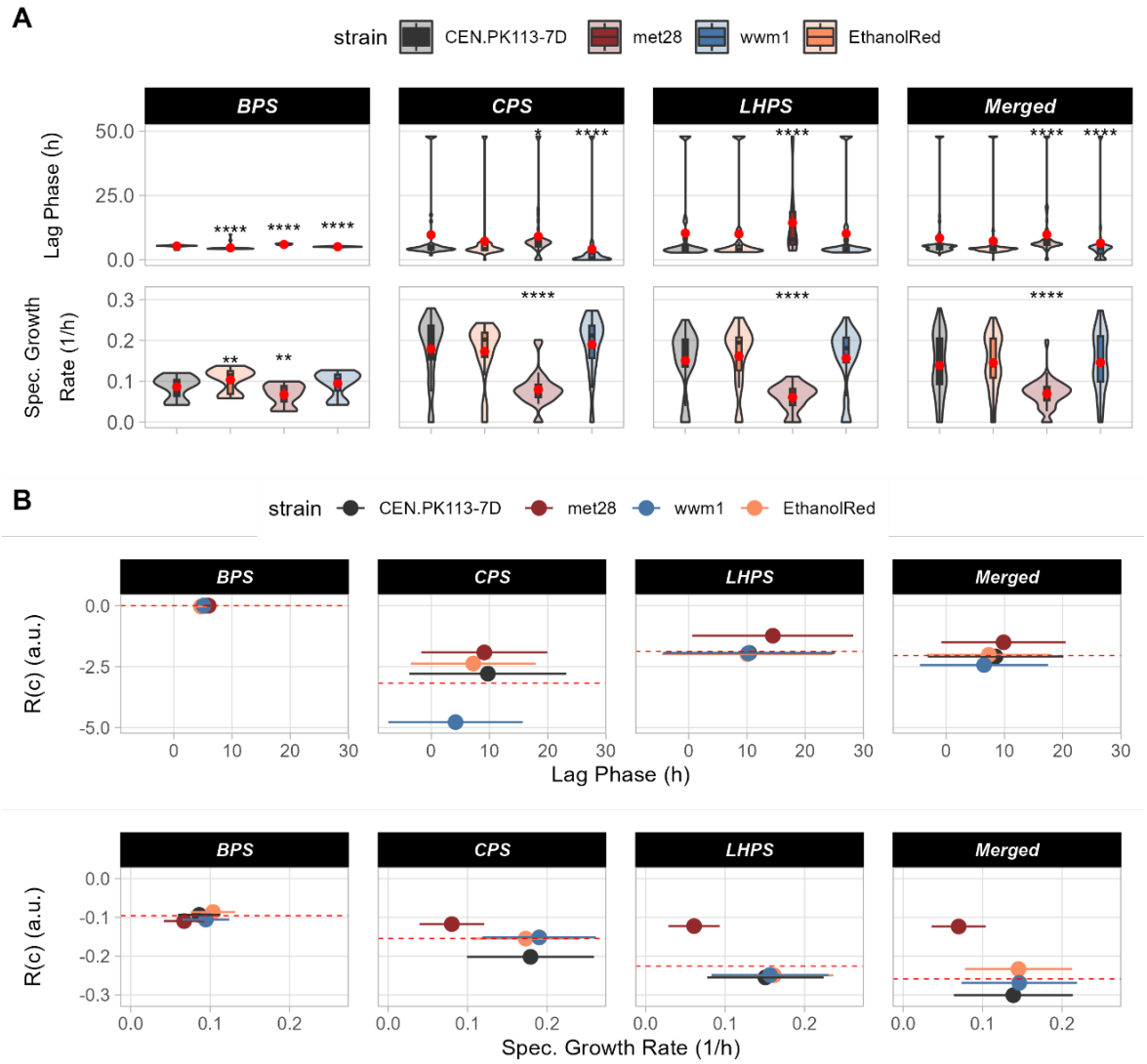


Figure 3.7. Strain comparison in multiple perturbation spaces. *Strain comparison among CEN.PK113-7D, its two single-gene deletions* met28 *and* wwm1*, and Ethanol Red. (A) Performance distribution of lag phase and specific growth rate in the individual perturbation spaces mimicking beer production (BPS), richness of antifungal agents (CPS), and LHs (LHPS), as well as across all of them (Merged). Wilcox test was performed to assess statistical differences between CEN.PK113-7D and each other strain; **p ≤ 0.01, and ****p ≤ 0.0001. (B) Scatter plots representing the correlation between lag phase and specific growth rate performance for the corresponding R(c). Horizontal lines represent the standard deviation of the function performance across all tested conditions. The dashed red line represents the average R(c) of all 16 strains screened. Data from Paper V (adapted for specific growth rate, unpublished for lag phase).*

The biological role of WWM1 is unknown, but it might be involved in apoptosis. While its overexpression blocks cells in G1 [181,182], its deletion leads to a shorter chronological lifespan and poor nitrogen utilisation [183,184]. Its regulation of the cell lifespan and cell cycle might explain the lower robustness observed with respect to lag phase and specific growth rate (Fig. 3.7). Met28 is a TF that controls sulphur metabolism together with Cbf1 and Met4 [185,186]. Sulphur metabolism involves multiple processes, including amino acids or glutathione synthesis as well as DNA replication, and correlates positively with the specific growth rate [187,188]. Slowing biosynthetic processes might slow down growth, allowing cells to use ATP and NAD(P)H+H^+ to better cope with multiple adverse conditions. For example, the deletion of MET28 might cause S-containing biomolecules to become a limiting factor in growth. Overall lower specific growth rate and longer lag phase result in more robust growth functions across different conditions (Fig. 3.7). Slow growth has been shown to be positive in some instances. Slow-growing bacterial subpopulations within a bulk population are less affected by antibiotics [146]; whereas in the case of yeast, they are less affected by temperature and acids [147]. Moreover, limiting growth and reducing metabolic costs was shown to boost lactic acid production in *Lactobacillus lactis* [189] and eGFP expression in *E. coli* [164]. Microorganisms grown in near-zero growth conditions are often used to study basal metabolism linked to maintenance, as cells are viable and active but limited by the scarcity of key compounds [190]. Zero-growth scenarios serve in bioprocesses to increase productivity, with the aerobic growth phase being used for biomass formation, followed by the production of the target molecule with maximal yield at high cell densities [168]. In both cases, growth is limited by environmental factors. Instead of using nutrient availability to hinder growth and decrease metabolic costs, the same could be potentially achieved by altering a genetic target. By repressing specific genes, cyanobacteria arrest growth and increase the output of lactic acid, n-butanol or ethanol [169,191].

Mining more phenomics datasets, screening gene deletions, and applying more perturbation spaces might unveil mechanisms underlying the robustness of functions. Strain background is certainly another key determinant, reinforcing the need to test deletion strains in multiple backgrounds and microorganisms to reveal universal trends. Although the identification of individual genes responsible for robustness is unlikely, single-gene deletions might uncover key mechanisms capable of improving future cell factories.

Chapter 4. Downscaling Reactor Dynamics

This chapter reviews ways to study reactor dynamic environments in the laboratory. The focus is on microfluidics single-cell cultivation, which allowed to downscale small time-constant dynamics to a picolitre scale. Its application and potential are discussed in combination with robustness quantification. A case study of feast-starvation oscillations is presented and then applied to industrially relevant conditions to compare three *S. cerevisiae* strains.

4.1. Mimicking and studying large-scale dynamics in the lab

The prevalent modes for cultivating microorganisms in the laboratory include microtiter plates, shake flasks, and lab-bench reactors. In these, environmental conditions are generally homogenous, stable, and controlled, allowing for only slow changes over time [23]. Constant conditions are achieved by control systems (pH or gas regulation in lab-bench reactors or use of pH buffers) or because of the small volumes employed (e.g. microtiter plates), which prevent gradients from forming. Dynamics in laboratory-scale cultivation conditions, such as changes in substrate and product concentrations, follow large time constants. Due to these slow dynamics, cells have time to adapt and respond. The same cannot be said for some industrial conditions, where insufficient mixing and large volumes cause the formation of substrate or pH gradients [24,25]. As cells move around the bioreactor, they experience rapidly changing environments, with dynamics in the order of seconds or minutes [24]. These conditions are poorly reproducible, requiring specific instrumentation. Moreover, data analysis relies on population-averaged measurements, which often neglect phenotypic heterogeneity [32]. To overcome these issues, experimental setups must attain single-cell resolution, as well as mimic the fast dynamics of large-scale cultivations.

4.1.1. Instruments for single-cell resolution analysis

Microscopy and flow cytometry are two of the most widely used techniques for single-cell analysis. Despite some similarities, they present many differences (Table 5). They can both detect and characterise cells as they are, without any manipulation, but they can also employ fluorescence to expand the range of research questions being investigated.

Table 5. Comparison between flow cytometry and microscopy.

	Flow cytometry	**Microscopy**
Throughput	High	Low / Medium
Speed of analysis	Rapid, thousands of cells in a short amount of time	Slow, manual image acquisition and processing
Resolution	Population, subpopulation, single-cell	Population, subpopulation, single-cell
Sample preparation	Simple and fast	Simple / Complex
Data type	Mainly quantitative	Quantitative and qualitative
Structural information	No / Limited	Yes
Automation	Common	Limited
Data analysis	Manual / Automated	Mainly manual
Single-cell tracking	No	Yes
Dynamics analysis	Limited	Yes
Cell sorting	Yes	No
3D analysis	No	Yes

Flow cytometry is a laser-based technology used for the quantitative analysis of single cells in suspension [192]. It measures multiple parameters, including cell size, granularity, and fluorescence intensity thanks to staining, dyes, and FPs. Through *at line* rapid analysis of thousands of cells per second, flow cytometry provides valuable insights at bulk-population level, but it can also discern cellular heterogeneity within populations [161,193]. Flow cytometry has been widely used to investigate *S. cerevisiae* at different scales, such as in flask cultivations for the identification of high glutathione-producing mutants [194], in 3-L continuous bioprocessing of spent sulphite liquor to investigate yeast physiology [195], and in 20-L fermentations to monitor yeast stress response [196]. Furthermore, it allows to sort and screen subpopulations based on the desired features [197], perform adaptive laboratory evolution [198] or develop biosensors [127]. Flow

cytometry has served as the springboard for multiple and more advanced techniques. ART-FCM allows for automated and *online* monitoring of cells with variable sampling frequency [104]. It was used to investigate the differences in growth and lipid accumulation in *Yarrowia lipolytica* caused by pH and/or aeration in bioreactors [199], the effects of acetate on growth and cell size in *S. cerevisiae* [200], and to select strains with improved tolerance to acetate [201]. Nevertheless, the use of ART-FCM in the laboratory remains limited and it has never been adapted for industrial scale due to the unavailability of some instrumentation parts (generally self-made) or the challenges associated with setting up and handling the systems [104]. Another technique is imaging flow cytometry, which combines flow cytometry with high-resolution microscopy, enabling the simultaneous analysis of cell morphology, structure, and fluorescence [202,203]. However, imaging flow cytometry has a much lower throughput than conventional flow cytometry. One of the biggest drawbacks for all types of flow cytometry is the impossibility of monitoring single cells over time, thus limiting the analysis of dynamic environments.

Microscopy is a powerful tool enabling the detailed visualisation and study of individual cells' morphology, behaviour, and interactions. Thanks to light microscopy, scientists can observe microbial replication, membrane and cell wall structures, and intracellular organelles, such as the nucleus and vacuoles [204]. Fluorescence microscopy uses stains, dyes, and FPs to quantitatively and qualitatively assess the desired parameters [204,205]. These include protein localisation [206], morphological changes to different organelles, such as mitochondria or vacuoles, in response to varying conditions [207,208], and yeast biofilms' intricate structure [209]. More advanced techniques allow for very high-resolution imaging of individual cells. Confocal microscopy offers 3D and 4D (3D over time) reconstruction of yeast cells [210–212]. Atomic force microscopy enables the investigation of surfaces, such as cell walls or membranes. In fact, this technique revealed heat-shock-induced circular structures on the yeast surface [213]. In more sophisticated setups, *online* monitoring of morphology and budding events over time has been achieved [105]. However, one of the biggest advantages of microscopy is the possibility to visualise and track cells and intracellular behaviours with single-cell resolution [214], as has been done for intracellular pH [215] or ATP concentrations [119].

The above techniques nevertheless remain only tools to analyse cells grown in other setups. In **Chapter 4.1.2**, two techniques that allow the study of dynamic environments without having to change setup are presented, namely scale-down reactors and microfluidics.

4.1.2. Cultivation systems simulating dynamic environments

Scale-down reactors and microfluidics possess distinct features and, in different ways, support the investigation of dynamic environments (Table 6). While both have been used for around 40 years and are constantly evolving [216,217], microfluidics entered the biology/biotechnology field only some 20 years ago [218].

Table 6. Comparison between scale-down reactors and microfluidics.

	Scale-down reactors	**Microfluidics**
Principle	Replication of large-scale systems	Controlled manipulation of fluids
Throughput	Low	High
Resolution	Mainly population-averaged	Single-cell
Growth conditions	Batch to continuous	Batch to continuous
Spatial information	Limited	Detailed
Automation	Limited	Possible
Scale	Medium / large volumes (mL–L)	Small volumes (fL–nL)
Time-constants	Large (hours)	Small to large (seconds – hours)
Parallelization	Limited	High
Real-time analysis	No / Limited	Yes
Single-cell tracking	No	Yes
Application flexibility	Specific applications (process development, optimization, and understanding large-scale behaviour)	Diverse applications (Used for a wide range of questions in various fields, including biology, chemistry, and diagnostics)

Scale-down reactors are designed to emulate large-scale bioprocessing conditions in a controlled environment, providing valuable insights into bioreactor dynamics, microbial growth, product formation, and metabolic activity [34,219]. In scale-down reactors, the culture is circulated between a main tank and additional compartments/vessels (one or more) with different conditions to represent large-scale operations [24]. Various studies have been conducted in *S. cerevisiae* to investigate population heterogeneity [220], the impact of oxygen oscillations on growth and sterol biosynthesis [221], and the role of cation concentrations and pH regulation in energy management under varying CO_2 pressures [222]. However, when operating scale-down reactors, some limitations arise. First, the data obtained are generally population-averaged. Even though it is possible to combine scale-down reactors with flow cytometry to achieve single-cell resolution, real-time

measurement of cells is rare. Fast quenching of cellular activity and methods for the extraction, quantification, and analysis of the desired parameters (e.g. metabolites, DNA, and mRNA) are essential, thereby prolonging and complicating data collection. More importantly, a limited number of conditions can be tested; whereas, in real large-scale processes, the number and extent of different zones change over time and space [24].

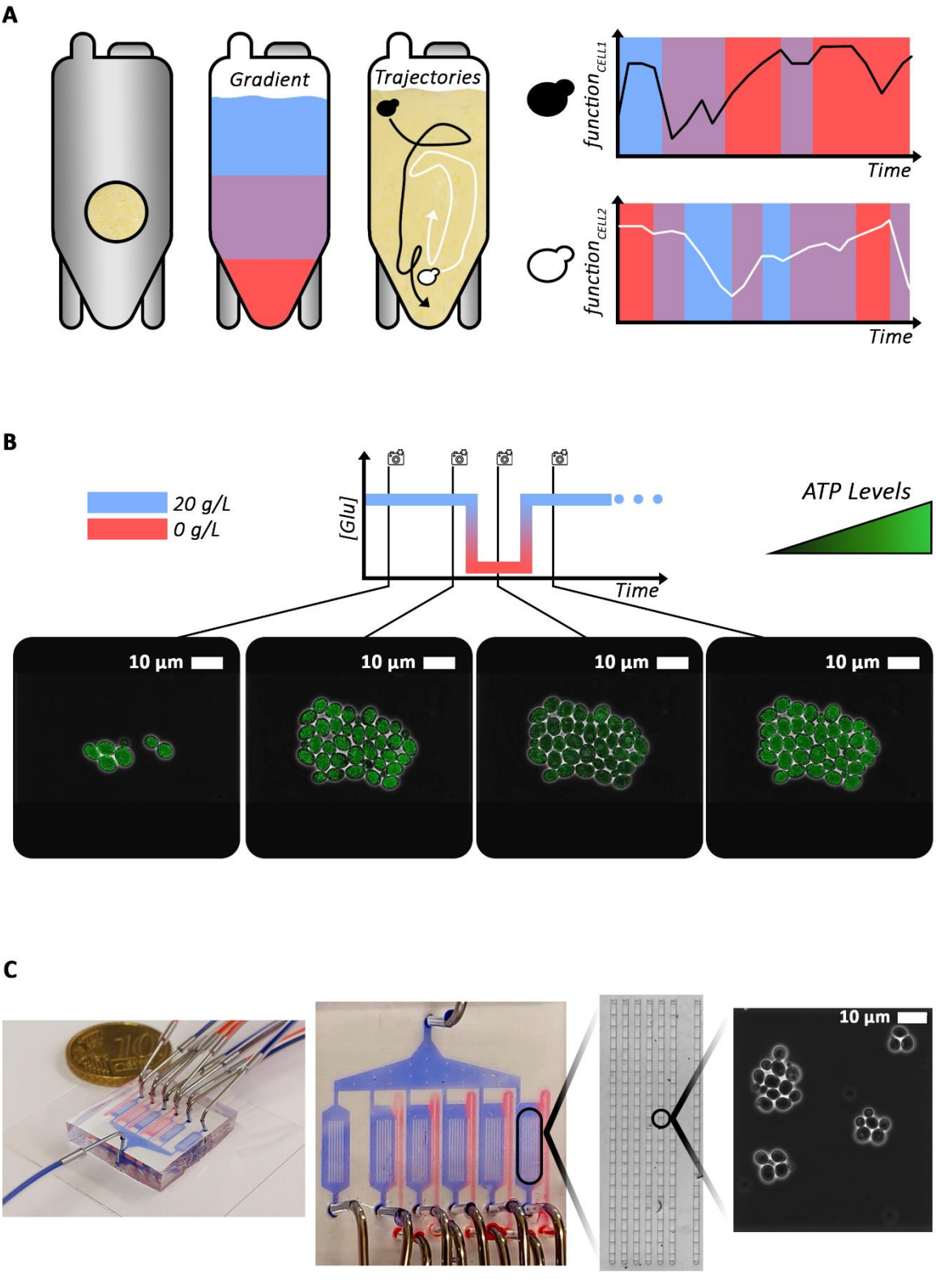
Microfluidics allows for the manipulation and control of small volumes of fluids at the microscale [217]. Miniaturized devices known as microfluidic chips enable the precise handling of liquids and particles, for applications in biology, chemistry, medicine, and engineering. These devices utilise microchannels, often on the scale of µm, to conduct experiments, perform chemical reactions, or analyse biological samples with high efficiency and accuracy [217]. Microfluidics is suitable for automation, high parallelisation, and high throughput thanks to rapid analysis [223]. Moreover, owing to live-cell imaging, individual cells can be followed over time, thereby supporting the investigation of growth or protein expression [214]. Based on how the microfluidic chip is structured, very different growth patterns (0D, 1D, 2D, and 3D) and cultivation types (batch, fed-batch, and chemostat) can be achieved [223]. In yeast, microfluidics setups have been used successfully to investigate single-cell gene expression over time [224] or ageing-associated changes [225], but also to simultaneously screen thousands of strains in a chemostat array [226]. Moreover, thanks to tracking tools, one can follow cells over time and reconstruct the mother-daughter lineage [227]. Using tailored setups, dynamic environments, whose conditions vary with small time constants can be explored (more on this in **Chapter 4.2**) [218]. Nevertheless, even microfluidics come with limitations. Live-cell imaging may trigger phototoxicity by promoting the generation of reactive oxygen species inside cells [214]. Moreover, it is not possible to extract the cells from the microchip to perform further characterisation or single-cell omics studies, and the maximum number of cells detected is limited by the size of the chamber used.

Although both techniques are extremely valuable and present compelling advantages, the research questions presented in this **thesis** were best addressed by microfluidics, which allowed the investigation of small time-constant dynamics, single-cell tracking over time, and the implementation of multiple conditions simultaneously.

4.2. Bioreactor dynamics at picolitre scale

Microbial lifelines are often used to describe from a spatial, extracellular or intracellular point of view what a cell experiences in a set environment over time [228]. Due to gradients in pH, gas, and substrate [24,25], cells experience different environments when growing within a large-scale bioreactor (Fig. 4.1A). These perceived environmental changes are generally in the order of seconds or minutes [24]. Replicating rapid changes in a laboratory setup is challenging because there must be sufficient knowledge of i) the trajectory of cells (spatial lifelines), ii) the environments perceived by each cell over time (extracellular lifeline), and iii) the physiological cell responses (intracellular lifeline). To experimentally identify the different spatial and extracellular lifelines, flow-following particles are generally used, such as radionuclides or Lagrangian sensors [229,230]. As the real-time tracking of individual cells and their physiological responses inside a reactor is impossible, spatial and extracellular lifelines are generally analysed by *in silico* models to predict cellular behaviour. The preferred approach is computational fluid dynamics, a branch of fluid mechanics that employs models and algorithms to simulate and analyse fluid flow and interactions in various systems [231,232]. Different types of models provide information on residence times, metabolic regimes, and yield/rate optimisations [33,233–235]. To experimentally assess the response to dynamic environments, the setup should be able to i) change the extracellular environment within seconds, and ii) monitor individual cells and their physiological behaviour over time. Microfluidics in combination with live-imaging microscopy is the most suitable technique to meet the second requirement, but some adaptations are required to meet the first one, too. Microfluidics has been used successfully to study gene expression in yeast at different resolution levels in dynamic environments [236], albeit with a limited throughput.

Figure 4.1. Microbial lifelines and dynamic microfluidics single-cell cultivation (Next Page ▶). *(A) Visual representation of lifelines cells experience when grown in a large-scale reactor with gradients. Spatial lifelines refer to the trajectory of cells in space. Extracellular lifelines encompass all the environments perceived by each cell over time (in the background of the graph). Intracellular lifelines refer to how specific functions (e.g. ATP levels) change over time. (B) Example of yeast cells grown in a dMSCC chamber with one starvation pulse, while monitoring ATP levels. (C) Pictures of a dMSCC chip and its compartments. Pictures of (left to right) chip overview, setup overview, a single region, and a chamber. Each chip has six regions, one with constant conditions (positive control), and five with switching zones. Each region has seven arrays, each containing 23 chambers. For the five switching regions, one array is always devoted to constant conditions (negative control).*


A
Gradient
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10 µm

Dynamic microfluidic single-cell cultivation (dMSCC) implements media switching within a few seconds due to pump-driven shifts in laminar boundary layers [237,238]. Thanks to live-cell imaging, the cells' growth and intracellular responses can be detected in real-time (Fig. 4.1B). Applications have included studies of *C. glutamicum* in feast-starvation [237] or pH [239] oscillations. The dMSCC setup was adapted for yeast cultivation and further improved to assess multiple oscillation frequencies simultaneously, thereby increasing the throughput (Fig. 4.1C and **Paper VI: Fig. 1**). Each chip was composed of six regions, each containing a total of 138 chambers, whereby cells were trapped and grown in a 2D monolayer (Fig. 4.1C). When analysing dMSCC image data, segmentation and tracking are crucial. Segmentation refers to the identification of individual cells and can be achieved via simple thresholding or more sophisticated neuronal network training [240]. Tracking refers to the process of monitoring individual cells over time and establishing mother-daughter relationships (lineage reconstruction). This task is difficult in 2D growing populations, so microfluidics setups generally monitor trapped mother cells while flushing away daughter cells [241], or allow cells to grow only in one direction in a narrow chamber for easier bud identification [227,242]. Moreover, thanks to the high throughput of dMSCC, automation becomes a necessity to analyse the large datasets generated. Therefore, a user-friendly semi-automated pipeline combining Fiji [243] for image analysis and R [244] for data analysis was developed (**Paper VI: Fig. 2**). Cell segmentation and tracking were achieved with the Fiji plugins Stardist-2D [245] and TrackMate [246], respectively, thereby overcoming the limitations of 2D growth.

4.3. Yeast response to fluctuations in carbon source

4.3.1. Combination of dMSCC and robustness quantification

Substrate gradients are extremely common in large-scale bioreactors [24]. These gradients alter substrate availability, which induces different metabolic responses in cells. Simulations in a bioproduction scenario suggested that yeast cells would experience substrate limitation conditions for 39% of the lifeline, conditions allowing for overflow metabolism for 3% of the lifeline, and severe starvation for 58% of the lifeline [30,247]. Therefore, exploring how cells experience and respond to these changes is of vital importance when developing or selecting a strain for industrial applications. Studies of cells exposed to feast-starvation oscillations in chemostats or scale-down reactors [30,171,172,248] have been constrained by population-averaged measurements, narrow fluctuation frequency, and limited screening throughput. Owing to time-resolved data output with single-cell resolution, dMSCC can complement the above studies, while also elucidating phenotypic population heterogeneity. Moreover, the same data can be easily used with equation 1 to assess different types of robustness of the desired functions alongside their performance.

One physiological function (intracellular ATP), two growth functions (budding ratio and specific growth rate), and two morphology functions (cellular area and circularity) of CEN.PK113-7D were monitored in feast-starvation oscillations (20 and 0 g/L glucose, respectively) over the course of 20 h using dMSCC (**Paper VI**). Together with a constant “feast” control condition, five symmetric oscillation frequencies ranging from 1.5 to 48 min were assessed, encompassing different biological timescales [249]. Longer oscillation frequencies were associated with a lower specific growth rate/budding ratio and smaller and rounder cells compared to short oscillations (Fig. 4.2A and **Paper VI: Fig. 2B**). Decreased specific growth rate/budding ratio in oscillating environments were probably due to increased demand for maintenance by CEN.PK113-7D in these environments [30,172]. Interestingly, the longer the oscillations, the higher the ATP level, with cells in 48-min oscillations displaying the highest intracellular ATP (Fig. 4.2A and **Paper VI: Fig. 2B**). However, when analysing R(t) and R(p), cells in 48-min oscillations showed the most unstable ATP levels and growth over time, as well as the highest level of population heterogeneity (Fig. 4.2B-C and **Paper VI: Fig. 6-8**). Due to a mutation in the CYR1 gene, CEN.PK113-7D does not mobilise carbon storage pools (e.g. trehalose) to maintain a stable glycolytic flux, likely affecting ATP levels over time [30,68]. Moreover, longer oscillations might favour the proper activation of pathways aimed at optimising energy production and usage in cells, thereby improving overall ATP availability [250,251].

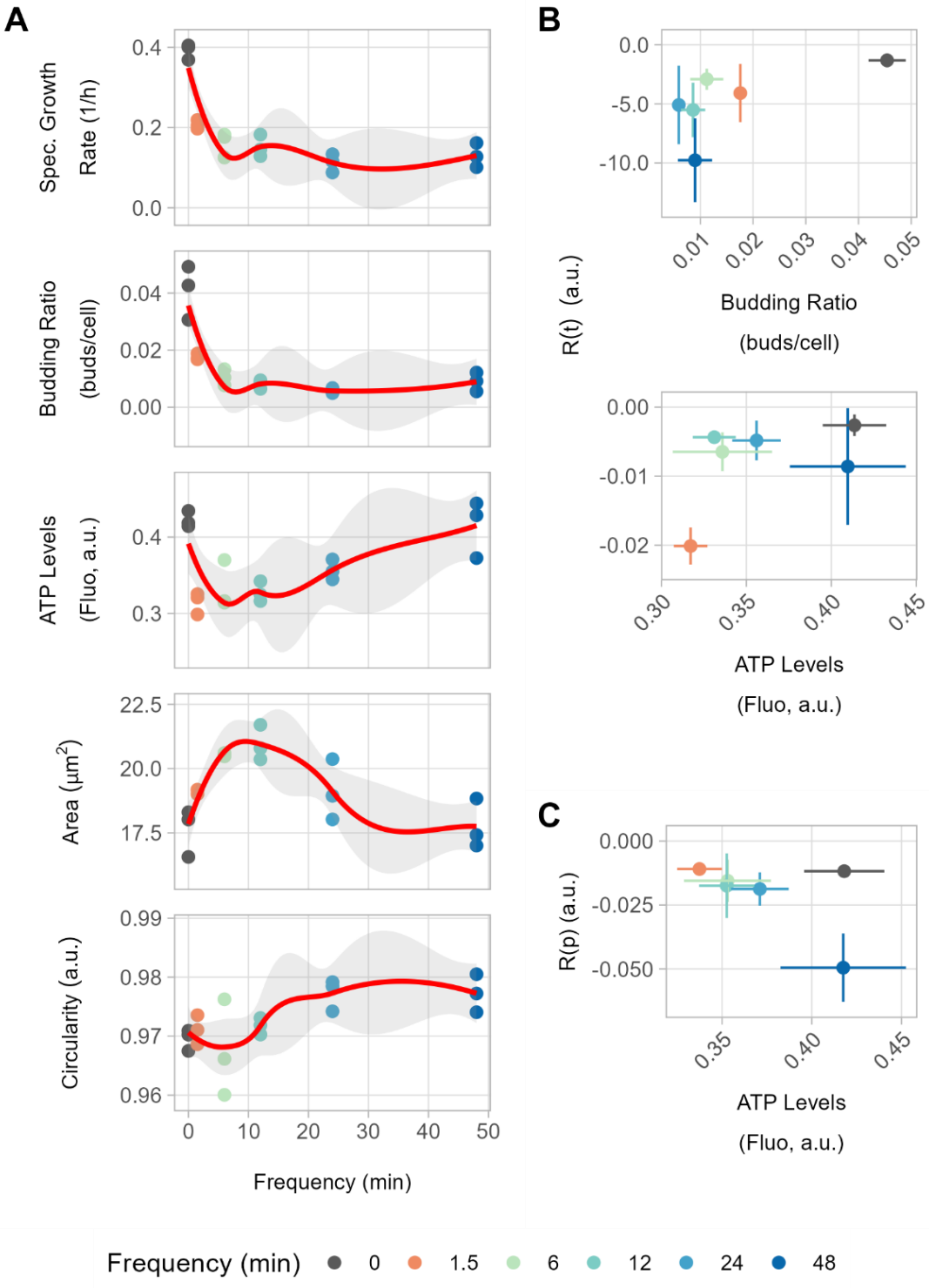

Figure 4.2. Function Performance and robustness of CEN.PK113-7D in feast-starvation oscillations. *(A) Trends with confidence interval (red lines with grey shadow) in performance for five functions of CEN.PK113-7D correlated with the length of feast-starvation oscillations. (B, C) R(t) at population level (B) and R(p) (C) of budding ratio and ATP levels in relation to the corresponding performance for each oscillation frequency. Data adapted from Paper VI.*

4.3.2. Strain comparison in feast-starvation oscillations

Industrial substrate concentrations might surpass 100 g/L, thus triggering osmotic stress in cells [35]. However, due to the presence of gradients in bioreactors, cells might experience different concentrations, including little to no substrate [24]. Therefore, besides different metabolic regimes, different degrees of osmotic pressure will be present. In feast-starvation studies carried out in chemostats, fed-batches or scale-down reactors, cells are generally subjected to substrate limitation or low glucose concentrations even during the "feast" condition. Moreover, strains are rarely compared, as only one is usually investigated. Here, dMSCC was used to study the three *S. cerevisiae* strains CEN.PK113-7D, Ethanol Red, and PE2 in feast-starvation oscillations. Notably, two changes in the setup were made with respect to **Paper VI**. First, the feast condition was based on 50 g/L glucose to better mimic elevated industrial substrate concentrations; whereas starvation was achieved with 1 mg/L glucose, which allowed cells to perform one or two replications within 16 h, but not die (data not shown). Second, the 12-min oscillation was replaced with 0.75 min to better simulate the rapid dynamics in the reactors.

When checking R(c), Ethanol Red exhibited the most stable budding ratio, ATP levels, and area, CEN.PK113-7D had the most stable circularity, and PE2 was the least stable for most functions (Fig. 4.3A). Whereas budding ratio and ATP levels were comparable across all strains; morphology descriptors varied substantially, with Ethanol Red having the largest and less circular cells overall (Fig. 4.3). The high R(c) for growth by Ethanol Red was in line with other screenings (**Paper IV**) [72]. Ethanol Red is more resistant to fluctuating environments and can adapt more easily than CEN.PK113-7D [30,171,172]. Specifically, Ethanol Red can mobilise the storage carbon pools (mainly trehalose) more quickly than CEN.PK113-7D [30,171,252]. The difference in carbon storage mobilisation is due to a mutation in the CYR1 gene in CEN.PK113-7D, which delays this process [68]. The easier mobilisation of storage carbon pools probably allowed Ethanol Red to have more stable ATP levels over time, as suggested also by R(t) values being higher than for CEN.PK113-7D and PE2 (Fig. 4.3B). Interestingly, Ethanol Red cells became rounder over time regardless of the oscillation they were facing (data not shown), while CEN.PK113-7D and PE2 had generally stable circularity, but a less stable area (Fig. 4.3B). Both PE2 and Ethanol Red were also bigger than CEN.PK113-7D, probably due to a higher ploidy than CEN.PK113-7D [253]. Ethanol Red had the lowest levels of population heterogeneity for ATP levels, but the largest heterogeneity for cell area (Fig. 4.3C). Finally, its low heterogeneity with respect to glycolytic flux in different hydrolysates further confirms stable ATP levels in this strain (**Paper IV: Supplementary Fig. S9, Additional File 1**) [72].

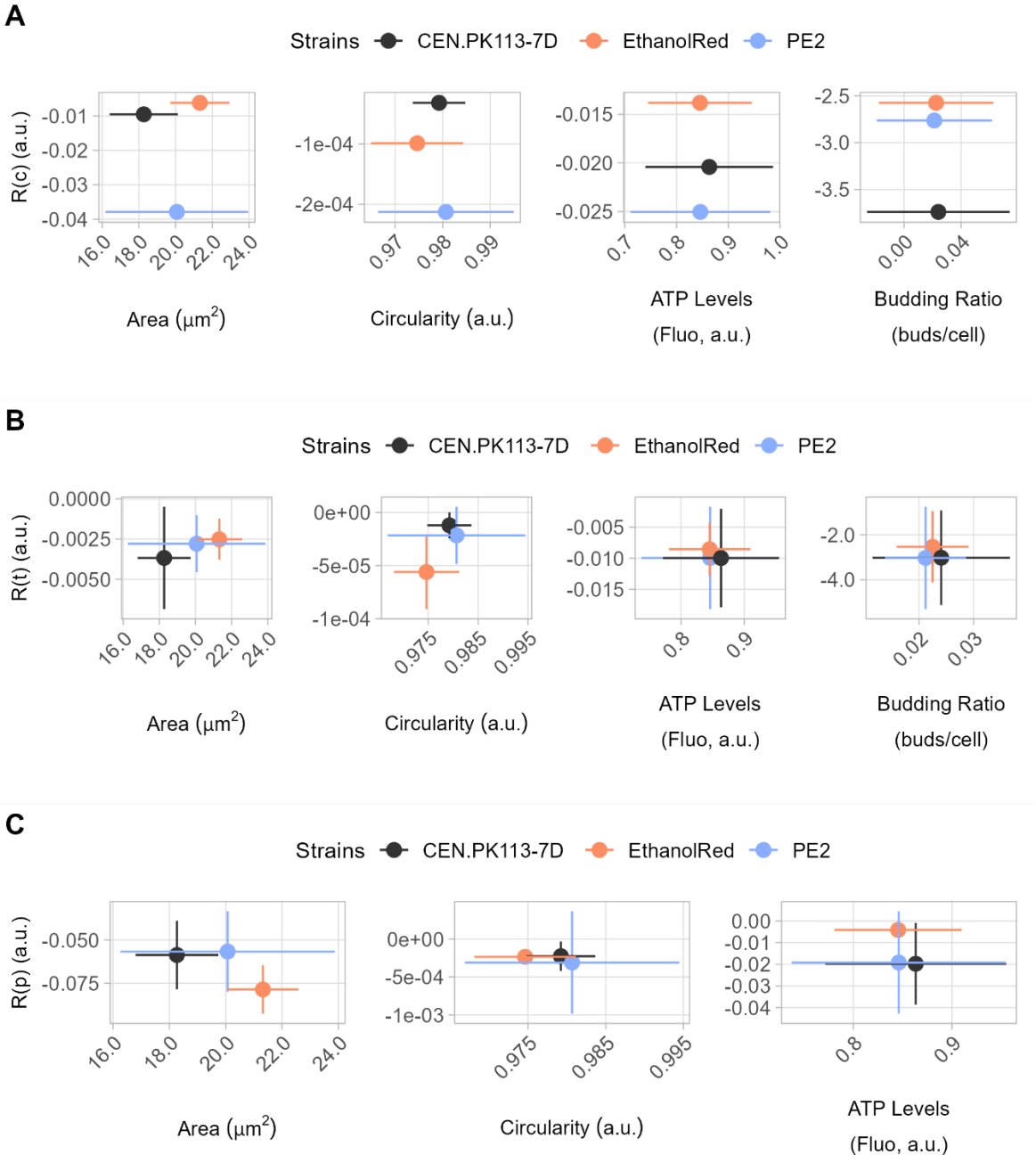

Figure 4.3. Relationship between function performance and different robustness types. *Three* S. cerevisiae *strains were grown under feast-starvation oscillations for 16 h and four functions were monitored (budding ratio, area, circularity, and ATP levels). For each function, the performance is presented in correlation to (A) robustness across conditions R(c), (B) robustness over time R(t), and (C) robustness across populations R(p). Unpublished data (Torello et al. 2024).*

Chapter 5. Conclusions and Outlook

This chapter summarises the main conclusions of the thesis and puts it in perspective with existing state-of-the-art bioprocesses. The chapter ends with a final outlook on future application and uses of the developed tools.

5.1. Conclusions

In this **thesis**, a series of tools were developed to improve the investigation of physiology and robustness within bioprocesses. The *Sc*EnSor Kit allowed for easy real-time monitoring of the yeast intracellular environment. Robustness quantification expanded the concept of robustness itself and revealed different aspects of function stability. A workflow combining robustness quantification and dynamic microfluidics single-cell cultivation (dMSCC) was set up to better investigate dynamic environments, especially the ones with small time constants. These tools were applied to study three *S. cerevisiae* strains in a 2G biofuels bioproduction scenario and address the following research questions posed in the **thesis** (**Chapter 1.3**).

How do microorganisms respond to the complex and dynamic environments found in large-scale bioreactors? The versatile and easy-to-use *Sc*EnSor Kit was developed to investigate the physiological responses of yeast cells and address the above question (**Papers I** and **II**). This kit uses fluorescent biosensors to easily monitor eight intracellular parameters in real-time, namely intracellular pH, ATP, glycolytic flux, oxidative stress, unfolded protein response, ribosome abundance, pyruvate metabolism, and ethanol consumption. In **Paper IV**, the toolkit along with standard specific growth rate and product yield measurements were used to investigate the responses of one laboratory strain (CEN.PK113-7D) and two industrial strains (Ethanol Red and PE2) to seven different LH substrates used for 2G biofuel production. These substrates are rich in sugars and contain a mixture of inhibitory compounds that affect microbial performance [52]. Therefore, these experiments reflected the complexity of LH substrates better than the more canonical microorganisms' characterisation performed in media containing single inhibitors. Ethanol Red stood out as a highly performing strain with robust growth functions, especially in oxygen-limited

conditions. Aerobic screenings pointed to PE2 having the most robust growth functions, although it was still outperformed by Ethanol Red. Substrate- and growth-phase-specific responses were identified. For example, increased OxSR and UPR were seen in non-woody and woody LHs, respectively. Most intracellular parameters were less stable during lag phase than exponential phase, highlighting how the former helps cells adapt to the new environment, in which metabolism is reset [138]. All physiological responses assessed in that part of the work follow large time-constant dynamics, thus happening in the order of hours or days. However, due to gradients formed in large-scale bioreactors, cells are also exposed to dynamics in the order of seconds to minutes [24]. To address these dynamics, a dMSCC setup was employed and the response of CEN.PK113-7D to feast-starvation oscillations was investigated (**Paper VI**). Application of oscillating environments reduced the specific growth rate and caused oscillation-dependent physiological responses and morphological changes, such as cells becoming rounder and smaller with longer oscillations or triggering pseudohyphal growth in oscillations of 1.5 and 6 min. Interestingly, longer oscillations led to higher intracellular ATP levels, probably because they induced metabolic and physiological rearrangements aimed at improving energy usage.

How can predictable and stochastic large-scale perturbations be studied at small scale?
One of the most common ways to investigate predictable perturbations (i.e. inhibitory compounds, pH, and temperature) is with high-throughput screenings, such as 96-well plates or setups allowing for the monitoring of multiple flasks simultaneously. In **Paper V**, two perturbation spaces deconstructed beer and 2G biofuel production into individual stresses. With this approach, it is possible to highlight, which conditions mostly affect the performance of desired functions. However, when the industrial setting encompasses a complex mixture of compounds and conditions, information on synergistic and antagonist effects is lost. Therefore, the response of two industrial and one laboratory yeast strains was assessed in a series of real LHs (**Paper IV**). Notably, as batch variability for these substrates is very high, the experiments are poorly reproducible across laboratories or once a new substrate batch needs to be prepared. Therefore, these complex substrates were mimicked by chemically defined model media containing a mixture of the main inhibitory compounds (**Paper I**). Other valid techniques to investigate predictable perturbations include bench-top bioreactors run as batch, fed-batch, and continuous cultures. They all guarantee better control of culture conditions and easy access to -omics investigations but are limited by the throughput of generated data.

While the above-mentioned screenings are widely used to investigate predictable perturbations, stochastic perturbations remain less studied. A known stochastic perturbation is the presence of gradients in large-scale bioreactors [24]; yet, it is one of the least investigated as there is no

instrumentation to faithfully mimic it at a small scale. To overcome these challenges, dMSCC was applied [237]. Owing to the ability to precisely control conditions and quickly switch them, dMSCC offered the means to investigate the response of CEN.PK113-7D to feast-starvation oscillations ranging from 1.5 to 48 min via live-cell imaging (**Paper VI**). Other stochastic perturbations, such as the quality of seed cultures, contaminations, mutations, and ageing [13], have not been investigated in this **thesis**. Nevertheless, they play a crucial role in bioprocesses, such as the positive effect on performance caused by short-term adaptation of seed cultures [135,140], the negative impact of *Lactobacillus* contaminations [254] or the emergence of mutations [255,256], and the differences in performance due to ageing of cells [22]. Although the investigation of those stochastic perturbations might not be as straightforward as that of predictable perturbations, it could be done by tweaking and adapting the techniques used in this thesis or elsewhere (e.g. bench-top bioreactors).

How can microbial robustness be defined and applied in academic research or in industry?

The term robustness has been used in different contexts over the years. In **Paper III**, robustness was defined as "*the ability of a system to maintain a stable performance in the face of perturbations*" and was distinguished from tolerance [13]. Thanks to Trivellin's robustness quantification method [69], the concept of robustness was then expanded in **Paper IV** to denote the "*stability of performance*" and explore different aspects of function stability. Besides assessing the stability of functions for multiple strains across different perturbations, Trivellin's robustness equation also helped identify the degree of population phenotypic heterogeneity, the stability of functions over time, and the impact of different conditions on strain metabolism (**Paper IV**).

In another approach, a phenotypic dataset of deletion strains was used as a starting point to identify genetic markers of microbial robustness (**Paper V**). As robustness is a function-specific feature highly dependent on the system considered, pointing out specific universal genes might be challenging. However, screening more strains and analysing other libraries and datasets might lead to the discovery of key genes important for performance and robustness.

In this **thesis** and other key publications [13,69,89], the emphasis was always on robustness not being a measurement of performance itself, but only of its stability. Whether the robustness of a function is a desired feature or not depends on the circumstances, the function, and the final goal. For example, a strain with a very low product yield but stable across different conditions might not be of interest to someone who is screening solely for the best-performing strain. Instead, selecting a strain with stable ATP levels across populations and conditions might be of interest when screening for the bioproduction of a secondary metabolite (generally elevated ATP demand). In

fact, knowing that ATP levels would not be a problem for that strain, might allow more flexibility in engineering the production of heterologous proteins and other products.
In general, obtaining a strain with only robust functions is practically impossible. First, robustness is a relative feature highly dependent on the specific system. Moreover, the robustness of one function might come at the expense of another one. Ethanol Red was shown to have a more stable glycolytic flux in feast-starvation oscillations than CEN.PK113-7D, as it could quickly mobilise trehalose [30,171]. Therefore, Ethanol Red has a more robust glycolytic flux than CEN.PK113-7D because the concentration of trehalose is not robust [30,171]. However, if the goal is selecting a strain with more stable trehalose concentrations over time, then CEN.PK113-7D becomes the candidate of choice. Overall, robustness quantification offers new insights into the systems investigated and how strains are screened, selected, and improved for industrial applications.

Concluding remarks. Altogether, the innovative tools and methods presented in this **thesis** address the challenges of scaling up bioprocesses while offering a new perspective on strain characterisation and physiology for both academic and industrial investigations. The parallel analysis of multiple resolution levels (population, subpopulation, and single cells) allowed for a better understanding of microbial performance and robustness in complex environments. Moreover, both the *Sc*EnSor Kit and the robustness equation can be used in a wide range of experimental systems other than the ones employed in this **thesis**. Strains developed with the *Sc*EnSor Kit can be grown in all cultivation systems and fluorescence can then be detected with other instruments, such as flow cytometry. The robustness equation can be applied to any setup with at least two systems, a function, and a series of conditions. Overall, the tools presented in this **thesis** can advance our understanding of microbial performance and robustness in complex environments to optimise bioproduction processes and their reliability.

5.2. Outlook

Considering the key role of bioprocesses in current as well as future socio-economic contexts, it is crucial to keep up with demand for increasingly more efficient bio-based industries. This requires constantly upgrading and evolving the ways bioprocesses are studied and developed, thus approaching this field with new perspectives and points of view. This PhD project started from the desire to explore more deeply "microbial robustness", a concept that is often talked of but rarely defined clearly. For this reason, this **thesis** focused predominantly on developing tools that would have allowed easier exploration of all the different categories that microbial robustness encapsulates (Fig. 5.1). Now that some tools for the quantification of robustness have been developed, downscaling bioprocesses and easy monitoring of yeast intracellular environment will help tackle new research questions.

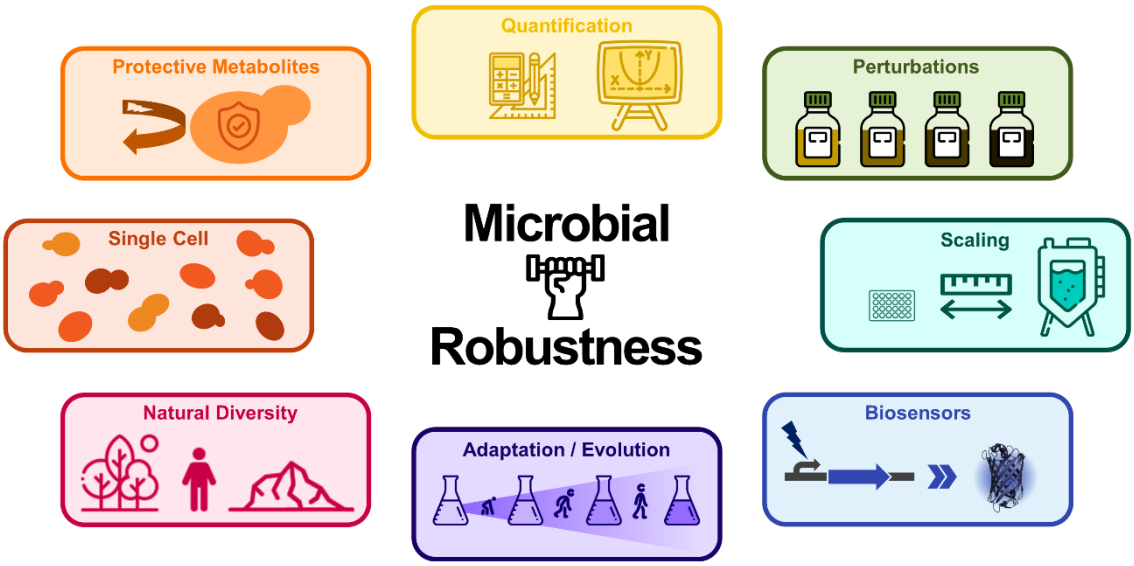

Figure 5.1. Future applications of microbial robustness. *Overview of the fields that can be explored within microbial robustness. Some of the fields, such as biosensors, single cells, and scaling, have been investigated in this PhD project, whereas others remain to be explored, such as natural diversity or protective metabolites.*

New biosensors are constantly being developed. The *Sc*EnSor Kit was built as a dynamic tool, in which new biosensors accommodating the desired needs could be easily implemented (**Paper II**) [111]. Following metabolite or protein profiles over time is crucial for accurate physiological studies. At present, transcriptome, proteome, and metabolome studies are limited in high-throughput setups, such as microtiter plates or microfluidics, due to the small number or inaccessibility of cells.

Biosensors can be developed and/or incorporated into the *Sc*EnSor Kit, thereby avoiding the need to extract cells. At the same time, they can offer real-time monitoring of key metabolites, such as trehalose, glutathione or ergosterol [257–260]. These molecules have become known as "cell protectants" because their presence has been associated with higher tolerance to stress [261–263]. Therefore, having tools to easily monitor them might reveal their association with robustness.

Tagging FPs to proteins with pivotal roles in specific contexts is another way of investigating physiology and robustness. For example, Rim15 can predict cell fate prior to exposure of yeast cells to starvation [264]; whereas proteins of the cyclin family can be used to distinguish between replicating and non-replicating cells [265]. Among non-replicating cells, there is a wide range of metabolic states, such as proliferation-arrested but metabolically active, senescent, or quiescent, all characterised by specific features that can be tracked via fluorescent reporters [266]. Tracking, characterising, and understanding these phenotypic differences in relation to production phenotypes might be valuable to further improve bioprocesses. During brewing, aged cells ferment at a higher rate than virgin or mixed cell cultures [22]. Biosensors aimed at monitoring the production of a desired compound are crucial in the context of bioproduction to screen strain libraries [98,267,268] or study phenomena such as phenotypic heterogeneity [157]. Moreover, the combination of these tools with Trivellin's robustness equation can lead to an easier evaluation of production hosts, thus directing strain selection/improvement and increasing throughput.

In this **thesis**, only a few strains have been characterised, as the focus was mainly on tool development. Therefore, expanding the analysis performed here to more strains would be the next step in identifying patterns and gaining a more complete overview of physiology and robustness. For example, trade-offs between robustness and performance of specific functions have been identified by analysing 24 *S. cerevisiae* strains [89]. Given that robustness is a complex feature, numerous studies are required before achieving a standardised protocol to easily and quickly "improve robustness". One interesting topic to explore would be the correlation between evolution and robustness. Often, when evolving a strain for a desired feature, only the final strain is carefully characterised and is generally defined as "superior" to others just because it was selected to optimise one specific feature. However, screening and characterising intermediate stages with the same meticulousness would be of high interest. For example, while evolving a strain to improve its product yields, the final strain would be selected just because of its performance. However, intermediate stages might give more robust product yields across different conditions at the cost of lower production, which might be of high interest, too. Advances in molecular and synthetic biology have made it easier to rationally engineer strains to increase product yields and rates. However, improving strains for tolerance and robustness is more difficult, probably due to the

complex nature of these two features. Therefore, obtaining a strain with inherently advantageous features might be considered of higher value than a better-producing one. Similarly, exploring natural diversity is another topic that would lead to a better understanding of the processes regulating robustness. New strains with peculiar features are constantly isolated from nature [269–273]. They represent a valuable source of knowledge for the identification of key robustness or tolerance pathways that can be mimicked in current production hosts. This might also lead to the discovery of new "cell protectants".

One of the most promising techniques for studying bioprocesses is dMSCC. Even though the oscillations tested in this **thesis** centred on feast-starvation of glucose, other key nutrients (such as nitrogen) or pH could be tested. Gas gradients are also common in bioprocesses, and they could affect productivity [24]. However, as the dMSCC chip is made of PDSM, it is still not possible to switch media with different gas compositions. Finally, only symmetric oscillations were applied in this **thesis** work, but asymmetric oscillations might better mimic the extracellular lifelines of cells in large-scale bioprocesses, where they experience different metabolic regimes [33]. Moreover, the data output generated from dMSCC experiments would be of high interest also to computer scientists for improving existing metabolic *in silico* models. For example, rather than considering only population-averaged predictions, single-cell predictions accounting for the levels of heterogeneity in the bulk population can be performed. As individual cells are tracked over time, key processes or conditions can be identified to better understand the genesis of subpopulations and their role in function performance and robustness. As cells are monitored through live-cell imaging, this technique is compatible with any type of fluorescent biosensor, thus increasing the amount of knowledge one can obtain from one single experiment. Even though dMSCC enables the investigation of rapid dynamics, it could be applied to also elucidate some slower phenomena. For example, by swapping the medium only once, the transition between seed culture and production can be simulated, thus unveiling short-term adaptation strategies at both single-cell and subpopulation levels [135,140]. Alternatively, near-zero growth conditions can be better investigated [168,189,274], obtaining single-cell and time-resolved data output.

The same concepts applied in this **thesis** can be extended from applications in yeast for 2G biofuel production to any bioprocess and research field. The *Sc*EnSor Kit is designed specifically for *S. cerevisiae*, but biosensors are available for every organism. Upon proper modifications of the chip design and pump profile, dMSCC can accommodate different organisms and scenarios. Instead, robustness quantification can be expanded to any field of science without the need for any modification of the equations. The applications are endless, and the only limit is the scientist's creativity and curiosity.

Acknowledgements

"I would like to thank the judges for picking me,

My parents who I love, I love you, Houston…

♫"

– *Ghost/Haunted*, Beyoncé

It has been a long time, but it is finally the time also for me to wrap up this PhD and thank some people for sharing this journey with me. Through the ups and downs, at the end of the day, it's the people you surround yourself with that will make the difference in the journey. Therefore, here are some "thank you, thank you" to all those people. Thanking will be in no particular order.

First, I would like to thank my supervisor, Lisbeth. I heard of too many supervisors being impossible to work with, being lowkey tyrants and not willing to help their students. However, I knew that from that point of view, I wouldn't have had any problems with you. Having a supervisor who was always open for discussions, scientific and not, reassured me and indirectly also led to better science. So, thank you for your patience with a (most of the times) strong-opinionated person and for guiding me through my growth in this journey.

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When I read the project description for the PhD vacancy, it looked interesting and a good chance to learn a lot for my future. However, I'm not going to lie, it was also a pretty big question mark. It was nice to have the freedom to do whatever we wanted due to the topic being broad, but definitely more challenging than other research projects for which the path was already clearly set.

However, thanks to the Robustness Team, diving into this underexplored field has been very nice and an extremely good learning curve. Therefore, thank you to Lisbeth, Peter, Nathalia, and Cecilia. And to Nathalia, although you were the last one joining the group, it has been super nice and fun working with you, pity you haven't joined before!

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No tea, no shade, but I think that it is fair to say that the Nordic culture is extremely different from the South European one. At times, it almost became alienating. Therefore, I need to thank all the South Europeans that I have met at work and outside. Having a part of my culture and that warmth has been very helpful in feeling understood, seen and less alone.

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Finally, I must make some final special thanks. I remember when, around 6 years ago, I was listening to a TED Talk by Anne Lamott entitled "*12 truths I learned from life and writing*". There, one of her truths, specifically the second one, stuck with me more than the others, probably because it was very short and I bet that EVERYONE would agree with. Here I quote: "*Number 2. Almost everything will work again if you unplug it for a few minutes. Including you.*". The PhD journey is overwhelming and finding ways to unwind and unplug myself from it has been extremely important to keep my interest and motivation up. One way was to dedicate myself to my crafty and artsy projects. However, after a long day of science, you might not want to make things, but rather do nothing and think about something else. Here Trixie and Katya come to play. All the laughter (also leading to either scream-laughing or cry-laughing) was and will always be invaluable. Shoutouts to UNHhhh episodes 136, 142 and 152, definitely my go-to whenever I was feeling down or overwhelmed.

Well, that's a wrap I would say.

* *bird noises* *

– Trixie Mattel

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