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Perruca Foncillas, Raquel

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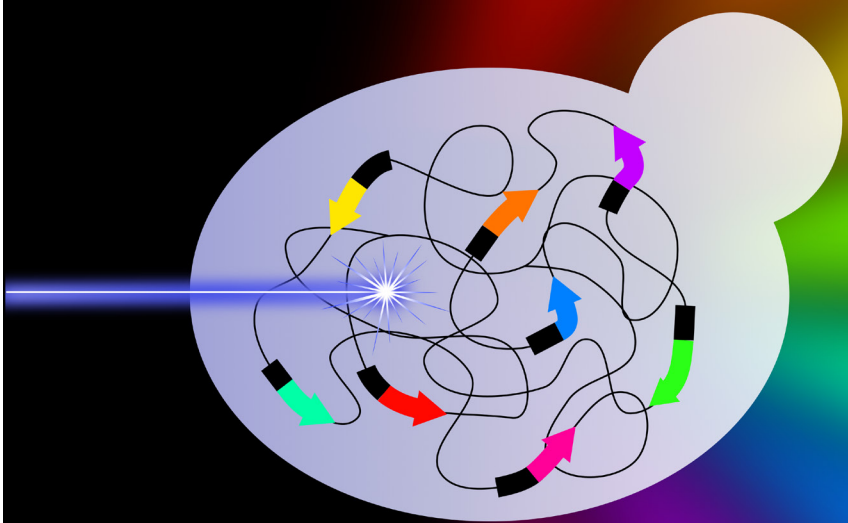

Evaluation of biosensors and flow cytometry as monitoring tools in lignocellulosic bioethanol production

Raquel Perruca Foncillas

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DOCTORAL DISSERTATION

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Title and subtitle: Evaluation of biosensors and flow cytometry as monitoring tools in lignocellulosic bioethanol production

Abstract: The significant environmental impact of the current fossil fuel-based industry is a major concern for society. Consequently, various initiatives are being undertaken to establish a more sustainable industrial model. One example is via the transition from conventional fossil fuel refineries to biorefineries, where renewable raw materials are utilised. Amongst these raw materials, the use of lignocellulosic biomass from agricultural residues or wood has been favoured, as it does not compete with food or land resources. In particular, extensive research has been conducted to produce biofuels such as bioethanol from lignocellulosic biomass, referred to as second-generation (2G) bioethanol.

In this thesis work, the goal was to develop and apply new tools to address challenges encountered in 2G bioethanol production. Specifically, the work focused on monitoring the impact of inhibitory compounds and mixed sugars on the fermentation performance of the yeast *Saccharomyces cerevisiae*.

Inhibitory compounds are released during the pretreatment of the lignocellulosic biomass, a crucial step necessary to break down its complex structure and to enhance sugar accessibility This thesis work specifically focused on the redox imbalance induced in cells exposed to furaldehydes such as furfural or HMF. To study this effect, a biosensor for redox imbalance, *TRX2*p-*yEGFP*, was introduced into the cells and its fluorescence signal was monitored in real-time using flow cytometry.

One potential strategy for enhancing the cells' tolerance to these inhibitors is to prepare them by introducing lignocellulosic hydrolysate in the feed during cell propagation. During this pre-exposure phase, a transient induction of the *TRX2*p-*yEGFP* biosensor signal for redox imbalance was observed, which gradually diminished. This indicated that, by the time of cell collection, the cells had adapted to the inhibitor concentration within the culture. To examine whether an increased induction level of the biosensor at the time of cell collection influenced the fermentation performance, an automated control system was devised. This system utilised data from the flow cytometry analysis to control the level of inhibitors in the cultivation feed. Consequently, when the biosensor signal began to decline, higher amounts of inhibitors were added, as long as the addition did not lead to an increase in the number of damaged cells.

A second biosensor was used in this thesis work to investigate the sugar signalling response of *S. cerevisiae* to the presence of xylose. Xylose is the second most abundant sugar in lignocellulosic biomass; however, naturally, *S. cerevisiae* cannot metabolise it. Genetically modified *S. cerevisiae* strains have been generated by introducing heterologous pathways such as the XR/XDH or XI pathways to enable xylose consumption. Nevertheless, xylose consumption rates remain lower compared to glucose. Sugar signalling emerged as a potential bottleneck in the efficient utilisation of xylose. In the present work, the response of the *SUC2*p-*yEGFP* biosensor for sugar signalling was found to vary significantly depending on the pathway employed. A higher induction for the strains carrying the XI pathway was associated with poorer growth on xylose.

Lastly, the effect of introducing a xylose epimerase capable of catalysing the conversion between the two anomers, α-D-xylopyranose and β-D-xylopyranose, as a strategy to improve xylose consumption was studied. The effect was enzyme-specific and proved to be particularly beneficial in strains utilising the xylose isomerase from *Lachnoclostridium phytofermentans*.

In conclusion, the results presented in this thesis demonstrate how biosensors can facilitate the understanding and monitoring of intracellular processes that occur within the cell under stress conditions and be a key tool for improving production processes.

Keywords: *Saccharomyces cerevisiae*; biosensors; flow cytometry; bioethanol; lignocellulosic biomass

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A mi familia,
en especial a su miembro más reciente, Daniel

Table of Contents

Abstract

The significant environmental impact of the current fossil fuel-based industry is a major concern for society. Consequently, various initiatives are being undertaken to establish a more sustainable industrial model. One example is via the transition from conventional fossil fuel refineries to biorefineries, where renewable raw materials are utilised. Amongst these raw materials, the use of lignocellulosic biomass from agricultural residues or wood has been favoured, as it does not compete with food or land resources. In particular, extensive research has been conducted to produce biofuels such as bioethanol from lignocellulosic biomass, referred to as second-generation (2G) bioethanol.

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abundant sugar in lignocellulosic biomass; however, naturally, *S. cerevisiae* cannot metabolise it. Genetically modified *S. cerevisiae* strains have been generated by introducing heterologous pathways such as the XR/XDH or XI pathways to enable xylose consumption. Nevertheless, xylose consumption rates remain lower compared to glucose. Sugar signalling emerged as a potential bottleneck in the efficient utilisation of xylose. In the present work, the response of the *SUC2*p-*yEGFP* biosensor for sugar signalling was found to vary significantly depending on the pathway employed. A higher induction for the strains carrying the XI pathway was associated with poorer growth on xylose.

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In conclusion, the results presented in this thesis demonstrate how biosensors can facilitate the understanding and monitoring of intracellular processes that occur within the cell under stress conditions and be a key tool for improving production processes.

Popular science summary

Microorganisms can help us mitigate the environmental impact of human activities on our planet. One way to do it is to use them for the production of biofuels, such as ethanol or butanol. Biofuels represent a more environmentally friendly alternative to conventional fossil fuels like petrol because their production and utilisation result in significantly lower greenhouse gas emissions.

To produce biofuels, one can use baker's yeast (*Saccharomyces cerevisiae*), a microorganism that is very good at fermenting sugars to ethanol. Traditionally, yeast has been used in the production of bread, beer or wine. But this microorganism can also help us produce bioethanol from agricultural residues like wheat straw or corn stover, a process commonly referred to as second-generation (2G) biofuels. The problem is that baker's yeast cannot directly consume wheat straw or corn stover, so it is necessary to process this complex material to extract and utilise its sugars. Unfortunately, this processing also leads to the release of toxic compounds that hinder the performance of the yeast. Additionally, different types of sugars are extracted from these materials and yeast is not able to naturally consume all of them.

In this thesis, my objective was to improve the performance of the yeast *Saccharomyces cerevisiae* for ethanol production from wheat straw. I first developed a tool that enables a better understanding of how the cells are "feeling" during their cultivation and to be able to adjust the cultivation conditions to obtain yeast cells that are more resistant to inhibitors. To do so, I genetically modified the yeast by introducing a fluorescent biosensor which indicates the cellular response to the presence of toxic compounds. So, for example, if the cells are "unhappy" because there are too many toxic compounds in the broth, they will start emitting a fluorescence signal that can be monitored and used to adapt the process conditions.

To obtain real-time fluorescence measurements, I connected an instrument capable of autonomously collecting periodic samples from the cultivation and transferring them to the flow cytometer, the instrument responsible for measuring the fluorescence. Additionally, a computer program was developed to interpret the analysis results and utilise the response to adjust the cultivation conditions.

Besides the presence of toxic compounds, another challenge for 2G bioethanol production is the yeast's inability to consume all types of sugars obtained from wheat straw. To address this limitation and make the process economically feasible, baker's yeast has been genetically modified to expand its substrate range. However,

the efficient conversion of xylose, the second most abundant sugar in wheat straw, remains problematic. In this work, I explored the possibility of further improving xylose consumption by introducing a new genetic modification. In parallel, another biosensor was used to measure how the cells “sensed” the presence of this sugar in the broth.

Finally, a part of the PhD work consisted of evaluating the possibility of detecting the fluorescence of several proteins at the same time. I identified a combination of proteins that open the possibility to introduce several biosensors in the cells and simultaneously gather information about different aspects such as the presence of toxic compounds and the response to xylose.

Populärvetenskaplig sammanfattning

Med hjälp av mikroorganismer kan miljöpåverkan på vår planet minskas från mänskliga aktiviteter. Ett sätt att använda mikroorganismer på är att producera biobränsle, så som etanol och butanol från dem. Biobränsle är ett mer miljövänligt alternativ till vanliga fossila bränslen så som bensin, eftersom produktionen och användningen av dem släpper ut mycket mindre växthusgaser jämfört med vanliga bränslen.

För att producera biobränsle kan man använda bagerijäst (*Saccharomyces cerevisiae*), en mikroorganism som är väldigt bra på att fermentera socker till etanol. Jäst har traditionellt sätt använts vid tillverkning av bröd, öl och vin. Den här mikroorganismen kan också hjälpa oss att producera bioetanol från restprodukter från jordbruk, till exempel halm av vete, så kallat andra generationens (2G) biobränslen. Problemet är att bagerijäst inte kan tillgodogöra sig halmen direkt, vilket gör det nödvändigt att bearbeta det komplexa material som halmen är för att kunna utvinna och använda sockret det innehåller. Tyvärr leder den här bearbetningen också till att giftiga sammansättningar frigörs, vilket begränsar jästens förmåga att omvandla socker till etanol. Därtill är flera olika sockerarter extraherade från halmen och jäst kan inte naturligt tillgodogöra sig alla sorter.

I den här avhandlingen var min målsättning att förbättra jästen *Saccharomyces cerevisiae* prestationsförmåga att producera etanol från halm av vete. Det första jag gjorde var att utveckla ett verktyg som gör att vi bättre kan förstå hur cellerna ”mår” under kultiveringen, odlingen av cellerna. Beroende på hur cellerna mår kan odlingsförhållandena anpassas för att få en mer resistent jäst som klarar av fler inhibitorer. För att uppnå det genmodifierade jag jästcellerna genom att introducera en fluorescerande biosensor som indikerar cellernas svar på närvaron av giftiga föreningar. Till exempel, om cellerna inte är ”glada”, då det är för mycket giftiga sammansättningar i odlingsmediumet, kommer de att avge en fluorescerande signal som kan övervakas och användas för att ändra processen.

För att mäta fluorescensen i realtid använde jag mig av ett instrument som med jämna mellanrum automatiskt kan ta prover från cellodlingen. Provet pumpas sedan direkt vidare till flödescytometern, ett instrument som kan mäta fluorescensen på en cell i taget. Dessutom utvecklades ett datorprogram som kan läsa av resultaten från analysen och därefter justera odlingsförhållandena efter behov.

Förutom förekomsten av giftiga sammansättningar är en annan utmaning med produktion av 2G bioetanol jästens oförmåga att tillgodogöra sig de många sockerarterna som extraherats från halmen. För att komma över det här hindret och göra processen ekonomiskt hållbar har forskare genmodifierat bakjäst för att möjliggöra fermentering av fler sockerarter som substrat. Effektiv omvandling av xylos, vilket är den näst vanligaste sockerarten i halmen, är dock fortfarande problematisk. I det här arbetet undersökte jag möjligheten att ytterligare förbättra xyloskonsumtion genom att introducera en ny genetisk modifiering. Parallellt med det så användes en annan biosensor för att mäta hur cellerna känner av närvaron av sockret i odlingsmediumet.

Slutligen bestod en del av doktorandarbetet i en utvärdering av möjligheten att detektera flourescensen av flera proteiner samtidigt. Jag identifierade en kombination av proteiner som öppnar upp möjligheten att introducera flera biosensorer i cellerna och samtidigt samla information om flera olika aspekter, exempelvis förekomsten av giftiga sammansättningar och förekomsten av xylos.

Resumen de divulgación científica

Los microorganismos pueden ayudarnos a reducir el impacto ambiental causado por la actividad humana en nuestro planeta. Una forma de hacerlo es utilizándolos para producir biocombustibles como etanol o butanol. Los biocombustibles son una alternativa más respetuosa con el medio ambiente que los combustibles fósiles tradicionales como la gasolina ya que se liberan muchos menos gases de efecto invernadero durante su producción y combustión.

La levadura *Saccharomyces cerevisiae* es un microorganismo con excelentes capacidades para la transformación de azúcares en alcohol (etanol) en un proceso de fermentación. Es por eso que, tradicionalmente, se ha usado para la producción de pan, cerveza o vino. Pero este microorganismo también se puede usar para producir bioetanol a partir de residuos agrícolas como la paja obteniendo lo que se conoce como biocombustibles de segunda generación (2G). El problema es que la levadura no puede consumir la paja directamente, así que es necesario procesarla para poder extraerle los azúcares. Lamentablemente, durante este tratamiento de la paja se liberan compuestos tóxicos que obstaculizan el óptimo rendimiento de la levadura. Además, la paja está formada por distintos tipos de azúcares y no todos ellos pueden ser consumidos de forma natural por la levadura.

En esta tesis, mi objetivo ha sido mejorar el rendimiento de la levadura *Saccharomyces cerevisiae* en la producción de etanol a partir de la paja. En primer lugar, he desarrollado una herramienta que permite entender mejor cómo "se sienten" las células durante su cultivo. En función de su estado, se ajustan las condiciones del cultivo para conseguir una levadura más resistente a los compuestos tóxicos. Para ello, he modificado genéticamente la levadura introduciéndole un biosensor fluorescente que indica la respuesta de las células a la presencia de compuestos tóxicos. Así, por ejemplo, si las células no están "contentas" porque hay demasiados compuestos tóxicos en el caldo de cultivo, empezarán a emitir una señal fluorescente que puede medirse y utilizarse para cambiar las condiciones del cultivo.

Para medir la fluorescencia en tiempo real, he implementado el uso de un instrumento capaz de tomar automáticamente muestras periódicas del cultivo y mandarlas al citómetro de flujo, que es el instrumento encargado de medir su fluorescencia de forma inmediata. También se ha desarrollado un programa de ordenador capaz de leer los resultados del análisis y utilizarlos para ajustar las condiciones del cultivo.

Además de la presencia de compuestos tóxicos, otro reto para la producción de etanol de 2G es la incapacidad de la levadura para transformar los múltiples azúcares obtenidos de la paja. Para hacer el proceso económicamente viable, la levadura debe convertir todos los azucares a alcohol en la fermentación. Los investigadores han conseguido modificar genéticamente la levadura para aumentar el número de azúcares que puede utilizar. Sin embargo, la conversión eficiente de xilosa, el segundo azúcar más abundante en la paja, sigue siendo problemática. En esta tesis, he investigado la posibilidad de mejorar el consumo de xilosa introduciendo una nueva modificación genética en la levadura. A su vez, he usado otro biosensor para medir cómo las células "sienten" la presencia de este azúcar en el caldo de cultivo.

Finalmente, como parte de este trabajo también he evaluado la posibilidad de medir la fluorescencia de varias proteínas a la vez y he identificado una combinación de proteínas que abre la puerta a la introducción de varios biosensores en las células permitiendo recopilar información de forma simultánea sobre distintos aspectos como la presencia de compuestos tóxicos o de xilosa.

List of Papers

This thesis is based on the following papers and manuscripts, which can be found at the end of the thesis and will be referred to by Roman numerals.

I. Assessment of the TRX2p-yEGFP biosensor to monitor the redox response of an industrial xylose-fermenting *Saccharomyces cerevisiae* strain during propagation and fermentation

Perruca Foncillas, R., Sanchis Sebastiá, M., Wallberg, O., Carlquist, M. & Gorwa-Grauslund, M. F. (2023). *Journal of Fungi*, *9*, Article 630. https://doi.org/10.3390/jof9060630

II. Automated yeast propagation control using a biosensor and flow cytometry

Perruca Foncillas, R., Magnusson, S., Wallberg, O., Gorwa-Grauslund, M. F. & Carlquist, M. *Manuscript*.

III. Impact of xylose epimerase on sugar assimilation and sensing in recombinant *Saccharomyces cerevisiae* carrying different xylose-utilization pathways

Persson, V. C., Perruca Foncillas, R., Anderes, T. R., Ginestet, C. & Gorwa-Grauslund M. F. (2023). *Biotechnology for Biofuels and Bioproducts*, *16*, Article 168. https://doi.org/10.1186/s13068-023-02422-z

IV. Assessment of fluorescent protein candidates for multi-color flow cytometry analysis of *Saccharomyces cerevisiae*

Perruca-Foncillas, R., Davidsson, J., Carlquist, M. & Gorwa-Grauslund, M. F. (2022). *Biotechnology Reports*, *34*, Article e00735. https://doi.org/10.1016/j.btre.2022.e00735

My contribution to the papers

I. I participated in the design of the study and performed the experimental work. The simultaneous saccharification and co-fermentation (SScF) experiments were performed with the assistance of Miguel Sanchis Sebastiá. I performed the data analysis and visualisation and wrote the manuscript.

II. I participated in the design of the study and performed the wet lab experiments together with the student, Sara Magnusson, whom I supervised. I performed the data analysis and visualisation and wrote the manuscript.

III. I participated in the design of the study and performed the experimental work related to the strains containing the xylose isomerase pathway, including strain construction, aerobic shake flask cultivations, anaerobic cultivations, flow cytometry and HPLC analysis. I supervised the student Clément Ginestet for the yeast strain construction. I contributed to the data analysis and co-wrote the manuscript.

IV. I participated in the design of the study and performed the experimental work together with the student, Johan Davidsson, whom I supervised. I performed the data analysis and visualisation and wrote the manuscript.

Abbreviations

1G	First generation
2G	Second generation
3G	Third generation
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	Adenosine monophosphate cyclic form
CBP	Consolidated bioprocessing
CER	Carbon dioxide evolution rate
CFP	Cyan fluorescent protein
CFU	Colony forming units
CO_2	Carbon dioxide
CPP	Critical process parameters
CQA	Critical quality attributes
DNA	Deoxyribonucleic acid
FCM	Flow cytometry
FDA	Food and Drug Administration
FP	Fluorescent protein
FRET	Fluorescence resonance energy transfer
FSC	Forward scatter
GC	Gas chromatography
GFP	Green fluorescent protein
HMF	5-hydroxymethylfurfural
HPLC	High-pressure liquid chromatography
K_M	Michaelis-Menten constant

LPMO	Lytic polysaccharide monooxygenases
MIR	Mid-infrared spectroscopy
MWF	Multiwavelength fluorescence
$NAD(P)^+$/NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) oxidised/reduced forms
NIR	Near-infrared spectroscopy
OUR	Oxygen uptake rate
PAT	Process analytical technology
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PI	Propidium iodide
PKA	Protein kinase A
PLA	Polylactic acid
PPP	Pentose phosphate pathway
ROS	Reactive oxygen species
RQ	Respiratory quotient
SHF	Separate hydrolysis and fermentation
SPR	Surface plasmon resonance
SS(c)F	Simultaneous saccharification and (co-)fermentation
SSC	Side scatter
UV	Ultraviolet
WIS	Water-insoluble solids
XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XK	Xylulose kinase
XR	Xylose reductase
YFP	Yellow fluorescent protein

Chapter 1
Introduction

The growing role of biorefineries

Our current industrial model heavily relies on the use of fossil resources, in particular oil and gas. Their extraction and use have led to increasing environmental problems which include the increasing emission of greenhouse gases that contribute to the climate change observed in recent decades (Lelieveld et al., 2019). As a response, efforts are being put into the development of a more sustainable industrial model. Notably, a transition into a bioeconomy-based society has been proposed, in which biorefineries play a key role. The biorefinery concept offers a similar approach to established petroleum refineries in which multiple products can be obtained through a combination of technologies and processes, but a major difference is that renewable biomass is used as a raw material instead of fossil resources (Fernando et al., 2006).

Biorefineries can be classified according to their goals, either as (i) energy-driven biorefineries, where the main product is a biofuel and co-products are considered as added-value; or (ii) product-driven biorefineries in which the main goal is the production of food, feed, chemicals or materials and the side-products are a source of energy (Cherubini et al., 2009).

The International Energy Agency, as part of their Task 42, has also established a classification system for biorefineries that is based on four main components: platforms, products, feedstock and processes, where the platforms are the key intermediates between raw material and product (Cherubini et al., 2009). Recently, this classification has been updated and complemented in the EU Biorefinery Outlook to 2030, especially regarding product-driven biorefineries (Figure 1) (Platt et al., 2021).

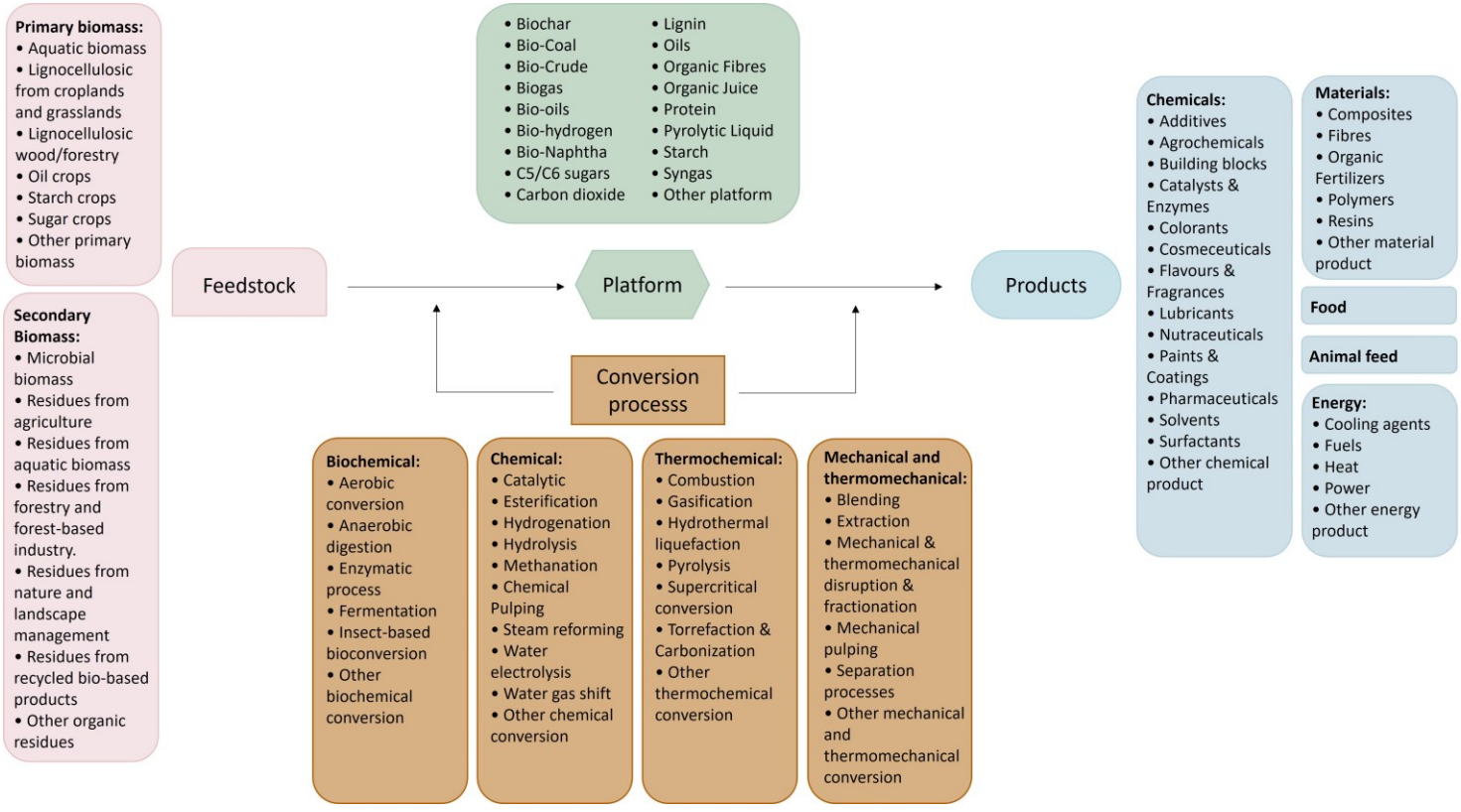


Figure 1. Biorefinery classification based on feedstock, conversion process, platform and products. Adapted from (Platt et al., 2021).

Lignocellulosic biomass: a major feedstock

Amongst the potential feedstock for biorefineries, lignocellulosic biomass represents a major source due to its abundance and limited cost. Examples of lignocellulosic biomass include agricultural residues (e.g., wheat straw, sugar cane bagasse or corn stover), forestry residues (e.g., wood or sawdust) and industrial waste streams (e.g., black liquor or spent grain) (Wyman, 1994). Traditionally, these materials have commonly been considered as waste or low value and they have been discarded or simply burnt for energy production. As such, their valorisation as raw materials for biorefineries still has great potential.

Three main polymers constitute lignocellulosic biomass: cellulose, hemicellulose and lignin (Figure 2) (Wyman, 1994). Cellulose is a linear polymer of cellobiose, two D-glucose residues linked by β-(1→4) glycosidic bonds. Several of these long cellulose chains interact with each other through weak forces, i.e., hydrogen bonds and van der Waals forces, and generate cellulose fibres (Pérez et al., 2002).

Hemicellulose is a much more diverse polymer as it consists of a variety of five-carbon and six-carbon sugars. Among them, monomers of xylose, arabinose, mannose, galactose or glucose can be found (Pérez et al., 2002). As in cellulose, the sugar residues can be linked by β-(1→4) glycosidic bonds but also by β-(1→3) glycosidic bonds creating ramifications in the polymer. The specific composition of the hemicellulose fraction varies widely, notably according to the plant species.

Lignin is a highly heterogeneous polymer of aromatic compounds. Based on the modifications observed in the aromatic ring, three different building block structures have been described: p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) and they are interlinked via a variety of C-C and C-O bonds (Abdelaziz et al., 2016).

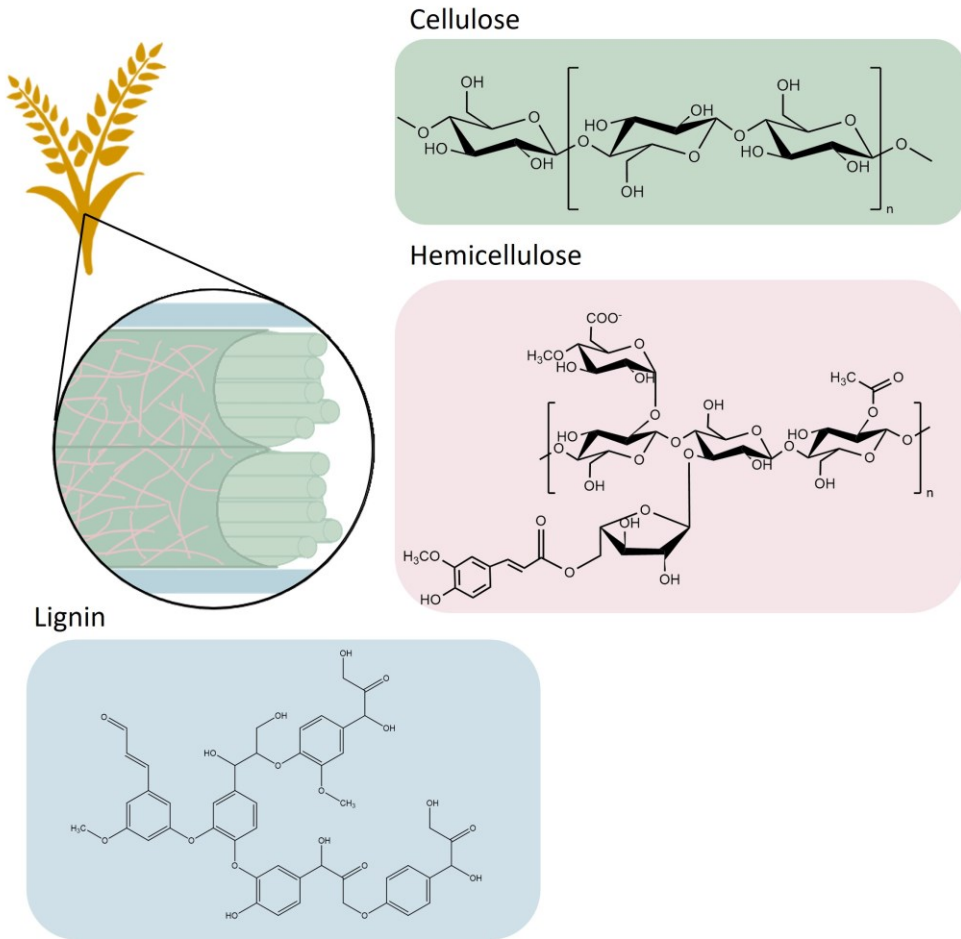


Figure 2. Schematic representation of the three main components of lignocellulosic biomass.

The abundance of each of these fractions varies according to the type of raw material (Table 1). Furthermore, within the same species, variations in the composition may also depend on the origin, growth stage and cultivation conditions (Bhatia et al., 2020).

In this thesis work, wheat straw was used as feedstock to produce bioethanol. It is an example of an underutilised and abundant material as it is obtained from wheat cultivation after the wheat grains have been recovered from the plant. In this agricultural waste, about a quarter of the material is hemicellulose in which xylan is the major component (Sun, Fang, & Rowlands, 1998; Sun, Fang, Rowlands, et al., 1998). Thus, the utilisation of xylose, the most abundant sugar in this fraction, is crucial for an economically feasible process based on its use as raw material.

Table 1. Example of lignocellulosic biomass and their reported composition.

Biomass	Cellulose	Hemicellulose	Lignin	References
Agricultural residues				
Corn stover	40%	22%	18%	(T. H. Kim et al., 2003)
Wheat straw	29.3%	25.2%	27.5%	(Bondesson & Galbe, 2016)
Rice straw	32-47%	19-27%	5-24%	(Binod et al., 2010)
Oat straw	37.6%	23.3%	12.9%	(Adapa et al., 2009)
Hard woods				
Poplar	49%	23%	27%	(Rego et al., 2019)
Birch	56.5%	24.8%	12.2%	(D. K. Shen et al., 2009)
Oak	54%	29%	9.4%	(D. K. Shen et al., 2009)
Soft woods				
Spruce	42.4%	17.5%	33,8%	(Frankó et al., 2015)
Pine	52.1%	15.4%	27.5%	(D. K. Shen et al., 2009)
Waste streams				
Eucalyptus black liquor	-	1-2%	40-42%	(Morya et al., 2022)
Agro-residue black liquor	-	8-18%	28-32%	(Morya et al., 2022)
Brewer spent grain	21.4%	30.6%	11.4%	(Zeko-Pivač et al., 2022)
Sugarcane bagasse	44%	28%	21%	(Ajala et al., 2021)

Industrial applications of lignocellulosic biomass

Through the implementation of both chemical and biological processes, many industrial applications have been explored to transform the different fractions of lignocellulosic biomass and exploit its potential as feedstock (Figure 3) (Okolie et al., 2021).

Biofuels are one of the main product categories because of the key role of fuels in the transportation and energy sectors. Biofuels can be obtained from lignocellulosic biomass in liquid form, like bioethanol (Periyasamy et al., 2023), biobutanol (Re & Mazzoli, 2023) or biodiesel (Chintagunta et al., 2021); or as gaseous form like biogas (Martínez-Gutiérrez, 2018), biohydrogen (Singh et al., 2015) or syngas (Ghodke et al., 2023).

Besides biofuels, many other relevant biochemicals can be obtained from lignocellulose. For example, the production of organic acids such as succinic acid (Lu et al., 2021) or lactic acid (Nwamba et al., 2021) can be accomplished through microbial fermentation. If chemical transformation of cellulose and hemicellulose is considered instead, relevant compounds such as levulinic acid and furfural (Biomass et al., 2021) or 5-hydroxymethylfurfural (5-HMF) (Thoma et al., 2020) can be formed. Both chemical and biological conversion processes have also been described for the production of xylitol from xylose, which is commonly used as an artificial sweetener (Venkateswar Rao et al., 2016). Large quantities of glycerol are

also produced from lignocellulosic biomass as a by-product of biodiesel production (Monteiro et al., 2018). Finally, vanillin can be obtained through depolymerisation of the lignin fraction (Fache et al., 2016).

The production of bioplastics from lignocellulosic materials has also gained attention. For example, polylactic acid (PLA) is used in biomedical applications for its biocompatibility (Singhvi et al., 2019). Microbial production of precursors of bioplastics such as polyhydroxyalkanoates (PHA) and polyhydroxybutanoate (PHB) has also been demonstrated (Al-Battashi et al., 2019).

More recently, the suitability of lignocellulosic biomass for the production of speciality materials has also been explored. Artificial fibres such as lyocell, which are nowadays used in the textile industry, can be obtained by dissolving cellulose (J. Y. Chen et al., 2017). Lignin-derived materials are also of interest as they can be used, for example, to fabricate rechargeable batteries (W. J. Chen et al., 2022). Other materials such as activated carbon, which can be used for environmental remediation (Hoang et al., 2022) or carbon nanotubes (Osman et al., 2020), which possess high strength, electrical and thermal conductivity properties, represent other promising alternatives for added-value products.

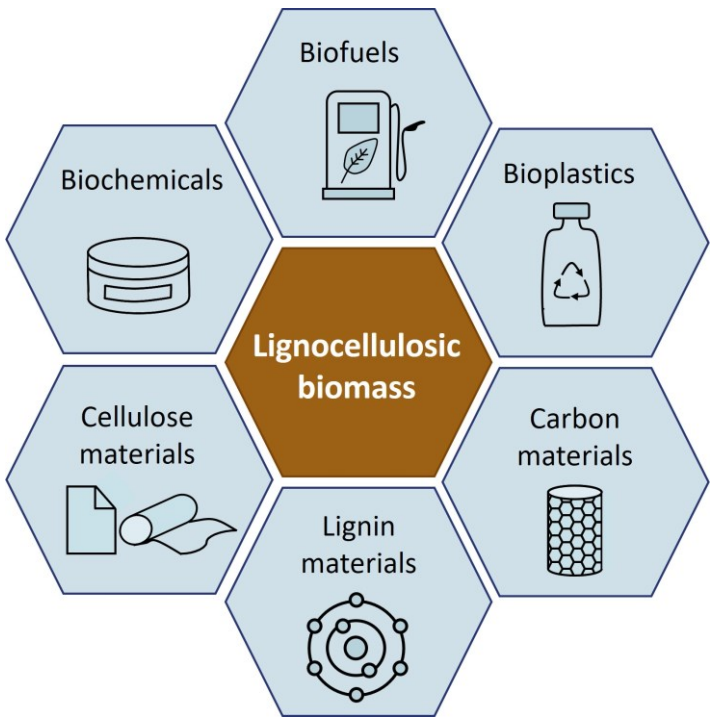


Figure 3. Industrial applications of lignocellulosic biomass. Adapted from (Okolie et al., 2021).

Lignocellulose for 2G-bioethanol production

The production of biofuels, in particular bioethanol, has historically been the driving force behind the development of technologies for the use of lignocellulosic biomass. The first bioprocesses developed for the production of biofuels, commonly referred to as first generation (1G), used edible biomass such as corn, maize or sugar cane as raw material (Naik et al., 2010). The most known examples of 1G biofuel are bioethanol production from sugar cane in Brazil or corn in the United States (Bothast & Schlicher, 2005; Rosillo-Calle & Cortez, 1998). However, due to the competing use of these raw materials as food resources, concerns were raised and alternative non-edible raw materials were searched for, leading to investigations on the use of lignocellulosic biomass instead. In this second generation (2G) of biofuels, agricultural wastes or forest residues are used as raw materials. They no longer compete with the use of resources that could be destined for food; instead, value is given to what, up to that point, was considered a waste (Naik et al., 2010). Since then, a third generation (3G) of biofuels has also been described, based on the use of microalgae cultivations (Chowdhury & Loganathan, 2019).

Due to the inherently complex nature of lignocellulosic biomass, several processing steps are needed to convert the raw material into an adequate substrate for microbial conversion to bioethanol. These steps and their respective challenges are summarised below.

Biomass pretreatment

Initially, the complex structure of the biomass needs to be broken down into smaller pieces to facilitate its processing in the later stages (Figure 4). A wide range of strategies have been developed for this purpose, comprising physical, chemical, physico-chemical and biological technologies (Brodeur et al., 2011) (Table 2).

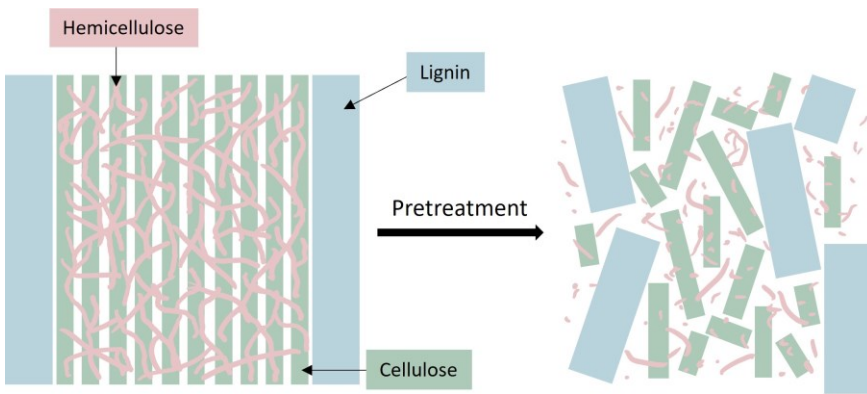


Figure 4. Schematic representation of the effect of pretreatment on lignocellulosic biomass.

In short, physical pretreatments rely on milling or grinding procedures to reduce particle size; these are energy expensive and often combined with other types of pretreatments. Biological pretreatment would be the most environmentally friendly alternative as fungal enzymes can be used at mild conditions to degrade the biomass. Unfortunately, due to the low hydrolysis rates, long and incomplete pretreatment is achieved. Thus, chemical and physico-chemical methods have been widely preferred (Table 2).

Regardless of the strategy, the following common goals are envisioned:

(1) Obtain solids with high digestibility for enzymatic hydrolysis.
(2) Avoid sugar degradation.
(3) Minimise inhibitor formation.
(4) Minimise heat and power requirements to improve cost efficiency.

Table 2. Examples of chemical and physico-chemical pretreatment methods. Adapted from (Brodeur et al., 2011).

Pretreatment	Compounds	Advantages	Disadvantages
Chemical			
Alkaline	NaOH, KOH, lime	Lignin removal Room temperature Low inhibitor formation	High cost Need for neutralisation
Acid	H_2SO_4, HCl, HNO_3	Hemicellulose and cellulose hydrolysis Room temperature	High cost Need for corrosion-resistant materials Inhibitor formation Need for neutralisation
Wet oxidation	O_2+H_2O	Lignin removal Hemicellulose solubilisation	By-product formation Inhibitor formation
Green solvents	Ionic liquids	Solvent recovery No toxic product formation Cellulose crystallinity reduced	High cost Cellulase inactivation
Physico-chemical			
Steam explosion	H_2O	Hemicellulose solubilisation Complete sugar recovery Cost-effective	Need for acid catalyst if high lignin content Inhibitor formation
Ammonia Fiber Explosion (AFEX)	NH_3	Lower temperatures Low inhibitor formation	High cost Need for ammonia recovery
Supercritical Fluid	Supercritical CO_2	Lower temperatures	Very high pressure No effect on lignin and hemicellulose

In the present thesis work, the physico-chemical steam explosion, one of the most commonly used methods for pretreatment (Brodeur et al., 2011), was used in combination with the addition of an acid catalyst (Paper II). First, the material was impregnated in dilute H_2SO_4 and further treated by continuous steam explosion at 195 °C for 10 minutes. Then, the pressured steam was rapidly depressurised causing the disruption of the structure. The acid was added because it has been shown to decrease the retention time and temperature needed while improving the recovery of sugars and their following hydrolysis (Ballesteros et al., 2006).

A major challenge of most pretreatments, including the one above, is the concomitant formation of compounds that inhibit the following biocatalytic steps. This will be further discussed in Chapter 2.

Enzymatic hydrolysis and fermentation

Once the complex structure of lignocellulosic biomass has been disrupted by the pretreatment step, it can be submitted to the action of specific hydrolytic enzymes, that enable the release of monomeric sugars from their polymeric form.

Multiple enzyme activities are needed to efficiently hydrolyse the pretreated material, which requires the development of dedicated enzyme cocktails. For the degradation of cellulose into glucose, endoglucanases, cellobiohydrolases and β-glucosidases are needed (Figure 5A). Hemicellulose hydrolysis typically requires the action of additional enzymes such as endoxylanases, xylosidases, acetylxylan esterases, arabinofuranosidases and glucuronidases to release the different sugars (Figure 5B) (Lopes et al., 2018). More recently, another group of enzymes called lytic polysaccharide monooxygenases (LPMOs) was identified and shown to possess auxiliary activities that improve the saccharification of lignocellulose (Johansen, 2016). These enzymes need the presence of oxygen or hydrogen peroxide as well as an electron donor for their activity. They are capable of initiating the degradation of cellulose by cleaving internal β-(1→4) glycosidic bonds without the need for prior de-crystallisation (Peciulyte et al., 2018). Their activity generates nicks in the crystalline structure of cellulose facilitating the action of traditional cellulases.

Different factors in the process may influence the choice of an optimal enzyme cocktail. On one hand, the type of substrate will determine the composition of the material and consequently which enzymatic activities are needed. On the other hand, the pretreatment method used is also relevant. For instance, the steam explosion pretreatment used in the present thesis work enables the hemicellulose fraction to get hydrolysed; as a consequence, most of the xylose present in the polymer is released as a monomer in the liquid fraction without the need for enzyme addition, whereas the cellulose fraction remains mostly untouched (Ballesteros et al., 2006). Thus, the addition of cellulases is the most relevant in this case.

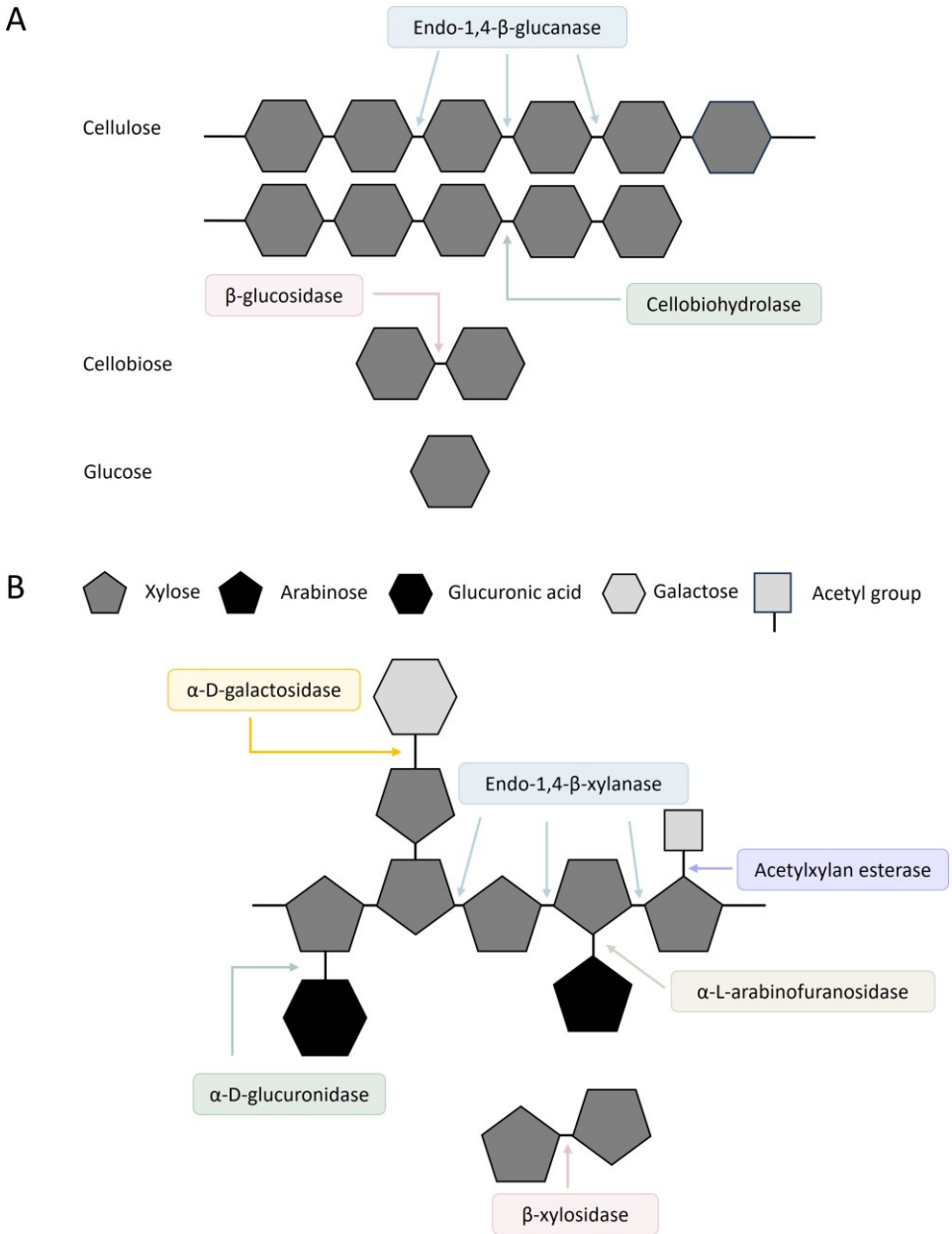


Figure 5. Enzymatic hydrolysis of cellulose (A) and hemicellulose (B).

After hydrolysis, the mixture of (mostly) monomeric sugars is used as substrate in the fermentation step. In this step, the sugars are taken up by the microorganism of choice and further converted into ethanol. Two microorganisms have originally been considered due to their high ethanol production yield: the baker´s yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis* (Olsson & Hahn-Hägerdal, 1993). However, with the introduction of lignocellulosic biomass as raw material, new challenges are faced, which need to be additionally considered for the selection of the appropriate microbial host. These challenges include e.g., the presence of inhibitory compounds and mixed sugars in the medium (cf. Chapter 2). As a result, other yeasts, such as the pentose-using yeast *Scheffersomyces stipitis* and *Spathaspora passalidarum* are now considered as potential candidates (Veras et al., 2017).

Ideally, the microorganism used in the fermentation step should be capable of withstanding the presence of inhibitors, consuming all the sugars in the mixture and efficiently producing ethanol. Unfortunately, finding a microorganism that fulfils all the requirements is not an easy task (Olsson & Hahn-Hägerdal, 1993).

An early study in 1993 compared the performance of the microorganisms *Escherichia coli*, *Lactobacillus brevis*, *Lactococcus lactis* spp. *lactis*, *Z. mobilis*, *Saccharomyces cidri* and *S. cerevisiae* in fermentations using lignocellulosic hydrolysates. *S. cerevisiae* was established as the fastest among them, especially in hydrolysates containing higher concentrations of inhibitory compounds (Olsson & Hahn-Hägerdal, 1993). Indeed, *S. cerevisiae* remains the most commonly used microorganism in industry (Almeida et al., 2007) and as such, it will be the microorganism of choice and focus of this thesis.

Process configurations

Several process configurations have been proposed, combining or not the three steps described above (Figure 6). When the enzymatic hydrolysis and fermentation steps are performed separately, the process configuration is commonly referred to as *separate hydrolysis and fermentation* (SHF). Alternatively, the two steps can be performed at once in the same tank in a *simultaneous saccharification and fermentation* (SSF) process. The possibility of combining yeast-based enzyme production, enzymatic hydrolysis and fermentation in a single bioreactor, known as *consolidated bioprocesses* (CBP) has also been recently explored (Periyasamy et al., 2023).

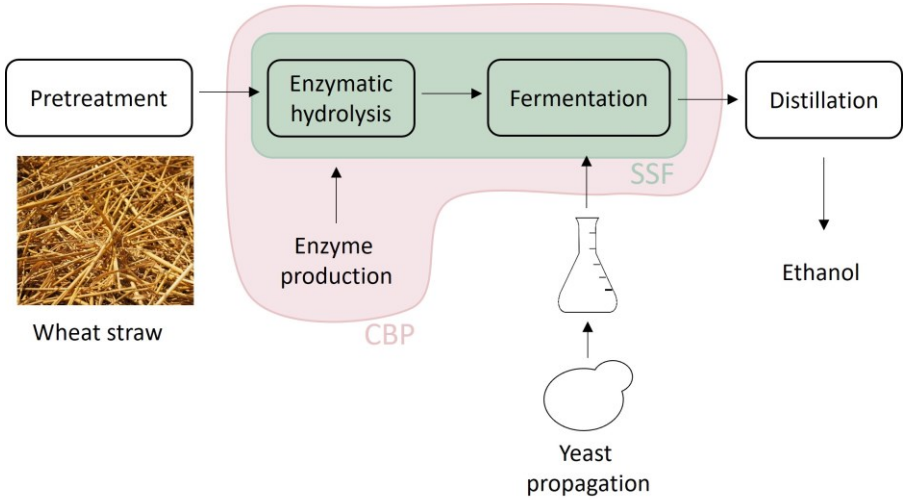


Figure 6. Possible process configurations for the production of lignocellulosic bioethanol. SSF: simultaneous saccharification and fermentation; CBP: consolidated bioprocess.

Besides decreasing the capital cost by reducing the number of vessels needed for the process, SSF presents advantages over SHF for enzymatic hydrolysis. Since both saccharification and fermentation happen simultaneously, the glucose residues released by the action of the enzymes are immediately consumed by the microorganism in the fermentation step, thus reducing the product inhibition otherwise faced in the enzymatic hydrolysis by the high concentrations of glucose (Stenberg et al., 2000). Nevertheless, when performing the two steps simultaneously, a major drawback is the difference in the optimal conditions for each of them (Stenberg et al., 2000). The optimal temperature for enzyme mixtures such as Cellic® CTec3 is within 50-55°C (Cellic® CTec3 application sheet, Novozymes, Denmark) while most microorganisms grow at temperatures around 30-37°C. Thus, finding a compromise between these temperature ranges in which to run the SSF is critical for its optimisation (Mutturi & Lidén, 2013). Additionally, the complex matrix generated in SSF processes represents a challenge for analytical purposes, which will be further discussed in Chapter 3.

Separation and purification

The last step in the production of bioethanol from lignocellulosic material is the recovery of the product from the fermentation broth. Distillation is the most commonly used method to separate ethanol from water.

In lignocellulosic bioethanol processes, it is estimated that an ethanol concentration of at least 4-5% in the broth at the end of the fermentation is needed to make the

process economically feasible (Galbe et al., 2007). This implies that high solid loadings, i.e., the ratio of solids compared to water, are needed to obtain such concentrations. However, the higher the solid loadings the more challenging the process becomes. A negative correlation between the efficiency of the enzymatic hydrolysis and the solid loadings has been observed (Jørgensen et al., 2007). Also, higher concentrations of inhibitory compounds are naturally obtained in a process with high solid loadings which negatively impact the fermentation efficiency.

Scope and outline of the thesis

The work presented in the present thesis is focused on the development of strategies for improving 2G bioethanol production from lignocellulosic biomass using *S. cerevisiae*, with a special focus on the fermentation step. Chapter 2 first summarises the main challenges faced during fermentation. Chapter 3 focuses on the use of monitoring and control strategies to optimise the yeast performance in the production process. The strategies used to obtain *S. cerevisiae* strains more tolerant to the presence of inhibitory compounds as well as the application of a biosensor to monitor the cellular response to these inhibitors are described in Chapter 4. In Chapter 5, key genetic modifications needed to obtain efficient xylose-consuming *S. cerevisiae* strains are summarised, including notably the role of xylose epimerase. Finally, the conclusions and outlook of the thesis work and its associated Papers I-IV (whose focus is described below), can be found in Chapter 6.

Paper I describes the use of a biosensor for measuring the cofactor deficiency related to the presence of inhibitory compounds and monitoring the redox state of *S. cerevisiae*. A system allowing automatic sampling and flow cytometric analysis of the biosensor response during cultivation is established. Furthermore, three different strategies for the improvement of yeast performance are compared. Finally, the relationship between the biosensor's response and the ethanol production capabilities of the strains is studied.

In **Paper II**, the applicability of the monitoring system developed in Paper I for automatic sampling and flow cytometric analysis as a control strategy is explored.

In **Paper III**, the impact of the addition of a xylose epimerase on xylose sensing and utilisation is studied in xylose-consuming *S. cerevisiae* strains carrying a biosensor for sugar signalling.

Paper IV reports on the assessment of several fluorescent proteins for their use as reporters in transcription factor-based biosensors in *S. cerevisiae*. Combinations of these fluorescent proteins are explored for the establishment of multicolour flow cytometry analysis.

Chapter 2
Challenges faced during the fermentation step in 2G bioethanol production

During the fermentation step, the monomeric sugars present in lignocellulosic biomass are converted into ethanol, making this step a key one in achieving an efficient production process. While *S. cerevisiae* is considered the most promising organism for this step (cf. Chapter 1), it still faces several challenges that are summarised in the following sections.

The presence of inhibitory compounds

The pretreatment of lignocellulosic biomass, which is needed to dissociate the different components of the material (Chapter 1), can also generate a range of compounds that inhibit microbial growth, hindering the fermentation step and in turn, the whole process.

Three major types of inhibitors have been described: (i) furaldehydes, (ii) weak acids and (iii) phenolic compounds (Figure 7). The furaldehydes furfural and 5-hydroxymethyl furfural (HMF) are formed by the degradation of pentose and hexose sugars, respectively (Dunlop, 1948; Ulbricht et al., 1984). Amongst the weak acids, acetic acid, formic acid and levulinic acid can be found. Acetic acid originates from the acetyl groups in the hemicellulose fraction, whereas formic acid and levulinic acid are formed by the further degradation of furfural and HMF (Ulbricht et al., 1984). Phenolic compounds are mostly obtained as a result of a partial breakdown of lignin and thus the specific compounds vary significantly as a function of the source of biomass (Palmqvist & Hahn-Hägerdal, 2000).

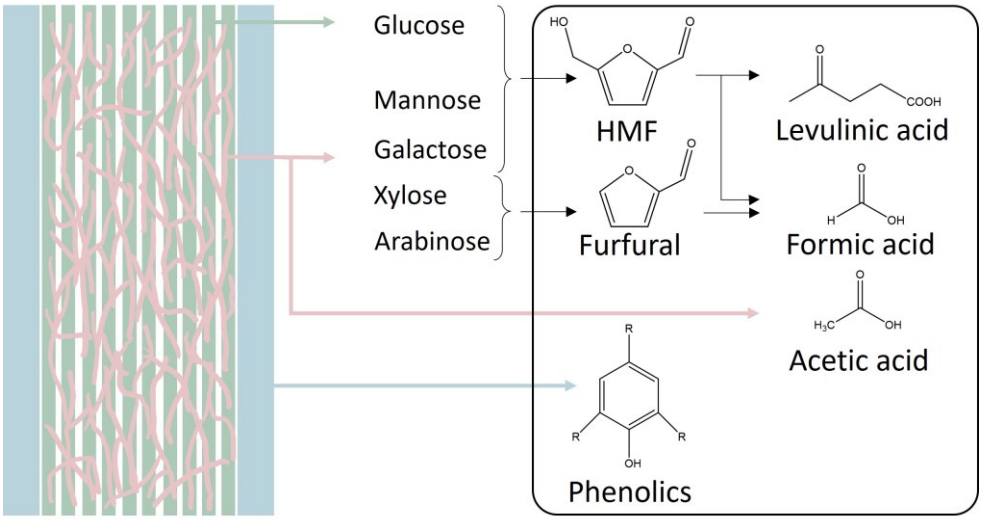


Figure 7. Inhibitors present in pretreated lignocellulosic material.

Furfural and HMF

The inhibitory effect of furfural and HMF on *S. cerevisiae* has been thoroughly studied. In the presence of furfural, inhibition of both growth and ethanol production has been observed. At the beginning of the fermentation, an increased lag phase is observed, as well as an increase in cellular death. During this lag phase, rapid removal of furfural is carried out by the cells (Boyer et al., 1992). *S. cerevisiae* can detoxify the medium by converting furfural into its less toxic furfuryl alcohol form (Figure 8A). The conversion is performed by NAD(P)H-dependent alcohol dehydrogenase enzymes (Diaz de Villegas et al., 1992; Villa et al., 1992). Similarly, HMF can be converted into 5-hydroxymethyl furfuryl alcohol (Taherzadeh et al., 1999) (Figure 8B). When furfural and HMF are converted, the lag phase is ended and the remaining cells can grow and ferment the sugars (Boyer et al., 1992; Palmqvist et al., 1999; Taherzadeh, Gustafsson, et al., 2000).

At the mechanistic level, furaldehydes have been shown to have a strong inhibitory effect on glycolytic enzymes such as alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase (Banerjee et al., 1981; Modig et al., 2002). Additionally, it has been suggested that cofactor imbalances happen during exposure to furfural and HMF (Almeida et al., 2007). On the one hand, the detoxification processes of furfural and HMF conversion entail the consumption of NAD(P)H (Wahlbom & Hahn-Hägerdal, 2002), thus decreasing their cytosolic availability. On the other hand, exposure to furfural induces the accumulation of reactive oxygen species (ROS) (Allen et al., 2010). This explains the crucial role that the pentose phosphate pathway (PPP) plays in the tolerance to furfural (Gorsich

et al., 2006) as the NADPH generated through the PPP is used as a cofactor by different stress protection enzymes to fight for example ROS accumulation (Allen et al., 2010).

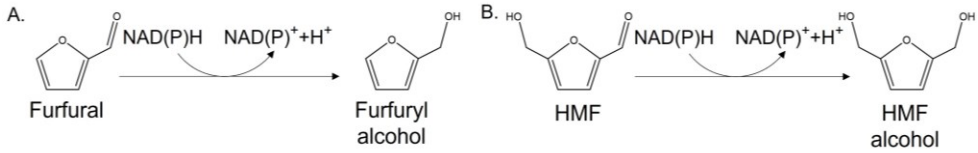


Figure 8. Transformation of the inhibitors furfural (A) and 5-hydroxymethyl furfural (HMF) (B) into their alcohol forms by *S. cerevisiae*.

Although the capability of detoxifying inhibitory compounds into less toxic forms confers *S. cerevisiae* an innate tolerance, different strategies from genetic engineering to process engineering have been proposed to further improve its robustness, and thereby the process (cf. Chapter 4).

Weak acids

Weak acids such as acetic acid, levulinic acid and formic acid have high pK_a values: 4.75 for acetic acid, 4.66 for levulinic acid and 3.75 for formic acid (Brown et al., 1955; Soni et al., 1982). This implies that at pH values lower than their pK_a, the acids remain undissociated. As such, they are permeable to the cell membrane and can enter the cells. Inside the cells, the neutral pH dissociates the acids into their anionic form and a proton (Figure 9), which results in a decrease in intracellular pH. Two different theories have been described to explain the associated inhibitory effect: (i) anion accumulation and (ii) uncoupling (Russell, 1992). The accumulation of anions to high levels can induce high turgor pressure in the cell and free radical production, which leads to severe oxidative stress (Piper et al., 2001). In the uncoupling mechanism, the cell utilises a plasma membrane ATPase to pump out the protons by hydrolysing ATP to maintain a neutral intracellular pH (Figure 9). Eventually, the action of this ATPase results in the depletion of the cellular ATP levels which leads to growth inhibition (Palmqvist & Hahn-Hägerdal, 2000; Russell, 1992). The intracellular acidification resulting from exposure to a medium containing acetic acid has also been shown to trigger a myriad of processes including the accumulation of reactive oxygen species (ROS), the release of cytochrome c or an increase of DNA fragmentation which results in programmed cell death (Guaragnella et al., 2011).

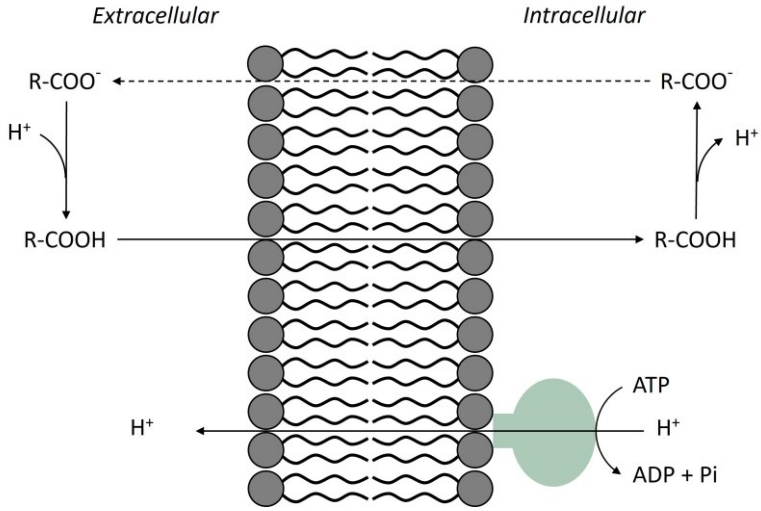


Figure 9. Uncoupling mechanism response to weak acid inhibition.

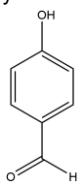
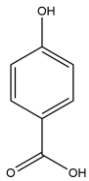
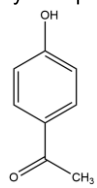
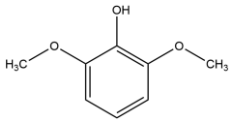
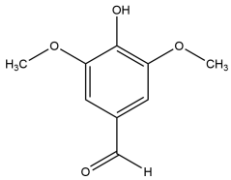
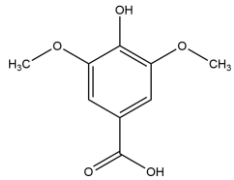
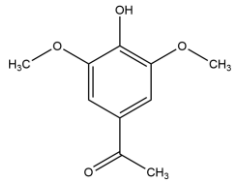
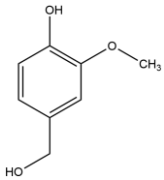
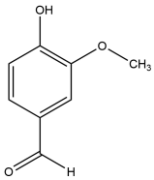
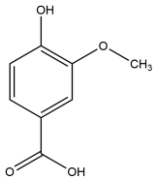
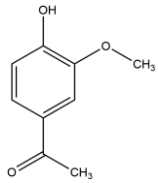
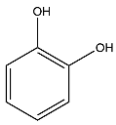
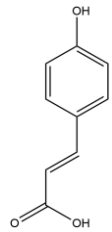
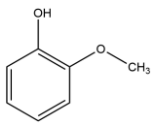
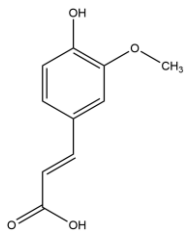
Phenolic compounds

The source of lignocellulosic biomass, as well as the pretreatment method utilised, determines the composition of the phenolic compounds found in the pretreated lignocellulosic material (Almeida et al., 2007; Klinke et al., 2004; S. Larsson et al., 1999). Still, some compounds are more commonly found in pretreated lignocellulosic biomass and they are listed in Table 3.

In the fermentation step, phenolic compounds with low molecular weight have a more severe inhibitory effect than those with high molecular weight (Klinke et al., 2004). It has also been observed that the toxicity is not determined by the category in which they are classified (alcohol, aldehyde, acid or ketone). Instead, their additional functional groups may play a more important role in their toxicity effects (Adeboye et al., 2014). The inhibitory effects of these compounds have been linked to a disruption of the cytoplasmic membrane together with the acidification of the intracellular cytoplasm (Gu et al., 2019).

Lignin and the derived phenolic compounds can also affect the SS(c)F fermentation process as they can also hinder the enzymatic hydrolysis. After pretreatment, most of the lignin remains insoluble and enzymes adsorb non-specifically onto lignin, which affects their efficiency. Solubilised simple and oligomeric phenolics can also inhibit or deactivate hydrolysing enzymes (Tejirian & Xu, 2011).

Table 3. Examples of phenolic compounds found in pretreated lignocellulosic biomass (Klinke et al., 2004).

Alcohols	Aldehydes	Acids	Ketones
	4-Hydroxybenzaldehyde 	4-Hydroxybenzoic acid 	4-Hydroxyacetophenone 
Syringol 	Syringaldehyde 	Syringic acid 	Acetosyringone 
Vanillylalcohol 	Vanillin 	Vanillic acid 	Acetovanillone 
Catechol 		Coumaric acid 	
Guaiacol 		Ferulic acid 	

The presence of mixed sugars

The inherently complex nature of lignocellulosic biomass implies that a mixture of sugars is available as a substrate for fermentation. Although the specific composition of this mixture depends on the source of the lignocellulosic material (cf. Chapter 1), both hexose sugars (i.e., glucose, galactose, mannose) and pentose sugars (i.e., xylose, arabinose) are to be expected. For the process to be profitable, all these sugars must be fully converted to ethanol (Saha, 2003).

Microorganisms have a system called carbon catabolite repression by which the presence of the preferred carbon source, typically glucose, represses the expression of genes related to the metabolisation of alternative carbon sources (Gancedo, 1992; Stülke & Hillen, 1999). This means that when presented with a mixture of sugars as substrate, like the one obtained from lignocellulosic biomass, microorganisms will preferentially consume the sugar that allows their fastest growth before they start consuming the next sugar. This sequential consumption of sugars is not ideal from a process point of view as it reduces the productivity of the process. Instead, simultaneous consumption of the sugars present in the medium would be preferred (J. H. Kim et al., 2010).

In the case of *S. cerevisiae*, the presence of mixed sugars adds an extra challenge, as this yeast species is not able to naturally utilise xylose, the second most abundant sugar in lignocellulosic biomass. This has led to a wide range of strategies to procure and optimise the capacity to use xylose in this species, which will be further discussed in Chapter 5.

The risk of contamination

The 2G ethanol production process presents some interesting specificities that limit the chances of microbial contamination. First, the pretreatment of lignocellulosic biomass is performed at high temperatures, which is expected to lead to a reduction of the contaminating microbial flora present in the raw material. In addition, the formation of inhibitors during the pretreatment step generates a harsh environment that helps prevent the growth of microorganisms with a low tolerance for these inhibitors. Finally, the ethanol produced in the fermentation broth acts as an inhibitor of microbial growth; accordingly, only microorganisms with high ethanol tolerance will be able to survive in such conditions (Tomás-Pejó et al., 2008).

Still, unwanted microorganisms can establish themselves and negatively affect the fermentation step due to the amount and diversity of sugars present in the broth. For instance, if the microorganism utilised for the conversion is not capable of consuming all the sugars present in the broth, other microorganisms may take over

and consume the remaining sugars (Muthaiyan et al., 2011). Schell et al., observed this phenomenon in the industrial conversion of corn fibre to ethanol, where *Lactobacillus* bacteria were found to consume the arabinose present in the mixture because it was not consumed by *S. cerevisiae* (Schell et al., 2007). Also, as the fermentation progresses, the level of inhibitors decreases (due to microbial detoxification by yeast), and, with it, their protective action against contamination.

The most popular approach to avoid contamination in small-scale biological processes is the sterilisation at 121°C for 20 minutes of both equipment and solutions before their use. However, in 2G ethanol production processes, sterilisation of the lignocellulosic material is not commonly performed (Skinner & Leathers, 2004), as it is an energy-consuming and thus too costly process for this low-value product. An alternative option is to use antibiotics such as penicillin and virginiamycin that target bacterial cells without compromising the yeast viability (Skinner & Leathers, 2004). However, here again, the increased cost of production, but also the appearance of resistant bacterial strains (Bischoff et al., 2009) and their presence in downstream products (Bischoff et al., 2016) call for the development of alternative solutions.

The choice of the process configurations can play a significant role in reducing the risk of contamination during fermentation. For example, in SHF, where a complete hydrolysis of the biomass is performed before fermentation high concentrations of different sugars are initially present. In contrast, in SS(c)F, xylose is the most abundant sugar present in the solution at the beginning of the fermentation, whereas glucose is gradually released and consumed; this keeps its concentration low throughout the fermentation and thus limits the number and type of contaminating microorganisms. In contrast, SS(c)F is usually performed at 37°C as a compromise between the optimal temperature for the enzymatic hydrolysis and the fermentation, which is the optimal growth temperature of lactic acid bacteria, the most common contaminant in ethanol fermentations (Skinner & Leathers, 2004).

Overall, the risks associated with bacterial contamination call for the development of engineered *S. cerevisiae* strains capable of consuming not only the hexose but also the pentose sugars in the broth (further developed in Chapter 5).

Chapter 3
Monitoring and control strategies for efficient yeast performance

To ensure the best results during yeast cultivation, optimal environmental conditions need to be guaranteed. Hence, it is crucial to be able to monitor and maintain these conditions during cultivation in bioreactors. Furthermore, the monitoring of relevant parameters in real-time gives insight into the evolution of the cultivation. This is the philosophy behind process analytical technologies (PAT), described by the FDA to ensure the quality of the products obtained in bioprocesses. Through the use of PAT, it is possible to identify the process's critical quality attributes (CQAs) and adjust the critical process parameters (CPPs) (Glassey et al., 2011). Although this framework is most used in biopharmaceutical applications, the concept is transferable to other bioprocesses, including the production of 2G bioethanol.

Monitoring methods in 2G ethanol bioprocesses

Monitoring methods can be classified as in-line, on-line, at-line or off-line depending on their position relative to the bioreactor (Figure 10) (Gargalo et al., 2020). In-line sensors are directly in contact with the broth and collect data continuously. If the sensor is not directly in contact with the broth it is often referred to as on-line instead (Gargalo et al., 2020). At-line sensors rely on the periodic collection of samples, either manually or automatically, which are analysed next to the bioreactor. However, small time delays in the data analysis are encountered. Finally, off-line measurements require the collected samples to be analysed in the laboratory with the consequent longer time delays. In-line and on-line sensors are usually utilised for the adequate implementation of PAT tools as they can collect data in real-time without experiencing time delays for the analysis of the sample (Gargalo et al., 2022). Still, at-line systems should not be disregarded since, as will be shown later on in this chapter, with an adequate time resolution, they can also be utilised as PAT tools.

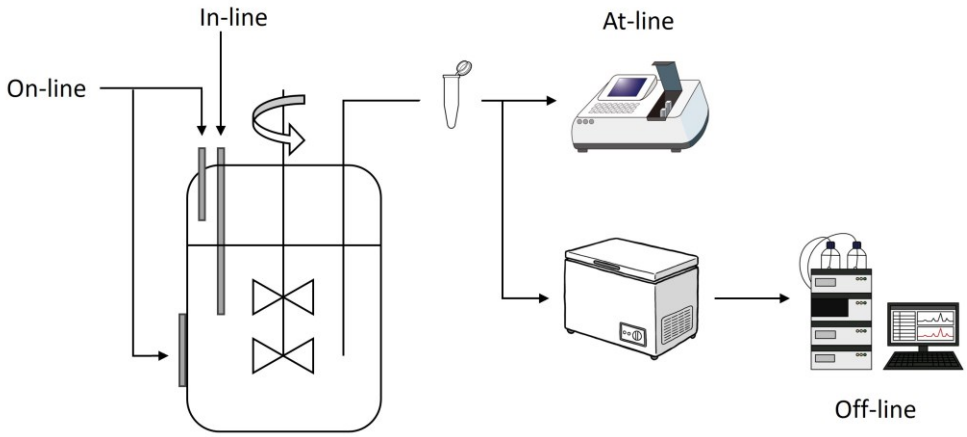


Figure 10. Overview of different types of monitoring methods in bioreactor cultivations.

Critical process parameters (CPPs) such as pH, temperature or dissolved oxygen are routinely monitored during bioreactor cultivations with the aid of in-line or on-line probes. However, biological parameters such as cell density, substrate consumption or product formation are often performed by off-line measurements analysed by optical density or cell dry weight, in the case of cell density, or using high-performance liquid chromatography (HPLC) and/or gas chromatography (GC) for metabolite concentrations. The compilation of real-time data for these parameters is limited by the lack of available sensors (Gargalo et al., 2022).

In lignocellulosic ethanol fermentations, the composition of the feedstock might vary from batch to batch (cf. Chapter 1) and consequently, the conditions faced by the yeast during the cultivation may also vary between fermentations. As such, the development and implementation of PAT tools that allow the monitoring of relevant parameters during bioreactor cultivation can be highly beneficial. Advances in the development of sensing technologies including UV-Visible spectroscopy, multiwavelength fluorescence (MWF), near- or mid-infrared spectroscopy (NIR/MIR) and Raman spectroscopy have opened the possibility of obtaining real-time measurements during cultivation (Gargalo et al., 2022).

The use of ultraviolet-visible spectroscopy (UV-Vis) to monitor fermentation processes is based on the absorption of electromagnetic radiation in the 200-780 nm region by chromophores. Although this method is limited by the fact that the sugars used as substrate in fermentation do not contain chromophores that absorb in that range (Gargalo et al., 2022), it has successfully been utilised for the quantification of inhibitory compounds such as vanillin, ferulic acid, HMF and furfural in lignocellulosic hydrolysates (Pinto et al., 2018).

MWF systems capable of on-line measurement of the fluorescence in the bioreactor have also been developed (Marose et al., 1998). These measurements rely on the fluorescent nature of several compounds such as NAD(P)H, flavins or aromatic amino acids. As such, they are usually considered as an indirect measurement of cell concentration in the bioreactor (Haack et al., 2004). However, with the development of chemometric approaches that combine MFW measurements and the compilation of other relevant bioprocess parameters, such as carbon dioxide evolution rate (CER) or oxygen uptake rate (OUR), mathematic predictive models for the concentration of glucose and ethanol have been developed (Ödman et al., 2009). A similar approach was used by Razan and colleagues to monitor the concentrations of sucrose, glycerol, ethanol and cell biomass in an ethanol fermentation from sugarcane juice by *Saccharomyces cerevisiae* (Ranzan et al., 2012).

NIR and MIR spectroscopy are methods based on the change of vibrational state generated in the molecules upon absorption of infrared light. NIR transitions occur in the 780-2500 nm region, whereas MIR transitions occur in the 2500 nm-25 μm region (Lourenço et al., 2012). Although less mature than NIR, MIR may represent a better choice due to the higher number of molecules measurable in this range as well as the lower interference of water in the measurements (Gargalo et al., 2022; Lourenço et al., 2012). Both methods have been successfully implemented in ethanolic fermentations by *S. cerevisiae* for at-line monitoring of cell biomass, glucose and ethanol concentrations (Finn et al., 2006; Mazarevica et al., 2004).

Finally, another methodology that has gained interest is Raman spectroscopy. This technique is based on the wavelength change between the light source and the light scattered by the molecules, which is dependent on the chemical bonds present in the molecule (Lourenço et al., 2012). One of the major advantages of Raman spectroscopy is the lack of interference from water in the measurements (Lourenço et al., 2012). In-line monitoring of glucose and ethanol concentrations in lignocellulosic bioethanol fermentations was achieved using an immersion Raman probe (Ewanick et al., 2013; Iversen & Ahring, 2014). More recently, Schalk and colleagues developed a non-invasive Raman probe for on-line monitoring based on the measurements in a small glass cell on the side of the bioreactor (Schalk et al., 2017).

Control strategies in 2G ethanol bioprocesses

The information compiled by the different monitoring methods can be used for the application of control strategies that guarantee the efficiency of the cultivation. If unforeseen results are detected through monitoring, control decisions can then be taken before the process becomes unrecoverable. For such purpose, it is of utmost importance that the monitoring method gives real-time information, which is often accomplished by using in-line or on-line sensors. The use of at-line sensors for control purposes might also be feasible if the sample preparation and analysis time are short enough. Finally, off-line measurements are often discarded for control strategies due to the time delay between sampling and data analysis.

In a 2G ethanol bioprocess, there are two steps in which the implementation of control strategies could benefit the optimal yeast performance: the propagation of the yeast and the following fermentation step (Figure 11). The main objective of yeast propagation is the obtention of high cell biomass, which is often performed in aerobic fed-batch cultivations (Papers I & II). *S. cerevisiae* exhibits a Crabtree effect by which ethanol fermentation is favoured over respiration, even under fully aerobic conditions, if there is an excess of carbon source (De Deken, 1966). To avoid such behaviour, which would diminish the biomass yield of the cultivation, the amount of carbon source introduced into the bioreactor is controlled and limited in the fed-batch mode. In contrast, in the case of the following fermentation step, the maximisation of ethanol production is the main objective. The use of fed-batch mode for yeast propagation can also be beneficial to limit the amounts of inhibitory compounds introduced into the bioreactor and reduce their impact on the cell population. Consequently, possible strategies for feed rate control in fed-batch mode for the improvement of 2G ethanol bioprocesses will be the focus of this section.

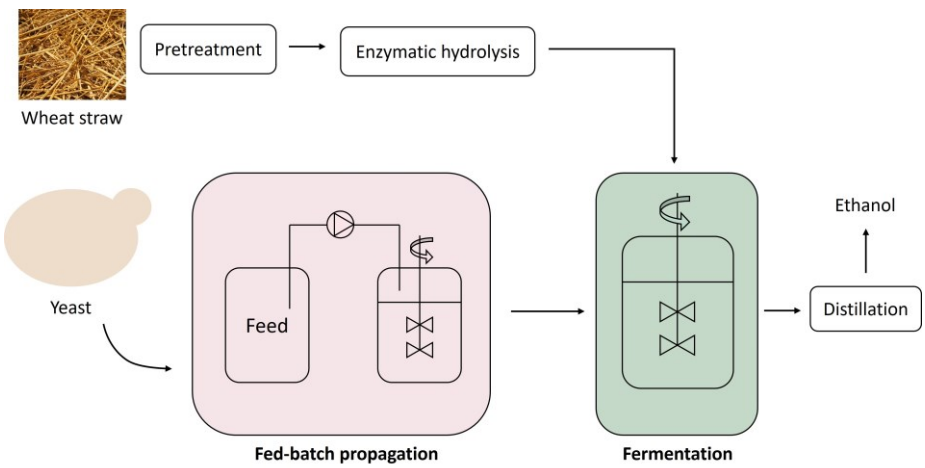


Figure 11. Schematic representation of the process steps involved in 2G bioethanol production.

Different process parameters can be used as input variables for the development of control strategies. An early method utilised on-line measurements of heat production rate, with the help of a microcalorimeter, to control the substrate feed (C. Larsson et al., 1991; Nilsson et al., 2001). The composition of the exhaust gas is routinely monitored in bioreactor cultivations using e.g., acoustic sensors (Christensen et al., 1995). The production of CO_2, often referred to as carbon dioxide evolution rate (CER) is directly related to microbial growth and the ethanol production rate. Thus, a control strategy in which the feed rate can be increased as long as the CER increases has also been developed and refined (Nilsson et al., 2001; Taherzadeh, Niklasson, et al., 2000). Its implementation in fermentations using lignocellulosic hydrolysates led to increased sugar conversion and ethanol productivity (Nilsson et al., 2001). The respiratory quotient (RQ), which is the ratio between the CER and the oxygen uptake rate (OUR), can also be used as the input variable to control the feed rate during propagation (Dobrescu et al., 2021). Finally, the concentration of ethanol in the gas exhaust can also be monitored. As such, ethanol concentration has also been used as an input variable for the development of a control strategy (Petersson & Lidén, 2007). This strategy was successfully applied to control the substrate feeding rate for maintaining low levels of ethanol during yeast propagation and high specific growth rates were obtained (Petersson & Lidén, 2007).

Additional monitoring methods and their suitability for control strategies have also been explored. For example, real-time fluorescence measurements have been used to maintain aerobic respiration in a feed-rate controlled fed-batch propagation (Hantelmann et al., 2006). The system was based on the differences in NADH and flavin concentrations between oxidative and oxidoreductive metabolism (Hantelmann et al., 2006). Different methods such as Raman spectroscopy (Hirsch et al., 2019), refractive index (Knudsen & Rønnow, 2020) and MIR (Cabaneros Lopez et al., 2020) have also been used to monitor glucose concentration in the fermentation and use this input variable to develop feed rate control strategies. Cabaneros Lopez and colleagues were able to improve sugar co-consumption during fermentation with the application of their MIR-based control strategy (Cabaneros Lopez et al., 2020).

Although all these examples have the potential to improve 2G bioethanol processes, they are mainly based on the measurement of extracellular metabolites such as CO_2, glucose or ethanol, but little is known about the intracellular processes during cultivation. In the present thesis, the application of intracellular biosensors to elucidate relevant cellular responses was explored. The following questions were addressed: Can biosensors be used to measure relevant intracellular properties? Is it possible to develop a system for real-time measurement of the biosensors' response? Can biosensors be used to follow dynamic responses? Can the biosensor response be used as an input variable for a control strategy?

Biosensors

What is a biosensor?

Biosensors are molecular devices based on biological recognition elements capable of interacting with a target compound and generating a specific signal. This response is sent to a transducer that converts it into a detectable signal (L. Su et al., 2011). Depending on how this signal is measured, different types of biosensors can be distinguished: optical, electrochemical or acoustical (Gargalo et al., 2022), the electrochemical and optical ones being the most widely used (L. Su et al., 2011).

In this thesis, fluorescent biosensors, a type of optical biosensors in which the fluorescence intensity is measured, were introduced into *S. cerevisiae* strains to measure intracellular redox imbalance (Papers I & II) and the sugar signalling response (Paper III).

Fluorescent biosensors in *S. cerevisiae*

Fluorescent biosensors have been developed to provide information about cell physiology and/or quantify metabolite production in *S. cerevisiae* (Table 4). These biosensors rely on the use of fluorescent proteins (FPs) as reporter molecules, the yeast-enhanced green fluorescent protein (yEGFP) being the most popular FP (Cormack et al., 1997). yEGFP is a variant of the wild-type GFP discovered in the jellyfish *Aequorea victoria* (Prasher et al., 1992) which was modified for optimal expression in yeast resulting in high fluorescence intensities (Cormack et al., 1997).

Amongst the biosensors used in *S. cerevisiae*, two types can be encountered: (i) fluorescence resonance energy transfer (FRET)-based biosensors and (ii) transcription factor-based biosensors. In FRET-based biosensors, two fluorescent proteins are used, typically a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) which are separated by the sensing region (Colombo et al., 2017) (Figure 12A). When the metabolite binds to the sensing region, a conformational change occurs bringing the two FPs together. This generates a shift in the fluorescence emission as the emission from the CFP is now used to further excite the YFP which will then emit its fluorescence (Figure 12A). In transcription factor-based biosensors the expression of a FP is controlled by an inducible promoter of interest. The induction or repression of the promoter is regulated by the binding of transcription factor(s) to it (Figure 12B). The possibility of using transcription factor-based biosensors to follow *S. cerevisiae* behaviour in 2G ethanol production was explored in the present thesis.

Table 4. Examples of fluorescent biosensors used in *S. cerevisiae*. FCM: flow cytometry.

Monitoring of	Reporter molecule	Application	Analysis method	Reference
Fluorescence resonance energy transfer (FRET)-based				
Maltose	CFP+YFP	Monitoring of maltose uptake	Molecular imaging	(Ha et al., 2007)
Methionine	CFP+YFP	Screening for production strains	Microscopy	(Mohsin & Ahmad, 2014)
cAMP	mTurquoise2 +dVenus/ tdTomato	Monitoring of intracellular cAMP	Microscopy + FCM	(Botman et al., 2021; Colombo et al., 2017)
ATP	ymTq2Δ11 +tdTomato	Monitoring of ATP levels	Microscopy	(Botman et al., 2020)
PKA activity	eCFP+ YPET	Monitoring of PKA activity	Microscopy + FCM	(Botman et al., 2023)
Transcription-factor based				
Growth	yEGFP	Monitoring of growth and cell membrane robustness	FCM	(Carlquist et al., 2012)
NADH/NAD^+ imbalance	yEGFP	Monitoring of redox imbalance	FCM	(Knudsen et al., 2014)
Malonyl-CoA	tdTomato	Screening of genomic library	Fluorometry	(S. Li et al., 2015)
cis, cis-Muconic acid (CCM)	GFP	Screening for production strains	FCM	(Skjoedt et al., 2016)
NADPH deficiency	yEGFP	Monitoring of redox imbalance	Fluorometry + FCM	(J. Zhang et al., 2016)
Sugar signalling	yEGFP	Monitoring of sugar signalling	FCM	(Brink et al., 2016)
Fatty acid	GFP	Screening for production strains	Fluorometry	(Baumann et al., 2018)
4-hydroxybenzoic acid (pHBA)	mCitrine	Screening for production strains	FCM	(Castaño-Cerezo et al., 2020)
Acetic acid	mCherry	Screening for production strains	Fluorometry	(Mormino et al., 2021)
Glucaric acid	GFP	Screening for production strains	Fluorometry	(R. Su et al., 2022)
S-Adenosylmethionine (SAM)	yEGFP	Screening for production strains	FCM	(Y. Chen et al., 2023)

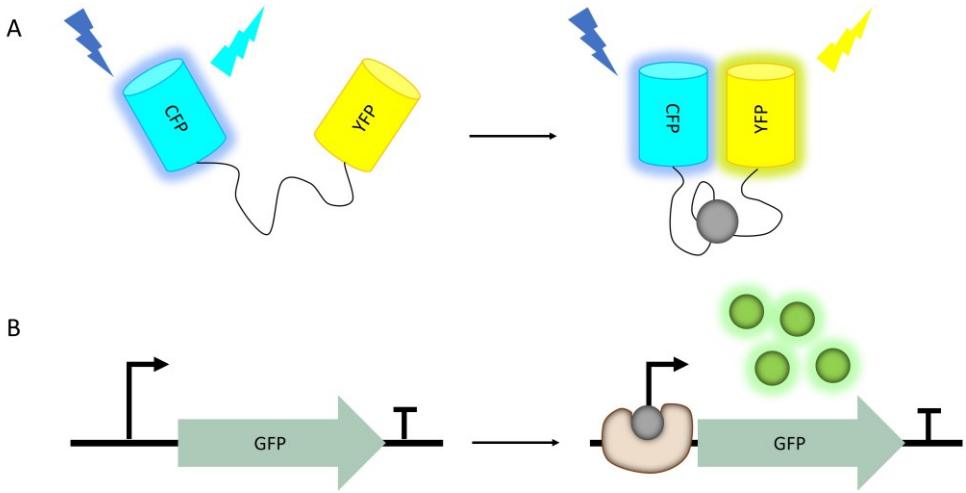


Figure 12. Schematic representation of a fluorescence resonance energy transfer (FRET)-based biosensor (A) and a transcription factor-based biosensor (B). Upon activation of the FRET biosensor, a conformational change occurs which brings the two FPs closer so that the fluorescent emission of CFP can be used to excite YFP. In the transcription factor-based biosensor, the inducing molecule activates a transcription factor which in turn induces the transcription of GFP. CFP: cyan fluorescent protein. YFP: yellow fluorescent protein. GFP: green fluorescent protein.

Flow cytometry

In order to maximise the data extracted from biosensors, it is essential to employ a suitable methodology for their measurement. The two most common methods used to measure the response of transcription factor-based biosensors are fluorometry and flow cytometry (FCM) (Table 4). The main difference between these two methods is that fluorometry measurements are based on the average fluorescence value of the cell population. In contrast, FCM has the advantage of providing single-cell measurements which allows the obtention of information on population heterogeneity. In this thesis work, FCM was preferred over fluorometry to measure the response of transcription factor-based biosensors (Papers I, II & III) in *S. cerevisiae* during cultivation.

In a flow cytometer, the cells are passed through and analysed one by one (Figure 13). For the analysis, lasers with different wavelengths can be used, including the standard 488 nm blue laser. After excitation, the fluorescence emitted by each cell in the different wavelength ranges can be measured with the use of filters that collect specific wavelengths in each channel (Figure 13). Besides the fluorescence intensity, other parameters can be elucidated from FCM analysis. For example, the light scattered in the same direction as the light source is known as forward scatter (FSC) and is considered an indication of the size of the cell, while the light scattered perpendicular to the light source is known as side scatter (SSC)

and it indicates the granularity of the cell (Shapiro, 2003). Moreover, the number of events measured in the sample is also recorded, thus FCM can also be used for cell quantification (Rao et al., 2023). FCM is also routinely used with fluorescent dyes to obtain additional information. Propidium iodide (PI), that is a common fluorescent dye, has been used in the present thesis work to measure cell membrane integrity. PI can only penetrate cells with damaged membranes. Once inside the cell, it binds to the DNA and results in staining of membrane-damaged cells (Davey & Hexley, 2011).

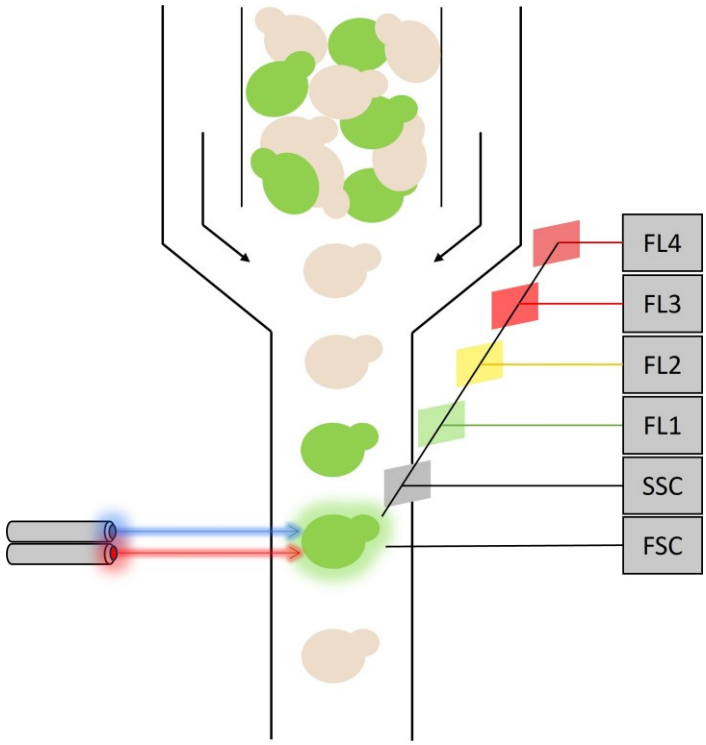


Figure 13. Schematic representation of flow cytometry (FCM) instrument. The sheath fluid is circulated on the sides to push the cells in a single line, this way, the cells reach the interrogation point one at a time where they are exposed to the blue and red excitation lasers and their light refraction and fluorescent emission is collected on the different channels. FSC: Forward scatter; SSC: side scatter; FL1-FL4: fluorescence collection channels.

In the present thesis, the possibility of measuring different FPs using the benchtop flow cytometer BD Accuri C6 (+) was explored (Paper IV). The objective was to identify the most suitable FPs for being used as reporter molecules in *S. cerevisiae*, both individually and in potential combinations for multicolour FCM.

Out of the six selected candidates, the monomeric variant of GFP mEGFP, the orange FP CyOFP1 and the red FP mBeRFP showed the best results; the

simultaneous measurement of these three FPs was also possible using a standard 488 nm blue laser for excitation (Paper IV). This finding opens up the possibility of simultaneously analysing up to three different properties in the same cell.

One of the biggest limitations of multicolour FCM is the spillover from one FP into other collection channels (Hawley et al., 2017). To minimise such a phenomenon, a narrower filter (510/15 nm) was utilised in the study above to measure the fluorescence of GFP - as opposed to the common 533/30 nm filter. The application of colour compensation strategies to mathematically transform the data can additionally be used for the successful implementation of multicolour FCM (Hawley et al., 2017).

Biosensors as monitoring and control tools

The combination of biosensors with FCM analysis has great potential for the compilation of information at the single-cell level on virtually any cellular property for which a specific biosensor is available. However, as detailed previously in this chapter, real-time measurements are needed to reach the full potential of FCM as a PAT tool. The application of FCM as a real-time monitoring method has been described in drinking water applications (Besmer et al., 2014; Hammes et al., 2012) and it has recently also been used to monitor the growth of microalgae (Haberkorn et al., 2021) and lactic acid bacteria (Rao et al., 2023). However, its application to yeast cultivations remains relatively unexplored. An early example showed the successful monitoring of cell count and viability as well as the constitutive expression of GFP throughout the cultivation of *S. cerevisiae* in bioreactors (Abu-Absi et al., 2003). Similarly, Bouchedja et al., used automated real-time FCM to study the cell physiology and lipid accumulation of *Yarrowia lipolytica* (Bouchedja et al., 2017). More recently, efforts have been made to develop an open-source platform to push forward the implementation of this methodology (Bertaux et al., 2022).

In the present work, at-line FCM was combined with the utilisation of a biosensor and PI fluorescent dye to monitor (Paper I) and control (Paper II) the response of a redox imbalance sensor in *S. cerevisiae* during cultivation in a bioreactor. In this setup, an automatic sampler was connected to the bioreactor for periodic sampling. The sample was then automatically diluted, stained with PI and sent for FCM analysis (Figure 14A). The rapid sampling and measurement obtained with this method allowed the monitoring of the dynamic response of the biosensor for redox imbalance in the presence of furfural, the compound responsible for the induction of the biosensor's response (Paper I).

In order to take it one step further, the possibility of utilising this automated FCM system as a feed control parameter was assessed (Paper II). In this case, such a setup was applied during the fed-batch propagation of *S. cerevisiae* in a 2G ethanol process context. A new program code was developed in Python to automatically analyse the information obtained from the FCM measurements of the biosensor's response and adjust the substrate feed accordingly (Figure 14B) (Paper II). The application of this system is further discussed in Chapter 4.

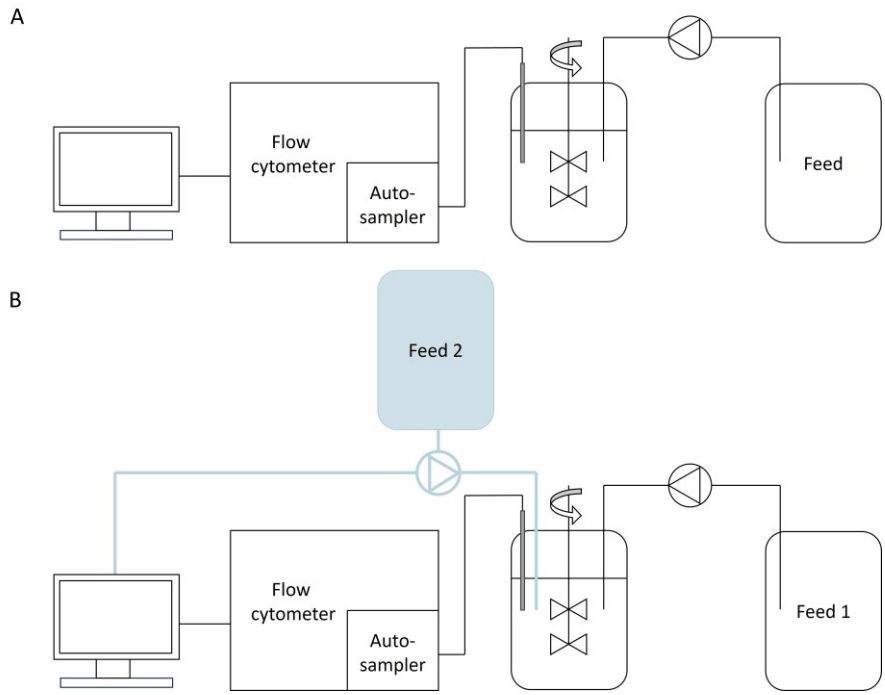


Figure 14. Schematic representation of the monitoring (A) and control (B) strategies based on FCM measurements.

Specific sampling challenges in 2G ethanol bioprocesses

One of the biggest challenges for the development of monitoring and control strategies in 2G ethanol bioprocesses is the complex nature of the substrate matrix. This is especially relevant in process configurations like SS(c)F where the pre-treated raw material is used as fermentation broth. In this process, initial high solid contents are usually present, even hindering mixing operations. Through the action of hydrolytic enzymes, the fermentation broth liquifies over time. However, solid lignin particles remain throughout the cultivation. These conditions render the

distinction between cells and particles challenging. Overestimation of cell concentration can be obtained due to false positives if material particles in the matrix are mistaken for cells. Cells might also adsorb on material particles and not be measured, which results in an underestimation of cell concentration (R. Wang et al., 2021).

A comparison of different methods for the quantification of cell concentration in SSF samples concluded that traditional methods such as cell counting in a hemocytometer and colony forming unit (CFU) remained the best option (R. Wang et al., 2021). However, these are time-consuming off-line methods that cannot be used for real-time monitoring. The determination of extracellular metabolite concentration is also mostly measured off-line by HPLC of the supernatant after the sample has been centrifuged. This is because the implementation of real-time methods such as UV-Vis, MWF or Raman spectroscopy has proven limited due to the interference of the solid particles with the measurements (Gargalo et al., 2022; Ranzan et al., 2012). Iversen and Ahring managed to obtain in-line Raman spectroscopy measurements during the fermentation of sugarcane bagasse by placing the probe in the top layer and reducing the stirring speed so that the particles would precipitate at the bottom of the bioreactor (Iversen & Ahring, 2014). Although ingenious, this strategy is not optimal as it is difficult to ensure the representativeness of the measured sample without a homogenous composition in the bioreactor.

Currently, sample preparation in 2G bioethanol processes mostly relies on centrifugation or filtration for the separation of the solids from the liquid in the sample. However, these methods are only valid for the analysis of the liquid fraction for extracellular metabolite concentration. Cells and particles will likely remain unseparated in the solid fraction. Also, delays between sample acquisition and data analysis make the application of analytical methods as monitoring methods very challenging.

In this thesis work, the potential of utilising FCM as a monitoring tool for SS(c)F was assessed. The presence of solid particles that could clog the lines in the instrument, did not make it possible to apply the developed monitoring method (Paper I) to the SS(c)F experiments. Instead, attempts were made to manually treat SS(c)F samples to separate the cells from the material particles before FCM analysis. Out of the tested methods, the most promising one involved the use of a syringe filter with a 10 µm pore size that allowed yeast cells (ca. 5-6 µm) as well as the smallest particles to pass through the filter while retaining bigger particles (Figure 15). The filtered samples were stained with the fluorescent dye SYBR Green, which binds to DNA. During FCM analysis, a minimum threshold in forward scatter (FSC-H) was then used to discard particles based on size (Figure 15A, D). Then the SYBR Green fluorescence in FL1-H was used to identify the cell

population (Figure 15B). The cell population was found to correspond to the subpopulation with a higher size (Figure 15C). This approach was successful up to 2% WIS samples (Figure 15A-C), but no cell population was distinguishable in samples with higher solid-loading (5% WIS) (Figure 15D-E). The higher number of solid particles in the 5% WIS samples likely resulted in the formation of a cake on the filter preventing cells from passing through.

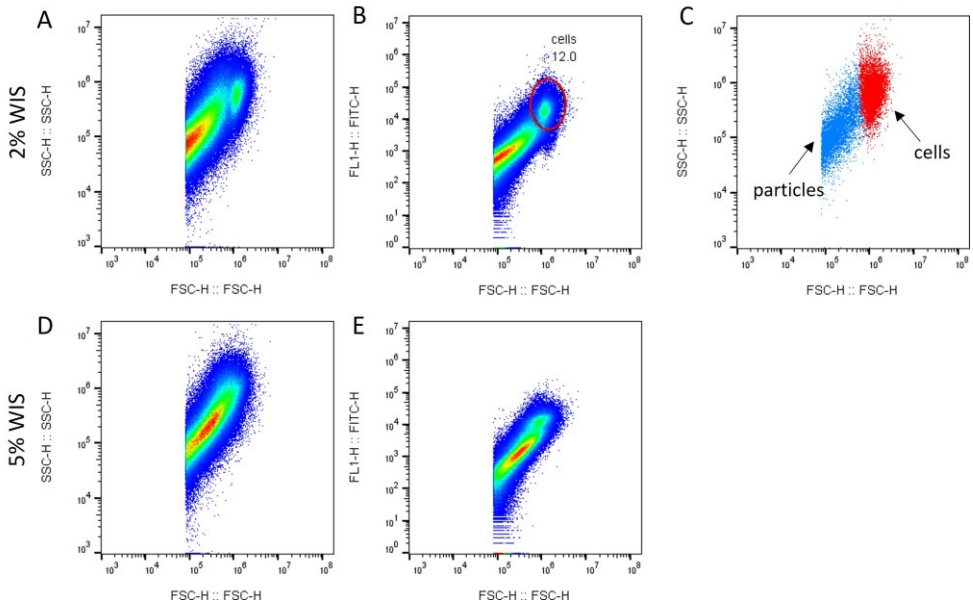


Figure 15. Flow cytometry analysis of 2% WIS sample (A-C) and 5% WIS sample (D-E). Samples were filtered using a 10 µm pore size filter and stained with SybrGreen prior to flow cytometry analysis. Samples are first analysed in forward (FSC-H) vs side (SSC-H) scatter (A, D), then the green fluorescence intensity is looked at (FL1-H) to identify the cell population (B, E). Finally, the cell population is coloured in red and the solid particles in blue in a FSC-H vs SSC-H scatter plot (C).

Alternatively, for samples in which the utilised strains carry a biosensor, the fluorescent signal of the biosensor could be used as the distinctive parameter between cells and particles without the need for a fluorescent dye.

In the future, further optimisation of the filtration strategy may enable the development of a method suitable for higher WIS concentrations, as well as for its automatic functioning, for FCM to become applicable as a monitoring tool for SS(c)F processes.

Chapter 4
Yeast engineering and monitoring for overcoming the effect of inhibitory compounds

Different types of inhibitors - furaldehydes, weak acids and phenolics - can be encountered in lignocellulosic hydrolysates (cf. Chapter 2). This has led to the development of several strategies to obtain *Saccharomyces cerevisiae* strains with enhanced tolerance to these compounds. This chapter discusses the most significant advances in this area.

Strain engineering approaches

Targeted engineering

When the mechanisms behind the inhibitory effects or the cellular response against the inhibitor are known, rational engineering targeting the corresponding gene(s) can be envisioned. This strategy has been employed with each type of inhibitor, as discussed below.

Improving tolerance to furaldehydes

Intracellular accumulation of furaldehyde compounds triggers the formation of reactive oxygen species (ROS) and redox imbalance in yeast (cf. Chapter 2). Therefore, genes encoding enzymes responsible for the conversion of these aldehydes into less toxic alcohol forms have commonly been targeted to speed up the detoxification capabilities in the modified *S. cerevisiae* strain and reduce the inhibitory effects (Figure 16). The first gene reported to encode an enzyme with such activity was *ADH6*. ADH6p is an NADPH-dependent alcohol dehydrogenase that can convert HMF into its less inhibitory alcohol form in *S. cerevisiae* (Petersson et al., 2006). Accordingly, *ADH6* gene overexpression has led to an increase in the conversion rate of HMF under both aerobic and anaerobic conditions (Petersson et

al., 2006) resulting in improved tolerance both in laboratory (Almeida et al., 2008; Park et al., 2011) and industrial strains (Lewis Liu et al., 2008). Since then, several other endogenous enzymes with the same activity have been identified including alcohol dehydrogenases, aldehyde reductases and aldose reductases. The overexpression of the genes encoding these enzymes has also been shown to improve growth in the presence of HMF and/or furfural (Table 5).

The need for NAD(P)H during the detoxification of furaldehydes as well as in the processes involved in protecting the cell from the oxidative stress caused by the presence of aldehyde compounds, generates a cofactor imbalance in the cell (cf. Chapter 2). So, another approach to increase the tolerance towards furaldehydes has focused on strategies aiming at maintaining the redox balance in the cell (Table 5). The main target of this approach has been *ZWF1*, a gene encoding glucose 6-phosphate dehydrogenase that is the first enzyme in the oxidative pentose phosphate pathway and a major source of NADPH (Figure 16). Strains overexpressing *ZWF1* were shown to grow at higher furfural concentrations than their control strains, likely due to the increased availability of NADPH (Gorsich et al., 2006). Similarly, other genes encoding activities involved in processes that could provide a source of NADPH or that are involved in maintaining redox balance in the cell, such as the glutathione metabolism, have also been overexpressed and have resulted in a higher tolerance to furaldehydes (Table 5).

Improving tolerance to weak acids

The presence of weak acids in the medium results in decreased intracellular pH, ATP depletion and accumulation of ROS species that lead to programmed cellular death in *S. cerevisiae* (cf. Chapter 2). Thus, genes involved in the membrane transport of weak acids or protons have been potential rational engineering targets for increased tolerance to weak acids (Table 5). The entry of undissociated weak acids such as acetic acid into the cells in *S. cerevisiae* is made by facilitated diffusion through the aquaporin channel Fps1p (Mollapour and Piper, 2007) (Figure 16). Consequently, *FPS1* was considered as a possible target to increase tolerance to weak acids. Indeed, the deletion of *FPS1* in an industrial strain resulted in increased growth rates and ethanol yields under acetic acid stress conditions (J. G. Zhang et al., 2011).

Another gene involved in the cellular response to acetic acid in *S. cerevisiae* is *PMA1*. Pma1p is the main proton pump utilised by *S. cerevisiae* to actively export protons out of the cells at the expense of ATP (Serrano et al., 1986), a process necessary to counteract the acidification caused by the dissociation into anionic acid and its proton inside the cell (Figure 16). Overexpressing *PMA1* resulted in an enhanced acetic acid tolerance characterised by an increased proton efflux and membrane integrity, as well as a reduction in the accumulation of ROS species (Y. Lee et al., 2017).

Several other genes encoding different functions have been shown to increase tolerance to acetic acid. Amongst these, other transporters, regulators or enzymes involved in counteracting the oxidative stress and intracellular acidification generated by acetic acid can be found (Table 5).

Improving tolerance to phenolic compounds

The inhibitory effect of phenolic compounds has been related to membrane damage as well as intracellular pH acidification (cf. Chapter 2). In order to help reduce these deleterious effects, strategies have focused on their conversion into less toxic derivatives as well as their transport outside of the cell (Table 5) (Figure 16). For example, increased tolerance to phenolic compounds such as ferulic acid or cinnamic acid has been achieved by overexpression of *PAD1*. Pad1p is a phenylacrylic acid decarboxylase capable of converting aromatic carboxylic acids into their corresponding vinyl derivatives in *S. cerevisiae* (Clausen et al., 1994). Accordingly, *PAD1* overexpression allowed a faster conversion of the inhibitors which improved the growth rate and ethanol productivity of the generated strain in the presence of ferulic acid and cinnamic acid, as well as when spruce hydrolysate was used (S. Larsson, Nilvebrant, et al., 2001).

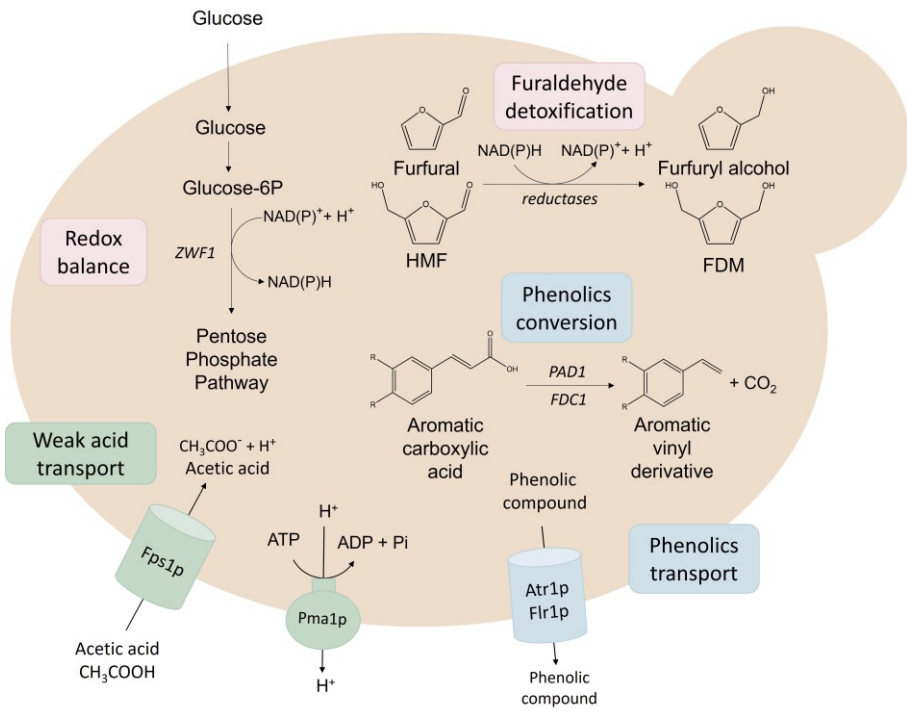


Figure 16. Main cellular processes targeted by rational engineering strategies to increase the inhibitor tolerance in *S. cerevisiae*.

Table 5. Review of major strain engineering strategies targeting single genes involved in the cellular response of *S. cerevisiae* to the different types of inhibitors to obtain increased inhibitor tolerance. Adapted from (Cámara et al., 2022; B. Li et al., 2022).

Gene	Modification	Enzymatic activity/Role	Reference
Furaldehyde detoxification			
ADH6/7	Overexpression	NAD(P)H-dependent alcohol dehydrogenase	(Lewis Liu et al., 2008; Petersson et al., 2006)
ALD6 *ARI1* *YDR541C* *YGL039W* *YNL134C* *OSI1* *GRE2/3*	Overexpression	NAD(P)H-dependent aldehyde reductase	(Heer et al., 2009; Jayakody et al., 2018; Z. L. Liu & Moon, 2009; Moon & Liu, 2015; Park et al., 2011; Zhao et al., 2015)
Improved redox balance			
ZWF1	Overexpression	Glucose-6-phosphate dehydrogenase	(Gorsich et al., 2006)
OYE2	Overexpression	NADPH-dependent oxidoreductase	(C. G. Liu et al., 2020)
POS5	Overexpression	Mitochondrial NADH kinase	(C. G. Liu et al., 2020)
GSH1	Overexpression	γ-glutamylcysteine synthetase	(D. Kim & Hahn, 2013)
GLR1	Overexpression	NADPH-dependent glutathione reductase	(D. Kim & Hahn, 2013)
IDP1 *IDH1*	Overexpression	NAD(P)H-dependent isocitrate dehydrogenase	(C. G. Liu et al., 2020; Unrean, 2017)
Weak acid transport			
FPS1	Deletion	Aquaporin channel	(J. G. Zhang et al., 2011)
ADY2	Deletion	Acetate transporter	(M. Zhang et al., 2017)
PMA1	Overexpression	Proton pump	(Y. Lee et al., 2017)
AZR1	Overexpression	Membrane transporter	(Tenreiro et al., 2000)
PDR12	Overexpression Deletion	Efflux pump	(Nygård et al., 2014)
Weak acid – other mechanisms			
WHI2	Overexpression	Cytoplasmic globular scaffold protein involved in general stress response by activating Msn2/4p	(Y. Chen et al., 2016)
PRS3	Overexpression	Phosphoribosyl pyrophosphate synthetase involved in amino acid biosynthesis	(Cunha et al., 2015, 2018)
JJJ1	Deletion	Co-chaperone involved in ribosome biosynthesis	(X. Wu et al., 2016)
ACS2	Overexpression	acetyl-coenzyme A synthetase involved in acetate conversion	(Ding et al., 2015)
GND1	Overexpression	6-phosphogluconate dehydrogenase involved in oxidative stress	(Y. Lee et al., 2015)
CCW12	Overexpression	Cell wall mannoprotein involved in cell wall composition	(Kong et al., 2021)
HOG1	Overexpression	Mitogen-activated protein kinase involved in osmoregulation	(Mollapour & Piper, 2007b)
ADE1/13/17	Overexpression	Adenine deaminases involved in purine biosynthesis	(M. M. Zhang et al., 2019)

RCK1	Overexpression	Protein kinase involved in oxidative stress	(Oh et al., 2019)
adhE	Heterologous expression	Acetylating acetaldehyde dehydrogenase involved in acetate reduction to ethanol in *Escherichia coli*	(Wei et al., 2013)
Phenolic conversion			
PAD1	Overexpression	Mitochondrial phenylacrylic acid decarboxylase	(S. Larsson, Nilvebrant, et al., 2001)
FDC1	Overexpression	Cytosolic ferulic acid decarboxylase	(Richard, Viljanen and Penttilä, 2015)
lcc2	Heterologous expression	Laccase involved in coniferyl aldehyde detoxification in white rot fungus *Trametes versicolor*	(S. Larsson, Cassland, et al., 2001)
ALD5	Overexpression	Aldehyde dehydrogenase	(Adeboye et al., 2017)
ATF1	Overexpression	Alcohol acetyl transferase	(Adeboye et al., 2017)
ATF2	Overexpression	Alcohol acetyl transferase	(Adeboye et al., 2017)
BDH2	Overexpression	Putative medium-chain alcohol dehydrogenase	(Ishida et al., 2016)
Phenolic transporter			
ATR1	Overexpression	Putative membrane-associated transport protein	(Alriksson et al., 2010)
FLR1	Overexpression	Putative membrane-associated transport protein	(Alriksson et al., 2010)
PDR5 *YOR1* *SNQ2*	Overexpression	ATP-binding membrane transporter	(X. Wang et al., 2017)
Other/unknown			
PHO13	Deletion	p-nitrophenylphosphatase	(Fujitomi et al., 2012)
MCR1	Overexpression	Mitochondrial NADH-cytochrome b5 reductase	(Wallace-Salinas et al., 2014)

Increasing the global response via transcription factors

Besides functional genes, transcription factors have also been targeted as potential strategies to increase inhibitor tolerance. Transcription factors regulate the expression of numerous genes; thus their modification may cause a more significant impact than when a single modification of a functional gene is used (Alriksson et al., 2010). Three main transcription factors have been reported to be involved in the inhibitor tolerance of *S. cerevisiae*: Haa1p, Msn2/4p and Yap1p. Haap1p is involved in the response to the stress induced by weak acids (Fernandes et al., 2005). In the presence of acetic acid, Haap1p is translocated to the nucleus where it induces the transcription of membrane transporters and protein kinases to reduce the concentration of intracellular acetate (Fernandes et al., 2005). Indeed, overexpression of *HAA1* has been shown to improve cellular growth and sugar consumption in industrial strains in the presence of acetic acid and lignocellulosic hydrolysates (Cunha et al., 2018; Inaba et al., 2013; Tanaka et al., 2012).

The homologous transcription factors Msn2p and Msn4p are involved in the response to environmental stress (Martínez-Pastor et al., 1996). Upon the presence of stress factors, Msn2p and Msn4p are dephosphorylated and translocated to the nucleus where they activate the transcription of numerous genes. Amongst the genes regulated by Msn2/4p are genes encoding heat shock proteins, antioxidant enzymes and genes involved in carbon metabolism such as the pentose phosphate pathway (Boy-Marcotte et al., 1999). Overexpression of *MSN2* has been shown to improve furfural tolerance and fermentation rate in an industrial strain (Sasano et al., 2012).

Finally, the transcription factor Yap1p is involved in the response to oxidative stress (Kuge et al., 1997). Furfural and HMF can directly activate Yap1p, by acting as thiol-reactive electrophiles (D. Kim & Hahn, 2013). Furthermore, they induce the accumulation of ROS species which, in turn, activates Yap1p (D. Kim & Hahn, 2013). Upon activation, Yap1p is translocated to the nucleus and induces the transcription of numerous genes encoding, among others, antioxidant enzymes, reductases, and efflux pumps (Jungwirth et al., 2000; J. Lee et al., 1999; Ma & Liu, 2010). Overexpression of *YAP1* has also been shown to improve tolerance to coniferyl aldehyde, furfural, HMF and lignocellulosic hydrolysates both in laboratory and industrial strains (Alriksson et al., 2010; D. Kim & Hahn, 2013; Wallace-Salinas et al., 2014; G. Wu et al., 2017).

Evolutionary engineering

The complex cellular response exhibited by *S. cerevisiae* in the presence of an inhibitor can make the identification and engineering of multiple target genes challenging (Oh et al., 2019). As an alternative, evolutionary engineering approaches might be preferred.

Evolutionary engineering, also known as adaptive laboratory evolution (ALE), is based on the growth of the microorganism under selective conditions over many generations, during which the microorganism acquires spontaneous or induced mutations that lead to increased variability in phenotype. Throughout the generations, the evolved strains with acquired mutations that confer enhanced survivability under the chosen conditions will take over the cultivation (Çakar et al., 2012). Through the application of different evolutionary set-ups, several evolved strains with improved tolerance towards a single or a mixture of inhibitors have been successfully obtained (Table 6). Generally, the strains showed shorter lag phases, higher biomass formation and increased sugar consumption, leading to higher ethanol yields.

In evolutionary engineering experiments, the design of the evolution strategy is crucial for obtaining the expected results. This has notably been exemplified for acetic acid tolerance where strains have first been evolved either using sequential batch cultivation with increasing concentrations of acetic acid or continuous

cultivation in the presence of acetic acid without pH control. Evolved strains with improved acetic acid tolerance have been obtained with both methods; however, they lost their phenotype after being cultivated in the absence of acetic acid and transferred back to fresh medium containing acetic acid (Wright et al., 2011). Instead, a strategy in which the presence of acetic acid in the medium was alternated in the cultivations was necessary to obtain a constitutive acetic acid tolerance (González-Ramos et al., 2016).

With evolutionary engineering, careful characterisation of the evolved strains is essential to avoid running into trade-off situations in which highly specialised strains are obtained for specific conditions at the cost of losing fitness for other conditions (Çakar et al., 2012). Such a situation was observed, for example, in an early evolutionary engineering study in which the obtained evolved strain showed the expected improved xylose and glucose co-consumption during batch cultivation, but had lost capabilities for optimal growth on glucose (Sonderegger & Sauer, 2003).

With the development of technologies such as transcriptomics or whole genome sequencing (WGS), the mutations acquired by the evolved strains can be identified, generating valuable insights into the molecular mechanisms behind inhibitor tolerance (Menegon et al., 2022). Often, the role of the genes involved in known mechanisms and modified by target engineering approaches is confirmed; but new seemingly unrelated genes can also be identified. For example, SNP mutations in four different genes have been identified through WGS of evolved strains with enhanced tolerance to acetic acid. The identified genes encoded a putative transcriptional regulator (*ASG1*), a mitochondrial alcohol dehydrogenase (*ADH3*), a protein kinase involved in adaptation to low glucose concentrations (*SKS1*) and a protein linked to ion homeostasis and glucose derepression of *SUC2* (*GIS4*). In that study, the effect of the identified mutations has been further validated by introducing them into the parental strain in a reverse engineering approach (González-Ramos et al., 2016; Pereira et al., 2020). However, this last step of validation through reverse engineering is often neglected (Menegon et al., 2022).

Regardless of the approach used to engineer yeast strains, targeted or evolutionary engineering, many of the reported studies have focused on the changes observed upon treatment with one specific inhibitor; however, it has been shown that the cellular response to the individual inhibitors can differ from the response observed when a mixture of inhibitors is used (Bajwa et al., 2013). As the development of tolerant strains towards not only one but the combined inhibitors present in lignocellulosic hydrolysates is highly desirable, studies in which lignocellulosic hydrolysates are used are more likely to generate more robust strains.

Table 6. Examples of evolutionary engineering studies of *S. cerevisiae* towards improved performance in lignocellulosic hydrolysate fermentations. Examples are listed in chronological order. Adapted from (Menegon et al., 2022).

***S. cerevisiae* parent strain**	**Strategy**	**Selection pressure**	**Evolved strain phenotype**	**Reference**
D5A K-1	Sequential batch transfer with increasing inhibitor concentrations	Softwood hydrolysate	Higher tolerance, shorter lag phase and higher ethanol productivity in SSF	(Keller et al., 1998)
NRRL Y-12632	Sequential batch transfer with increasing inhibitor concentrations	Furfural or HMF	Shorter lag phase and higher ethanol productivity	(Z. L. Liu et al., 2005)
TMB3001	Continuous cultivation with increasing inhibitor concentrations	Sugarcane bagasse	Faster furfural conversion, higher ethanol yield and productivity	(Martín et al., 2007)
TMB3400	Sequential batch transfer with increasing inhibitor concentrations	Furfural	Higher tolerance, shorter lag phase and higher ethanol productivity	(Heer & Sauer, 2008)
F12	Sequential batch transfer with increasing inhibitor concentrations	Wheat straw hydrolysate	Higher xylose consumption, higher ethanol productivity and titer	(Tomás-Pejó et al., 2010)
RWB218	Sequential batch transfer with increasing inhibitor concentrations and continuous cultivation without pH control	Acetic acid	Transient higher acetic acid tolerance and specific xylose consumption rate	(Wright et al., 2011)
XR122N	Sequential batch transfer	Pretreated pine wood	Higher growth at high solid loadings	(Hawkins & Doran-Peterson, 2011)
TMB3400	Sequential batch transfer and continuous cultivation	Inhibitor cocktail and spruce hydrolysate	Improved growth, sugar consumption and ethanol productivity	(Koppram et al., 2012)
FY2	Sequential batch transfer with increasing inhibitor concentrations	Corn stover hydrolysate	Increased relative fitness in the presence of inhibitors	(Almario et al., 2013)
Ethanol Red	Sequential batch transfer	Spruce hydrolysate	Shorter lag phase, higher inhibitor tolerance, higher ethanol yield, higher thermotolerance	(Wallace-Salinas & Gorwa-Grauslund, 2013)
D5A^{+}	Continuous cultivation with 60% (v/v) hydrolysate	Triticale straw hydrolysate	Improved xylose consumption and SSF performance	(Smith et al., 2014)
NAN-27	Sequential batch transfer with	Corn stover hydrolysate	Higher vanillin tolerance and vanillin reduction capacity	(Y. Shen et al., 2014)

	increasing inhibitor concentrations			
DQ1	Sequential batch transfer	Corn stover hydrolysate	Improved SSF performance at high solid loadings	(Qureshi et al., 2015)
CEN.PK113-7D	Sequential batch transfer with alternating presence of acetic acid	Acetic acid	Higher acetic acid tolerance	(González-Ramos et al., 2016)
TMB3500	Continuous cultivation at low pH	Inhibitor cocktail	Transient tolerance to low pH and inhibitors	(Narayanan et al., 2016)
Y8	Sequential batch transfer with increasing inhibitor concentrations	Acetic acid	Higher ethanol levels	(Gurdo et al., 2018)
SyBE005 (E7)	Sequential batch transfer with increasing inhibitor concentrations	Inhibitor cocktail	Higher ethanol yield	(W. C. Li et al., 2019)
CEN.PK 113–7D	Sequential batch transfer with increasing inhibitor concentrations	Coniferyl aldehyde	High tolerance to phenolic compounds	(Haclsalihoglu et al., 2019)
XUSE	Sequential batch transfer with increasing inhibitor concentrations	Acetic acid	Higher tolerance to acetic acid and xylose consumption	(Ko et al., 2020)
GL01	Sequential batch transfer with increasing inhibitor concentrations	Coumaric acid and ferulic acid	Higher tolerance to coumaric acid and ferulic acid	(Pereira et al., 2020)
TTY23	Sequential batch transfer with increasing inhibitor concentrations	pH, acetic acid	Higher acetic acid and temperature tolerance	(Salas-Navarrete et al., 2022)

Strain propagation approaches

Besides the above strategies that are based on genetic modifications of the strain, the process configuration can also impact the performances of the microorganism in response to the presence of inhibitors. A major example concerns the so-called "short-term" adaptation strategy, which is based on the propagation of the microorganism under specific conditions that will transiently pre-expose it to the conditions faced later in the process, thus decreasing the time needed for the microorganism to adapt to those conditions.

Short-term adaptation is of special relevance in processes like simultaneous saccharification and (co-)fermentation (SS(c)F) when the propagation of the cell culture is performed prior to its utilisation in the SS(c)F step (Almeida et al., 2023;

Nielsen et al., 2015). The yeast propagation is routinely performed under aerobic conditions and in fed-batch mode to obtain high cell densities (Paper I) (Chapter 3). The addition of a feed gives the possibility to incorporate additional compounds, in addition to the growth medium, in a continuous manner. This strategy has been used for the short-term adaptation of *S. cerevisiae* to lignocellulosic inhibitors by the addition of diluted lignocellulosic hydrolysate into the feed during the fed-batch propagation (Paper I). During this process, the cells are exposed to low concentrations of the inhibitors present in the hydrolysate and develop a cellular response to combat their inhibitory effects. As such, when the cells are later used for inoculation of SS(c)F, they are already pre-adapted to the conditions, which reduces the lag phase and improves ethanol productivity (Paper I) (Almeida et al., 2023).

Besides lignocellulosic hydrolysate in the feed, other propagation alternatives have been suggested to improve cell fitness. For example, the use of glycerol and ethanol as carbon sources during propagation was reported to positively affect the xylose consumption rate in the subsequent fermentation of softwood hydrolysate (Dobrescu et al., 2021).

In the present work, this last strategy was compared for a xylose-engineered industrial strain, with the addition of diluted lignocellulosic hydrolysate in the feed, as well as a control propagation using only glucose and xylose as carbon sources (Paper I). Overall, cells propagated using hydrolysate supplementation in the feed (H propagation) outperformed the other strategies (Figure 17) (Paper I); when inhibitor levels corresponding to those present in a 10% WIS SS(c)F were used, an ethanol yield of 91% of the maximum theoretical yield was achieved after 72 hours (Paper I). These results highlight the key role of cell pre-exposure to lignocellulosic hydrolysate prior to fermentation.

A few recent studies have focused on the mechanisms involved in this cellular response during short-term adaptation. A comparison between the transcriptome of non-adapted and pre-adapted cells using wheat straw hydrolysate (van Dijk et al., 2021) or spruce hydrolysate (Almeida et al., 2023) during propagation, showed significant changes. Amongst them, up-regulation of furaldehyde detoxification genes, oxidative stress response genes, biotin and thiamine metabolism genes as well as genes of the DHA1 multidrug proton antiporter family was observed, in agreement with the targets already identified via targeted and evolutionary engineering.

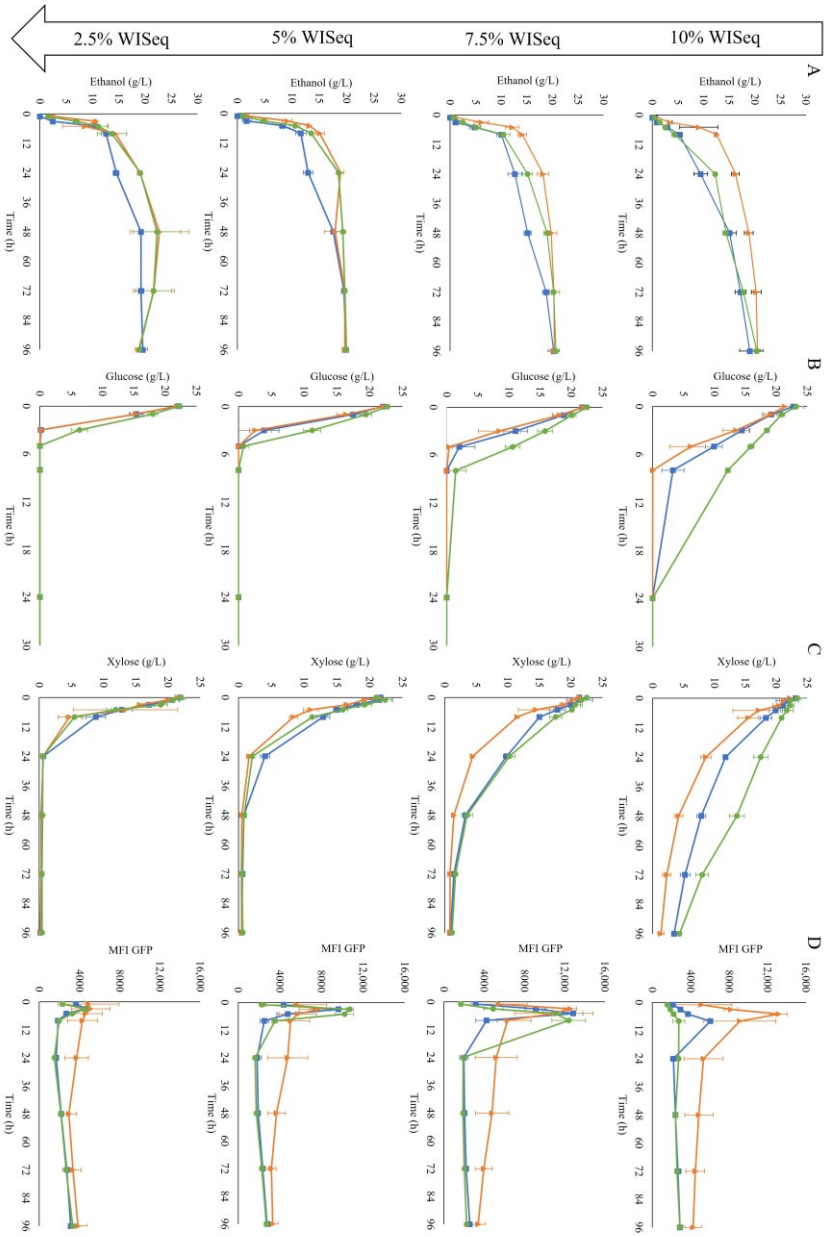


Figure 17. Fermentation profiles of cells collected after GX propagation (■), H propagation (▲), and GE propagation (●) in inhibitor levels corresponding to 10% WIS (first row), 7.5% WIS (second row), 5% WIS (third row), and 2.5% WIS (fourth row). Concentrations of ethanol (A), glucose (B) and xylose (C) over time are shown. The final column (D) shows the mean fluorescence intensity (MFI) of the GFP response from the biosensor for redox imbalance. Two biological replicates were performed. Taken from Paper I.

Biosensor-based monitoring and control for improved tolerance

The performance of strains developed for lignocellulosic biomass utilisation is generally evaluated using external parameters such as improved sugar consumption, inhibitor detoxification or ethanol production. However, little is known about the intracellular responses of the cell and its fitness in response to the faced conditions.

In order to shed light on it, fluorescence-based biosensors (cf. Chapter 3) have been developed to monitor intracellular properties that can be affected by the presence of lignocellulosic inhibitors. Some examples of such properties include intracellular pH (Miesenböck et al., 1998; Reifenrath & Boles, 2018), ATP concentration (Yaginuma et al., 2014) or redox imbalance (Knudsen et al., 2014; J. Zhang et al., 2016). Among these, the monitoring of redox imbalance sensed by the cell is of special relevance for inhibitor tolerance as the detoxification of aldehyde inhibitors is known to require NAD(P)H (cf. Chapter 2).

Zhang et al., have previously developed a transcription factor-based biosensor capable of reporting on NADPH deficiency (J. Zhang et al., 2016). The sensor is based on the response of *S. cerevisiae* to oxidative stress that involves the oxidation of the transcription factor Yap1p which triggers the induction of several genes, including the thioredoxin gene *TRX2*. The levels of oxidised and thus active Yap1p are regulated by reduced thioredoxin, which in turn is regulated by the NADPH-dependent thioredoxin reductase (Trr1p) (Figure 18). Thus, if there is a lack of NADPH in the cell, no reduced form of thioredoxin will be available and Yap1p will remain active and induce *TRX2*. Consequently, induction of *TRX2* expression level can be used as an indirect measure of NADPH deficiency (J. Zhang et al., 2016).

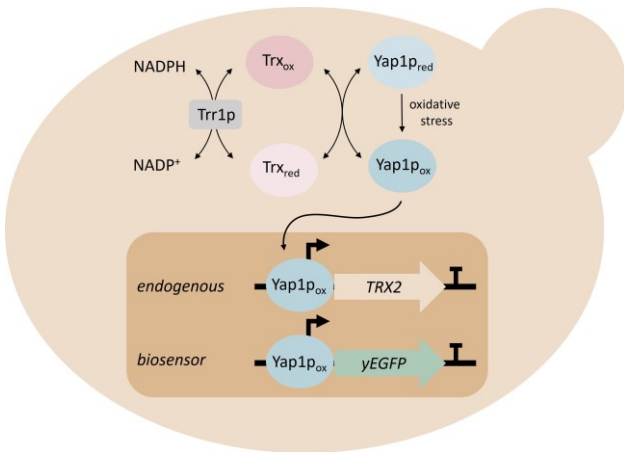


Figure 18. Oxidative stress response in *S. cerevisiae* by the transcription factor Yap1p. Adapted from (J. Zhang et al., 2016).

Based on this knowledge, the *TRX2* promoter has been used to control the expression of a GFP-encoding gene in the *TRX2*p-*yEGFP* biosensor and to monitor cellular NADPH deficiency. Its induction has been indeed confirmed when the cells have been exposed to different compounds known to cause redox imbalance such as diamide, hydrogen peroxide or furfural, one of the main inhibitors present in lignocellulosic hydrolysates (J. Zhang et al., 2016).

In the present work, the *TRX2*p-*yEGFP* biosensor was introduced into a xylose-fermenting industrial strain and its response to different concentrations of furfural was assessed (Paper I). A correlation between the furfural concentration and the fluorescence response was confirmed. Furthermore, when the cultures were supplemented with 2.5 g/L of furfural, no growth was observed for 24 hours whereas the biosensor was still induced, showing that the biosensor response was not growth-dependent (Paper I). The industrial strain carrying the *TRX2*p-*yEGFP* biosensor (TMBRP011) was propagated in fed-batch mode while samples were automatically taken and analysed by flow cytometry (cf. Chapter 3) to monitor its redox state (Paper I & II). During fed-batch cultivation, induction of the sensor was observed when the feed was supplemented with wheat straw hydrolysate (Paper I). However, this induction was lost before the end of the feeding phase, suggesting that the cells were adapted to those concentrations of inhibitors and no longer suffered from NADPH deficiency (Paper I & II) (Figure 19A). It was also observed that the initial response of the *TRX2*p-*yEGFP* sensor in anaerobic fermentation of wheat straw hydrolysate correlated to the ethanol production rate of the strain (Paper I), suggesting that the monitoring of the *TRX2*p-*yEGFP* sensor response could be used to predict the duration of the fermentation.

The *TRX2*p-based biosensor is part of a recently developed and expanded biosensor toolbox for real-time monitoring in *S. cerevisiae* (Torello Pianale et al., 2022; Torello Pianale & Olsson, 2023). This toolbox includes seven other biosensors targeting the monitoring of ATP levels, intracellular pH, glycolytic flux, ribosome production, unfolded proteins, pyruvate metabolism and ethanol consumption (Torello Pianale & Olsson, 2023), which are relevant parameters for the study of the cellular response of *S. cerevisiae* to the presence of lignocellulosic inhibitors. In that study, the biosensors were combined in pairs and introduced into the same cell allowing the simultaneous measurement of two fluorescent responses in real-time and the study of possible interactions between the parameters. A correlation between ATP concentration and intracellular pH was notably observed (Torello Pianale et al., 2022).

The studies above highlight how biosensors can help continuously monitor strain performance and fitness through the analysis of relevant intracellular processes. It also paves the way for the development of process control tools based on these responses. The latest approach was initiated in the present thesis work with the development of a dynamic control system based on the response of *TRX2*p-*yEGFP* biosensor (Paper II). As the response of the biosensor was found to decrease over

time during fed-batch propagation - due to yeast adaptation - (Paper I & II) (Figure 19A), it was hypothesised that a reinduction of the biosensor response could be achieved at that point by further increasing the inhibitor feeding; this may, in turn, result in a stronger cell adaptation and consequently increase fermentation performance. To test this hypothesis, a computer program was developed to read the results obtained from the at-line flow cytometry analysis in real-time and communicate with an Arduino to control the feed composition of the fed-batch cultivation (Paper II). The program was designed to start the secondary pump responsible for the introduction of a highly concentrated furfural solution when a decrease in the fluorescent response was observed (Paper II).

Indeed, the developed control strategy resulted in the reinduction of the *TRX2*p-*yEGFP* response in the propagation step, without any negative effect on cell integrity, measured by PI staining (Figure 19B). However, in the preliminary trials, no improvement in fermentation performance was observed using the cells propagated with the developed control strategy (Paper II).

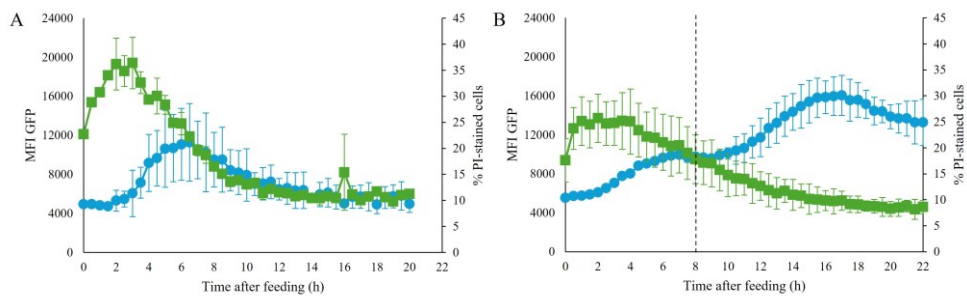


Figure 19. Mean fluorescence intensity (MFI) of GFP (●) and percentage of PI-stained cells (■) during the feeding phase of yeast propagation in fed-batch cultivations with fixed feed rate (A) and sensor-controlled feed rate (B). The dotted line marks the time at which the secondary pump was automatically initiated by the program. Taken form Paper II.

Nevertheless, the developed system portrays the use of biosensors and flow cytometry as a promising tool for the implementation of automated control systems in microbial cultivations.

Chapter 5
Yeast engineering and monitoring for improving pentose utilisation

The presence of different types of hexose and pentose sugars in lignocellulosic biomass represents a challenge for the development of optimised bioprocesses using *S. cerevisiae* (cf. Chapter 2). Whereas the hexose sugars glucose, fructose, galactose and maltose can be converted to ethanol by *S. cerevisiae*, the pentose sugars xylose and arabinose are not naturally fermented. In this chapter, an overview of the different strategies developed to enable and improve the consumption of xylose, the major pentose sugar in lignocellulosic biomass, is presented.

Xylose consumption pathways

Although *S. cerevisiae* possesses aldose oxidoreductases that can convert xylose to xylitol and xylulose (Träff et al., 2002), it is not naturally able to catabolise and grow on xylose as the sole carbon source. As bacteria and other yeasts in Nature do have this capability, metabolic engineering techniques have been used to introduce and test these utilisation pathways in *S. cerevisiae* (Figure 20).

The first successful approach included the introduction of two enzymes: a xylose reductase (XR) and a xylitol dehydrogenase (XDH) from the yeast *Scheffersomyces (Pichia) stipitis* (Kötter et al., 1990). XR allows the conversion of xylose into xylitol while XDH oxidises xylitol into xylulose (Figure 20). The combination of these two enzymes is commonly referred to as the XR/XDH pathway. Alternatively, some bacteria and fungi carry out the conversion of xylose into xylulose in a single step by utilising a xylose isomerase (XI) (Figure 20). Several XI enzymes have been tested in *S. cerevisiae*, and the first anaerobic xylose conversion to ethanol was achieved using multiple copies of the xylose isomerase (XI) gene from the anaerobic fungus *Piromyces* (Kuyper et al., 2003). This is commonly known as the XI pathway. Both alternatives require the upregulation of the endogenous xylulose kinase (XK) for an efficient phosphorylation of xylulose into xylulose-5-phosphate.

At this point, xylulose-5P is metabolised using the native non-oxidative pentose phosphate pathway (PPP).

Apart from these two common pathways, two oxidative pathways have been described, where the assimilation of xylose is not based on phosphorylation but on xylose oxidation (Figure 20). Xylose is first converted in three steps to 2-keto-3-deoxyxylonate, the common intermediate for these oxidative pathways. In the Dahms pathway, 2-keto-3-deoxyxylonate is further converted to pyruvate and glycolaldehyde (Stephen Dahms, 1974), whereas in the Weimberg pathway, α-ketoglutarate is produced instead (Weimberg, 1961). As these oxidative pathways are better suited for the aerobic production of other products than ethanol (Francois et al., 2020), they will not be further discussed in the present thesis that focuses on anaerobic ethanol production.

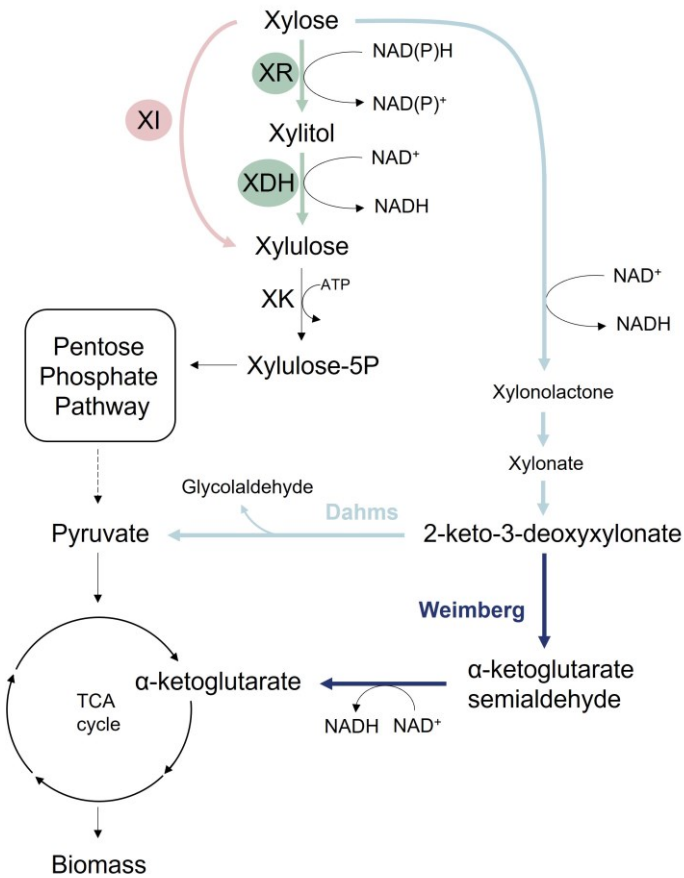


Figure 20. Heterologous pathways introduced for xylose utilisation in *S. cerevisiae*. XI: xylose isomerase; XR: xylose reductase; XDH: xylitol dehydrogenase; XK: (endogenous) xylulose kinase; TCA cycle: tricarboxylic acid cycle.

The introduction of the XR/XDH and XI pathways into *S. cerevisiae* has proven challenging. The main drawback of the XR/XDH pathway is that both XR and XDH are cofactor-dependent enzymes. And, whereas the available XRs are mainly NADPH-dependent, XDHs are strictly NAD^+-dependent, which generates a cofactor imbalance in the cell (Kotter & Ciriacy, 1993). Different strategies have been developed to address this issue including varying the levels of XR and XDH (Eliasson et al., 2001; Jin & Jeffries, 2003; Karhumaa et al., 2007; Walfridsson et al., 1997), adding acetoin as an external electron acceptor (Wahlbom & Hahn-Hägerdal, 2002), modifying the redox metabolism in the cell (Jeppsson et al., 2002; Roca et al., 2003; Verho et al., 2003) or altering the cofactor affinity of XR (Jeppsson et al., 2006; Petschacher & Nidetzky, 2008) or XDH (Watanabe et al., 2005).

In the case of XI, the enzymatic activity is strongly inhibited by xylitol (Yamanaka, 1969), the product of the endogenous aldose reductase encoded by the *GRE3* gene (Kuhn et al., 1995). Therefore, deletion of *GRE3* in XI-carrying strains has been performed to avoid xylitol formation and thus XI inhibition (Träff et al., 2001). Still, the *in vivo* activity of the XIs remains a challenge and high copy numbers of the XI-encoding gene are needed to reach sufficient enzymatic activity (Matsushika et al., 2009). To counteract this challenge, new XI variants with higher activities are constantly being screened for and/or engineered. In the present work, two new XI variants, one from *Lachnoclostridium phytofermentans* (ClosXI) (Brat et al., 2009) and another one from *Parabacteroides* spp. (ParaXI) (Silva et al., 2021) were compared to the established XI from *Piromyces* sp. (PiroXI) (Paper III). As two gene copies integrated into the chromosome were not sufficient to enable growth on xylose, the use of multicopy plasmids was still needed to allow xylose consumption; the study also showed that ClosXI gave the best performance amongst the three tested XIs (Paper III).

Regardless of the chosen xylose pathway, some additional hurdles need to be overcome to achieve efficient utilisation of xylose in *S. cerevisiae*. The role of transport, PPP, xylose structural form and sugar signalling routes will be further discussed in this chapter.

Xylose transport

The transport of extracellular xylose into the cell has been identified as one of the key steps for the efficient utilisation of xylose by *S. cerevisiae* (Hector et al., 2008). Since *S. cerevisiae* does not express xylose-specific transporters, its uptake is carried out by hexose transporters encoded by the *HXT* gene family (Kruckeberg, 1996) and by the galactose transporter Gal2p (Boles & Hollenberg, 1997). This implies that in a mixed sugar scenario, competition for transport will occur between

the different sugars, i.e., glucose and xylose. However, this will always favour glucose, because the transporters have a higher affinity for glucose than for xylose, with up to two orders of magnitude difference in K_M (Kotter & Ciriacy, 1993). Studies in which strains were adapted to grow on xylose also showed an increase in the expression of hexose transporters associated with improved xylose uptake kinetics (Kuyper et al., 2005; Wahlbom et al., 2003), further corroborating transport as a limiting factor for xylose optimal consumption.

One possible strategy to improve the xylose uptake rates is the heterologous expression of pentose-specific transporters. Through extensive screening, a wide range of pentose transporters have been identified in natural xylose-consuming microorganisms and expressed in *S. cerevisiae* (Table 7). However, this strategy did not yield the expected results as the generated strains generally suffered from low activity and stability of the transporters, likely due to poor expression and degradation by the yeast protein degradation machinery (Sen et al., 2016).

Table 7. Heterologous expression of pentose transporters in *S. cerevisiae*.

Microorganism of origin	Transporter	Reference
Scheffersomyces stipitis (also known as *Pichia stipitis*)	Xut1p-Xut7p	(E. Young et al., 2011)
	Sut1p	(Katahira et al., 2008)
	Sut4p	(Moon et al., 2013)
	Xyp29p	(Du et al., 2010)
	AraTp	(Subtil & Boles, 2011)
	Rgt2p	(Moon et al., 2013)
	Hxt2.6p	(de Sales et al., 2015)
	Qup2p	
Candida intermedia	Gxs1p	(Leandro et al., 2006)
	Gxf1p	
Debaryomyces hansenii	XylHPp	(E. Young et al., 2011)
	2D01474p	(E. M. Young et al., 2014)
	2C02530p	
Arabidopsis thaliana	Stp2p	(Hamacher et al., 2002)
	At5g17010p	(Hector et al., 2008)
	At5g59250p	
Ambrosiozyma monospora	Lat1p and Lat2p	(Verho et al., 2011)
Neurospora crassa	An25p	(Du et al., 2010)

To avoid possible complications associated with the expression of heterologous genes, the use and engineering of native hexose transporters have also been explored. Initial attempts showed that overexpression of the endogenous xylose transporters Hxt4p, Hxt7p and Gal2p in a xylose-fermenting strain did not improve the growth rate nor the xylose consumption rate (Hamacher et al., 2002). However, through evolutionary engineering, several mutations were identified in native transporters, which either contributed to an improved xylose specificity or a reduced

sensitivity towards glucose inhibition (Nijland & Driessen, 2020). In particular, a conserved asparagine which was present in all Hxt transporters was identified as a key residue in glucose recognition. The substitution of this asparagine in position 376 in Gal2p for a phenylalanine (N376F) not only increased its affinity for xylose but also resulted in the reduction of its ability to transport glucose (Farwick et al., 2014). Nowadays, the most efficient transporter is based on the synergistic effect of two mutations in Gal2p, the N376Y/M435I variant (Rojas et al., 2021).

Upregulation of the pentose phosphate pathway genes

Another limiting factor for the optimal consumption of xylose in *S. cerevisiae* has been the limited flux through the non-oxidative pentose phosphate pathway (PPP), which connects xylulose-5P into the central carbon metabolism (Figure 21). It was notably observed that strains carrying the XR/XDH pathway accumulated some of the intermediates formed in the PPP such as sedoheptulose-7-phosphate, xylulose-5P and 6-phosphogluconate (Kotter & Ciriacy, 1993). This suggested that the metabolic flux through the non-oxidative PPP was limiting for efficient metabolisation of xylose.

This was further confirmed by several studies in which strains with improved xylose consumption showed an upregulation of the transaldolase (*TAL1*) and/or the transketolase (*TKL1*) genes (Figure 21) (Becker & Boles, 2003; Wahlbom et al., 2003). Indeed, overexpression of the genes *TAL1* and *TKL1,* as well as its combination with the other genes from the non-oxidative PPP, *RPE1* and *RKI1* (Figure 21), has been shown to be beneficial for strains carrying either XR/XDH or XI pathway (Walfridsson et al., 1995; Karhumaa, Hahn-Hägerdal and Gorwa-Grauslund, 2005; Karhumaa et al., 2007). Nowadays, upregulation of non-oxidative PPP genes is routinely performed in most strains engineered for xylose utilisation worldwide.

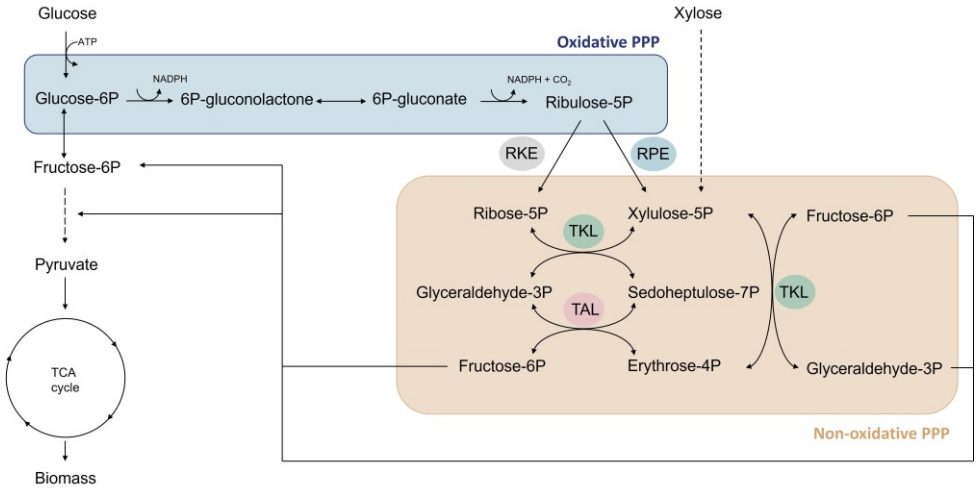


Figure 21. The pentose phosphate pathway (PPP) in *S. cerevisiae*.

The role of xylose anomerisation

In lignocellulosic biomass, most of the xylose present in the xylan polymer is in the β-D-xylopyranose form (Gírio et al., 2010). However, it has been shown that some xylose isomerases (Miyamoto et al., 2022; Schray & Rose, 1971) and xylose reductases (Vogl & Brecker, 2013) have a preference for the opposite anomer, α-D-xylopyranose. Thus, the introduction of a xylose epimerase/mutarotase that is capable of catalysing the conversion between the anomers α-D-xylopyranose and β-D-xylopyranose (Figure 22) has been considered as a possible strategy for strains using lignocellulose-derived xylose as substrate.

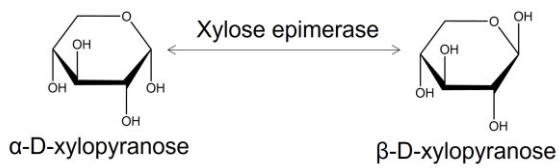


Figure 22. Conversion between the α-D-xylopyranose and β-D-xylopyranose anomer by mutarotase/xylose epimerase.

After the mutarotase gene *xylM*, was identified in *Lactococcus lactis* and further characterised for its activity on different sugars, including D-xylose (Erlandson et al., 2000, 2001), it was found that its addition improved xylose consumption in a strain carrying one of the XI pathways (Sibbesen et al., 2009). In the present thesis,

the role of the addition of the same xylose epimerase XylM in strains carrying various XI or XR/XDH pathways was investigated (Paper IV). Among the three tested XI variants, the highest benefit was obtained with the fastest XI isolated from *L. phytofermentans* (ClosXI), with i.e., ethanol yields increasing from 0.18 g/g xylose to 0.38 g/g xylose by the addition of the xylose epimerase gene. For the XI isolated from *Parabacteroides* spp. (ParaXI), a smaller impact was observed since the maximum growth rate under aerobic conditions was only increased by 5%, as compared to the 14% observed for ClosXI. Finally, no effect was observed for the less efficient XI isolated from *Piromyces* sp. (PiroXI), where the addition of the xylose epimerase did not enable growth on xylose. No significant improvement was observed either in strains carrying the XR from *Spathaspora passalidarum* upon the addition of xylose epimerase (Paper IV). It is possible that some of the tested enzymes have no preference for one of the anomers, which could then render the epimerase role irrelevant. But, as the spontaneous conversion between the anomers is possible, the enzyme may also only benefit strains in which the conversion of xylose is the main controlling step.

In conclusion, although the benefits of adding the xylose epimerase are enzyme-dependent, it should be considered as a potential strategy for strain improvement, especially for strains with fast xylose conversion rates.

Sugar signalling

Despite all the metabolic engineering efforts to generate *S. cerevisiae* strains with optimised xylose consumption, the co-consumption of xylose in the presence of glucose remains a challenge; also xylose is still used at a lower rate than glucose. Using a transcriptomic approach, the cellular response to xylose was shown to resemble that of a non-fermentable sugar, suggesting that *S. cerevisiae* may not recognise the foreign xylose sugar as a fermentable carbon source (Matsushika et al., 2014). *S. cerevisiae* has a complex system for sugar signalling and regulation, which regulates for example the carbon catabolite repression system. Accordingly, only the cellular machinery required for a particular environmental condition will be made available, thus optimising resources.

Three main sugar signalling routes are present in *S. cerevisiae* (Figure 23): (i) the Snf3p/Rgt2p pathway recognises the presence of hexoses in the medium and induces the expression of hexose transporter genes for their uptake (Santangelo, 2006); (ii) the SNF1/Mig1p pathway induces the use of alternative carbon sources during glucose depletion (F. Moreno et al., 2005) and is involved in the carbon catabolite repression system (Gancedo, 1992); and (iii) the cAMP/PKA pathway responds to both extracellular and internalised glucose and regulates environmental stress response (Santangelo, 2006).

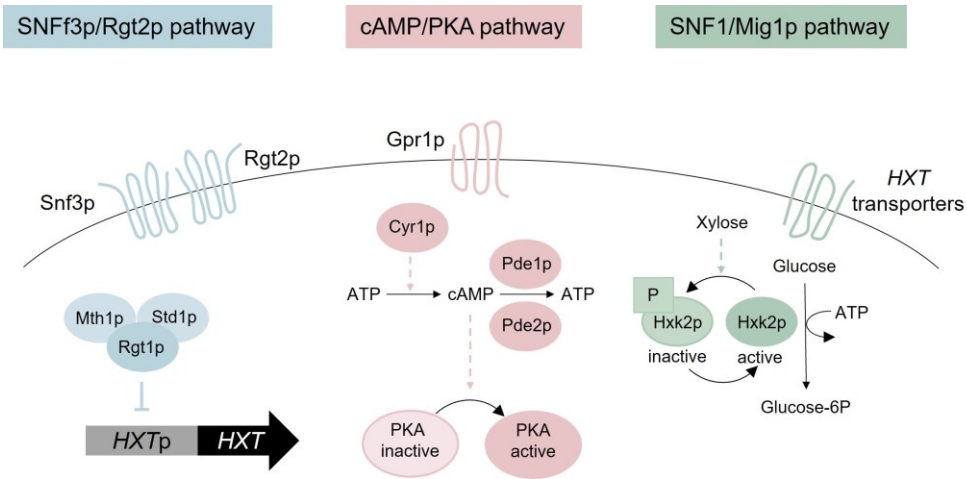


Figure 23. Simplified representation of the three main sugar signalling pathways in *S. cerevisiae*. Adapted from (M. Wu et al., 2020).

To further elucidate the sugar signalling response of *S. cerevisiae* towards the non-natural xylose sugar, Brink et al., (2016) have developed reporter strains that carried transcription factor-based biosensors for the three main sugar signalling routes (Table 8). Using these biosensor strains, it has been found that the presence of *external* xylose did not lead to a signalling response (Brink et al., 2016). Instead, signalling has only been observed upon xylose assimilation in XR-XDH strains; also, the response that was mostly observable in high xylose concentrations (50 g/L), has been similar to the one observed in low glucose concentrations (≤5 g/L) (Table 8) (Osiro et al., 2018).

Table 8. Summary of the biosensors developed for the three sugar signalling routes and their induction state in different conditions. Adapted from (Osiro et al., 2018)(Brink et al., 2021)

Signalling route	Biosensor	Low glucose	High glucose	High xylose
Snf3p/Rgt2p	*HXT1*p-y*EGFP3*	Repressed	Induced	Repressed
SNF1/Mig1p	*SUC2*p-y*EGFP3*	Induced	Repressed	Induced
cAMP/PKA	*TPS1*p-y*EGFP3*	Induced	Non-induced	Induced

As these studies only focused on strains carrying the XR/XDH pathway, the sensor *SUC2*p-*yEGFP* was utilised in the present work to compare the sugar signalling response of strains carrying either the XR/XDH pathway or the XI pathway (Paper III). In the case of the XR/XDH strains, previous studies had shown an induction after 6 hours of cultivation (Osiro et al., 2018). In the present study, the response that was monitored during the whole cultivation showed that, after this initial induction, the signal response decreased over time (Figure 24) (Paper III).

The decrease in fluorescence signal could indicate a deactivation of the SNF1/Mig1p pathway which would correspond to the response with high glucose present in the medium, thus alleviating the possible hurdles generated by the sugar signalling response.

Induction of the *SUC2*p-*yEGFP* biosensor was also observed in the XI strains grown in 50 g/L xylose; however, the induction profile was very different from the one observed for the XR/XDH strains: the signal kept increasing for a long period, leading to much higher induction levels than for the XR/XDH strains (Figure 24) (Paper III). Generally, an induction of *SUC2* indicates that the cells are sensing a lack of fermentable sugars and thus a non-optimised metabolism (Brink et al., 2021). Thereby the higher induction of *SUC2* for the XI strains corroborates the slower xylose consumption observed for these strains as compared to the XR/XDH strains.

Besides the effect of the metabolic pathway on the sugar signalling, the influence of the different anomeric forms of xylose on sugar signalling was analysed. The strains in which a xylose epimerase was introduced showed a similar biosensor response to those without it (Figure 24) (Paper III). This indicated that the possible differences generated by the xylose epimerase in the balance of xylose anomers did not influence the sugar signalling response of the cells.

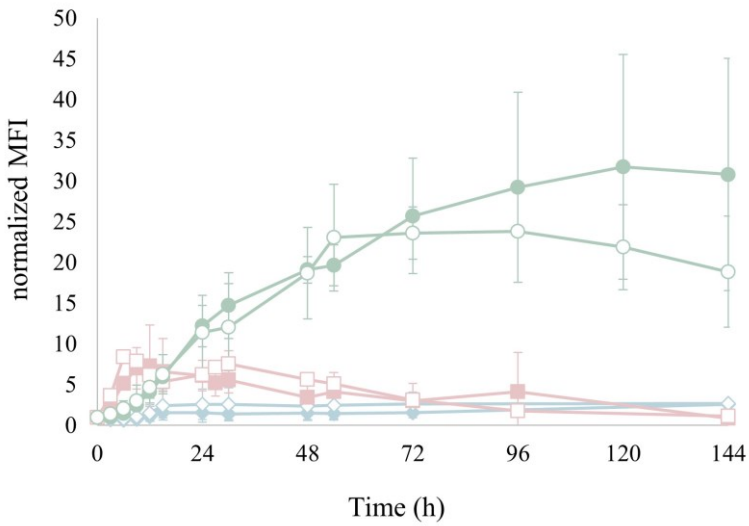


Figure 24. Normalised mean fluorescence intensity of the *SUC2*p-*yEGFP* biosensor over time during anaerobic cultivation of XR/XDH strains TMBRP024 (XR/XDH, gre3Δ) (■) and TMBRP025 (XR/XDH, gre3::epimerase)(□); XI strains TMBRP026 (ClosXI, gre3Δ) (●) and TMBRP027 (ClosXI, gre3::epiemrase) (○); and control strains TMBVP1005 (gre3Δ) (◆) and TMBVP1105 (gre3::epimerase) (◇) in YNB medium supplemented with 50 g L^{-1} xylose. Taken from Paper III.

Regardless of the pathway, the sugar-sensing response observed in the presence of xylose indicates that the cellular resources activated are not optimal for its consumption. Accordingly, current efforts are being made towards the modification of signalling targets for the improvement of xylose-engineered strains. Modifications involving the SNF1/Mig1p pathway have proven to be the most challenging and efforts in this route have mainly targeted Hxk2p, as its interaction with xylose leads to autophosphorylation and inactivation (Figure 23) (Fernández et al., 1986); however, no notable improvements have been reported so far (Brink et al., 2021). On the contrary, it has been recently demonstrated that modifications in the other two signalling pathways that aim to mimic the activation of glucose sensing in the presence of xylose have given promising results (M. Wu et al., 2020). Deletion of the *PDE1* and *PDE2* genes, which encode the cAMP phosphodiesterases in charge of regulating the levels of cAMP in the cell (Figure 23), has resulted in increased cAMP levels, leading to the activation of PKA which upregulates glycolysis (Figure 23). The generated strain (*pde1*Δ*pde2*Δ) had a 51% higher specific xylose consumption rate and a 72% higher specific ethanol production rate compared to the control strain (M. Wu et al., 2020). Another modification involved the Snf3p/Rgt2p pathway by deleting *RGT1*, a transcription repressor of hexose transporters (*HXTs*) (Figure 23). In this case, an increase in the levels of *HXT1* and *HXT2* has been observed together with a 24% increase in specific xylose consumption rate (M. Wu et al., 2020).

Besides engineering the native sugar signalling routes, the introduction of synthetic regulation strategies has also been investigated (Gopinarayanan & Nair, 2018). The GAL regulon, which is responsible for the induction of a number of genes involved in galactose catabolism in the presence of galactose in the medium, has been modified to respond to the presence of xylose as well (Gopinarayanan & Nair, 2018). Furthermore, the genes involved in xylose catabolism have been expressed under *Gal1*p and *Gal10*p promoters so that their expression would be controlled by the GAL regulon (Gopinarayanan & Nair, 2018). As a result, xylose catabolism genes were expressed upon activation of the GAL regulon in the presence of xylose which led to higher growth rates and better xylose consumption than the control strain with constitutive expression of the xylose catabolism genes (Gopinarayanan & Nair, 2018).

Chapter 6
Summary and outlook

The work presented in this thesis focused on the use of transcription factor-based biosensors together with flow cytometry analysis to study two main challenges faced by *S. cerevisiae* in the 2G bioethanol process: the presence of inhibitory compounds and the consumption of xylose. The main findings and possible future strategies are summarised below.

Inhibitory compounds

The biosensor *TRX2*p-*yEGFP* was used to measure the deficiency of NADPH generated by the presence of inhibitory compounds such as furfural or HMF. The implementation of an automated at-line flow cytometry analysis during the cultivation of *S. cerevisiae* in bioreactors allowed the continuous monitoring of the biosensor's response to the presence of lignocellulosic hydrolysate. With such a system, it was observed that induction of the sensor's response during the propagation with lignocellulosic hydrolysate was correlated with a better performance in the consequent fermentation. It was also confirmed that propagation with lignocellulosic hydrolysate for short-term adaptation remained the best strategy as it resulted in a shorter lag phase during fermentation which improved the ethanol productivity.

The initial biosensor response in the fermentation step was also found to be correlated with ethanol productivity. This correlation, if confirmed, could be useful as a tool to predict the most cost-effective duration for each fermentation run.

Although the focus of the present work has been on the effects of furaldehydes, the methodology utilised here could be easily applied for the study of biosensors responding to the presence of other inhibitory compounds such as acetic acid or phenolic compounds. For example, the previously developed *Haa1*p-based biosensor for acetic acid (Mormino et al., 2021) could be an interesting biosensor to use. Different biosensors responding to the presence of phenolic compounds have already been developed in recent years (Augustiniene et al., 2023; Flachbart et al., 2021); however, the majority of these biosensors are based on bacteria such as *E. coli* or *Pseudomonas putida*, so they would need to be tested for their application in *S. cerevisiae*.

Xylose metabolism

Regarding xylose consumption, the role of a xylose epimerase capable of catalysing the interconversion between the anomers α-D-xylospyranose and β-D-xylospyranose was studied. It was shown that its addition was especially relevant for the strain utilising the xylose isomerase from *L. phytofermentans* (ClosXI) as it resulted in better xylose consumption and ethanol yields. In order to better understand the impact of xylose anomerisation in xylose consumption, the binding mechanism of xylose into the specific catalysing enzymes (XI or XDH) should now be elucidated.

The response of the sugar signalling biosensor *SUC2*p-*yEGFP* showed significant differences between XI strains and XR/XDH strains. The higher and longer signal observed in XI strains was correlated with a lower xylose consumption, pointing to sugar signalling as a possible bottleneck for efficient xylose consumption in these strains. Finally, the presence of the xylose epimerase did not affect the sugar signalling of the strains, regardless of the pathway used for xylose consumption, which indicates that the anomer form has no impact on the extracellular sensing of xylose.

To complement the information about sugar signalling in XI strains, the fluorescent signal of the *HXT1*p-*yEGFP* and *TPS1*p-*yEGFP* biosensors for the Snf3p/Rgt2p and cAMP/PKA signalling routes, respectively, could be studied. That would allow to obtain a more wholesome view of the three main sugar signalling routes. Also, the use of chemostat cultivation with both pathways could enable to compare the sensing response under similar growth conditions.

Biosensors for monitoring and control

In this thesis work, transcription factor-based biosensors were used to successfully monitor the intracellular response of *S. cerevisiae* strains to lignocellulosic inhibitors and xylose in real-time. The results observed with the use of automated at-line flow cytometry to monitor the response of the redox biosensor also led to the development of a process control strategy based on the biosensor response. As a result, it was possible to automatically adjust the concentration of furfural in the feed of a fed-batch cultivation depending on the cellular integrity and redox status. One could also envision a system in which the biosensor detects the main metabolic product of the selected strain so that the cultivation conditions can be dynamically adjusted according to the biosensor response to ensure maximal production.

Due to the real-time nature of the at-line flow cytometry system employed during this thesis work, large datasets can be obtained for every run. If this methodology was routinely implemented in microbial cultivations, it is likely that sufficient data would be collected to train an artificial intelligence model to learn to distinguish the patterns observed in the biosensor response and their corresponding cultivation outcome. The model could then be applied to adjust the cultivation parameters to optimise the outcome of the cultivation.

Remaining challenges

A long-term goal of the present research is to exploit the potential of biosensors and flow cytometry as monitoring and control tools to improve the production of bioethanol from lignocellulosic biomass in industrially relevant conditions. The knowledge gained through the implementation of these tools is also expected to help in the development of optimal strains and process configurations. To reach this goal, some challenges remain to be solved.

Multiple biosensors on the same cell

It would be ideal to use as many biosensors as possible in the same cell since simultaneous information about different parameters could be obtained. This requires multicolour FCM to be optimised. During this thesis work, the simultaneous measurement of the three FPs mEGFP, CyOFP1 and mBeRFP utilising solely the most common blue laser at 488 nm for excitation was shown to be possible. However, the second excitation laser available in the flow cytometer, a standard 640 nm red laser, was not used due to the lack of suitable FPs excitable by that laser. This is a known issue that limits the implementation of multicolour FCM to its full potential (Piatkevich & Verkhusha, 2011). Therefore, the field could highly benefit from the development of suitable far-red FPs as it would allow the expansion of the current working spectrum.

Nevertheless, the at-line flow cytometry monitoring system presented in this thesis work can already be expanded for the simultaneous measurement of up to three cellular properties on the same cell. For example, the two biosensors utilised in this work could be combined to measure redox imbalance and sugar signalling simultaneously during yeast cultivation. This could eventually lead to the development of a control system for optimal yeast propagation in which not only the levels of inhibitors are adjusted but also the introduction of xylose as a carbon source, all based on the biosensors responses of the cells.

Application in solid-containing matrix

So far, the developed system could be applied in the propagation step or fermentation steps in which hydrolysate, the liquid fraction of the pretreated lignocellulosic material, was used. However, the current system was not applicable to bioreactor cultivations in which solid matter is present. Also, mimicking the SS(c)F conditions with the use of liquid hydrolysate did not yield the same results as true SS(c)F (Paper I), which indicated that the presence of solid particles themselves also influenced the fermentation efficiency. This makes the development of tools for monitoring fermentations with complex matrixes even more vital.

One possibility would be to introduce a filtration step prior to the analysis in the system. However, it is likely to be clogged making it necessary to replace the filter which is hardly doable. Collaboration with process engineers to develop suitable alternative separation methods is thus essential for the efficient collection of cells in the future.

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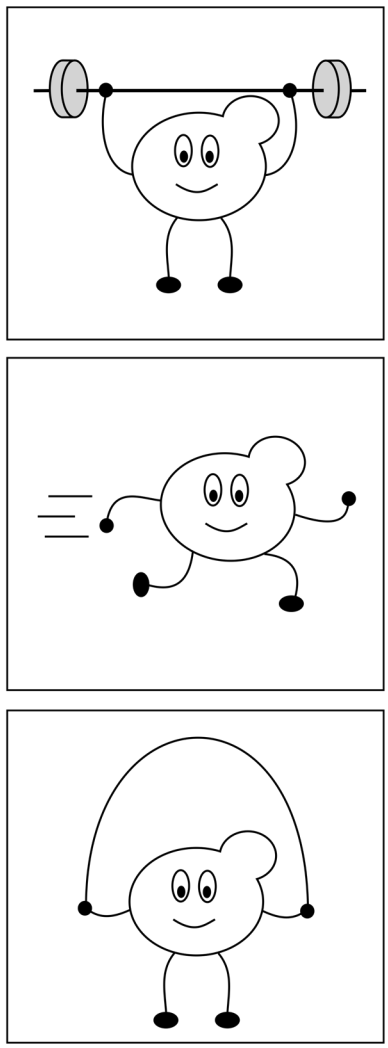

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