Universidade do Minho
Escola de Engenharia

Cristiana Fernandes Martins

Engineering *Saccharomyces cerevisiae* for the production of value-added compounds from lignocellulosic biomass

outubro de 2023

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Engineering *Saccharomyces cerevisiae* for the production of value-added compounds from lignocellulosic biomass

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Trabalho efetuado sob a orientação do(a)
Professora Doutora Lucília Maria Alves Ribeiro Domingues

outubro de 2023

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Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

A crescente demanda de energia e produtos químicos essenciais em diversas atividades do dia-a-dia, bem como os efeitos das alterações climáticas e o aumento das emissões dos gases de efeito de estufa, tem vindo a impulsionar a necessidade de substituir os combustíveis fósseis por processos biológicos. Para atingir esse objetivo, é essencial o estabelecimento de biorrefinarias para a produção de biocombustíveis e outros produtos químicos a partir de matérias-primas renováveis, incluindo a biomassa lignocelulósica. O hidroximetilfurfura é um composto versátil e um produto químico atrativo devido às suas propriedades únicas e ampla gama de aplicações. Pode ser reduzido ou subsequentemente oxidado a uma variedade de produtos que possuem uma vasta gama de aplicações, como a síntese de polímeros, síntese de surfactantes e resinas ou para a produção de plastificantes, poliuretano, poliéster ou poliamida, entre outros. A levedura *Saccharomyces cerevisiae* tem sido extensivamente estudada pela sua capacidade para destoxificar o HMF. No entanto, a sua capacidade como biocatalisador para gerar derivados de oxidação de HMF não foi suficientemente investigada.

Uma estirpe industrial e uma estirpe laboratorial de *S. cerevisiae*, ambas geneticamente modificadas para produzir HMF/furfural oxedorredutase (HMFH) ou HMF oxidase (HMFO) foram utilizadas como hospedeiras para a oxidação de HMF. Tanto as estirpes recombinantes como as selvagens foram avaliadas quanto à sua capacidade de detoxificar o HMF e a melhor estirpe demonstrou uma conversão eficaz de 50 mM de HMF, resultando na produção de aproximadamente 11 mM de FDCA. Posteriormente, as condições de bioconversão foram otimizadas através da avaliação do impacto das condições de oxigenação. Além disso, o FDCA foi produzido utilizando um hidrolisado hemicelulósico sintético com glucose e frutose como fontes de carbono. Os efeitos do furfural no meio de conversão foram também analisados revelando que a levedura é altamente inibida pela mistura HMF e furfural, demonstrando uma tendência para detoxificar o furfural mais rapidamente.

Finalmente, um hidrolisado de bagaço de maçã, previamente submetido a um pré-tratamento com um solvente eutéctico e micro-ondas, foi utilizado como fonte de HMF para bioconversão. A estirpe com melhor desempenho foi capaz de converter aproximadamente 60% de HMF, resultando na produção de derivados com um rendimento de aproximadamente 16% de FDCA, para a melhor condição testada. Estes resultados demonstram que a utilização de um hidrolisado de bagaço de maçã, pela estirpe selecionada, é uma opção viável e sustentável para a bioconversão de HMF usando células inteiras.

Palavras-chave: *Saccharomyces cerevisiae*, HMF, FDCA, Biocatálise com células inteiras, Biomassa lignocelulósica.

Abstract

The escalating demand for energy and chemicals necessary in various daily routines coupled with the effects of climate change and the increase in greenhouse gas emissions has been pushing the need to shift away from fossil fuels to biological processes. To achieve this aim, establishing a biorefinery is essential for producing biofuels and other chemicals from renewable feedstocks, including lignocellulosic biomass. Hydroxymethylfurfural is a versatile compound and an attractive chemical because of its unique properties and multiple applications. It can be reduced or subsequently oxidized to a variety of products, and its derivatives have a wide range of applications such as synthesising polymers, synthesising surfactants and resins and producing plasticisers, polyurethanes, polyesters or polyamides, among others. The yeast *Saccharomyces cerevisiae* has been extensively investigated for its ability to detoxify HMF, a toxic by-product of hexoses degradation. However, its capability as a whole-cell biocatalyst to produce HMF derivatives, has not been sufficiently investigated.

A robust industrial and a laboratory *S. cerevisiae* strain, both genetically modified to produce HMF/furfural oxidoreductase (HMFH) or HMF oxidase (HMFO) were utilised as hosts for HMF oxidation. The recombinant and wild-type strains were evaluated for their ability to detoxify HMF and I-msHMFO strain demonstrated effective detoxification of 50 mM of HMF, resulting in the production of 11 mM of FDCA. Afterwards, the bioconversion conditions were optimised by evaluating the impact of oxygenation conditions. Furthermore, FDCA was produced using a synthetic hemicellulosic hydrolysate with glucose and fructose as carbon sources. The effects of furfural in the conversion medium were also analysed, revealing that the yeast is highly inhibited by the HMF and furfural mixture and has a tendency to detoxify furfural more rapidly.

Finally, an apple pomace hydrolysate that underwent a pretreatment with a deep eutectic solvent and microwave, was utilised as a source of HMF for the bioconversion. The best-performing strain was able to convert approximately 60% of HMF resulting in the production of derivatives with a yield of approximately 16% of FDCA, for the best condition tested. These results demonstrate that the use of apple pomace hydrolysate by the selected engineered strain is a viable sustainable option for the whole-cell bioconversion HMF.

Keywords: *Saccharomyces cerevisiae*, HMF, FDCA, Whole-cell biocatalysis, Lignocellulosic biomass.

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List of abbreviations

BHMF	2,5-bis(hydroxymethyl)furan
DES	Deep eutectic solvents
DFF	2,5-diformylfuran
FDCA	2,5-furandicarboxylic acid
FFCA	5-formyl-2-furancarboxylic acid
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HMF	5-Hydroxymethylfurfural
HMFCA	5-hydroxymethyl-furan-2-carboxylic acid
HMFH	HMF/furfural oxidoreductase
HMFO	Hydroxymethylfurfural oxidase
HPLC	High-performance liquid chromatography
NADES	Natural deep eutectic solvents
PEF	poly (2,5-furandicarboxylate)
PET	poly (ethylene terephthalate)
UHPLC	Ultra-High-Performance Liquid Chromatography
YPD	Yeast extract-Peptone-Dextrose

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1. Introduction

Climate change and the increase in greenhouse gas emissions, combined with population growth and consequent growing demand for energy and chemicals needed for many different activities of daily life, have fostered the need to replace energy from fossil fuels with energy derived from biological processes.

The concept of biorefinery incorporates a range of technologies, such as thermochemistry, biochemistry, combustion, and microbial bioconversion, which, used separately or in combination can transform renewable biological resources, including lignocellulosic biomass, agricultural feedstocks, algae and various forms of organic waste, into the necessary building blocks for producing biofuels, chemicals or other value-added compounds. Unlike the conventional petroleum-based refinery, which predominantly exploits natural resources and generates colossal amounts of waste, biorefineries represents a significant transformation through the integration of systems that facilitate the complete utilisation of resources (Cherubini, 2010).

In this context, the correct implementation of a biorefinery based on renewable bioresources, especially lignocellulosic biomass represents an important aspect (Singh et al., 2022). Most recently, the conversion of all biomass factions into value-added products has been recognised as an essential element to enhance the value and profitability of biorefinery operations and to cover their two main objectives. The first is the energetic one being focused on the replacement of oil with renewable carbon sources in the production of fuels, while the economic objective focuses on directing efforts towards the efficient production of chemicals of biological origin and providing the economic incentive for the maintenance and expansion of this sustainable industry (Baptista et al., 2021; Bozell & Petersen, 2010).

1.1. Lignocellulosic Biomass

The replacement of fossil resources with renewable resources has been extensively studied, with a focus on the conversion of lignocellulosic biomass into industrially relevant products. Numerous studies have indicated that lignocellulosic biomass has a significant potential for sustainable production of chemical products or fuels and is a renewable substrate that is widely available in nature (Isikgor & Becer, 2015).

Lignocellulosic biomass may derive from energy crops, aquatic flora, forest residues and waste and agricultural remains (Kumar et al., 2009; Limayem & Ricke, 2012). It consists mainly of three

components: cellulose, hemicellulose, and lignin and these polymers appear in varying quantities depending on the type of biomass. The content in cellulose is generally between 40% and 60%, while the hemicellulose content is around 15% to 30% and the lignin content varies between 10% and 25%, Furthermore, lignocellulosic biomass may also contain extractives and ash (Wang et al., 2017).

The cellulosic and hemicellulosic factions constitute the main carbon sources utilized in the production of value-added products. Cellulose is the world's most abundant polymer and the major constitute of lignocellulosic biomass. The crystalline matrix structure, formed by extensive hydrogen bonding between glucose molecules, makes it resistant to depolymerisation and insoluble in water (Zhao et al., 2012). On the contrary, hemicellulose is a heteropolymer consisting of short linear and branched chains of monomers such as hexoses (glucose, galactose, and mannose) and pentoses (xylose and arabinose). The backbone of hemicellulose is predominantly composed of xylan (β-1,4-linked xylose) which can constitute up to 50% of the composition of certain grasses and cereals (Gírio et al., 2010).

Lignin is an aromatic polymer with a three-dimensional network structure produced from fundamental phenylpropanolic monomers, namely *p*-comaryl, coniferyl and sinapyl alcohols, and is mainly found in the cell wall of hardwood and softwood, contributing to the rigidity of plants (Yuan et al., 2021).

1.1.1.Apple Pomace

Apples are one of the most consumed fruits worldwide, not only due to its pleasant taste, but also to its proven beneficial effects in the prevention of chronic cardiovascular diseases, respiratory and pulmonary disorders, diabetes, obesity, and cancer, among others (Tu et al., 2017). A considerable portion of apples are also highly processed in food production sector industries, generating a significant amount of industrial by-products known as apple pomace (Barreira et al., 2019).

Apple pomace is the main by-product from the processing of apples for cider ad juice production, accounting for approximately 25 to 30% of the total apple mass (Gullón et al., 2008). Every year, millions of tonnes of apples are processed for the food industry, resulting in large amounts of waste (Fernandes et al., 2019).

Pomace and its extracts constitute a significant waste product in the apple processing industry and offer substantial potential within the biotechnology industry. This waste is generally referred to as a heterogeneous mixture of peels, pulp, and seeds, which comprises simple sugars (glucose, fructose and

sucrose), high water content and contains insoluble carbohydrates such as hemicellulose, cellulose and lignin, resulting in its classification as a valuable by-product (Mirabella et al., 2014).

The appropriate disposal of this by-product can incur high costs for industries. Its utilisation as a fertiliser without any pre-treatment or its discharge in landfills cannot be classified as environmentally favourable options (Suárez et al., 2010). Currently, there are multiple methods of utilising apple pomace, such as in animal feed. Nonetheless, its low protein content and seasonally restricted availability do not make it the optimal choice for this substrate. Furthermore, a number of studies have examined the possibility of using this by-product for various purposes, including the production of biofuels (González-García et al., 2022), aromatic compounds (Bramorski et al., 1998) or organic acids (Gullón et al., 2008), pectin extraction (Wang et al., 2014) and in the food and cosmetic industry (Barreira et al., 2019). The limited utilisation of its potential makes the susceptibility of this by-product an area of concern, even though the ideal application for this by-product has not yet been found, according to Kennedy et al. (1999).

1.2. 5-Hydroxymethylfurfural (HMF)

One chemical product obtained from lignocellulosic biomass is 5-hydroxymethylfurfural (HMF). This compound consists of an aromatic furan ring and two reactive functional groups (aldehyde group and alcohol group) (Figure 1), being attractive due to its versatility, unique properties and various applications (Menegazzo et al., 2018). This chemical was identified in 2010 as one of the most promising compounds to be derived from biomass to support the successful development of biorefining industry (Bozell & Petersen, 2010).

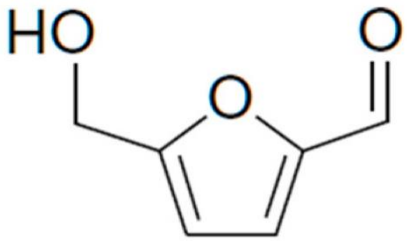


Figure 1. 5-hydroxymethilfurfural (HMF) structure (adapted from (Menegazzo et al., 2018))

HMF can be obtained from dehydration of hexoses such as fructose or glucose. It is usually produced during the pretreatment of lignocellulosic biomass and its accumulation depends not only on

the type of biomass used, but also on the type and severity of the treatment applied to it (Steinbach et al., 2017).

1.2.1. HMF production

Since this chemical has been attracting more attention, several studies have prioritised economic and environmental sustainability and the optimisation of the pretreatment process to increase the accumulation of HMF from different types of biomass (Steinbach et al., 2017). Therefore, several catalysis media and reaction environments have been evaluated, such as Lewis and Bronsted acids, ionic liquids, deep eutectic solvents (DES) or two-phase systems, as well as alternative mechanical methods as microwaves and ultrasound (Chen et al., 2020; Ji et al., 2021).

There are a variety of methods for producing HMF. This study concentrates on the sustainable generation of HMF via deep eutectic solvents utilizing microwaves.

Choosing the right solvent significantly affects the cost, safety and environmental impact of the process, given this, DES have emerged as a new class of green solvents that first gained recognition as alternative ionic liquids in 2001 (Abbott et al., 2001). Subsequently, numerous studies have explored the applications of these solvents in various fields, including material synthesis and biotransformation (Carriazo et al., 2012; García et al., 2015). It is noteworthy that DES have been extensively investigated due to their exceptional properties and unique characteristics, which make them a promising alternative to conventional solvents.

DES consist of two individual chemicals that blended in a particular molar ratio act as a hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD). The resulting eutectic solvent is characterised by having a melting point lower than that of its individual components and being able to remain in a liquid state even at low temperatures (Tiago et al., 2022). The primary HBA used is choline chloride, a quaternary ammonium salt with a high melting point. As for HBD, the most commonly used are organic acids, urea, sugars among other (Rodríguez-Martínez et al., 2022).

A specific combination of naturally occurring substances in a solid state that results in a eutectic mixture is referred to as a natural eutectic solvent (NADES). These solvents are found in the natural world and participate in several cellular processes in living organisms. As they are made up of natural components NADES are considered fully biodegradable and sustainable, making them increasingly appealing (Choi et al., 2011).

Eutectic solvents find extensive application in extraction processes, food and enzyme analyses, purification and fermentation techniques among others. Describing particularly, DES can extract both hydrophilic and lipophilic compounds form different substrates (Obluchinskaya et al., 2021). Additionally, a range of DES has been recently implemented in various fields, including health, pharmaceuticals, chemical production and biofuel production (García et al., 2016).

The production of HMF using DES is a recent technique. If used correctly, these solvents serve as a low-cost and non-toxic alternative, given their low toxicity, biodegradability and renewability.

The synthesis of value-added compounds such as furans, commonly involves traditional heating techniques such as oil baths or reactors. Nevertheless, microwave radiation provides a faster and cleaner alternative, providing good yields. The energy emitter by the microwave interacts directly with the molecule via ionic conduction, resulting in quick, uniform and efficient heating. In addition, the microwave can create an environment with extreme conditions of high temperature and pressure in a safer manner (Gomes & Pastre, 2020).

1.2.2. HMF derivatives

HMF can be reduced to 2,5-bis(hydroxymethyl)furan (BHMF) or undergo sequential oxidation to produce a variety of compounds with a with range of applications (Figure 2). Two different routes exist for the oxidation process of HMF: (1) oxidation of the aldehyde group of HMF resulting in 5-hydroxymethyl-furan-2-carboxylic acid (HMFCA), and (2) oxidation of the hydroxymethyl group leading to 2,5-diformylfuran (DFF). The resulting compounds can be further oxidised to generate 5-formyl-2-furancarboxylic acid (FFCA), which can then be oxidised to produce 2,5-furandicarboxylic acid (FDCA). These HMF derivatives have a wide range of uses in synthesis of polymers and esters (BHMF), producing polymers and fungicides (DFF), synthesising surfactants and resins (FFCA) and the production of bioplastics (FDCA) (Saikia et al., 2022)

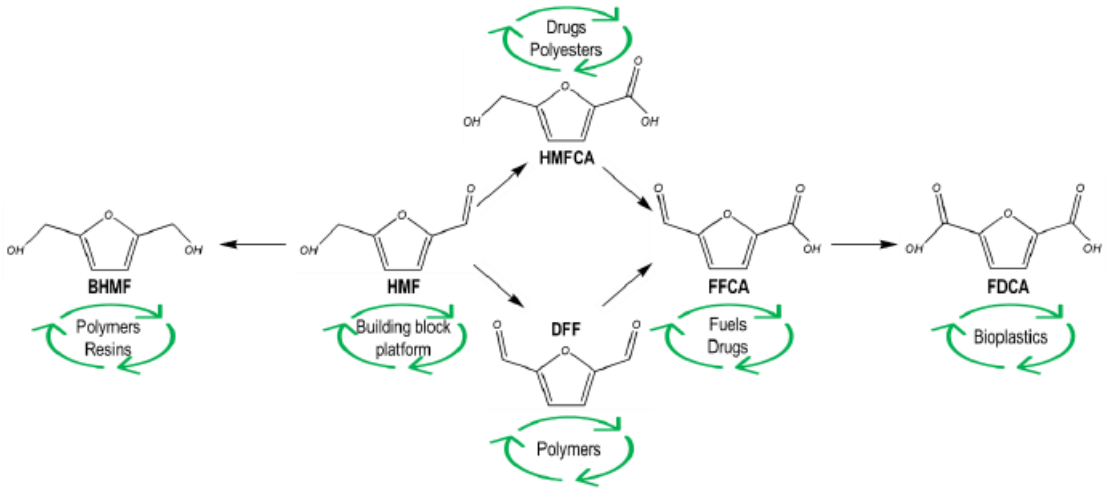


Figure 2. HMF derivatives and its applications. BHMF: 2,5-bis(hydroxymethyl)furan, HMF: 5-hydroxymethylfurfural, HMFCA: 5-hydroxymethyl-furan-2-carboxylic acid, DFF: 2,5-diformylfuran, FFCA: 5-formyl-2-furancarboxylic acid, FDCA: 2,5-furandicarboxylic acid (Cunha et al., 2022).

1.2.2.1. 2,5-bis(hydroxymethyl)furan (BHMF)

BHMF is a valuable chemical that has potential for application in polymer, resin and ether synthesis (Hu et al., 2017). This compound was initially reported by Liu et al., (2004) as a reduction product of HMF by the yeast *Saccharomyces cerevisiae*, being identified later as a product from HMF biocatalyst from fungi, other yeast and bacteria.

For the conversion of HMF into BHMF, NAD(P)H functions as an essential cofactor. The regeneration of this cofactor relies on the co-substrates used for the reaction, and hence, glucose has extensively been employed in the biotransformation of HMF into BHMF (Sehnem et al., 2013).

In recent years, there has been extensive research on the whole-cell biotransformation of HMF into BHMF. Liu and colleagues have published findings on the effectiveness of two *S. cerevisiae* stains, 307-12H120 and 307-12H60, in producing BHMF, their research revealed a 100% yield of BHMF from 60 mmol/L of HMF in 48 hours (Liu et al., 2004). Subsequently, Xu and colleagues (2018) identified new yeast strain *Meyerozyma guilliermondii* SC1103, suitable for reducing HMF. BHMF yields of 86% were achieved after 12 h when 100 mmol/L of HMF was used. The report suggests that immobilising *M. guilliermondii* SC1103 on calcium alginate can enhance its tolerance, the immobilised strain was capable of reducing 200 mmol/L of HMF in order to produce BHMF with an 82% yield in 7 hours (Xu et al., 2018).

More recently, the non-conventional yeast *Kluyveromyces marxianus* was also explored as a whole-cell biocatalyst for the bioconversion of HMF to BHMF, achieving a high yield (99.7%) from 55 mM HMF after 12 h with a productivity of 0.6 g/(L.h) (Baptista et al., 2021)

An approach to genetic engineering involved a recombinant strain of *S. cerevisiae* expressing an aryl alcohol dehydrogenase from *M. guilliermondii* exhibited a high tolerance towards HMF along with a high level of selectivity and activity during the HMF reduction process. BHMF was produced with a high yield (94%) within 24 hours with glucose supplemented culture medium (Xia et al., 2020). Additionally, considering environmental concerns, the low-cost hydrolysate form corn cob was identified as a potentially favourable substitute to glucose as a co-substrate for the biocatalyst (Xia et al., 2020).

1.2.2.2. 5-hydroxymethyl-furan-2-carboxylic acid (HMFCA)

HMFCA is derived from the oxidation of the aldehyde group of HMF. This compound is a multifunctional precursor in the production of polyesters (Hirai, 1984) and is an inhibitor of interleukin (Braisted et al., 2003). Furthermore, there have been reports stating that HMFCA exhibits anti-tumour activity (Munekata & Tamura, 1981). Obtaining high yields of HMFCA during HMF oxidation presents a challenge due to its susceptibility to further oxidation to 5-formyl-2-furancarboxylic (FFCA) acid and 2,5-furandicarboxylic acid (FDCA) in aerobic conditions, particularly at elevated temperatures (Gao et al., 2022).

Chemoselective oxidation techniques are primarily utilised to produce HMFCA from HMF (Davis et al., 2014). Noble metals catalysts are commonly employed, for example, a molybdenum complex immobilised in toluene was recently used to selectively oxidise HMF to HMFCA in 3 hours with a yield of about 87% (Z. Zhang et al., 2014). The biocatalytic oxidation of HMF to HMFCA is a promising alternative to chemical methods due to its range of benefits. This includes mild an environmentally friendly reaction conditions, as well as excellent selectivity and high efficiency (Cang et al., 2019). More recently, Qin and colleagues (2015) have used a molybdenum-dependent enzyme for the biocatalytic oxidation of HMF to form HMFCA with a 94% yield. Krystof and colleagues (2013) utilized immobilized lipase B derived from *Candida antactica* (CaLB) in combination with acyl donors and hydrogen peroxide for the oxidation of HMF. With t-butanol as the acyl donor, a yield of 76% HMFCA was achieved within a time span of 24 hours, thereby establishing the favourable nature of lipase-mediated oxidation of HMF in producing HMFCA (Krystof et al., 2013).

Compared to using isolated enzymes, utilizing whole-cell biocatalysts for the oxidation of HMF proves to be more advantageous. More recently, there has been significant interest in HMFCA and in the discovery of whole-cell biocatalysts capable of efficiently producing it from HMF (Cunha et al., 2022). Many bacterial species have been investigated for this purpose. It was found that *Serratia marcescens* displayed tolerance to HMF. Optimal conditions resulted in the production of 790.2 mg/L of HMFCA from crude HMF through the implementation of a fed-batch strategy (Muñoz et al., 2020). In another study, a substrate adaptation strategy was implemented to enhance the catalytic performance of *Comamonas testoteroni* SC11588 cells. The cells were able to produce HMFCA in a quantitative yield from 200 mM HMF within a 24-hour time frame. Furthermore, HMFCA yields ranging from 71% to 81% were attained at substrate concentrations of up to 250-300 mM (Wen et al., 2020).

Although satisfactory results have been achieved with wild-type strains, attempts have been made to genetically modify bacteria to produce HMFCA. A synthetic *E. coli* strain containing an *hmfo* enzyme from *Methylovorus* sp. Strain M688 was mutated for specificity towards HMFCA which result in a 97% yield of conversion in an aqueous reaction media (Wang et al., 2020).

1.2.2.3. 2,5-diformylfuran (DFF)

DFF, which features two symmetrical aldehyde groups, is exclusively formed through the oxidation of the hydroxymethyl group, while FFCA and FDCA are produced through subsequent oxidation. This compound has the potential to act as a precursor in the synthesis of polymers, fungicides and pharmaceuticals (Yadav & Sharma, 2014).

The first report of biocatalytic transformation of HMF to DFF was presented by Van Deurzen and co-workers (1997) wherein chloroperoxidase from *Caldariomyces fumago* oxidised HMF under the influence of hydrogen peroxide, ensuing in a 53% yield in 2.5 hours. However, chloroperoxidase cannot achieve complete conversion of HMF, another study utilised an alcohol oxidase from *Candida boidinii* in conjunction with catalase to oxidise HMF, ultimately resulting in a DFF yield of 43% after 72 hours of conversion under non-optimized conditions (Qin et al., 2015). McKenna and colleagues (2017) implemented a biocatalytic system which comprised horseradish peroxidase, galactose oxidase and catalase resulting in a DFF yield of 88% at pH 7.5 compared to a 77% yield at pH 6.5. The biocatalytic conversion of HMF under the exact same rection conditions in deionized water yielded 56% DFF, highlighting the decisive role of pH in the biocatalytic conversion of HMF to DFF.

The transformation of HMF to DFF is primarily achieved through chemical catalysis. Although there have been investigations involving microorganism-derived enzymes for biocatalysis, there are no published reports of whole-cell biocatalysis.

1.2.2.4. 5-formyl-2-furancarboxylic acid (FFCA)

Following the partial oxidation of HMF, another noteworthy component is FFCA, which possesses a carboxylic acid and an aldehyde group rendering it suitable for numerous additional applications in the synthesis of surfactants and resins (Moreau et al., 2004; Qin et al., 2015). However, there have been a limited number of studies focusing on FFCA production due to its selective oxidation from HMF.

FFCA can be produced via partial oxidation of HMF with the aid of catalysts like heteropolyacid, mixed metal oxides and transition metals. The first studies on the biocatalytic production of FFCA used CaLB in combination with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) as a catalytic system for HMF oxidation. The use of CaLB in the oxidation process of HMF resulted in a yield of FFCA of 52% over a period of 24 hours within a t-butanol system (Domínguez de María & Guajardo, 2017). In a similar way, in another study, laccase and TEMPO were used to oxidise HMF and a yield of 82% of FFCA was obtained within 96 hours (Qin et al., 2015). Aryl-alcohol oxidase (AAO) was capable of catalysing two oxidations from 3 mM of HMF and achieved a FFCA yield of 100% after 4 hours at room temperature (Carro et al., 2015). Dijkman & Fraaije, (2014) also found that hydroxymethylfurfural oxidase (HMFO) produced an excellent yield starting from 2 mM of HMF. To improve the productivity of FFCA production a laccase known as CotA-TJ102, derived from *Bacillus subtilis*, was identified. This laccase, whether in its free form or immobilised form, has shown potential to selectively oxidise HMF, as a result of it, 98% yield of FFCA was achieved within 12 hours with a conversion rate of 100% (Zhang et al., 2019).

In general, biocatalytic production of FFCA seems to be relevant and further studies to optimise, design proteins and develop processes may be of high interest.

1.2.2.5. 2,5-furandicarboxylic acid (FDCA)

2,5-furandicarboxylic acid (FDCA) is a diacid and considered the most significant furan-derived compound used in polymer chemistry. FDCA is listed as one of the molecules in the “Top 10 + 4” list by the US Department of Energy (Bozell & Petersen, 2010). FDCA has generated interest because it serves as an aromatic monomer for producing bio-based poly(2,5-furandicarboxylate) (PEF), which could be an

environmentally friendly alternative to the widely used poly(ethylene terephthalate) (PET), a non-degradable petrochemical. Furthermore, it can also serve as a fundamental component in the production of medicines and polyamides (Hu et al., 2018).

At an industrial scale, the production of FDCA has been initiated by Avantium through their "YXY®" project. Despite widespread production of FDCA, most of these mechanisms are chemical technologies that use metal salts and organic solvents at high temperatures and pressures, that contribute to environmental toxicity and hazardous conditions. Therefore more eco-friendly and cost-effective alternatives are recommended to address environmental and global warning concerns (Han et al., 2017; S. Xu et al., 2017). In this context the microbial conversion of HMF to FDCA is of high importance and acceptability due to its ability to resolve most of the environmental issues. Nevertheless, the biological conversion methods also raise issues related to the economic challenges, which could be partially addressed by the different optimisation approaches (Rajesh et al., 2019a).

The first whole-cell biotransformation of HMF to FDCA was based on a bacterial *Pseudomonas putida* S12 strain modified with the *hmfH* gene from *Cupriavidus basilensis* which encodes an HMF/furfural oxidoreductase. The conversion was managed via two sequential procedures, in which the alcohol group of HMF is initially oxidised to the corresponding aldehyde, which is subsequently oxidised to FDCA, this strategy produced 193 mM FDCA with a 97% yield (Koopman et al., 2010a). Recently, *hmfo* from *Methylovorus* sp. strain MP688, which encodes an HMF oxidase, has been expressed in *P. putida* S12, resulting in the production of 545 mM of FDCA in a fed-batch system (Hsu et al., 2020). Tan et al. (2020) reported a whole-cell cascade reaction in which an engineered *E. coli* was paired with a wild-type *P. putida* for stepwise reactions. The HMF substrate was first oxidized into DFF by the *E. coli* and then further oxidized into FDCA by adding a *P. putida* strain. The reaction successfully converted 100 mM of HMF into FDCA with a yield exceeding 99%, however was necessary the addition of commercial enzymes such as horseradish peroxidase and catalase was required. The roles of these are to activate enzymes and decompose H_2O_2, respectively (Tan et al., 2020a).

In a study conducted by Hossain et al., (2017) *a Raoultella ornithinolytica* BF60 strain was isolated and engineered to remove the *dcaD* gene in order to decrease FDCA degradation. To further improve the yield, different promoters and plasmids were utilized to express *hmfH* and *hmfO* genes and this approach yielded a 96.2% FDCA in 144 hours. Another study involved the introduction of two genes that encode for HMF oxidase (from *Methylovorus* sp. Strain MP688) and HMF/furfural oxidoreductase (from *C. basilensis*

HMF14) into *Raoultella ornithinolytica* BF60. Through this method, the FDCA yield reached 93.6% (H. Yuan et al., 2018).

Employing the bacteria *E. coli* as a cell host, FDCA production was achieved through heterologous expression of HMF/furfural oxidoreductase (HMFH) form *C. basilensis* HMF14 and vanillin dehydrogenase (VDH1) from *Comamonas testoteroni* SC1588, this yielded 96% of FDCA (Wang et al., 2020). These and other examples of whole cell biocatalysis of HMF in FDCA are compiled in Table 1.

Table 1. Examples of whole cell biocatalyst of 5- hydroxymethylfurfural (HMF) into 2,5-furandicarboxylic acid (FDCA).

Strain	Modifications	Conditions	Initial HMF concentration	Final FDCA concentration	Yield (%)	Reference
Pseudomonas putida S12	*hmf*H from *Cupriavidus basilensis* HMF14, encoding HMF/furfural oxidoreductase	30 °C; pH 7; 150 rpm;	0 (fed batch; total concentration of HMF 188 mM)	30.1 g/L (238.7 mM)	97	(Koopman et al., 2010a)
P. putida S12	*hmfO* from *Methylovorus* sp. strain MP688 encoding HMF oxidase	30 °C; pH 8.0;	0 (fed batch)	545 mM	nd	(Hsu et al., 2020)
P. putida S12	*hmfH*, encoding HMF/furfural oxidoreductase and *HMFT1* encoding an HMF transporter from *C. basilensis* HMF14	30 °C; pH 7; 180rpm	250 mM	196 mM	78	(Pham et al., 2020)
Burkholderia cepacian H-2	none	28 °C, pH 7	2000 mg/L	1276 mg/L (10.1 mM)	64	(Yang & Huang, 2016)
Aspergillus flavus APLS-1	none	30 °C; pH 6.5; 200 rpm	8 mM	6.6 mM	82	(Rajesh et al., 2019b)
Raoultella ornithinolytica BF60	none	30 °C; pH 8; 150 rpm	100 mM	50.7 mM	51	(Hossain et al., 2017)
R. ornithinolytica BF60	Mutation of *dcaD*, encoding dicarboxylic acid decarboxylase Mutation of *aldR*, encoding aldehyde reductase.	30 °C; pH8; 150 rpm	100 mM	89.0 mM	89	(Hossain et al., 2017)

	Overexpression of the gene encoding aldehyde dehydrogenase					
R. ornithinolytica BF60	*Hmfo* from *Methylovorus* sp. Strain MP688, encoding HMF oxidase and *hmfH* from *C. basilensis* HMF14 encoding HMF/Furfural oxidoreductase	30 °C; pH 8; 220 rpm	100 mM	93.6 mM	94	(Yuan et al., 2018)
Escherichia coli	HMFH from *Methylovorus* sp. Strain MP688 encoding HMF oxidase. VDH encoding vanillin dehydrogenase	30 °C; pH 7.0; 150 rpm	150 mM	144 mM	96	(Wang et al., 2020)
E. coli and *P. putida* KT2440	Galactose oxidase in *E. coli*	37°C; pH 7.0; 200 rpm	100 mM	>99 mM	>99	(Tan et al., 2020b)

1.3. Whole-cell Biocatalysis

Chemical catalysts can adversely impact the environment and can result in unwanted by-products which decrease efficiency an incur additional costs (J. Choi et al., 2015). Considering this, looking for innovative approaches to achieve a sustainable and biobased economy, biocatalysis has a significant impact on several fields ranging from the production of mass chemicals to the highly particular synthesis of fine chemicals or pharmaceutical products (Wachtmeister, 2016). Biocatalysis refers to the use of isolated enzymes or cells containing enzymes for chemical transformations. Generally, whole cell biocatalysis is preferred over stand-alone enzymes because it can provide distinctive benefits over the usage of raw, refined or immobilized enzyme preparations. In addition to its cost-effectiveness as a catalyst formulation, the whole-cell provide the necessary cofactors for biotransformation, regenerate the necessary enzymes, can use crude feedstocks and are cheaper (Lin & Tao, 2017). Whole-cell catalysis approaches can be broadly divided into biotransformation (biocatalysis) and fermentation bioprocess (Sheldon & Pereira, 2017). In fermentations, products are synthesized from growth substrates using the host cells native metabolism, in fermentation broth, metabolic intermediates accompany the products making the downstream process more complicated (Ladkau et al., 2014). In biotransformation, the cell growth and production phases are separated, the substrate is converting to the desired product by resting cells (Carvalho, 2020).

Efficient whole-cell biocatalysts are crucial for economically feasible biocatalysis processes with optimal titre, yield and productivity. It is worth noting that the approach to design whole-cell biocatalysts for bioconversions differs significantly from that employed when designing microbial cell factories for fermentation (Lin & Tao, 2017). To create whole-cell biocatalyst to produce value-added chemicals, single or multiple enzymes must be introduced into host cells, depending on the complexity of the synthetic pathway that produces the desire product. To fully exploit the synthetic potential of microbial biocatalysts, it is important to consider the whole cell as a catalyst rather than focusing on a single active enzyme, optimizing the flux through a synthetic pathway is also essential in achieving optimal volumetric productivity for bioconversion, resulting in reduced production costs. The recent inclusion of synthetic biology tools combined with comprehensive omics data have facilitate metabolic engineering of microbial cells at an unprecedented capacity. This technology reaffirms the potential towards the creation of a well-balanced, stable, productive and efficient whole cell workhouse platform (Lee et al., 2011).

The biocatalytic upgrading of biobased furans has garnered significant interest. Numerous whole cells have been utilized for the selective oxidation of these furans to furan carboxylic acids, however, the catalytic efficiencies of biocatalytic conversion of furans are generally unsatisfactory, particularly ate high substrate concentrations that are necessary for industrial production (Cheng et al., 2020). Previously, HMF and furfural, which are furan derivatives, were extensively studied due to their common inhibitory properties in lignocellulosic hydrolysates. These inhibitory properties affect microbial fermentation that results in a decreased microbial growth and metabolism, consequently reducing production of metabolites (Cunha et al., 2019). Research has shown that these compounds inhibit dehydrogenase activity (MODIG et al., 2002), reduce intracellular ATP and NAD(P)H levels (Sa et al., 2003) and damage DNA membranes (Khan & Hadi, 1994).

1.3.1.Whole-cell biocatalysis using *Saccharomyces cerevisiae*

Considering the limitations associated with the pretreatment and hydrolysis procedures, the economical and productive use of lignocellulosic biomass for biofuels and value-added products is reliant on a robust microorganism to execute the fermentation process.

The yeast *S. cerevisiae*, commonly referred to as GRAS (Generally Regarded as Safe) yeast, finds widespread use in the biotechnology field such as in the manufacturing of beer, bread, wine, bioethanol, nutraceuticals, chemicals and pharmaceuticals (Nielsen & Jewett, 2008). It is a model eukaryotic system with extensively studied molecular and cellular biology, along with a variety of available genetic tools. As a eukaryotic organism, it has several organelles that are suitable for compartmentalizing biosynthesis of different compounds. Furthermore, *S. cerevisiae* displays high tolerance towards harsh industrial conditions, reinforcing its popularity as an industrial cell factory (Cunha et al., 2020; Lian & Zhao, 2015).

The utilisation of strains extracted from industrial environments, including those with high sugar concentration, high temperatures, pH variations and presence of toxic and inhibitory compounds, has been subject of many studies lately, particularly in ethanol production (Cunha et al., 2020; Pereira et al., 2010) and in furan detoxification (Pereira et al., 2014) . Additionally, it has been acknowledged that industrial settings serve as a source of *S. cerevisiae* strains which possess higher robustness, fermentation capacity and resistance against stress factors commonly encountered in severe industrial processes in contrast to laboratory strains which are typically believed to have lower generalised tolerance (Pereira et al., 2010).

Selecting and characterising optimal strains for withstanding common fermentation inhibitors present in lignocellulosic hydrolysates, such as 5-hydroxymethylfurfural (HMF), furfural and acetate, poses

a challenge and is time-consuming. These characteristics have given rise to the advancement of *S. cerevisiae* as a chassis microorganism for metabolic engineering with the goal of exploiting lignocellulosic biomass (Costa et al., 2017; Lian et al., 2018; Romaní et al., 2015). Genetically modified strains have been equipped with a diverse range of molecular tools to proficiently withstand various arduous fermentation conditions, such as elevated temperatures, acidic environments and the presence of inhibitory compounds (Cunha et al., 2020).

S. cerevisiae has been extensively researched for its ability to detoxify HMF or furfural. However, the potential of *S. cerevisiae* as a whole-cell biocatalyst for the generation of value-added HMF or furfural derivatives has yet to be adequately explored. Therefore, the reduction of these compounds into the corresponding alcohol requires further investigation (Cunha et al., 2019). Liu et al. (2005) reported on two *S. cerevisiae* strains (307-12H60 and 307-12H120) that were adapted for higher HMF tolerance and could convert 60 mM of HMF into BHMF. Furthermore, a recent study investigated the heterologous expression of alcohol dehydrogenases (ADHs) from *M. guilliermondii* in *S. cerevisiae* to produce BHMF from HMF. The recombinant *S. cerevisiae* strain achieved higher productivities reaching a BHMF yield of 94% within 24 hours (Xia et al., 2020).

Given the yet unexplored potential of *S. cerevisiae* in bioconverting HMF into higher-value compounds, particularly its oxidized derivatives, our research team has, previously, conducted an investigation into the resistance of multiple industrial and laboratory strains to inhibitors commonly found in lignocellulosic biomass, such as HMF. Different *S. cerevisiae* strains from various sources demonstrated unique characteristics, including improved robustness and diverse capacities to detoxify inhibitors (Pereira et al., 2014). Consequently, three strains of *S. cerevisiae*, Ethanol Red, a thermotolerant strain developed for the ethanol industry (Fermentis, S.I. Lesaffre), PE-2 and CAT-1, derived from first-generation bioethanol plants and CA11, extracted from “cachaça” distillery, were assessed in terms of their ability to convert 50 mM of HMF into its derivatives (Cunha, 2021). As anticipating, the strains exhibited varying abilities to convert HMF. The strain Ethanol Red achieved complete HMF conversion within 24 hours, while CAT-1 and CA11 required 48 hours to reach full conversion, while PE-2 could only convert 86.8% of the initial HMF concentration even after 120 hours. Ethanol Red and CAT-1 exhibited higher HMFCA titres, indicating that these strains may favour oxidation reactions, whilst Ethanol Red resulted in a faster HMF conversion and higher HMFCA productivity. Moreover, the use of Ethanol Red also resulted in slightly higher FDCA titres (Cunha, 2021).

1.3.2.Genetic Modifications

Genetic engineering has become a means of boosting productivity and providing cells with resistance to the severe conditions of fermentations and to the inhibitors present in the fermentation medium (Cunha et al., 2019). The recent developments in this filed is enabling fast and precise engineering of microorganisms boosting the development of novel microbial factories to produce a diverse range of valuable products.

Considering previous studies on FDCA bioproduction from HMF, two enzymes have gained special attention for heterologous expression in other microorganisms, HMF/Furfural oxidoreductase from *C. basilensis* (CbHMFH) (Koopman et al., 2010a) and HMF oxidase from *Methylovorus* sp. (MsHMFO) (H. Yuan et al., 2018). Both these enzymes belong to the class of 5-hydroxymethylfurfural oxidases (HMFOs; EC .1.1.3.47) that are flavoenzymes of biotechnological significance, classified within the superfamily of glucose-methanol-choline oxidases and dehydrogenases (Cavener, 1992). These enzymes are crucial as they can catalyse the three successive oxidation steps required for producing 2,5-furandicarboxylic acid (FDCA) from HMF, with molecular oxygen acting exclusively as co-substrate, which generate hydrogen peroxide as a by-product. HMF/Furfural oxidoreductase (HMFH) activity was originally detected in *C. basilensis* strain HMF14 (Koopman et al., 2010a), and more recently times, in *Pseudomonas putida* strain ALS1267 (Crigler et al., 2020) with both metabolic pathways being characterized. More recently a gene from *Methylovorus* sp. strain MP688 showed potential to encode an HMFH homolog which was named HMFO (HMF oxidase). This enzyme is also a flavin-dependent enzyme that catalyses the oxidation of many aldehydes, primary and secondary alcohols, and is a relatively thermostable and active over a wide pH range, these characteristics make this enzyme more suitable for use under harsh reaction conditions (Dijkman & Fraaije, 2014).

Results obtained previously by our research group indicate the efficacy of an industrial strain (I) to be a promising biocatalyst for HMF oxidation. Given this the strain was engineered to produce heterologous enzymes (HMF/Furfural oxidoreductase from *C. basilensis* HMF14 (CbHMFH) and HMF oxidase from *Methylovorus* sp. Strain MP688 (MsHMFO)). The modified strains (I-cbHMFH and I-msHMFO) were used as biocatalyst in a bioconversion assay with 50 mM of HMF. The CbHMFH enzyme oxidizes HMF through the HMFCA intermediate (Figure 3), meanwhile the MsHMFO enzyme was described to oxidize HMF via DFF (Figure 3), which could be beneficial to increase HMF oxidation by having both oxidation routes active in the yeast (Cunha, 2021).

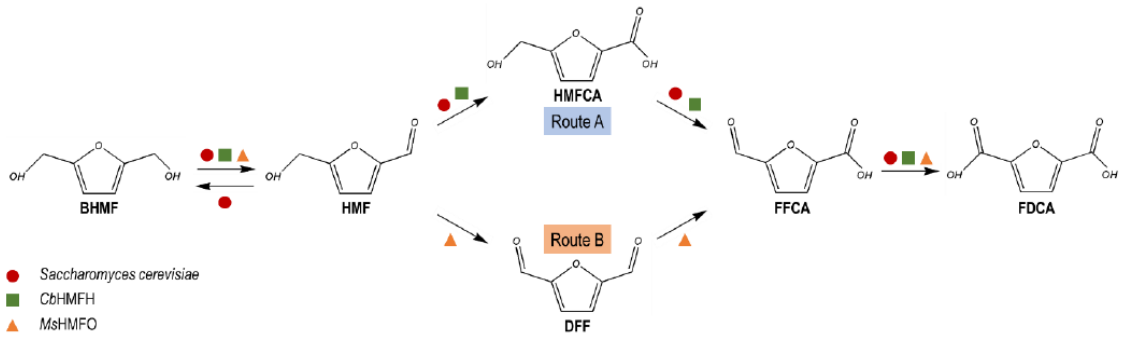


Figure 3. Routes for the reduction or oxidation of HMF to its derivatives, BHMF (2,5-bis(hydroxymethyl)furan), HMF (5-hydroxymethylfurfural), HMFCA (5-hydroxymethyl-furan-2-carboxylic acid), DFF (2,5-diformylfuran), FFCA (5-formyl-2-furancarboxylic acid), FDCA (2,5-furandicarboxylic acid). Red circles indicate reactions possibly catalysed by wild-type *Saccharomyces cerevisiae* strains. Green squares indicate the reactions catalysed by HMF/Furfural oxidoreductase from *Cupriavidus basilensis* HMF14 (CbHMFH). Orange triangles indicate the reaction catalysed by HMF oxidase from *Methylovorus* sp. strain MP688 (MsHMFO) (Cunha, 2021)

Given the environmental crescent necessity of implementing biorefineries for the production of high-value chemicals derived from industrial or agricultural waste, it is necessary to explore the yet unexplored potential of *S. cerevisiae* to heterologous express enzymes responsible for HMF oxidation. The proper selection of a whole-cell for the optimal production of FDCA from apple pomace hydrolysate should be approached from a global perspective, considering the limitations of the process associated with utilising a lignocellulosic hydrolysate.

2. Objectives

The establishment of biorefineries for the sustainable production of value-added chemicals from renewable substrates, such as lignocellulosic biomass, plays a central role in the bioeconomy field. Despite the widely use of yeast *S. cerevisiae* in industrial processes the data on its potential as a whole-cell biocatalyst for generating HMF derivatives is scarce. Hence, genetically modified strains of *S. cerevisiae* that express heterologous enzymes responsible for transforming HMF into its derivatives will be utilised to investigate the bioconversion of HMF into high-value derivatives.

The primary objective of this thesis is to assess the capability of a laboratory strain and an industrial strain of *S. cerevisiae*, both genetically modified to express the enzymes HMF/furfural oxidoreductase (HMFH) and HMF oxidase (HMFO) to convert HMF into its derivatives, specially FDCA, primarily applied in the production of bioplastics. Additionally, to exploit industrial waste, and hence economic feasibility and sustainability, an apple pomace hydrolysate was utilised as a HMF source for bioconversion. More specifically, the aims of this work are:

1) Characterise the wild-type and modified *S. cerevisiae* strains in relation to their physiological state, oxygenation requirements and tolerance to HMF and furfural inhibitors.
2) Study the potential of both, modified and wild type *S. cerevisiae* strains to convert synthetic HMF into its derivatives, namely FDCA.
3) Optimise the bioconversion parameters, such as conversion time and oxygenation.
4) Testing different bioconversion optimisation strategies for increase the yields and decrease the time of bioconversion through co-culture with both modified strains and continuous addition of hydrolysate pulses, respectively.
5) Investigate the capacity of the *S. cerevisiae* strain that demonstrates a higher HMF-oxidizing potential to convert HMF from an apple pomace hydrolysate into its derivatives.

3. Materials and methods

3.1. Sterilization of material, solutions and culture media

Glass material, pipette tips and culture media for yeast growth were sterilized in autoclave at 121 °C for 20 minutes. Thermolabile solutions (Glucose, Fructose and phosphate buffer) were sterilized by filtration through 0.22 μm filters.

After pretreatment and neutralization, the hydrolysate underwent pasteurization in a 60 °C bath for 1 hour to achieve sterilization.

3.2. Media

S. cerevisiae strains were grown in liquid YPD medium (2% (w/v) Dextrose, 2% (w/v) Peptone, 1% (w/v) Yeast Extract). Solid medium for agar plates was made by adding 2% (w/v) agar to the YPD media.

3.3. Strains

S. cerevisiae strains used in this work are listed in Table 2.

Table 2. *Saccharomyces cerevisiae* strains used during this work and their respective relevant features and source

Strain	Relevant features	Source
S. cerevisiae industrial strain (I)	Yeast for ethanol production	(Cunha, 2021)
S. cerevisiae laboratory strain (L)	Laboratory haploid strain	(Pereira et al., 2010)
S. cerevisiae I-cbHMFH-I	I, pHMFH_cb_I	(Cunha, 2021)
S. cerevisiae I-msHMFO-I	I, pHMFO_ms_I	(Cunha, 2021)
S. cerevisiae L-cbHMFH-I	L, pHMFH_cb_I	(Cunha, 2021)
S. cerevisiae L- msHMFO-I	L, pHMFO_ms_I	(Cunha, 2021)

3.4. Cell storage

Yeast cultures were maintained for up to 4 weeks at 4 °C in the appropriate selective medium, on inverted agar plates sealed with parafilm. Long-term storage was ensured by the preparation of permanent stocks. A culture grown overnight in proper selective liquid medium was diluted 10-fold in fresh medium and grown for a further 5 to 6 hours. 0.3 mL of sterile glycerol were then added to 1 mL of

culture, mixed by vortexing and incubated on ice for 10 minutes. Finally, the tubes were stored at -80 °C. For culture recovery, the frozen cells were scraped and plated on an appropriate agar medium plate. The permanent stock was stored and reused.

3.5. Whole-cell bioconversion of HMF with phosphate buffer

S. cerevisiae strains were pre-inoculated in either 100 mL or 1 L Erlenmeyer flasks, capped with a cotton plug, and containing 30 or 300 mL of growth medium YPD, respectively. An isolated colony was transferred into each flask using aa loop and were incubated at 30 °C and with orbital agitation at 200 rpm during 24 or 72 h. The cells were then collected by centrifugation at 4000 rpm for 5 minutes, washed with ultra-pure sterilized water and resuspended in 50 mM phosphate buffer at a pH of 7.

The assay media was comprised of 50 mM of HMF in 50 mM of phosphate buffer at a pH of 7 inoculated with 100 g of wet yeast cell/L resuspended in phosphate buffer at pH 7. The bioconversions were performed in a 6-well microplate or in a 100 mL Erlenmeyer flask with cotton plugs with a working volume of 4 mL and 30 mL respectively. The incubation temperature was 30 °C with orbital agitation between 120 and 300 rpm, depending on the experiment. Samples of 0.5 mL were collected through the experiment for HPLC analysis.

3.6. Whole-cell bioconversion of HMF with synthetic hydrolysate without furfural

To investigate the potential of the hydrolysate for HMF whole-cell bioconversion, a synthetic medium was formulated. This medium contained 24.9 g/L of glucose, 8.9 g/L of fructose and 11.5 g/L of HMF, concentrations similar to apple pomace hydrolysate after the microwave pretreatment. Initially furfural was not added to be able to test the HMF conversion without its presence. The synthetic hydrolysate was used as culture medium for the bioconversion process in the conditions outlined in section 3.5.

3.7. Effects of oxygenation on bioconversion

Experiments for evaluating the effect of the oxygenation levels on bioconversion were carried out in different types of flasks. The highest oxygenation tests were carried out in 250 mL baffled Erlenmeyer flask and a working volume of 30 mL, while the remaining tests, which comprise orbital shaking at 120, 150 and 200 rpm, took place in a 100 mL Erlenmeyer flask with a 30 mL of working volume.

The bioconversion experiments were conducted according to the procedures outlined in section 3.5, utilizing both synthetic hydrolysate and phosphate buffer supplemented with 50 mM HMF as culture media.

3.8. Whole-cell bioconversion od HMF with synthetic hydrolysate with furfural

Experiments to evaluate the effect of furfural on bioconversion were carried out in 100 mL Erlenmeyer flasks and 6-well microplate in synthetic medium replicating the apple pomace pretreated hydrolysate (as described in section 3.11) containing 24 mM of furfural, which is the actual concentration on the hydrolysate and conducted identically to those described in section 3.5.

3.9. Optimisation strategies

3.9.1. Co-culture with modified strains

S. cerevisiae I-msHMFO cells were inoculated in diluted synthetic apple pomace hydrolysate and the bioconversion was executed as stated in section 3.5. After 120 hours, the bioconversion medium was centrifuged at 4000 rpm for 5 minutes to remove the cells and the inoculated with 100 g wet yeast cell/L of *S. cerevisiae* I-cbHMFH. The bioconversion process was then conducted under the same conditions as before for an additional 48 hours.

3.9.2. Pulse addition of synthetic apple pomace hydrolysate with furfural

The experiment to evaluate the effect of pulse addition of synthetic apple pomace hydrolysate consisted of an initial bioconversion media of 10 mL of 50 mM phosphate buffer (pH 7) with 2 mL of synthetic apple pomace hydrolysate with furfural. Additional aliquots of 2 mL of synthetic apple pomace

hydrolysate to the medium every 12 h interval until a total volume of 10 mL of synthetic apple pomace hydrolysate was added to the bioconversion medium. In parallel, an experiment with a medium consisting of 10 mL of 50 mM phosphate buffer (pH 7) and 10 mL of synthetic apple pomace hydrolysate was conducted. These assays were conducted at 30 °C, with orbital agitation of 200 rpm, and the inoculum was prepared as described in section 3.5.

3.10. Preparation of Apple pomace hydrolysate

Enzymatic hydrolysis was conducted using commercial enzymes Celluclast, β-glucosidase and Viscozyme in an Erlenmeyer flask at 50 °C and orbital shake at 150 rpm for 24 to 24 hours. Before adding the enzymes, the apple pomace samples were suspended in citrate buffer to keep a constant pH and were sterilised for 20 minutes at 121 °C. Hydrolysis were carried out with a liquid-solid ratio of 4 (Gullón et al., 2007). One batch of 100 mL of hydrolysate was prepared and employed for the microwave-base HMF synthesis.

3.11. Preparation of Deep Eutectic Solvent (DES)

To prepare a DES ([CH]Cl:LA) with a molar ratio of 1:9, the quantities of [Ch]Cl (choline chloride) and LA (lactic acid) were accurately weighed into sealed glass flasks and heated with constant stirring until a clear liquid was obtained. After, reaching the liquid phase, the mixture was kept at the heating temperature for 1 hour before being cooled to room temperature.

3.12. Microwave treatment of apple pomace hydrolysate

A pre-prepared apple pomace hydrolysate was mixed with a DES ([Ch]Cl:LA) (1:9) to a total of 16 g in a standard 30 mL Pyrex vessel (G30) suitable for microwaves. The experiments were performed in a Monowave 450 single-mode microwave reactor (Anton Paar GmbH, Austria) combined with an air compressed as a cooling system. The selected heating time was 5 minutes, while the cooling time depended on the air compressor and was generally 5 to 6 minutes. The temperature was maintained at 160 °C and controlled with an infrared detector and stirring was maintained at 700 rpm using a magnet (del Río et al., 2021). Several experiments were carried out to obtain a total of approximately 250 mL of pretreated hydrolysate.

3.13. Hydrolysate neutralisation

Two aliquots of acid hydrolysate were neutralised by two different neutralising agents, sodium hydroxide (NaOH) and potassium hydroxide (KOH). These chemicals were directly added in pellets to the hydrolysate until a pH of approximately 6.5 – 7 was reached. The final pH was further fine-tuned to 7 using concentrated solutions (10 mM) of both chemicals.

3.14. Total phenolic content (TPC)

The determination of the total phenolic content (TPC) was determined by the Folin-Ciocalteau method as outlined by Teixeira-Guedes et al. (2019). In a 96-well microplate, 20 μL of samples or standard of gallic acid were initially added followed by 100 μL of Folin-Ciocalteau:H20 (1:20) and 80 μL of sodium carbonate (7.5%). The reaction was incubated for 30 minutes at 42 °C in the absence of light and the absorbance was measured at a wavelength of 750 nm using a microplate reader. Results were determined using a standard curve and expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAEq/g DW).

3.15. Whole-cell bioconversion of HMF with apple pomace hydrolysate

S. cerevisiae I-msHMFO strain was pre-inoculated in 100 mL Erlenmeyer flasks, capped with a cotton plug containing 30 mL of growth medium YPD and incubated at 30 °C and with orbital agitation at 200 rpm for 24 hours. The cells were then collected by centrifugation at 4000 rpm for 5 minutes, washed with ultra-pure sterilized water and resuspended in 50 mM phosphate buffer at a pH of 7.

The assay media was consisted of neutralised apple pomace hydrolysate diluted to 50% and 25% of the total volume inoculated with 100 g of wet yeast cell/L. The bioconversions were performed in a 6-well microplate with a working volume of 4 mL, at a temperature of 30 °C in an orbital shaker at 200 rpm. Samples of 0.5 mL were collected through the experiment for HPLC analysis.

3.16. Analytical methods

Samples from bioconversion assays were diluted appropriately, filtered sterilised (0.22 μm pore) and analysed for quantification of sugars, lactic acid, HMF, HMFCA, DFF, FFCA and FDCA by HPLC (High performance liquid chromatography) using a Bio-Rad Aminex HPX-87H column, operating at 60 °C with 5 mM H_2SO_4 as mobile phase and at a flow rate of 0.6 mL/min. For sugars and lactic acid peak detention was performed using a Knauer-IR intelligent refractive index detector, and the retention times were 8.8 min for Glucose, 9.5 min for Fructose and 12.4 min for Lactic acid. For the quantification of FDCA, HMFCA, HMF and DFF, the UV detector was set at 260 nm and the retention times were 13.9 min, 18.9 min, 27.7 min and 33.8 min respectively. For the quantification of FFCA, the UV detector was set at 300 nm and the retention time was 18.8 min.

For BHMF separation and quantification, the samples were analysed by a reverse phase UHPLC (Ultra-high performance liquid chromatography) using a Zorbax Eclipse XDB-C18 column (4.6 mm x 250 mm, 5 μm), operating at 25 °C, with a mixture of acetonitrile/ 0.4% $(NH_4)_2SO_4$ (10:90, v/v) at pH 3.5 and with a flow rate of 0.6 mL/min. The UV detector was set at 223 nm (maximum absorption wavelength for BHMF) and the retention time for BHMF was 6.8 min.

Quantification was achieved by comparing the peak area with the calibration curves of pure compounds constructed by injecting standards with known concentration for each compound in the study.

Yield (%) was determined as a percentage of the amount obtained of a specific HMF-derivative in relation to the maximum theorical amount that could be produced from the initial quantity of HMF. HMF conversion (%) was defined as the percentage of the converted HMF in relation to the initial amount of HMF.

4. Results and discussion

4.1. Screening of different wild-type and genetically modified *Saccharomyces cerevisiae* strains for FDCA production

Firstly, the ability of different wild-type and genetically modified *S. cerevisiae* strains to convert HMF into FDCA was tested. For this purpose, the laboratory and industrial strain, previously engineered to express the enzymes HMF/Furfural oxidoreductase from *C. basilensis* HMF14 and HMF oxidase from *Methylovorus* sp. were screened (Cunha, 2021). The assays were performed in a 6-well microplate to enhance oxygen transfer and HMF oxidation, while the bioconversion medium consists in 50 mM phosphate buffer pH 7 supplemented with 50 mM of HMF.

In a previous study using the wild-type strains, a 24 hour pre-inoculation period was utilised, whereas a 72 hour period was used for the modified strains (Cunha, 2021). This stage is key for the propagation and multiplication of yeast cells and, therefore, to produce the enzymes responsible for the bioconversion of HMF into its derivatives. Here both scenarios were evaluated for all the strains tested. Table 3 shows the HMF conversion and FDCA yield after 120 hours of bioconversion for both pre-inoculation periods. The results showed no differences regarding HMF conversion and FDCA yield when comparing the 24-hour pre-inoculation period with the 72-hour pre-inoculation period. All strains were able to completely convert HMF up to 120 hours, with both wild-type and modified strains behaving similarly. Furthermore, as anticipated, the modified strains yielded higher FDCA values compared to the wild-type strains. Moreover, the strain that underwent modification with the HMFO enzyme demonstrated greater yields of FDCA, reaching between 8.9% (L-msHMFO) and 11.5% (I-msHMFO) within 24 hours of pre-inoculation, in comparison with the yields obtained with strains modified with the HMFH enzyme (6.4 - 6.6%). This outcome is expected primarily due to the fact that the HMFO enzyme is capable of oxidising HMF via DFF, which could potentially enhance the oxidation process, particularly since there are two oxidation pathways active in yeast (Figure 3) and indicates that these strains are highly effective for the bioconversion of HMF to FDCA.

Table 3. Conversion of HMF and FDCA yield with *S. cerevisiae* industrial and laboratory wild-type and modified strains, with phosphate buffer supplemented with 50 mM of HMF as bioconversion medium, at 120 h with 24- and 72-hours period of pre-inoculation. Data represent mean ± standard deviation of two biological replicates.

***S. cerevisiae* strain**	**HFM conversion (%)**	**Yield FDCA (%)**
24 h pre-inoculation		
Industrial (I)	99.8 ± 0.1	0.6 ± 0.0
I-msHMFO	99.8 ± 0.1	11.5 ± 2.7
I-cbHMFH	99.8 ± 0.1	6.6 ± 4.2
Laboratory (L)	99.9 ± 0.0	0.2 ± 0.0
L- msHMFO	99.9 ± 0.1	8.9 ± 1.0
L- cbHMFH	99.8 ± 0.0	6.4 ± 1.3
72 h pre-inoculation		
Industrial (I)	99.8 ± 0.1	0.5 ± 0.3
I-msHMFO	100 ± 0.0	12.0 ± 0.6
I-cbHMFH	99.9 ± 0.0	5.4 ± 1.1
Laboratory (L)	99.9 ± 0.1	0.6 ± 0.4
L- msHMFO	99.9 ± 0.0	10.3 ± 0.7
L- cbHMFH	99.9 ± 0.0	5.6 ± 0.8

Given the fact that no noticeable discrepancies were observed regarding HMF consumption and FDCA production between both pre-inoculation periods evaluated, all subsequent tests in this study were carried out with a pre-inoculation of 24 hours, reducing this stage by 2 days. Then, a bioconversion profile of HMF conversion into FDCA was assessed (Figure 4). It is observed that the strains under investigation exhibit a very similar bioconversion profile of HMF, and this compound was completely converted after 10 hours of bioconversion (Figure 4A). It should also be noted that from 48 to 120 hours, there is no HMF present in the medium (Figure 4A), indicating that the yeast is no longer converting. Therefore, in this case, it will not be necessary 120 hours to complete the bioconversion process, and the time can be reduced to 48 hours. However, it is also possible to observe that the accumulation of FDCA primarily begins at the 48-hour mark (Figure 4B).

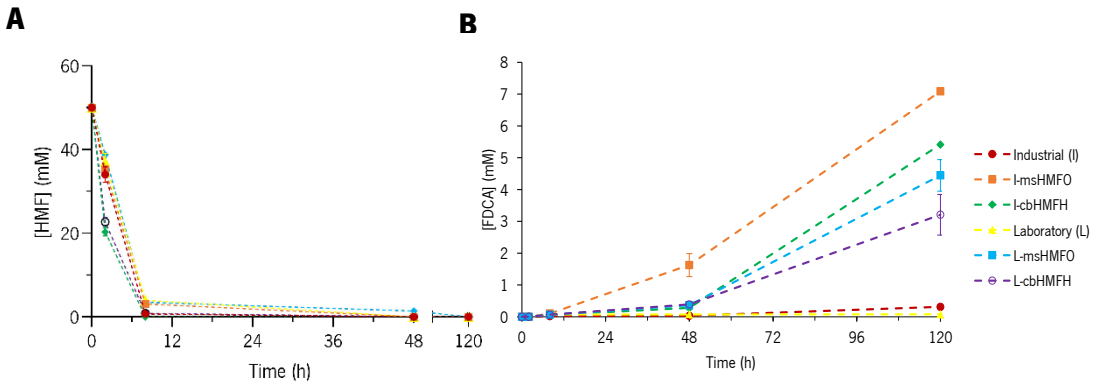
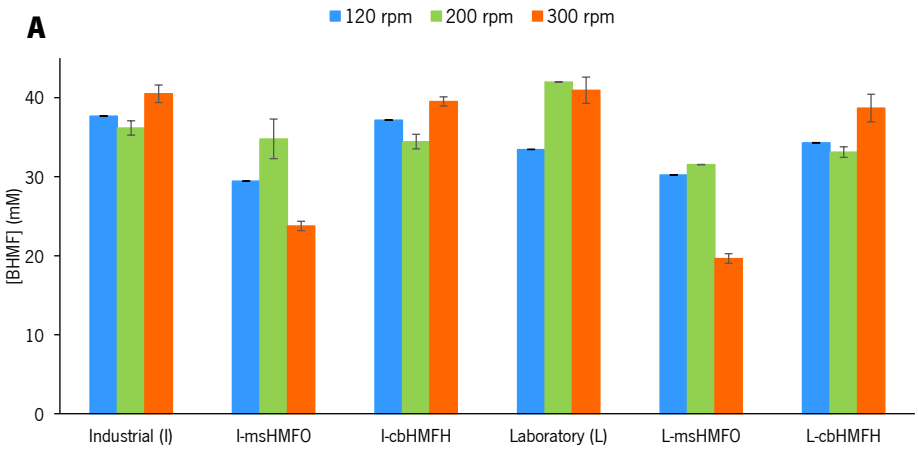


Figure 4. Bioconversion profile of HMF in FDCA with *S. cerevisiae* wild-type and modified strains in phosphate buffer supplemented with 50 mM HMF. The error bars represent the standard deviation of two biological independent replicates.

4.1.1. Effects of oxygenation on bioconversion

Studying the effects of oxygenation on bioconversion, particularly in modified strains, is crucial since the enzymes use oxygen as a co-substrate. Therefore, a range of tests were conducted with various orbital agitations (120, 200, 300 rpm) to evaluate the optimal levels of oxygenation.

Figure 5 shows the final concentration of derivatives and Table 4 presents the HMF conversion data and derivatives yields after 48 hours of bioconversion for varying levels of oxygenation examined in this study. Notably, DFF values were absent from the medium, a deviation from expectation, especially for the strains modified with the HMFO enzyme.



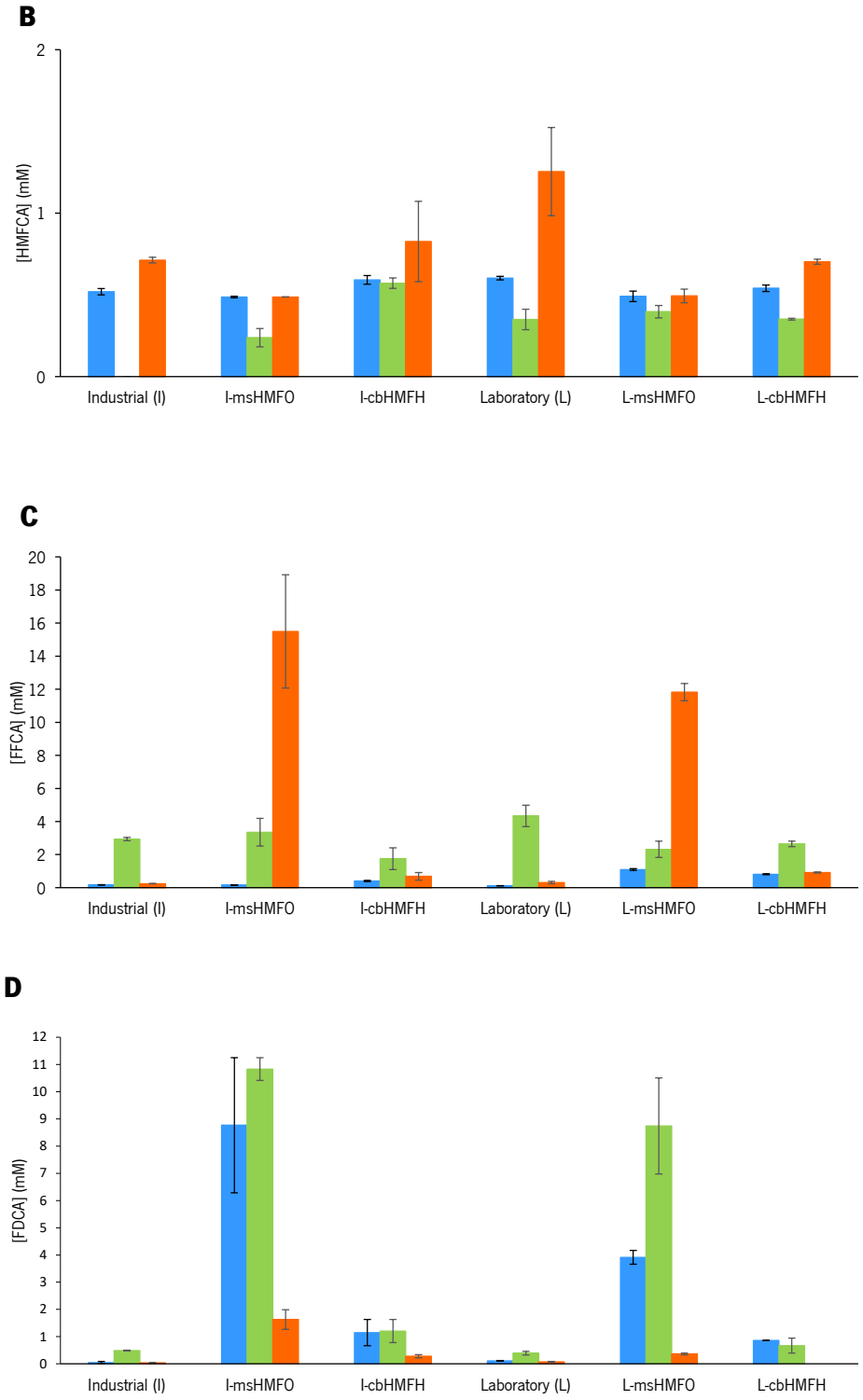


Figure 5. Concentration of derivatives with *S. cerevisiae* wild-type and modified (with HMFO and HMFH enzymes) strains in phosphate buffer supplemented with 50 mM of HMF at 48 hours with different orbital agitations in Erlenmeyer flasks. The blue bars represent bioconversion carried out at 120 rpm, while the green and orange bars reflect the use of 200 rpm and 300 rpm, respectively. The error bars represent the standard deviation of two biological independent replicates.

Upon analysis of the data, it is evident that all the strains demonstrated capability in detoxifying HMF up to 48 hours in each of the tested conditions (Table 4). In theory the condition that had the highest oxygenation (300 rpm orbital agitation) should result in greater amounts of FDCA since the conversion enzyme utilise molecular oxygen as a co-substrate. However, this conjecture was not supported, and a final concentration of 1.6 mM FDCA (Figure 5D) was achieved, with an agitation of 300 rpm, for I-msHMFO strain. Interestingly, high amounts of FFCA were observed, 15.5 mM and 11.8 mM for the strains industrial and laboratorial, respectively, both modified with HMFO enzyme, and for the 300 rpm condition in comparison to the other conditions (120 and 200 rpm orbital agitation) (Figure 5C). This indicates that oxygen plays a critical function in enzyme activation and bioconversion.

Table 4. Bioconversion of HMF yield with *S. cerevisiae* wild-type and modified (with HMFO and HMFH enzymes) strains in phosphate buffer supplemented with 50 mM of HMF at 48 h with orbital agitation of 300, 200 and 120 rpm in Erlenmeyer flasks. Data represent mean ± standard deviation of two biological replicates.

***S. cerevisiae* Strain**	**HFM conversion (%)**	**Yield BHMF (%)**	**Yield HMFCA (%)**	**Yield FFCA (%)**	**Yield FDCA (%)**
Orbital agitation: 300 rpm					
Industrial (I)	100 ± 0.0	81.0 ± 2.2	10.0 ± 0.9	0.5 ± 0.0	0.1 ± 0.0
I- msHMFO	100 ± 0.0	47.5 ± 1.1	6.8 ± 0.0	31.0 ± 6.8	3.3 ± 0.7
I-cbHMFH	100 ± 0.0	79.1 ± 1.1	11.6 ± 0.0	1.4 ± 0.5	0.6 ± 0.1
Laboratory (L)	100 ± 0.0	81.8 ± 3.3	17.6 ± 3.8	0.6 ± 0.1	0.2 ± 0.0
L- msHMFO	100 ± 0.0	39.3 ± 1.1	6.9 ± 0.6	23.6 ± 1.0	0.7 ± 0.1
L- cbHMFH	100 ± 0.0	77.4 ± 3.5	9.9 ± 0.2	1.8 ± 0.1	0.8 ± 0.0
Orbital agitation: 200 rpm					
Industrial (I)	100 ± 0.0	72.3 ± 1.8	0.0 ± 0.0	5.9 ± 0.2	1.0 ± 0.0
I- msHMFO	100 ± 0.0	69.0 ± 5.0	0.5 ± 0.1	5.0 ± 1.7	21.7 ± 0.8
I-cbHMFH	100 ± 0.0	84.0 ± 1.9	1.1 ± 0.1	3.5 ± 1.3	2.4 ± 0.8
Laboratory (L)	100 ± 0.0	69.6 ± 0.0	0.7 ± 0.1	8.7 ± 1.3	0.8 ± 0.1
L- msHMFO	100 ± 0.0	63.1 ± 0.0	0.8 ± 0.1	4.6 ± 1.0	17.5 ± 3.5
L- cbHMFH	100 ± 0.0	66.2 ± 1.3	0.7 ± 0.0	5.3 ± 0.3	1.3 ± 0.5
Orbital agitation: 120 rpm					
Industrial (I)	98.9 ± 0.5	75.3 ± 1.5	7.3 ± 0.3	0.4 ± 0.0	0.1 ± 0.0
I- msHMFO	100 ± 0.0	58.9 ± 0.0	6.8 ± 0.1	0.3 ± 0.0	17.5 ± 5.0
I-cbHMFH	100 ± 0.0	74.3 ± 1.1	8.3 ± 0.4	0.8 ± 0.1	2.3 ± 1.0
Laboratory (L)	100 ± 0.0	66.9 ± 0.0	8.5 ±0.1	0.2 ± 0.0	0.2 ± 0.0
L- msHMFO	100 ± 0.0	60.5 ± 0.0	6.9 ± 0.5	2.2 ± 0.1	7.8 ± 0.5
L- cbHMFH	100 ± 0.0	68.6 ± 2.6	7.6 ± 0.3	1.6 ± 0.1	1.7 ± 0.0

The HMFO enzyme employs molecular oxygen as an electron acceptor and produces hydrogen peroxide (H_2O_2) as a by-product. This co-product could possibly contribute to the inhibition. In fact, a similar inhibition was previously noted by Serrano et al. (2019) for an aryl-alcohol dehydrogenase (AAO). The enzyme has the ability to oxidise HMF into FFCA, although it is also capable of oxidising FFCA into FDCA, this reaction is highly inhibited by the presence of H_2O_2 produced in the first two oxidation steps. Additionally, the toxic effect of H_2O_2 was also confirmed by the successful conversion of HMF to FDCA when the AAO reaction was carried out in the presence of catalase (Serrano et al., 2019). Also, a novel enzymatic cascade similar to this was used by Wu et al. (2020) where, HMFO was used to convert HMF into DFF and was combined with a lipase that can efficiently oxidised DFF in FDCA in the presence of H_2O_2. The use of enzymes that only require O_2 as a co-substrate are undeniable advantageous for the bioconversion process, however, it is crucial to consider the co-products that may be resulting, such as H_2O_2, and their inhibitory effects. Thus, employing *S. cerevisiae* as a whole-cell biocatalyst can provide advantages, as it possesses an inherent catalase that can decompose some of the H_2O_2 (Martins & English, 2014).

Regarding the results obtained from the trial with the least oxygenation level (120 rpm orbital agitation) it can be observed that there was an increase in the production of FDCA, resulting in 8.8 mM FDCA when compared with the results obtained with the higher oxygenation level (Figure 5D). However, the amounts generated by 200 rpm orbital stirring, were greater, reaching 10.8 mM. Therefore, this once again demonstrates the indispensability of oxygen in the bioconversion process. In the condition with an orbital agitation of 200 rpm, the *S. cerevisiae* I-msHMFO strain accumulated the highest amount of FDCA (Figure 5D), this proves that 200 rpm is the optimal oxygenation condition for bioconversion in this scenario.

Furthermore, similar to the results obtained in the previous section, the strains modified with the HMFO enzyme obtained higher FDCA yields than the other strains, reaching 21.7% and 17.5% for I-msHMFO and L-msHMFO (Table 4), respectively, so it is noteworthy that the I-msHMFO strain exhibit higher yield values relative to the L-msHMFO strain. This confirms that the use of industrial strains has been shown to be more advantageous when compared to the use of laboratorial strains, due to their robustness and resistance to inhibitors commonly found in lignocellulosic hydrolysates, such as HMF. Previous research conducted by Pereira et al. (2014) also demonstrates the effectiveness of industrial

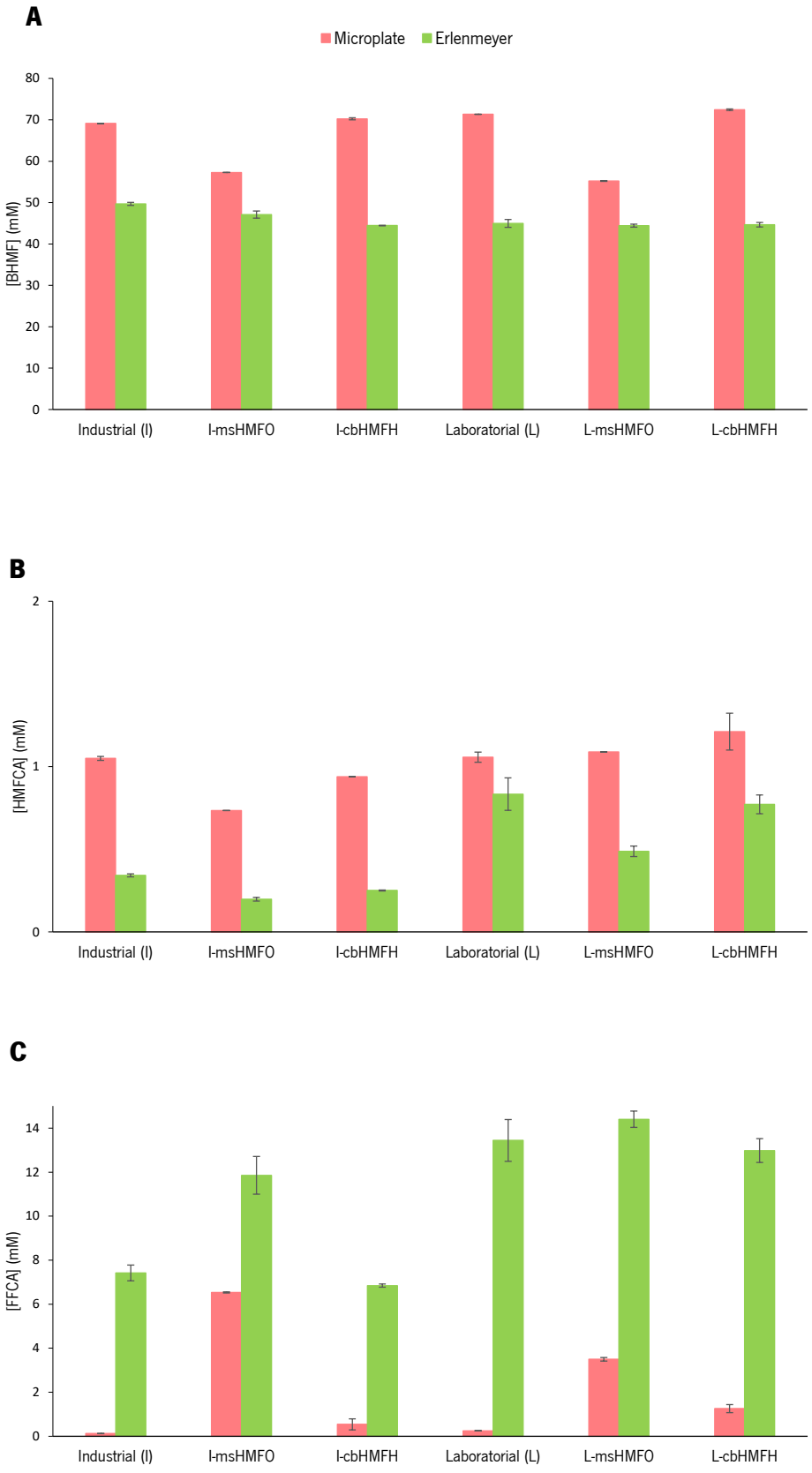
strains in detoxifying HMF present in a eucalyptus hydrolysate, while the laboratorial strains were incapable of achieving.

4.2. Whole-cell bioconversion of HMF with synthetic hydrolysate without furfural

To investigate the potential of the apple pomace hydrolysate for HMF whole-cell bioconversion, a synthetic medium was formulated. This medium contained glucose, fructose and HMF in the same concentrations as the apple pomace hydrolysate after the microwave pretreatment

Firstly, we investigated if glucose and fructose had any impact on bioconversion, despite being present in low concentrations. For this purpose, two assays were conducted in microplate and Erlenmeyer flasks using synthetic hydrolysate as the biotransformation medium.

It is evident that the presence of glucose and fructose in the medium has no effect on the bioconversion and accumulation of FDCA, whereby, these sugars are completely consumed by the yeast during the initial hours of bioconversion. Figure 6 displays the concentrations of derivatives obtained after 48 hours of bioconversion. Consistent with previous tests, it is apparent that strains engineered with HMFO enzyme produce increased quantities of FDCA. Specifically, the I-msHMFO strain and L-msHMFO strains attained 6.7 mM and 7.3 mM of FDCA, respectively in microplate while when Erlenmeyer flasks were used, 4.5 mM and 3.5 mM of FDCA were achieved by both strains, respectively. The strain I-msHMFO continues to excel, producing FDCA yields of 8.4% and 6.1% for the 6-well microplate and Erlenmeyer flasks tests, respectively (Table 5). Remarkably, considerable differences can be observed in the yield values obtained for the microplate tests compared to the Erlenmeyer flask tests, with higher yields being observed when the experiments are carried out in microplate (Table 5). This is also evident in Figure 6D where greater amounts of FDCA are produced during tests conducted on microplate while higher quantities of FFCA were obtained for the tests performed in Erlenmeyer flasks (Figure 6C). This variability may be attributed to the potentially more favourable oxygen levels for biocatalysis in microplates. Additionally, it is notable that wild-type strains tend to detoxify HMF to BHMF, with an accumulation of 69.2 mM and 71.3 mM observed for the industrial and laboratorial strains, respectively, in the microplate assays. Interestingly, lower amounts of BHMF were accumulated in the Erlenmeyer flasks assays, with levels of 49.7 mM and 45.0 mM, respectively (Figure 6A).


A
Microplate
Erlenmeyer
[BHMF] (mM)
0
10
20
30
40
50
60
70
80
Industrial (I)
I-msHMFO
I-cbHMFH
Laboratorial (L)
L-msHMFO
L-cbHMFH
B
[HMFCA] (mM)
0
1
2
Industrial (I)
I-msHMFO
I-cbHMFH
Laboratorial (L)
L-msHMFO
L-cbHMFH
C
[FFCA] (mM)
0
2
4
6
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10
12
14
Industrial (I)
I-msHMFO
I-cbHMFH
Laboratorial (L)
L-msHMFO
L-cbHMFH

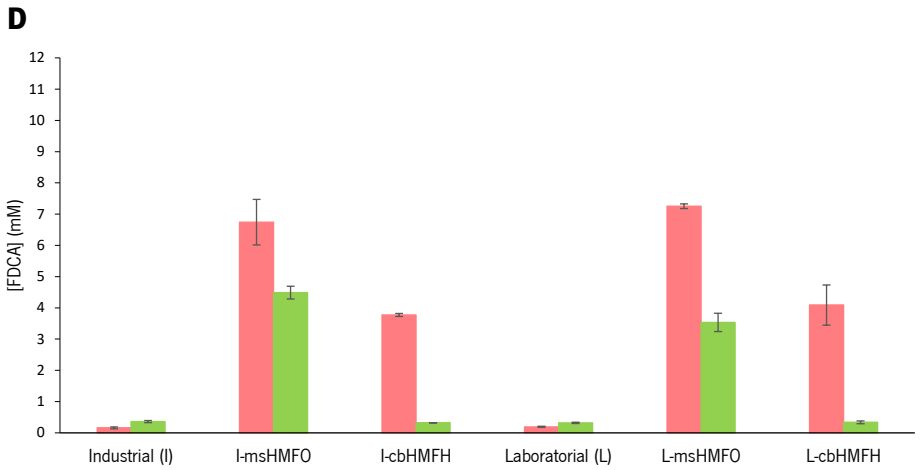

Figure 6. Concentration of derivatives at the end of 48 hours of bioconversion in synthetic hydrolysate without furfural with *S. cerevisiae* wild-type and modified (with HMFO and HMFH enzymes) strains. The pink bars represent bioconversion carried out in microplate, while the green bars represent the use of Erlenmeyer flasks. The error bars represent the standard deviation of two biological independent replicates.

Furthermore, these assays were conducted utilising synthetic hydrolysate medium supplemented with a higher level (80 mM) of HMF compared to earlier trials. This successfully demonstrates that this concentration of HMF is non-inhibitory and non-toxic to the yeast because of its complete detoxification through the bioconversion process which lasted up to 48 hours. However, it was expected that similar or higher yields of FDCA would result when compared with the tests carried out using 50 mM of HMF, yet this is not observed. Since HMF is oxidised to its corresponding acids, a higher concentration of HMF can cause an increased accumulation of acids in the medium, promoting the acidification. As a result, raising HMF levels appears not to be an advantageous solution, since it fails to increase yields, this is not only because of the toxic nature of HMF, but also because the acidification of the culture can inhibit the oxidation (Koopman et al., 2010a). To prevent the adverse impact of medium acidification on bioconversion, pH-regulating agents may be incorporated into the medium. The former research indicates that the addition of 0.5 g/L Na_2CO_3 significantly increase the HMF conversion and increased the concentration of FDCA from 0.23 to 0.31 g/L, whereas the addition of a larger amount of Na_2CO_3 was not beneficial and reduced the HMF conversion and the concentration of FDCA (Sheng et al., 2020).

Due to discernible variations in the yield values obtained from microplate or Erlenmeyer flasks, further testing was conducted to investigate these disparities. Additionally, as stated earlier, it was found

that the I-msHMFO strain has the ability to entirely detoxify HMF and also produced the higher FDCA yields in all the conducted tests. Consequently, for subsequent experiments this strain was selected.

Table 5. Bioconverison of HMF yield with *S. cerevisiae* wild-type and modified (with HMFO and HMFH enzymes) strains at 48 hours in synthetic hydrolysate without furfural as biotransformation medium in 6-weel microplate and Erlenmeyer flasks. Data represent mean ± standard deviation of two biological replicates.

***S. cerevisiae* Strain**	**HFM conversion (%)**	**Yield BHMF (%)**	**Yield HMFCA (%)**	**Yield FFCA (%)**	**Yield FDCA (%)**
Microplate					
Industrial (I)	99.2 ± 0.2	86.4 ± 3.7	9.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
I- msHMFO	99.6 ± 0.0	71.1 ± 2.2	6.5 ± 0.0	8.2 ± 0.0	8.4 ± 0.9
I-cbHMFH	99.6 ± 0.0	87.8 ± 1.6	8.3 ± 0.0	0.7 ± 0.3	4.7 ± 0.1
Laboratory (L)	98.8 ± 0.0	89.2 ± 0.9	9.3 ± 0.3	0.3 ± 0.0	0.2 ± 0.0
L- msHMFO	99.3 ± 0.1	69.0 ± 0.4	9.6 ± 0.0	4.4 ± 0.1	9.1 ± 0.1
L- cbHMFH	98.8 ± 0.2	90.6 ± 2.3	10.7 ± 1.0	1.6 ± 0.2	5.1 ± 0.8
Erlenmeyer flask					
Industrial (I)	100 ± 0.0	68.1 ± 1.4	0.5± 0.0	10.2 ± 0.5	0.5 ± 0.0
I- msHMFO	100 ± 0.0	64.5 ± 0.9	0.3 ± 0.0	16.2 ± 1.2	6.1 ± 0.3
I-cbHMFH	100 ± 0.0	60.9 ± 4.7	0.3 ± 0.0	9.4 ± 0.1	0.4 ± 0.0
Laboratory (L)	100 ± 0.0	61.6 ± 0.5	1.1 ± 0.1	18.4 ± 1.3	0.4 ± 0.0
L- msHMFO	100 ± 0.0	60.9 ± 0.0	0.7 ± 0.0	19.7 ± 0.5	4.8 ± 0.4
L- cbHMFH	100 ± 0.0	61.2 ± 0.2	1.1 ± 0.1	17.8 ± 0.7	0.4 ± 0.1

Figure 7 presents the final titres of HMF, and its derivatives obtained from the tests performed with a 50% diluted synthetic hydrolysate in both, microplate and Erlenmeyer flasks. No concentrations of DFF were detected in the medium, indicating probable production of this compound during the initial stages of bioconversion and at 72 hours it was fully oxidised to FFCA. Also, in a previous study, DFF was detected during the early stages of bioconversion (Cunha, 2021). Unexpectedly, the yeast accumulated high amounts of HMFCA, particularly in the Erlenmeyer experiments, where 8.7 mM of this compound was obtained, whereas 4.6 mM was achieved when the assay was conducted in microplate (Figure 7), suggesting activity in the HMFCA pathway despite HMFO enzyme modification (Figure 3).

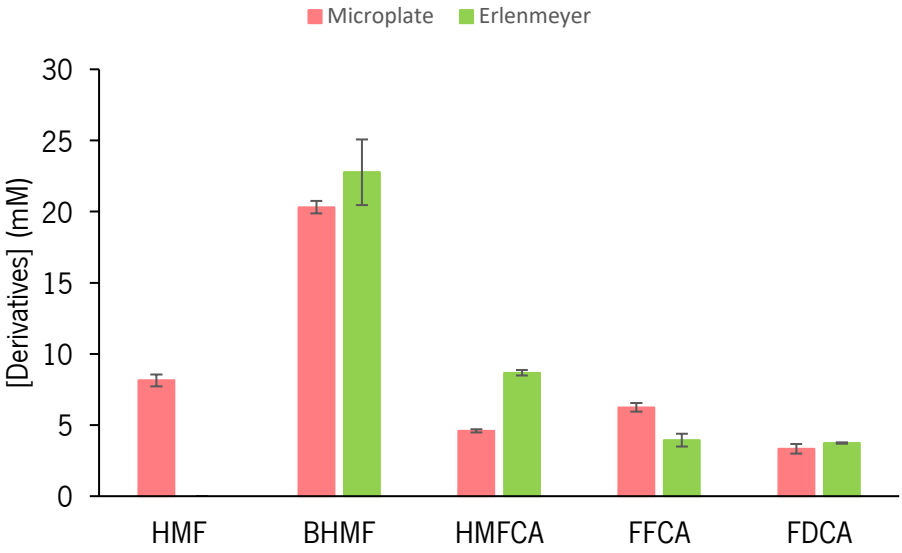

Figure 7. Concentration of HMF and derivatives at 72 hours with I-msHMFO strain using as substrate in synthetic hydrolysate without furfural with initial HMF concentration of 40 mM in 6-well microplate and Erlenmeyer flasks. The pink bars represent bioconversion carried out in microplate, while the green bars represent the use of Erlenmeyer flasks. The error bars represent the standard deviation of two biological independent replicates.

Notably, discrepancies were observed between this and the previous test, where the microplate outperformed. In the microplate tests, HMF was not fully detoxified, leaving 8.1 mM still to be detoxified, however, an amount of 3.3 mM FDCA was attained (Figure 7) resulting in a yield of 8.3%. In the Erlenmeyer flasks test, the yeast successfully detoxified all HMF, resulting in an FDCA yield of 9.3%.

4.2.1. Effects of oxygenation in whole-cell bioconversion with synthetic hydrolysate

In section 4.1.1 it was suggested that high oxygenation levels are not ideal for complete HMF bioconversion, with tests using orbital agitation of 120 and 200 rpm being more favourable. To explore the ideal agitation for oxygenation levels during bioconversion, further investigation was necessary. The aim of this experiment was to determine if intermediate oxygenation, between 120 and 200 rpm would be even more effective. As a result, agitation of 150 rpm was also tested alongside the retesting of 120 and 200 rpm.

Figure 8 displays the values for the amount of derivative present in the media after 120 hours of bioconversion tests carried out utilising the three orbital agitations under examination.

The optimal condition for achieving the highest titter of FDCA, 1.3 mM can be observed for orbital agitation at 200 rpm, producing the highest yield of FDCA at 3.4%, which is considerably lower when compared to previous experiments. Nevertheless, these conditions exhibited the best outcomes and henceforth, all the ensuing tests will employ 200 rpm orbital agitation, proving, once again, the significance of oxygen in bioconversion, furthermore, the study also found that orbital agitations of 120 and 150 rpm are inadequate for optimal enzyme functioning due to insufficient oxygen levels.

Additionally, there is a greater accumulation of BHMF observed in orbital agitations of 120 and 150 rpm (Figure 8), indicating that these conditions do not provide sufficient levels of oxygenation for enzyme activation. As a result, the yeast detoxifies HMF to BHMF through its intrinsic mechanism.

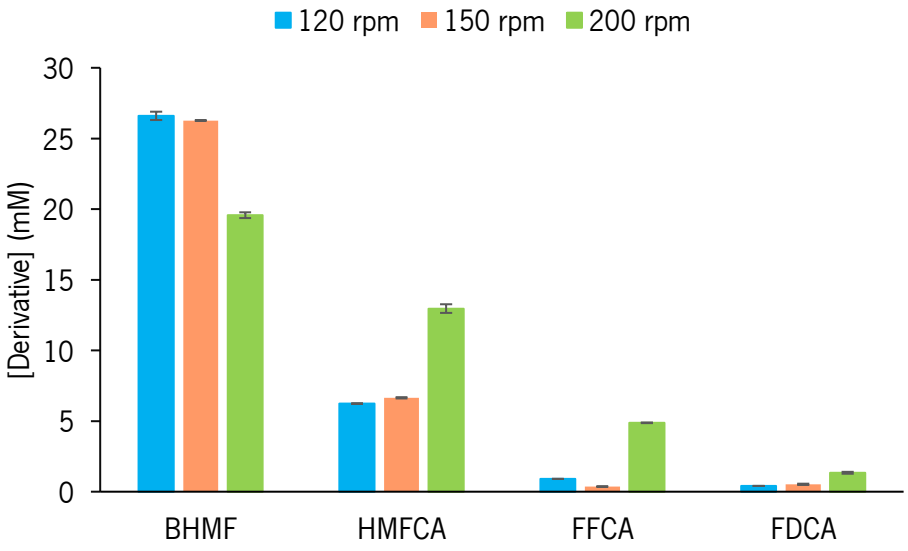

Figure 8. Concentration of derivatives at 120 hours of bioconversion with I-msHMFO in synthetic hydrolysate without furfural (50% diluted) for different orbital agitations. The blue bars represent bioconversion carried out at 120 rpm, while the orange and green bars reflect the use of 150 rpm and 200 rpm, respectively. The error bars represent the standard deviation of two biological independent replicates.

4.3. Whole-cell bioconversion with synthetic hydrolysate with furfural

Since apple pomace hydrolysate contains furfural in its composition after pretreatment and this compound has shown to be an effective microbial activity inhibitor, it is necessary to investigate the tolerance of wilt type and modified strains to furfural. To that end, the synthetic medium replicating the apple pomace pretreated hydrolysate was supplemented with 24 mM of furfural.

Both HMF and furfural belong to the furan class and inhibit microbial activity. In a prior study conducted by Liu & Ma (2020) was shown that a mixture of 1.9 g/L of furfural and 2.5 g/L HMF can inhibit an industrial *S. cerevisiae* strain for up to 76 hours of culture. As a result, the bioconversion time was extended from 72 hours to 120 hours.

Table 6 presents the influence of furfural on the bioconversion of HMF. It is worth highlighting that after 72 hours of culture, the yeast had already completely detoxified the furfural initially present in the medium (1.2 g/L), but this concentration inhibits the detoxification of HMF, so that after 120 hours of conversion only 60.9% of the total HMF had been detoxified, resulting in a yield of 1.9% of FDCA. In addition, there was a considerable accumulation of HMFCA and FFCA, which reached yields of 26.1% and 6.2% respectively (Table 6).

Table 6. Bioconversion of HMF with I-msHMFO strain at 72 hours and 120 hours in synthetic hydrolysate with furfural (50% diluted). Data represent mean ± standard deviation of two biological replicates.

S. cerevisiae **strain**	**HFM conversion (%)**	**Yield BHMF (%)**	**Yield HMFCA (%)**	**Yield FFCA (%)**	**Yield FDCA (%)**
At 72 hours					
I- msHMFO	50.0 ± 0.3	23.3 ± 0.0	22.7 ± 0.5	4.8 ± 0.3	1.2 ± 0.1
At 120 hours					
I- msHMFO	60.9 ± 0.7	29.0 ± 0.0	26.1 ± 0.2	6.2 ± 0.3	1.9 ± 0.0

HMF and furfural share similar characteristics, and, as such can be classified as furans and microbial inhibitors, however, their individual impacts and effects on microorganisms differ, with furfural having a more instant impact on yeast metabolism than HMF. This may explain why, in this scenario, furfural was entirely detoxified after 120 hours of bioconversion while HMF was not, suggesting that furfural's more instant impact causes the yeast to metabolise it more quickly than HMF. The impact of HMF and furfural on *S. cerevisiae* was studied previously by Taherzadeh et al. (2000), this study employed

a combination of 2 g/L of HMF and 2 g/L of furfural. The findings indicated that furfural was mainly converted before HMF, furthermore, the conversion rates of both compounds were considerably lower than those obtained when the compounds were used independently, thus demonstrating the inhibitory effect of furfural on HMF bioconversion.

4.3.1. Pulse addition of synthetic apple pomace hydrolysate

To optimize the bioconversion conditions, focusing on the necessary culture time required for the yeasts to detoxify furfural and HMF, a feeding strategy was considered to enhance the FDCA yields. Here, we continuously fed the culture with aliquots of 10% concentrated synthetic apple pomace hydrolysate containing with furfural at 12-hour intervals until a 50% dilution was achieved.

The addition of aliquots containing 10% diluted synthetic hydrolysate may provide a means to circumvent the high inhibition caused by the presence of high concentrations of HMF and furfural in the synthetic medium, thus optimising the time factor.

In fact, the results presented in Figure 9B demonstrate furfural complete detoxification after 10 hours of conversion, before the second pulse was added. Additionally, Figure 9A indicates partial detoxification of HMF and accumulation of derivatives, particularly BHMF at 10 hours of bioconversion (Figure 9C). Significantly, when further aliquots are added, the yeast's ability to detoxify both furfural and HMF is impaired, leading to an accumulation of these compounds. Consequently, the yeast's activity is inhibited (Figure 9A and 9B).

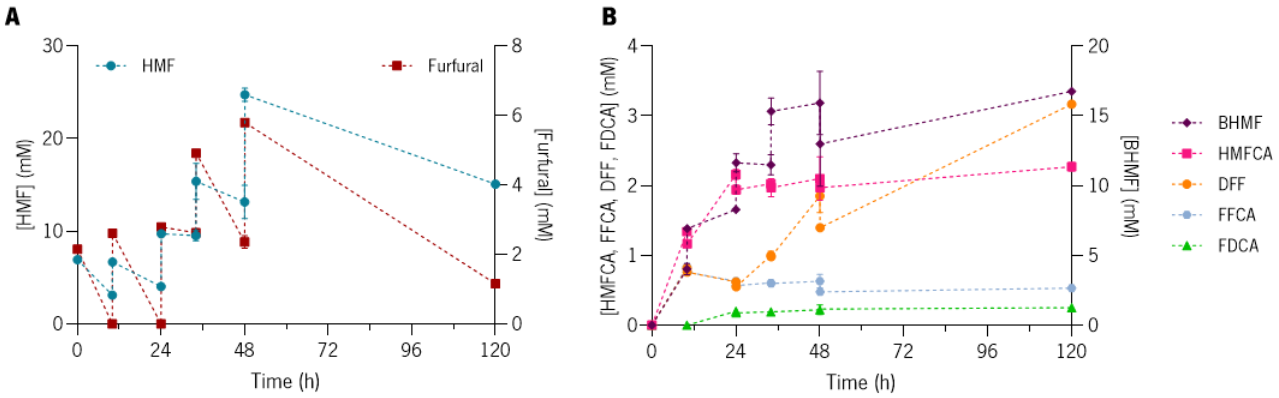

Figure 9. Bioconversion profile of HMF and furfural detoxification with I-msHMFO strain at 120 hours with continuous pulses of synthetic hydrolysate containing furfural. The error bars represent the standard deviation of two biological independent replicates.

Furthermore, it is evident from Figure 9A that significant levels of HMF remain in the detoxification medium even after 120 hours of conversion, this indicates that a longer timeframe is necessary to accomplish the complete bioconversion of HMF into its derivatives.

Table 7 shows the HMF conversion, and its derivatives yields at 120 hours of bioconversion.

Table 7. Bioconversion of HM with I-msHMFO strain at 120 hours with pulses of 10% diluted synthetic hydrolysate containing furfural. Data represent mean ± standard deviation of two biological replicates.

***S. cerevisiae* strain**	**HFM conversion (%)**	**Yield BHMF (%)**	**Yield HMFCA (%)**	**Yield DFF (%)**	**Yield FFCA (%)**	**Yield FDCA (%)**
At 120 hours						
I- msHMFO	62.3 ± 0.0	41.9 ± 0.4	6.5 ± 0.2	7.9 ± 0.0	1.3 ± 0.1	0.6 ± 0.0

When comparing this data to the results obtained in the previous section, where a test was conducted using 50% diluted synthetic hydrolysate as the initial concentration (Table 6), it is evident that the conversion of HMF is similar, with only 60 to 62% of the total HMF being detoxified in both cases (Table 6 and Table 7). It is noteworthy that despite the comparable HMF conversion values, there was a reduced accumulation of derivatives in this case, resulting in a yield of 0.6% FDCA. Furthermore, it was observed that DFF remained in the medium at a yield of 7.9% after 120 hours of fermentation. The presence of this compound suggests that the process is not fully complete, as it is generally detected in early stages of bioconversion.

Given the low FDCA yields and incomplete HMF detoxification, the addition of less concentrated synthetic hydrolysate aliquots did not prove to be an efficient way to optimise the time required for bioconversion.

4.3.2. Co-culture with modified strains

Since the synthesis of FDCA from HMF is a process comprising a multi-step reaction, the use of a combination of oxidative enzymes may prove to be advantageous for bioconversion. For instance, Wang et al., (2020) conducted a study in which a novel whole-cell biocatalyst was created through the co-expression of vanillin dehydrogenase (VDH1) and HMF/furfural oxidoreductase (HMFH) in *E. coli*. HMF was converted into HMFCA by VDH1, which was then oxidised to FFCA by HMFH and finally, VDH1 and/or HMFH transformed FFCA into FDCA, with a resulting FDCA yield of 96% (Wang et al., 2020).

Despite the use of enzyme combination approaches the HMFH enzyme and its homologue HMFO enzyme have been shown to be capable of catalysing the 3 consecutive reactions of the HMF oxidation pathway (Hsu et al., 2020; Koopman et al., 2010a). However, the addition of modified strains in succession may increase the efficiency of bioconversion, given that the enzymes involved exhibit disparate activities in the different steps of the bioconversion pathway. Additionally, in an enzymatic bioconversion study was found that HMFO was capable of oxidise HMF in FFCA but not beyond in FDCA, raising the hypothesis that this enzyme has incomplete ability to oxidise HMF in FDCA (Wu et al., 2020).

Therefore, since HMFO enzyme showed little efficiency in oxidising FFCA in FDCA and the HMFH enzyme proved to be more efficient at catalysing this step, a co-culture with modified strains was studied, Initially, I-msHMFO was inoculated to catalyse the oxidation HMF into its derivatives, namely FFCA, subsequently I-cbHMFH was then inoculated to enhance the oxidation of FFCA into FDCA.

Observing the data presented in Figure 10, it is possible to confirm that the co-culture strategy with the two engineered strains proved to be advantageous, as the yield in FDCA increased from 2% to 4%, although more expressive difference was expected.

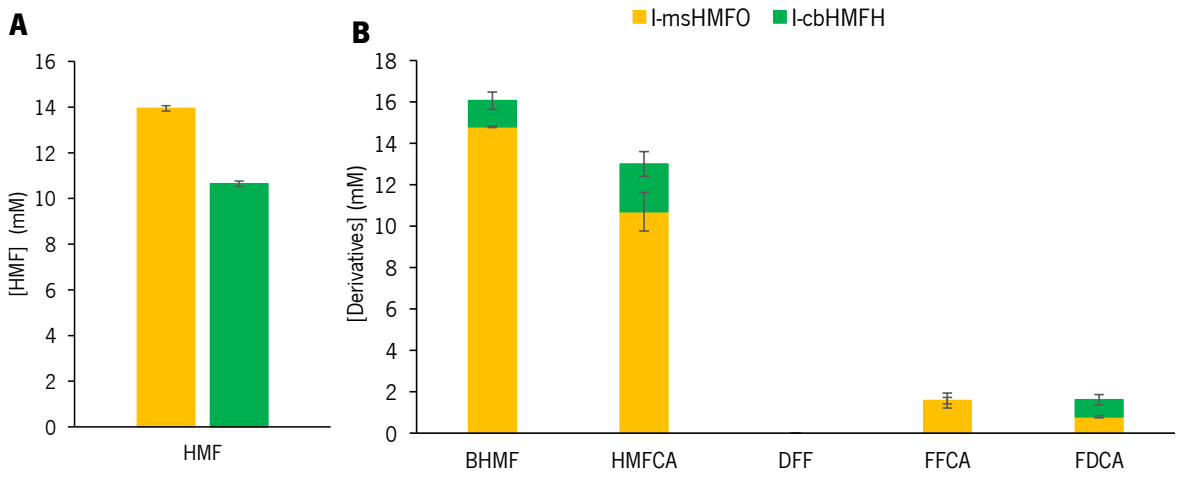

Figure 10. Concentration of HMF (A) and derivatives (B) at the end of the bioconversion for co-culture with the modified strains I-msHMFO (0-120h) and I-cbHMFH (120-144h) in diluted synthetic hydrolysate with furfural. The yellow bars represent bioconversion with I-msHMFO for 120 hours and green bars represent further 24-hour conversion with I-cbHMFH. The error bars represent the standard deviation of two biological independent replicates.

Similarly, to previous experiments, it can be observed that HMF is not completely detoxified after 120 hours of conversion remaining 13.9 mM in the medium (Figure 10A), thus confirming once again the inhibitory effect of the HMF and furfural mixture on yeast, remarkably, at 120 hours of culture, furfural was completely detoxified. Interestingly, after further 24 hours, the conversion of HMF increased from 65.1% to 73.4%, with an approximately 6% increase in yields of HMFCA (Figure 10B). This indicates that the addition of the strain I-cbHMFH continued to detoxify HMF through the HMFCA pathway (Figure 3).

Considering that the bioconversion process continued for an extra 24 hours following the addition of the I-cbHMFH strain and no studies have been conducted to evaluate the sequential addition of these two enzymes, there is lack of evidence to support the efficacy of this approach. Thus, the raised level of HMF conversion and FDCA yield could be attributed to the HMFH enzyme's activity or the prolonged time of bioconversion for an additional 24 hours.

However, Yuan and colegues (2018) investigated the expression and co-expression of both enzymes in *Raoutella ornitinolytica* BF60, they demonstrated the efficiency of using the enzymes separately, yielding 80.8% and 76.9% respectively when using the HMFH and HMFO enzymes. Furthermore, the co-expression of both enzymes proved to be more efficient than when the enzymes were used separately, achieving a yield of 93.6% (Yuan et al., 2018).

4.4. Apple pomace

The apple pomace was submitted to an enzymatic hydrolysis process to break down the polysaccharides into simple sugars and then subjected to a microwave treatment using a deep eutectic solvent to convert the monosaccharides, namely fructose, into HMF. Table 8 shows the apple pomace hydrolysate composition after the pre-treatment.

The utilization of microwave treatment alongside the eutectic solvent has demonstrated efficacy in the synthesis of HMF leading to the attainment of 11.5 g/L (Table 8). The eutectic solvent serving as both the reactant and catalyst was selected based on a study by Mankar and co-workers (2021), proving to be the most efficient in yielding HMF. The solvent contained choline chloride as HBA and lactic acid as HBD in a molar ratio of 1:9.

Table 8. Composition of apple pomace hydrolysate after DES and microwave treatment.

Compound	Concentration
Glucose	24.9 g/L
Fructose	8.9 g/L
HMF	11.5 g/L
Furfural	2.4 g/L
Lactic Acid	344.8 g/L
Phenolic Compounds	3.3 g/g

4.4.1. Whole-cell bioconversion with apple pomace

Finally, the capability of I-msHMFO for detoxifying the HMF present in the apple pomace hydrolysate was evaluated.

Neutralising lignocellulosic biomass hydrolysates is a crucial stage in the production of valuable compounds from biomass feedstocks, being essential to adjust the pH of the hydrolysate to a suitable range for subsequent fermentation or bioconversion. Some commonly used method for neutralising hydrolysates from lignocellulosic biomass includes adding strong bases like sodium hydroxide (NaOH) or potassium hydroxide (KOH), weaker bases such sodium carbonate (Na_2CO_3) or calcium carbonate ($CaCO_3$) or using microorganism that consume organic acids (Kumar et al., 2020).

Here, NaOH and KOH were assessed as neutralising agents for the hydrolysate and the capacity of I-msHMFO strain for detoxifying the HMF present in the apple pomace hydrolysate after neutralisation was evaluated.

Incomplete detoxification of the initial HMF was observed, akin to the observations made in tests using synthetic hydrolysate (Figure 11A). Only approximately 60% of the HMF was detoxified, however, by-products were formed, and it is feasible to yield around 16% and 15% of FDCA for the experiments conducted with apple pomace hydrolysate (25%) neutralised with NaOH and KOH, respectively. This indicates that the use of apple pomace hydrolysate is viable for the bioconversion of HMF to its derivatives.

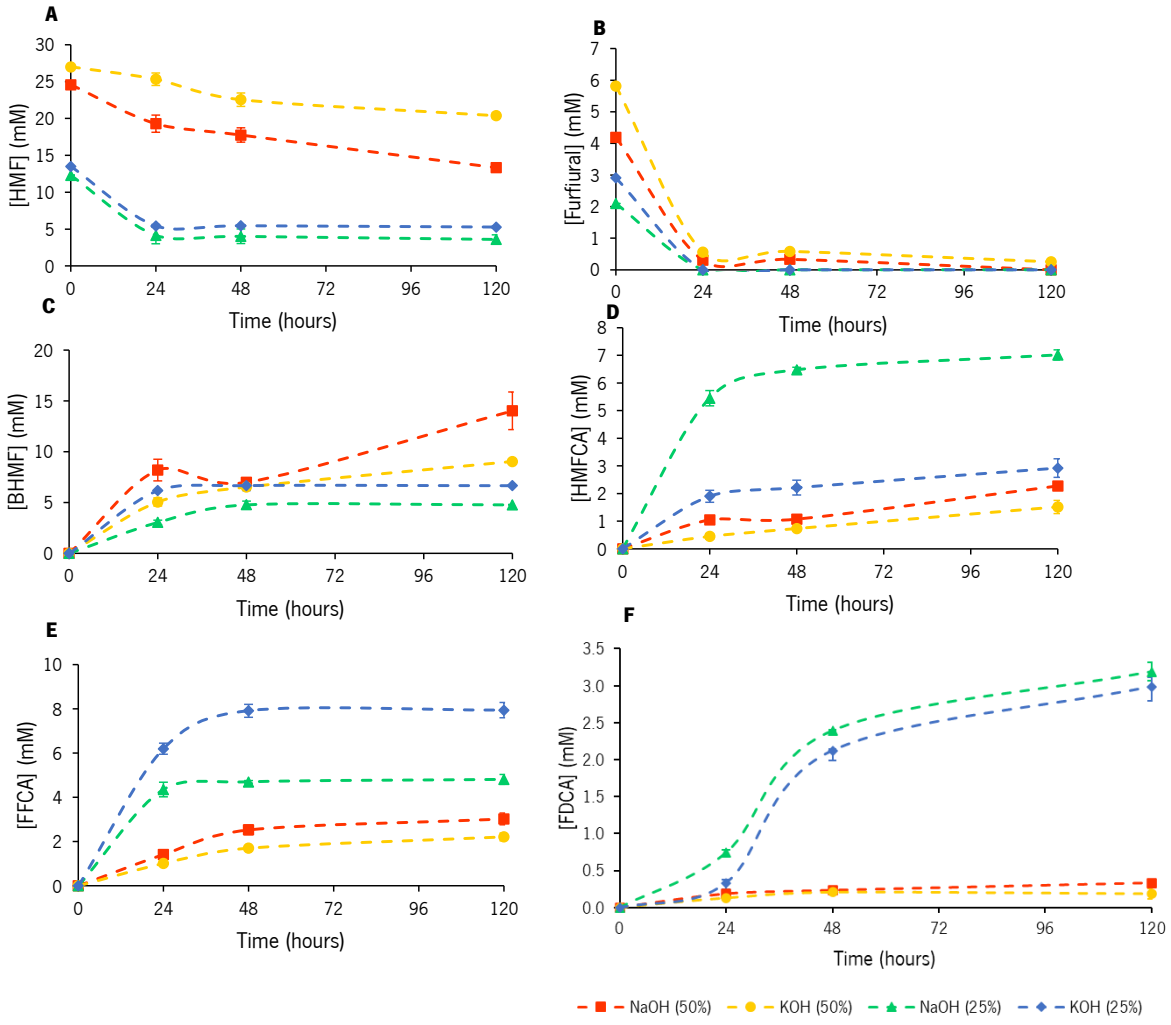

Figure 11. Bioconversion profile of HMF and furfural detoxification with the strain I-msHMFO with apple pomace hydrolysate (50% and 25% diluted) neutralised with NaOH and KOH. The error bars represent the standard deviation of two biological independent replicates.

Furthermore, it is evident that an accumulation of FDCA occurs only when the hydrolysate is diluted to 25% (Figure 11F). On the other hand, when the hydrolysate is diluted by 50%, detoxification of HMF can be observed (Figure 11A), but no increase of FDCA takes place (Figure 11F), instead, there is an accumulation of BHMF (Figure 11C), which could be linked to a higher concentration of inhibitors present in the medium. This implies that yeast tends to detoxify HMF to BHMF when exposed to an elevated level of inhibitors. Focusing on the hydrolysate diluted to 25%, both neutralisations, with NaOH and with KOH exhibit similar behaviour, resulting in practically the same yield of FDCA. Nevertheless, upon analysis, it appears that a greater buildup of HMFCA can be discerned in the hydrolysate neutralised with NaOH (Figure 11D), whilst a greater accumulation of FFCA can be observed in the hydrolysate neutralised with KOH (Figure 11E).

Furthermore, consistent with preceding experiments, furfural (figure 11B) is entirely detoxified and an accumulation of HMFCA is detectable (figure 11D), with no levels of DFF observed in the medium at any stage during the bioconversion.

Additionally, it is observable that high yields of FFCA are achieved, making up approximately 50% of the HMF that has been converted. This result supports the previous statement regarding the limited ability of the HMFO enzyme to oxidise FFCA to FDCA. Alternatively, as no studies of whole-cell biocatalysts for FFCA have been conducted to date (Saikia et al., 2022), the l-msHMFO strain could also be a promising candidate for this purpose.

A comparable investigation employing HMF derived from pineapple peel hydrolysate demonstrated that *Aspergillus flavus* APLS-1 could entirely detoxify 1 g/L of HMF and transform it into its derivatives resulting in a 16% FDCA yield (Rajesh et al., 2023). Furthermore, it has been demonstrated that the transformation of HMF and its intermediates into FDCA was substantially decelerated, perhaps due to the existence of impurities like levulinic acid or humins, which remained in the hydrolysate even after purification (Rajesh et al., 2023). Additionally, in a study conducted by Yang and Huang (2016) an acid algae hydrolysate, containing small amounts of HMF, was supplemented with 2 g/L of HMF and *B. cepacia* H-2 was able to detoxify it into 0.98 g/l of FDCA. In another study, a renewable media for the growth of *M. radiotolerans* G-2 was created using a diluted algal hydrolysate containing HMF, which result in 0.45 g/L of FDCA being produced (Yang & Huang, 2018).

The eutectic solvent chosen might not be optimal for the intended use of apple pomace hydrolysate as a culture medium for whole-cell biocatalysis of HMF due to its excessively acidic pH (below 1), which inhibits the yeast's activity. Lignocellulosic hydrolysates are generally known to be acidic, and additionally, this hydrolysate underwent pretreatment with an acidic eutectic solvent (lactic acid). Other eutectic solvents with lower acidity, such as CHCl:Urea, could potentially be utilised as a viable alternative. However, no research has been conducted on microwave-mediated HMF production with this solvent. Current studies primarily employ CHCl as the HBA and an organic acid such as lactic, citric or malic acid as the HBD (Mankar et al., 2021), which result in a DES with a pH of approximately 1 (Lemaoui et al., 2021). Given this, there is room for improvement regarding the selection of the pretreatment to integrate in a bioconversion process.

5.Conclusion and future perspectives

HMF is a potentially significant molecule because it can be produced from lignocellulosic biomass and serves as a building block to produce several value-added chemicals. Among these derivatives, FDCA is one of the most prominent, finding its main application in the bioplastic industry. Nonetheless, HMF can act as an inhibitor of microbial activity, impacting the feasibility of using whole-cell catalysts to generate its derivatives.

This study aims to elucidate the potential of *S. cerevisiae* strains genetically modified to express HMFH and HMFO enzymes, for the oxidation of HMF. The strains were screened in a phosphate buffer medium supplemented with 50 mM HMF and the results indicate that all strains, wild-type and modified, successfully detoxified HMF with higher FDCA levels observed in modified strains. Furthermore, the industrial recombinant strains (I-cbHMFH and I-msHMFO) exhibited higher efficacy in comparison to the laboratory recombinant strains reiterating the significance of screening different host chassis strains. Also, the importance of fine-tuning oxygen levels to achieve the optimal enzyme activity was demonstrated, given that low oxygen levels are not conducive to bioconversion, but, high oxygen levels also inhibit the conversion, namely the oxidation of FFCA to FDCA due to the formation of by-products such as H_2O_2.

Furthermore, in order to utilise a lignocellulosic hydrolysate, a comparable synthetic medium was developed and assessed. This led to the conclusion that the sugars within the medium had no negative effect on the bioconversion. Additionally, it was determined that out of the six studied strains, I-msHMFO exhibited the highest yields of FDCA. On other hand, due to the existence of furfural in lignocellulosic hydrolysates, the influence of this substance on bioconversion was intensively examined and it was established that the presence of furfural interferes with the detoxification of HMF by the yeast.

Subsequently, an apple pomace hydrolysate, which was appropriately neutralised was used as a source of HMF for bioconversion and it was demonstrated that, as in the synthetic medium, yeast tended to detoxify furfural more rapidly than HMF. Furthermore, it is possible to observe a higher conversion of HMF and a greater yield of FDCA when the hydrolysate is diluted 4-fold. Consequently, from a biotechnological point of view it has been proved the potential of *S. cerevisiae* I-msHMFO as a whole-cell biocatalyst for the production of FDCA utilising apple pomace as an HMF source, thus valorising an industrial by-product.

5.1. Future Perspectives

The present study offers a novel insight into the biotechnological synthesis of FDCA by a recombinant strain of *S. cerevisiae* (I-msHMFO), using an apple pomace hydrolysate as HMF source, contributing to the optimization of crucial parameters that influences the bioprocess. Nonetheless, there is still room for improvement for future research and developments.

Oxygenation has been identified as a critical factor in bioconversion and therefore, it is imperative to conduct further investigations to fine-tune its level. Evaluation of other process configurations such as different types of bioreactors, which enable a precise and more efficient control of process conditions can be valuable to achieve optimal conditions for the bioconversion process. Also, performing a scale-up for the bioconversion would have significant advantages from an industry standpoint.

Additionally, increasing the number of copies of the *hmfo* gene from *Methylovorus* sp. may be a strategy worth exploring as it can possibly enhance the production of FDCA. Furthermore, considering a study by Yuan et al. (2018), the co-expression of both enzymes (HMFO; from *Methylovorus* sp. strain MP688 and HMFH; from *C. basilensis* HMF14) in *S. cerevisiae* could be advantageous and effectively increase the production of FDCA, as both oxidation pathways are active in the yeast.

Sustainability and environmental issues related to the consumption of fossil fuels are on the agenda, thus increasing the demand for high-value chemical products such as FDCA. The finding in this thesis highlight the importance of industrial *S. cerevisiae* as a robust microbial platform for the production of bioproducts, contributing to a more sustainable bioeconomy and providing opportunities for a more environmentally friendly future.

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