Novel biocatalytic approaches for the valorization of furfural and 5-hydroxymethylfurfural

Alberto Millán Acosta

Universitat de Lleida

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Alberto Millán Acosta

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Programa de Doctorat en Ciència i Tecnologia Agrària i Alimentària

Director/a

Ramon Canela i Garayoa

Núria Sala Martí

Tutor/a

Ramon Canela i Garayoa

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The most exciting phrase to hear in science,

the one that heralds new discoveries, is not "Eureka!" but "That's funny…"

Isaac Asimov

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Summary

The current dependence on fossil resources to produce energy and commodity chemicals poses a major concern to the environment. Finding renewable alternatives is crucial to support and sustain future life on planet Earth. Biomass, specifically in the form of lignocellulose, presents as a promising alternative because it is abundant as waste, it does not compete for food supplies, and it is the most copious carbon feedstock of the planet, containing high amounts of sugars in the forms of cellulose and hemicellulose. Monosaccharides can be released through pretreatment and hydrolysis of the lignocellulosic material and further transformed into fuels such as ethanol. Furfural (FF) and 5-hydroxymethylfurfural (HMF) are furaldehydes generated by dehydration of sugars during the pretreatment of lignocellulose and are inhibitors for the sugar fermenting strains. The study of the transformation pathways that lead to their fewer toxic derivatives has opened a new line of research on the upgrade of FF and HMF, given the value of their derivatives at biorefineries. The main objective of this thesis is to contribute to the biocatalytic valorization of FF and HMF.

First, an optimization of the enzymatic oxidation of HMF to 2,5-diformylfuran (DFF) via galactose oxidase, catalase, and horseradish peroxidase was attempted. A high yield (>90 %) was achieved by adjusting the ratio between galactose oxidase and catalase and omitting the addition of horseradish peroxidase. However, it was concluded that the process had some limitations and that whole cells would be a more feasible (and yet not described) approach.

In the search for whole-cell biocatalysts, a screening of seven *Fusarium* species, natural producers of the enzyme galactose oxidase, was considered. Five of the species evaluated showed a high capability to reduce HMF to 2,5-di(hydroxymethyl)furan (DHMF), and two of them showed the capability to oxidize HMF to DFF with low yields and selectivities. The whole-cell production of DHMF was considered of interest and studied using *F. striatum*. It showed high tolerance towards HMF when using small inoculum sizes, transforming 75 mM HMF within 24 h with a high yield and selectivity. A fed-batch approach allowed a higher concentration of DHMF in the media. Finally, the feasibility of the scale-up of the process was evaluated, and a quantitative DHMF yield (95 %) and selectivity (98 %) were obtained in a bench-scale bioreactor (1.3 L), concluding that *F. striatum* is a promising candidate for DHMF production.

Among the two *Fusarium* species that showed HMF oxidation to DFF (*F. culmorum* and *F. sambucinum*), the former showed more encouraging preliminary results and was selected to optimize the process further. It was found that the nitrogen source and the concentration of glucose and peptones in the media highly influenced the redox capability of the strain. The concentration of both nutrients was carefully optimized through Response Surface Methodology by building two successive Central Composite Designs. A high DFF yield (92 %) and selectivity (94 %) were obtained under the optimized conditions starting from 50 mM HMF, thus describing for the first time an efficient whole-cell production of DFF and opening a new line of investigation.

Finally, the last Chapter of this thesis was focused on the use of *F. striatum* as a biological detoxification method in bio-based ethanol production from lignocellulosic hydrolysates containing high concentrations of FF and HMF. A co-culture between *F. striatum* and a xylose-consuming *S. cerevisiae* showed better performance than other biological detoxification methods previously reported in the literature. Moreover, it overcame the main drawbacks of biological detoxification: it was able to handle significantly higher inhibitor concentrations with higher degradation rates, the detoxification and fermentation steps were performed simultaneously, there was complete detoxification of the inhibitors, and there was no consumption of sugars during the detoxification process, meaning that the presence of *F. striatum* did not influence the ethanol yield. A high ethanol yield (0.40 g/g) and productivity (0.46 g/L/h) were obtained in a bench-scale bioreactor in the presence of 2.5 g/L FF and 3.5 g/L HMF, a concentration of furaldehydes that inhibited the fermentation in the absence of *F. striatum*. Moreover, the added-value alcohol derivatives of FF and HMF were produced during the detoxification process with high yields, adding more value to the lignocellulosic hydrolysate.

The biocatalytic approaches developed throughout this thesis provide novel sustainable methods to produce high-value compounds from biomass and open new lines of investigation in the biocatalytic valorization of FF and HMF.

Resumen

La actual dependencia en los recursos fósiles para producir energía y productos químicos supone una gran preocupación medioambiental. Encontrar alternativas renovables es crucial para sostener la vida futura en el planeta Tierra. La biomasa, específicamente en forma de lignocelulosa, es una alternativa prometedora porque es abundante como residuo, no compite con el suministro alimentario y es la fuente de carbono más copiosa del planeta, conteniendo altas cantidades de azúcares en forma de celulosa y hemicelulosa. Los monosacáridos se pueden liberar mediante el pretratamiento y la hidrólisis del material lignocelulósico y posteriormente se pueden transformar en combustibles como por ejemplo etanol. El furfural (FF) y el 5-hidroximetilfurfural (HMF) son furaldehídos generados por la deshidratación de azúcares durante el pretratamiento de lignocelulosa y son inhibidores de las cepas encargadas de la fermentación. El estudio de las vías de transformación que conducen a sus derivados menos tóxicos ha abierto nuevas líneas de investigación centradas en la valorización de FF y HMF, dado el elevado valor de sus derivados en las biorrefinerías. El principal objetivo de esta tesis es contribuir a la valorización biocatalítica del FF y del HMF.

Inicialmente, se intentó optimizar la oxidación enzimática de HMF en 2,5-diformifurano (DFF) vía galactosa oxidasa, catalasa y peroxidasa de rábano picante. Se obtuvo un rendimiento elevado (>90%) mediante el ajuste de la ratio entre galactosa oxidasa y catalasa y omitiendo la adición de peroxidasa. Sin embargo, se concluyó que el proceso tenía algunas limitaciones y que el uso de microorganismos sería un enfoque más factible (y aún no descrito).

En la búsqueda de microorganismos, se consideró un cribado de siete especies de *Fusarium*, que son productoras naturales de la enzima galactosa oxidasa. Cinco de las especies evaluadas mostraron una alta capacidad para reducir HMF en 2,5-di(hidroximetil)furano (DHMF), y dos de ellas mostraron capacidad para oxidar HMF a DFF con bajos rendimientos y selectividades. La producción de DHMF mediante microorganismos se consideró de interés y se estudió utilizando *F. striatum*. Mostró una elevada tolerancia hacia el HMF al utilizar tamaños de inóculo pequeños, transformando 75 mM de HMF en 24 h con un rendimiento y selectividad altos. La adición de HMF por lotes permitió una mayor concentración de DHMF en el medio. Finalmente, se evaluó la viabilidad del escalado del proceso y se obtuvo un elevado rendimiento (95%) y selectividad

(98%) en un biorreactor a escala de laboratorio (1.3 L), concluyendo que *F. striatum* es un candidato prometedor para la producción de DHMF.

Entre las dos especies de *Fusarium* que mostraron oxidación de HMF a DFF (*F. culmorum* y *F. sambucinum),* la primera mostró resultados preliminares más prometedores y fue seleccionada para la optimización del proceso. Se observó que la fuente de nitrógeno y la concentración de glucosa y peptonas en el medio influyeron en la capacidad de la cepa de transformar el HMF. La concentración de ambos nutrientes se optimizó cuidadosamente a través de la Metodología de Superficie de Respuesta mediante la construcción de dos Diseños Centrales Compuestos sucesivos. Se obtuvo un alto rendimiento (92%) y selectividad (94%) de DFF en las condiciones óptimas a partir de 50 mM de HMF, describiendo por primera vez una producción eficiente de DFF mediante el uso de microorganismos y abriendo una nueva línea de investigación.

Finalmente, el último capítulo de esta tesis se centró en el uso de *F. striatum* como método de desintoxicación biológica en la producción de etanol a partir de hidrolizados lignocelulósicos que contienen altas concentraciones de FF y HMF. El co-cultivo de *F. striatum* y una cepa de *S. cerevisiae* que consume xilosa mostró un mejor rendimiento que otros métodos de desintoxicación biológica previamente reportados en la literatura. Además, superó los principales inconvenientes de la desintoxicación biológica: degradó concentraciones de inhibidor significativamente más altas con tasas de degradación superiores, la desintoxicación y fermentación se realizaron simultáneamente, hubo una desintoxicación completa de los inhibidores y no hubo consumo de azúcares durante el proceso de desintoxicación, lo que significa que el rendimiento de etanol no se vio afectado por la presencia de *F. striatum*. Se obtuvo un alto rendimiento (0.40 g/g) y productividad (0.46 g L/h) de etanol en un biorreactor a escala de laboratorio en presencia de 2.5 g/L de FF y 3.5 g/L de HMF, una concentración de furaldehídos que inhibió la fermentación en ausencia de *F. striatum*. Además, los derivados alcohólicos de valor añadido del FF y HMF se produjeron durante el proceso de desintoxicación con rendimientos elevados, añadiendo más valor al hidrolizado lignocelulósico.

Los procesos biocatalíticos desarrollados en esta tesis proporcionan nuevos métodos sostenibles para producir compuestos de valor a partir de biomasa y abren nuevas líneas de investigación en la valorización del FF y HMF.

Resum

L'actual dependència en els recursos fòssils per a produir energia i productes químics suposa una gran preocupació mediambiental. Trobar alternatives renovables és crucial per sostenir la vida futura al planeta Terra. La biomassa, específicament en forma de lignocel·lulosa, és una alternativa prometedora perquè és abundant com a residu i és la font de carboni més copiosa de la planeta, contenint altes quantitats de sucres en forma de cel·lulosa i hemicel·lulosa. Els monosacàrids es poden alliberar mitjançant el pretractament i la hidròlisi del material lignocel·lulòsic i posteriorment es poden transformar en combustibles com l'etanol. El furfural (FF) i el 5-hidroximetilfurfural (HMF) són furaldehids generats per la deshidratació de sucres durant el pretractament de la lignocel·lulosa i són inhibidors de les soques encarregades de fermentar els sucres. L'estudi de les vies de degradació que condueixen als seus derivats menys tòxics ha obert noves línies d'investigació centrades en la valorització de FF i HMF, donat l'elevat valor dels seus derivats en les biorrefineries. El principal objectiu d'aquesta tesi és contribuir a la valorització biocatalítica del FF i de l'HMF.

Inicialment, es va intentar optimitzar l'oxidació enzimàtica d'HMF a 2,5-diformifurà (DFF) via galactosa oxidasa, catalasa i peroxidasa de rave picant. Es va aconseguir un alt rendiment (>90%) mitjançant l'ajust de la ràtio entre galactosa oxidasa i catalasa i ometent l'addició de peroxidasa. No obstant, es va concloure que el procés tenia algunes limitacions i que l'ús de microorganismes seria un enfoc més factible (i encara no descrit).

En la recerca de microorganismes, es va considerar un cribratge de set espècies de *Fusarium*, que són productors naturals de l'enzim galactosa oxidasa. Cinc de les espècies avaluades van mostrar una alta capacitat per reduir HMF a 2,5-di(hidroximetil)furà (DHMF), i dues d'elles van mostrar capacitat per oxidar HMF a DFF amb baixos rendiments i selectivitats. La producció de DHMF mitjançant microorganismes es va considerar d'interès i es va estudiar utilitzant *F. striatum*. Va mostrar una elevada tolerància cap al HMF a l'utilitzar mides d'inòcul petites, transformant 75 mM d'HMF en 24 h amb un rendiment i selectivitat alts. L'adició d'HMF per lots va permetre una major concentració de DHMF en el medi. Finalment, es va avaluar la viabilitat de l'escalat de el procés i es va obtenir un elevat rendiment (95%) i selectivitat (98%) en un bioreactor a escala de laboratori (1.3 L), concloent que *F. striatum* és un candidat prometedor per a la producció de DHMF.

Entre les dues espècies de *Fusarium* que van mostrar oxidació d'HMF a DFF (*F. culmorum* i *F. sambucinum),* la primera va mostrar resultats preliminars més prometedors i va ser seleccionada per a l'optimització del procés. Es va trobar que la font de nitrogen i la concentració de glucosa i peptones en el medi van influir en la capacitat de la soca de transformar l'HMF. La concentració de tots dos nutrients es va optimitzar acuradament a través de la Metodologia de Superfície de Resposta mitjançant la construcció de dos Dissenys Centrals Compostos successius. Es va obtenir un alt rendiment (92%) i selectivitat (94%) de DFF en les condicions òptimes a partir de 50 mM d'HMF, descrivint per primera vegada una producció eficient de DFF mitjançant l'ús de microorganismes i obrint una nova línia d'investigació.

Finalment, l'últim capítol d'aquesta tesi es va centrar en l'ús de *F. striatum* com a mètode de desintoxicació biològica en la producció d'etanol a partir d'hidrolitzats lignocel·lulòsics que contenen altes concentracions de FF i HMF. El co-cultiu de *F. striatum* i una soca de *S. cerevisiae* que consumeix xilosa va mostrar un millor rendiment que altres mètodes de desintoxicació biològica prèviament reportats en la literatura. A més, va superar els principals inconvenients de la desintoxicació biològica: va degradar concentracions d'inhibidor significativament més altes amb taxes de degradació superiors, la desintoxicació i fermentació es van realitzar simultàniament, hi va haver una desintoxicació completa dels inhibidors i no hi va haver consum de sucres durant el procés de desintoxicació, el que significa que el rendiment d'etanol no es va veure afectat per la presència de *F. striatum*. Es va obtenir un alt rendiment (0.40 g/g) i productivitat (0.46 g/L/h) d'etanol en un bioreactor a escala de laboratori en presència de 3.5 g/L d'HMF i 2.5 g/l de FF, una concentració de furaldehids que va inhibir la fermentació en absència de *F. striatum*. A més, els derivats alcohòlics de valor afegit de l'FF i HMF es van produir durant el procés de desintoxicació amb alts rendiments, afegint més valor a l'hidrolitzat lignocel·lulòsic.

Els processos biocatalítics desenvolupats en aquesta tesi proporcionen nous mètodes sostenibles per a produir compostos de valor a partir de biomassa i obren noves línies de investigació en la valorització del FF i HMF.

List of abbreviations

ANOVA	Analysis of variance
BBD	Box Behnken design
CCD	Central composite design
CS	Corn stover
DFF	2,5-Diformylfuran
DHMF	2,5-Di(hydroxymethyl)furan
DM	Dry matter
DOE	Design of experiments
DW	Dry weight
FA	Furfuryl alcohol
FB	Fed-batch
FDCA	2,5-Furandicarboxylic acid
FF	Furfural
FFCA	5-Formyl-2-furancarboxylic acid
FID	Flame ionization detector
FOA	Furoic acid
GC	Gas chromatography
GO	Galactose oxidase
GOM_{3-5}	Galactose oxidase mutant
HMF	5-Hydroxymethylfurfural
HMFCA	5-Hydroxymethyl-2-furancarboxylic acid
HPLC	High-pressure liquid chromatography

HRP	Horseradish peroxidase
ME	Malt extract
MEA	Malt extract agar
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NMR	Nuclear magnetic resonance
OFAT	One factor at a time
PPP	Pentose phosphate pathway
ROS	Reactive oxygen species
RSM	Response surface methodology
TCA	Tricarboxylic acid cycle
UV/VIS	Ultraviolet/visible
WS	Wheat straw
WW	Wet weight
YPX	Yeast peptone xylose media

List of contributions

Manuscripts

A. Millán, N. Sala, M. Torres, R. Canela-Garayoa, Biocatalytic transformation of 5-hydroxymethylfurfural into 2,5-di(hydroxymethyl)furan by a newly isolated *Fusarium striatum strain, Catalysts*. 11 (2021) 216. https://doi.org/10.3390/catal11020216.

A. Millán Acosta, D. Cosovanu, P. Cabañeros López, S.T. Thomsen, K. V Gernaey, R. Canela-Garayoa, Co-cultivation of a novel *Fusarium striatum* strain and a xylose consuming *Saccharomyces cerevisiae* yields an efficient process for simultaneous detoxification and fermentation of lignocellulosic hydrolysates, *Chemical Engineering Journal*. 426 (2021) 131575. https://doi.org/10.1016/j.cej.2021.131575

A. Millán Acosta, C. Cuesta Turull, D. Cosovanu, N. Sala Martí, R. Canela-Garayoa, A novel and efficient biotechnological approach to produce 2,5-diformylfuran from biomass-derived 5-hydroxymethylfurfural, *ACS Sustainable Chemistry & Engineering*. 9, 43 (2021) 14550-14558. https://doi.org/10.1021/acssuschemeng.1c05308

Conference participations

A. Millán, N. Ortega, C. Bernal, M. Balcells, M. Torres, R. Canela, Production of terpenes with interest as flavor agents by fungi isolated from food waste, Poster contribution at Congreso Nacional de Biotecnología, Murcia, Spain, 2017.

A. Millán, R. Canela-Garayoa, N. Sala, M. Balcells, M. Canudas, Fungal biotransformation of 5-(hydroxymethyl)furfural into 2,5-di(hydroxymethyl)furan, Oral presentation at European Congress on Biotechnology, Geneva, Switzerland, 2018.

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CHAPTER 1. INTRODUCTION AND OBJECTIVES

1. Biorefineries

The increase of the world population and the economic expansion are challenging the current production of energy and commodity chemicals from fossil resources (such as petroleum, coal, and natural gas), which are not considered renewable because their formation requires millions of years. They have been the most used feedstock for decades, and nowadays, there is still complete reliance on them. Their depletion and the emission of greenhouse gases to the atmosphere derived from their use have led to an alarming increase in environmental worries such as climate change, global warming, waste disposal, and natural resource reduction. These concerns are challenging the Sustainable Development Goals, and the human being is the main responsible due to their exploitation, with detrimental consequences for the planet. For these reasons, environmental, political, and economic concerns manifest the need for sustainable alternatives [1–4]. This has promoted the concept of "Biorefinery", in which renewable feedstocks are converted into fuels, energy, and commodity chemicals through chemical and/or biological catalysis [5–7]. In the search for fossil substitutes, the renewable nature of the raw material and high availability are imperative to ensure a sustainable and prolonged supply. Biomass, which is available in different forms as dead plants, crops, algae, marine organisms, and agri-food and forestry wastes, is a renewable source that represents the most abundant carbon feedstock on the planet. Therefore, it has a high potential to substitute fossil resources to prepare compounds of interest and produce energy. However, only cheap, abundant, and convertible feedstocks are suitable for biorefinery. Among the different forms of biomass available, lignocellulosic biomass is the most promising because it does not compete for food supply (as opposed to starch-based biomass) and is abundant as waste [4,7–9].

2. Lignocellulosic biomass

Lignocellulosic biomass is often a by-product of industrial processes in different sectors such as agriculture (like straw) and forestry (like wood waste) and is the most abundant form of biomass with an annual production of 170 billion metric tons [4]. Its use does not compete with the food supply, and therefore it provides an efficient solution to the problems caused by starch-based biorefineries. It is expected to become a great source of both fuels and chemicals in the coming years, although its recalcitrant structure still hinders its potential valorization. Lignocellulose

comprises a mixture of cellulose (30-50 wt%), hemicellulose (20-35 wt%), and lignin (15-30 wt%) as principal components. Cellulose is a linear polysaccharide of glucose monomers linked by β-(1,4)-glycosidic bonds and constitutes an essential component of plant cell walls. It can be broken down into glucose units, although it is hard to hydrolyze due to its crystalline structure. Hemicellulose is a linear or branched heteropolysaccharide containing pentoses (xylose and arabinose) and hexoses (mannose, galactose, and glucose). It is easier to hydrolyze than cellulose because the chains are shorter, and the polymers do not aggregate. Therefore, high amounts of sugar are present in lignocellulosic material in the form of polysaccharides. Lignin is an aromatic polymer and highly condensed macromolecule that provides rigidity to the structure and protection to cellulose and hemicellulose chains, and it is closely bound to them by covalent bonds. It is not a source of monosaccharides; however, it is a large source of biobased aromatic compounds. Its recalcitrant structure makes it resistant to most degradation treatments [4,9–12]. The direct use of lignocellulosic biomass to produce fuel and chemicals is limited by the lignin and water content, cellulose crystallinity, degree of polymerization, and available surface area [7,13].

2.1. Pretreatment of lignocellulose

The pretreatment of lignocellulosic material is a necessary step to make the components of lignocellulose available for their valorization. It consists of the breakdown of lignocellulosic fibers to reduce their recalcitrant structure, and it is mainly achieved by exposing them to high temperatures and/or extreme pH (acid or alkaline). After the pretreatment, cellulose is more exposed to enzymatic or chemical hydrolysis thanks to the solubilization of the hemicellulosic and/or lignin fractions. Monosaccharides (glucose, xylose, galactose, mannose, and arabinose) are then obtained through the hydrolysis of the exposed polysaccharides, obtaining the lignocellulosic hydrolysate, which is an essential step in the sugar platform concept processing of lignocellulose. These sugars can be further transformed into various products of interest by chemical or microbial catalysis, being ethanol one of the most common and extensively studied (**Figure 1**) [14,15].

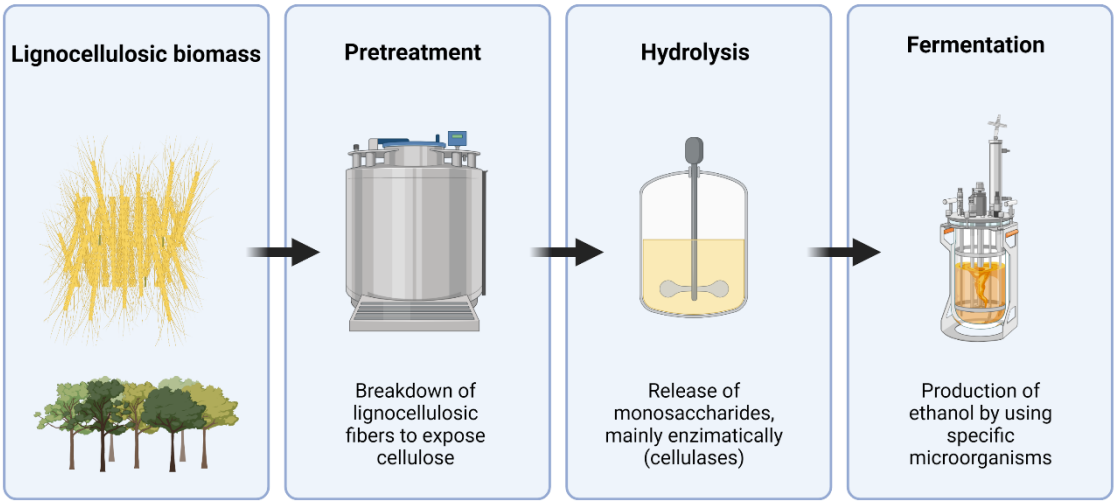

Figure 1. Steps in the production of ethanol from lignocellulosic biomass. Adapted from [16]. Created with BioRender.com

Due to the different nature of the lignocellulosic feedstocks, there exist a variety of different pretreatment methods that can be combined, including but not limited to chemical methods (pH variation by addition of acid or base, ozonolysis, organosolv, and ionic liquids), physical methods (grinding, milling, and extrusion), physico-chemical methods (steam explosion, liquid hot water, ammonia fiber explosion, wet oxidation, and microwave pretreatment), and biological methods (use of specific microorganisms, mainly fungi due to their capability to degrade lignin and hemicellulose). Among them, steam explosion is one of the most employed. It is based on the heating of the lignocellulose with pressurized steam followed by a sudden decompression, which can be assisted by an acid catalyst such as H_2SO_4 [13,14,16,17].

2.2. Formation of inhibitory compounds

The harsh conditions applied in the pretreatment lead to the formation of by-products that are inhibitors for the sugar fermenting strains. Those can be classified according to their chemical structure in weak acids (such as acetic, formic, or levulinic acids), phenolic compounds (such as vanillin or 4-hydroxybenzoic acid), and furaldehydes (such as furfural (FF) and 5-hydroxymethylfurfural (HMF)) (**Figure 2**) [18,19].

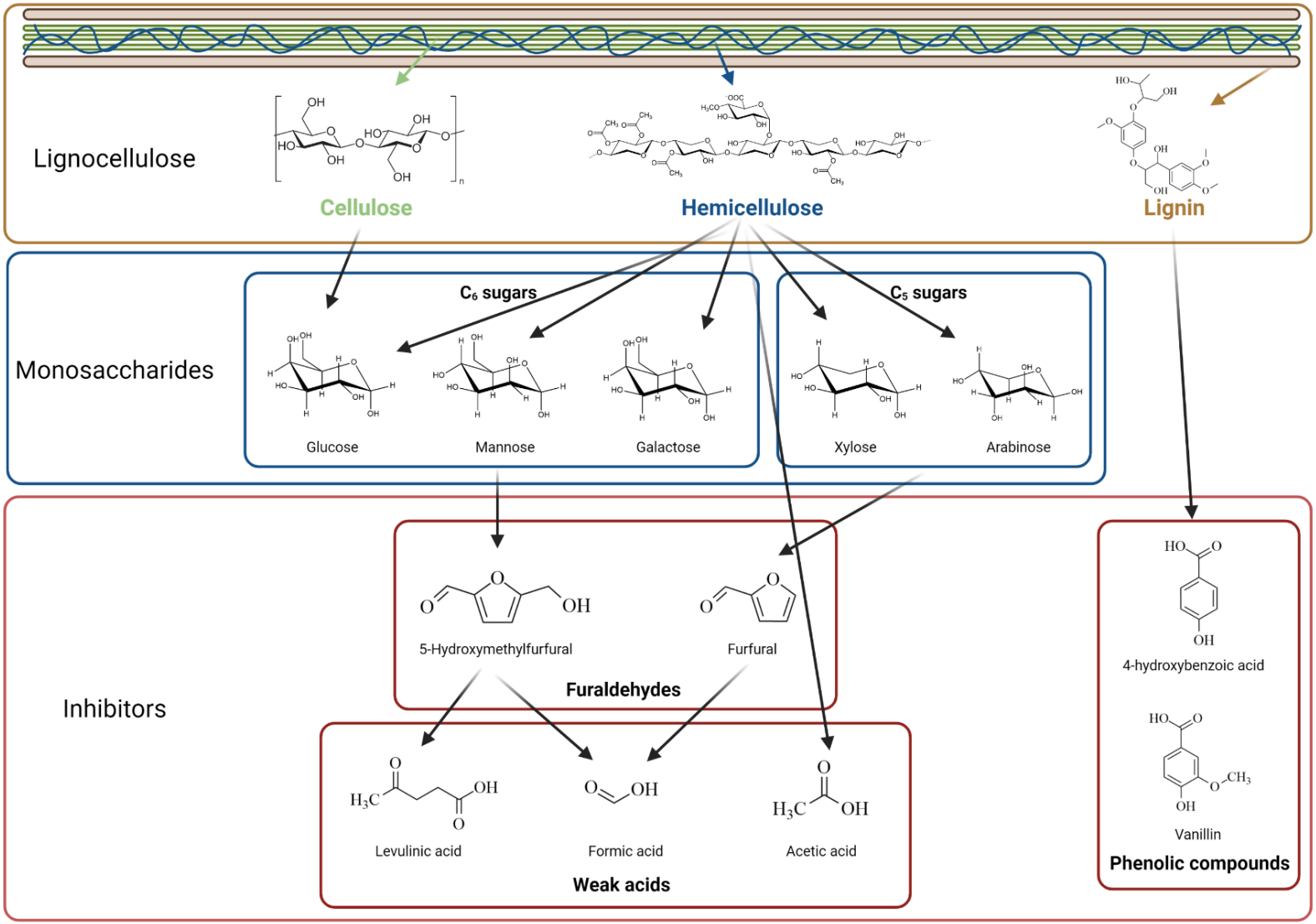

Figure 2. Compounds derived from the pretreatment of lignocellulosic material. Adapted from [15]. Created with BioRender.com

3. Furfural and 5-hydroxymethylfurfural

Among the different compounds formed during the pretreatment, FF and HMF are produced through dehydration of pentoses and hexoses, respectively, and deserve special attention for several reasons: i) they are considered among the most inhibitory compounds and can be present at high concentrations in the lignocellulosic hydrolysates, ii) they have a wide range of derivatives of commercial interest and therefore are considered highly valuable platform chemicals, iii) they can be produced exclusively by thermochemical conversion of the lignocellulosic biomass, and iv) they are carbon-efficient: HMF maintains all six carbon from hexoses and FF all five carbon from pentoses. For these reasons, they belong to the list of the top "10+4" added-value bio-based chemicals by the US Department of Energy [10,20,21].

FF is produced by dehydration of pentoses, being xylose the most studied due to its higher presence on lignocellulosic biomass, although it can also be produced from arabinose or ribose. HMF is produced through dehydration of hexoses (mainly glucose via fructose). First, acid hydrolysis of lignocellulosic biomass under high temperatures is performed to release the pentoses and hexoses from the cellulose and hemicellulose fractions. Once the sugars are released, their conversion into FF or HMF is produced through acid-catalyzed triple dehydration, also performed at high temperatures, which is a slower process. FF and HMF formation mechanisms during the pretreatment of lignocellulosic material are out of the scope of this thesis and are extensively reviewed elsewhere [22–26].

FF comprises a furan ring with an aldehyde group. It is a viscous and colorless liquid that turns brown on exposure to air and has a characteristic odor. It is produced naturally from the combustion of wood and coal. The first large-scale production of FF dates to 1922 by the Quaker Oats company due to the excessive amounts of oat hulls that remained unused after the industrial process. Due to the furan ring and the aldehyde group, it has excellent properties as a selective solvent, mainly in petroleum processing [10,23].

HMF comprises a furan ring, an aldehyde group, and a hydroxyl group. At room temperature, it is a yellow-brown solid with the odor of chamomile flowers. The first HMF report dates to 1875 as an intermediate in the formation of levulinic acid. HMF is more reactive than FF due to the

extra functional group, and therefore it has even more potential as a platform chemical [22,26,27].

The interest in them is increasing in the last years, as shown in **Figure 3**.

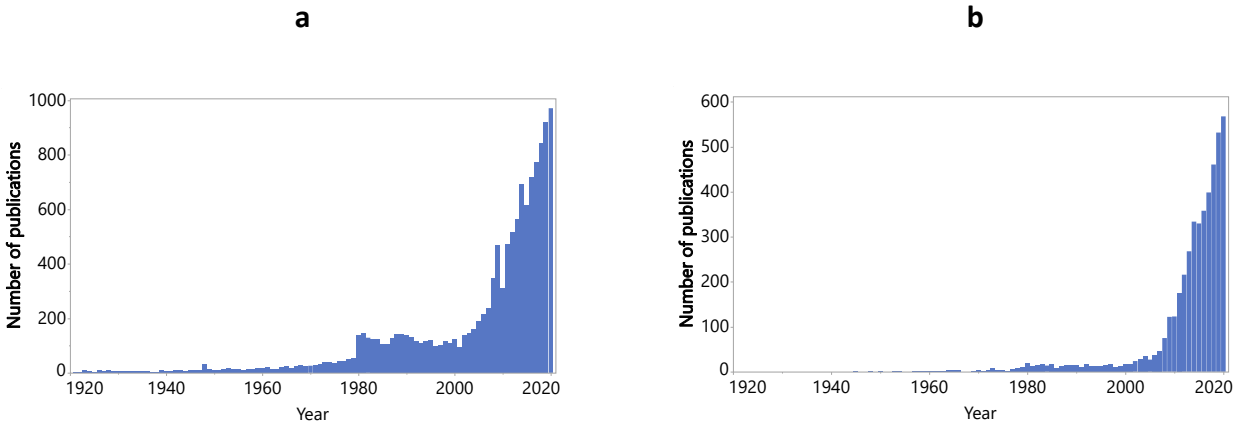

Figure 3. Number of publications on furfural (a) and 5-hydroxymethylfurfural (b) per year. Source: Web of Science, accessed in April 2021.

Given their value, the preparation of FF and HMF directly from the lignocellulosic biomass is also of interest and has been studied as an alternative to the preparation of fermentable sugars [25,28]. Therefore, the pretreatment method and the raw material used highly depend on the final product desired. If the aim is ethanol production, the final composition of the pretreated material should contain a high concentration of mono- and polysaccharides and a low concentration of inhibitors. For that, mild reaction conditions are used to minimize the formation of inhibitors, although the presence of some of them (mostly FF and HMF) at inhibitory concentrations may be inevitable (**Table 1**). On the other hand, if the aim is to prepare FF and HMF, the pretreatment should be focused on the obtention of these compounds in high concentrations, which typically requires harder reaction conditions, such as longer reaction times and higher concentration of homogeneous catalysts (**Table 1**). It should be noted that both approaches, ethanol and FF/HMF production, have the same intermediate products (monosaccharides), as HMF and FF are produced from the dehydration of hexoses and pentoses, respectively (**Figure 2**). This dehydration is achieved by the adjustment and application of more arduous conditions [25].

Table 1. Raw material, conditions of the pretreatment, and final composition depending on the aim product (ethanol or furfural).

Raw material	Conditions	Sugars (g/L)					Furaldehydes (g/L)		Product	Ref
		Glucose	Mannose	Galactose	Xylose	Arabinose	HMF	FF		
WS		11.1	4.2	3.1	54.2	5.6	0.2	2.2		
75% WS, 25% CS		9.4	3.6	2.9	41.7	5.0	0.3	2.4		
50% WS, 50% CS	0.2 wt % H_2SO_4, 190 °C, 10 min	5.9	2.9	2.6	37.6	4.0	0.2	1.7	Ethanol	[29]
25 &WS, 75%CS		8.0	3.6	3.4	39.5	4.7	0.3	2.0		
CS		5.0	2.9	3.1	36.4	4.0	0.3	1.8		
Pine	I: 0.7 % H_2SO_4, 190 °C, 3 min	27.8	33.9	13.4	25.4	9.8	2.4	2.5	Ethanol	[30]
	II: 0.4 % H_2SO_4, 215 °C, 3 min	74.0	3.0	1.7	0.6	0	5.8	0		
Wood[a]	I: 0.5 % H_2SO_4, 190 °C, 10 min	24.3	11.8	3.4	5.7	1.5	2.1	0.4	Ethanol	[31]
	II: <0.5 % H_2SO_4, 215 °C, 7 min	19.9	15.9	4.0	7.4	1.7	1.8	0.5		
Wood	5 g/L H_2SO_4, 215 °C, 7 min	30.5	19.8	-	4.7	-	5.9	1.3	Ethanol	[32]
WS	190 °C, 15 min	14.5	1.8	-	32.6	3.5	1.3	7.7	Ethanol	[33]
Maple, poplar, and birch	H_2SO_4 (pH 2), 190 °C, 71 min	-	-	-	-	-	-	23.5	Furfural	[34]
Palm fiber	I: 5.7 % H_2SO_4, 120 °C, 31 min II: 5.7 % H_2SO_4, 135 °C, 90 min	-	-	-	-	-	-	8.7	Furfural	[35]

[a]Two different batches. Roman numbers indicate different stages, WS: wheat straw, CS: corn stover. Adapted from [10].

3.1. Biotechnological relevance

The biotechnological relevance of FF and HMF comes from their inhibitory effect toward the sugar fermenting strains employed mainly in the production of bio-based ethanol. It has consequences on the process because the severity of the pretreatment must be balanced. This results in lignocellulosic biomass that is not sufficiently pretreated to avoid high concentrations of FF and HMF, and therefore the sugar concentration is also lower, decreasing the feasibility of the process. Moreover, it limits the fraction of accessible feedstocks, as some of them inherently produce high concentrations of furaldehydes due to the harsh pretreatment needed [10]. The relationship between the total concentration of furaldehydes and ethanol productivity is well established and has been widely studied [36]. Further, their inhibitory effect increases when both compounds are present in the hydrolysate, which is very common [10,37]. Therefore, the study of the metabolic effects of FF and HMF and their elimination from lignocellulosic biomass has attracted much attention in the last decades [38].

3.2. Metabolic effects

FF and HMF are toxic for most organisms. FF induces DNA mutations in different microorganisms and the formation of tumors and tissue damage in mice. HMF, nonetheless, seems to have a lower mutagenic effect, although its cytotoxic effect has been well established [10].

The biological bases of these effects have been studied in yeast and come mainly from the inhibition of the synthesis of protein and RNA, break-down of DNA, and damage to yeast cell walls and membranes [39–41]. There are several key enzymes inhibited by the presence of furaldehydes, such as the glycolytic enzymes hexokinase and triose-phosphate dehydrogenase [42], and the enzymes alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase [43], causing a decrease in cellular energy [10]. In contact with FF and HMF, the yeast enters in an extended lag phase to focus on the detoxification of the furaldehydes, and the growth only resumes once they are entirely metabolized (**Figure 4**).

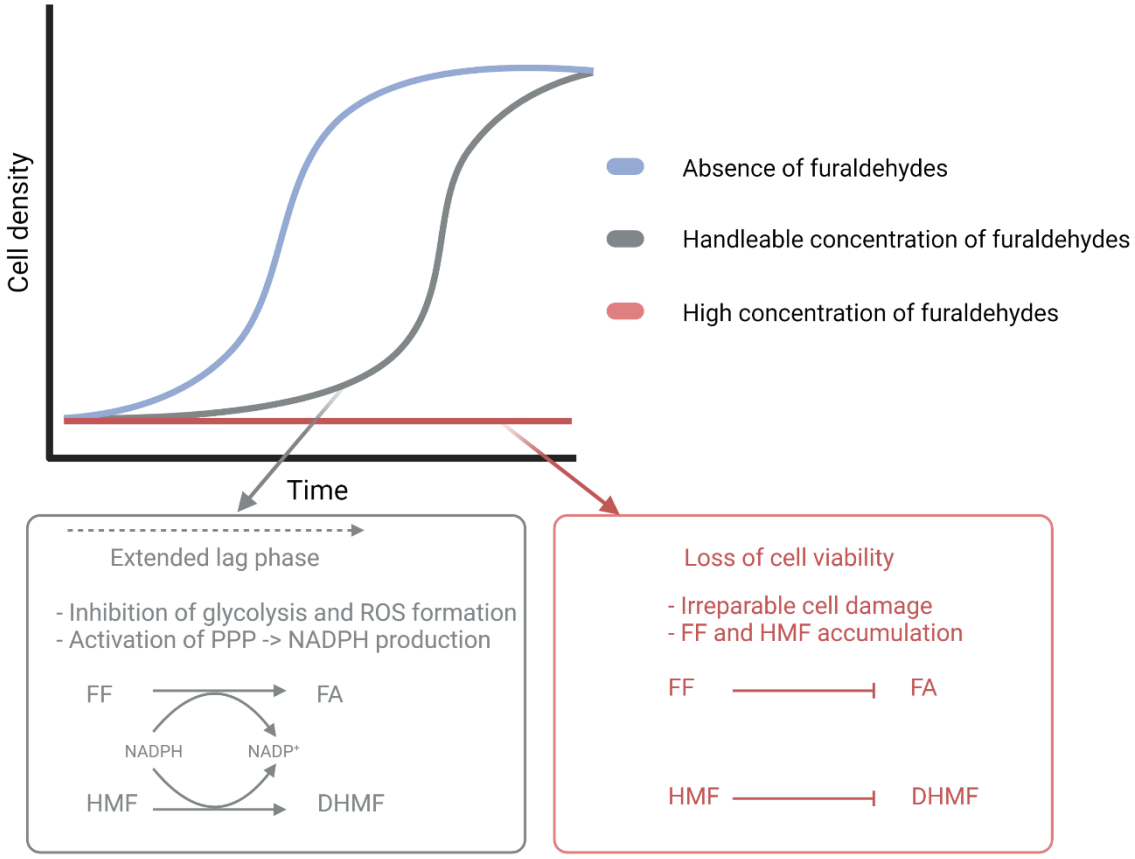

Figure 4. Effect of furaldehydes on cell growth. Adapted from [10,44]. Created with BioRender.com.

A correlation between FF and Reactive Oxygen Species (ROS) such as hydrogen peroxide, superoxide anion, and hydroxyl radicals has been reported, causing several damages to the cell, including: i) aggregation of tubular mitochondria, ii) fragmentation of vacuoles, iii) damage to cell membranes, iv) loss of actin cytoskeleton structure, and v) apoptosis, among others. Only if FF is completely metabolized, the cellular damage is repaired, and the amount of ROS decreases. The cells protect themselves from ROS by activating some genes of the Pentose Phosphate Pathway (PPP). This leads to the production of NADPH (**Figure 4**), an essential cofactor for both oxidative stress enzymes and enzymes that catalyze the transformation of FF into its less toxic derivative, furfuryl alcohol (FA), resulting from the reduction of the aldehyde group to a hydroxyl group [10,38,44–46]. The NAD(P)H shortage caused by the presence of FF and HMF affects the glycolysis, and glucose is not consumed for energy purposes until the concentration of the inhibitors decreases (**Figure 4**) [39,44]. The toxic effects of HMF toward the cells are similar, although lower, and have been long known. However, the lack of knowledge about the product resulting from its metabolization was limiting the studies. The conversion product was finally

identified as furan-2,5-dimethanol, or 2,5-di(hydroxymethyl)furan (DHMF), resulting from the reduction of the aldehyde group to a hydroxyl group [47]. Therefore, the detoxification mechanism is similar to that for FF and confirms that the toxic agent is the aldehyde group, not the furan ring or the hydroxyl group. The chemical structure of DHMF as an HMF conversion product by yeasts was first described by Liu *et al.* (2004) [47]. Lewis *et al.* (2008) [39] identified an aldehyde dehydrogenase (ALD4), an aldo-keto reductase (GRE3), and two alcohol dehydrogenases (ADH6 and ADH7) responsible for the conversion of both FF and HMF into their corresponding alcohol derivatives using NAD(P)H as a cofactor. Although NADH has also been shown to be a cofactor implied in reducing furaldehydes, it seems that only NADPH-dependent enzymes can perform the reaction efficiently [44]. The inhibitory effect of FF and HMF is increased when both compounds are present due to their synergistic effect and the competition for NAD(P)H [39]. It is worth mentioning that the knowledge on yeast adaptations to FF and HMF is still little and that further work is needed.

3.3. Effects on biotechnological processes

Furaldehydes constitute a significant problem in biotechnological processes starting from lignocellulosic biomass, such as ethanol, cellulase, or xylitol production [10,38]. Among the different processes, the production of bio-based ethanol from lignocellulosic hydrolysates is one of the most common and extensively studied. The lab and pilot-scale production of ethanol from lignocellulosic material has been successfully achieved. However, the industrial scale is not well-implanted yet. One of the main bottlenecks is precisely the presence of inhibitors formed during the pretreatment (**Figure 2**) [13,40]. FF and HMF are considered the major ones among all the inhibitors due to their acute toxicity and high concentrations [39,47,48]. The main effects on biotechnological relevant microorganisms are the extension of the lag phase, the reduction of the fermentation rate, and the loss of cell viability, depending on the concentration at which they are found (**Figure 4**). These cause a significant reduction in the productivity of the biotechnological processes [10,20,49].

3.4. Detoxification of the inhibitors

Several methods are reported to eliminate the inhibitors found in lignocellulosic hydrolysates: addition of chemicals (like alkali or reducing agents), liquid-solid extraction (like treatment with activated carbon or ion exchange), liquid-liquid extraction, or lignin-blocking agents. However, these methods have several drawbacks, like the addition of extra steps to the process, high costs of implementation, use of excessive fresh water and chemicals, and loss of fermentable sugars. Moreover, most of them are not sustainable due to the addition of chemicals, which is associated with fossil inputs [13,14,50,51].

Biological detoxification methods, also known as bioabatement, are an exciting alternative based on the use of microorganisms that completely metabolize the inhibitors or transform them into less toxic compounds that do not interfere with the fermentation process. Therefore, they are an environmentally friendly alternative [46,52–54]. However, several conditions must be met to be efficient and feasible: pH must be between 4-6, temperature between 25-50 °C, maximum 10 g/L inoculum size, and maximum 144 h detoxification time [55]. It can be approached in two different ways: detoxification of solid pretreated material before hydrolysis or detoxification of the pretreated liquid hydrolysate. The detoxification of the solid pretreated material has been achieved with promising results [56,57], with complete conversions of 5.5 mg/g DM of FF and 2.3 mg/g DM of HMF in 36 h using a heterozygous diploid structure of *A. resinae* ZN1 [58]. However, little progress has been achieved in the detoxification of inhibitors in lignocellulosic hydrolysates. The ascomycete *Coniochaeta ligniaria* NRRL30616 partially removed a total concentration of furaldehydes up to 3.6 g/L within 24 h [53,59,60]. The bacterium *Bordetella sp.* BTIITR removed a low concentration of furaldehydes (1.5 g/L) within 26 h [37]. Later, the same microorganism was immobilized within chitosan beds with the advantage of reusability, partially degrading 2 g/L of furaldehydes within 20 h [61]. Another bacterium, *Enterobacter sp.* FDS8 partially removed 2.1 g/L of furaldehydes within 3 h, showing the higher degradation rates reported [62]. However, all these processes were performed in a separate extra step, which poses a major drawback. Some studies reported simultaneous detoxification and fermentation of the lignocellulosic hydrolysates, mainly using strains of *S. cerevisiae* under a low concentration of furaldehydes (< 2 g/L) and with long detoxification times (>16 h) [63–65]. The co-culture of two different microorganisms in which one is specialized in the detoxification of the inhibitors and the other in

the fermentation of the sugars is an exciting alternative. However, reports in the literature are scarce because of some inherent difficulties, such as the competition among the different sugars between the microorganisms, which must be overcome [55]. There is one example of a co-culture between *A. nidulans* FLZ10 and *S. cerevisiae*, in which the co-cultivation of both microorganisms allowed a 3-fold increase in ethanol productivity. However, 72 h were needed to detoxify a total concentration of furaldehydes of 0.4 g/L, among other inhibitors [66]. Therefore, biodetoxification of lignocellulosic hydrolysates needs further investigation and development for several reasons: i) the detoxification rates and the concentration of inhibitors evaluated are low, ii) there may be consumption of fermentable sugars by the microorganism during the detoxification process, leading to a lower amount of sugars available for the fermentation, iii) the inhibitors are often not wholly metabolized nor transformed into less toxic derivatives, and v) the detoxification is usually performed in a separate step, reducing the productivity of the process.

4. Furfural and 5-hydroxymethylfurfural as platform chemicals

Platform chemicals are molecules with one or more functional groups that can be transformed into several added-value derivatives of interest with diverse final applications [67]. As stated before, FF and HMF are considered valuable platform chemicals and belong to the list of the top "10+4" added-value bio-based chemicals by the US Department of Energy. The study of the metabolic effects of FF and HMF and the degradation pathways that lead to their fewer toxic derivatives has opened new lines of investigation on the upgrade of FF and HMF. Due to the presence of one aldehyde group (FF) and one hydroxyl group and one aldehyde group (HMF), there is a wide range of derivatives that can be obtained through oxidation and reduction reactions (**Figure 5**), which have a higher market value than that of FF and HMF [20,23,68,69].

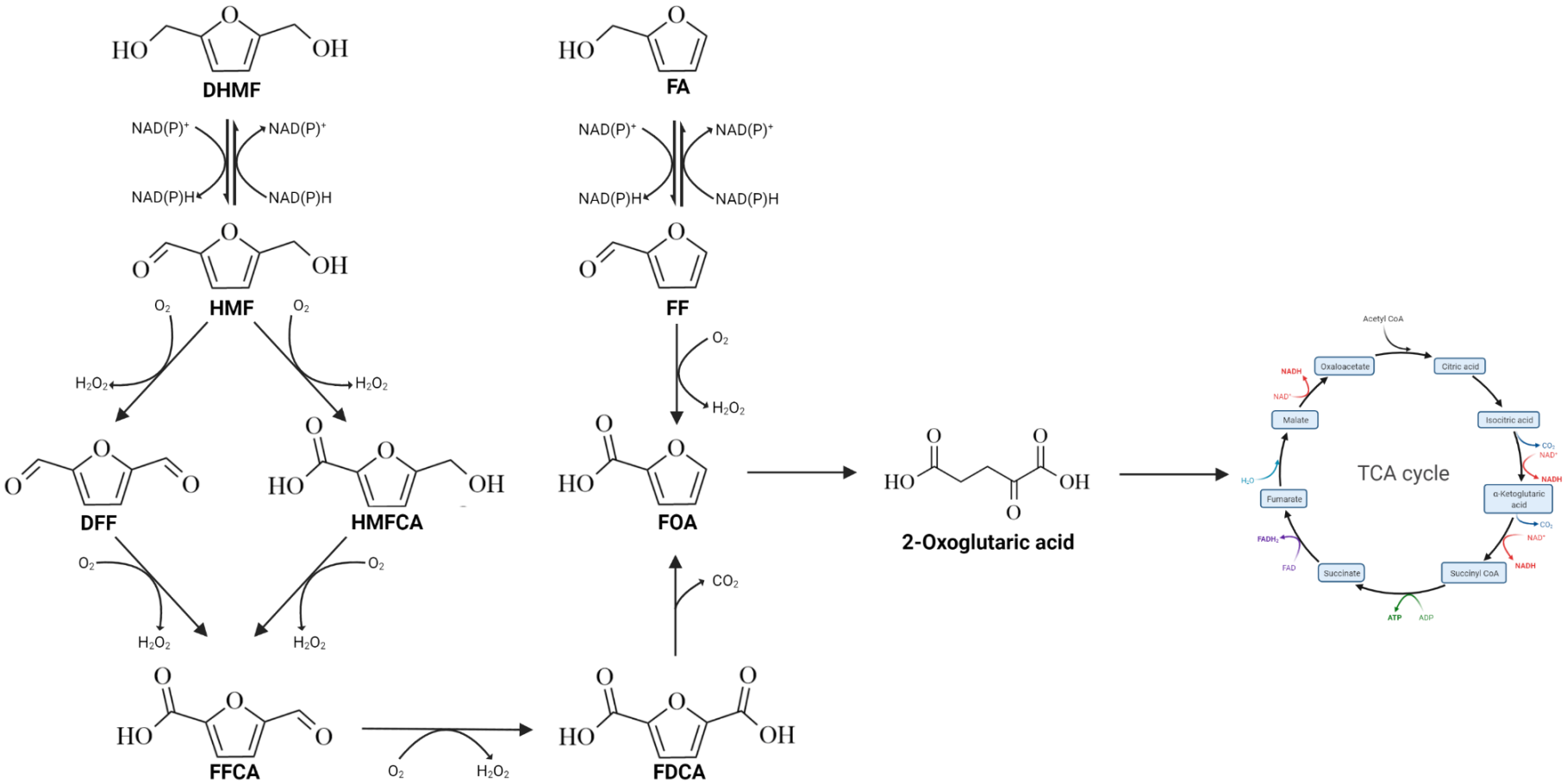

Figure 5. Metabolic pathways of FF and HMF [20,68,70,71]. Created with BioRender.com.

4.1. Furfural and 5-hydroxymethylfurfural derivatives

The reduction of the aldehyde group of FF and HMF leads to the formation of FA and DHMF, respectively (**Figure 5**). Besides being fewer toxic derivatives of FF and HMF, FA and DHMF are high-value compounds of interest for different industries.

FA is a high-value building block that can be transformed into different products such as 1,2-pentanediol, 1,5-pentanediol, 2-methylfuran, 2-methyltetrahydrofuran, and tetrahydrofurfuryl alcohol, and is an intermediate in the synthesis of vitamin C, lysine, plasticizers, lubricants, and dispersing agents. In addition, it serves as a monomer to produce furan resins, which can replace phenolic resins, and is used as a reactive solvent and viscosity reducer for phenolic and epoxy resins in the manufacture of polyurethane foams [28,72].

DHMF is a high-value building block mainly used as an intermediate to synthesize fibers, resins, foams, drugs, bio-based polymers, fuel additives, and crown ethers [73]. Due to the presence of two double bonds and two hydroxymethyl groups, it has a high potential as a substitute for petroleum-based aromatic diols in polymerization reactions for the manufacturing of polyurethanes and polyesters [74,75].

Although the common detoxification pathways involve the reduction of FF and HMF into their corresponding alcohol derivatives, these can be reoxidized into FF or HMF under aerobic conditions. Subsequently, FF can be oxidized into furoic acid (FOA), and HMF can be oxidized into 2,5-diformylfuran (DFF), 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), 5-formyl-2-furancarboxylic acid (FFCA), and finally 2,5-furandicarboxylic acid (FDCA), all of them promising building blocks. FDCA can be further decarboxylated to FOA, joining the FF metabolism. FOA can enter the tricarboxylic acid cycle (TCA) through conversion into 2-oxoglutaric acid in six steps (**Figure 5**) [68]. FOA is the oxidation product of furfural, and it has applications in the agrochemical and pharmaceutical industries [76]. The oxidation of the hydroxyl group of HMF leads to DFF formation, a dialdehyde that is used as a precursor in the synthesis of polymers, fluorescent materials, bio-based polyurethane thermosets, and pharmaceuticals, among others [20,68,77–80]. Instead of oxidizing the hydroxyl group to an aldehyde group, HMFCA results from the oxidation of the aldehyde group present in HMF to a carboxylic group. It has applications mainly in the synthesis of polymers and therapeutics and has also been described as an antitumor

agent [20,68]. FFCA can be produced either by the oxidation of one aldehyde group of DFF or by the oxidation of the hydroxyl group of HMFCA and has applications in the synthesis of resins and surfactants [48,68]. FDCA results from the complete oxidation of the two functional groups of HMF, yielding a symmetric compound with two carboxyl groups. It is a building block for the synthesis of pharmaceuticals, polyamides, and coordination compounds. Moreover, it has potential as a substitute for oil-derived terephthalic acid (TPA). Polyethylene furanoate (PEF), a candidate to substitute the petroleum-based polyethylene terephthalate (PET), can be produced through the polymerization of FDCA and ethylene glycol [20,68,81].

5. Biocatalytic valorization of furfural and 5-hydroxymethylfurfural

The chemocatalytic conversion of FF and HMF into their derivatives has been widely studied [26,69,82,83]; however, biocatalytic approaches offer many advantages such as environmental friendliness, milder reaction conditions, use of aqueous solvents and biodegradable catalysts, higher selectivity, and lower energy costs [20,68,81]. Biocatalysis is the use of biological systems, such as enzymes and microorganisms, to perform chemical reactions. It can be divided into two main types: growth-associated whole-cell catalysis, where the substrate is used for the growth of the cells and the reaction of interest (for example, fermentation of sugars into ethanol); and biocatalytic processes, where the production of the biocatalyst and the reaction step are performed separately. In the case of FF and HMF, biocatalytic processes are the way to go. They can be further divided into two subcategories: whole-cell biocatalysis and enzyme biocatalysis (isolated or immobilized). Each type has its advantages, as shown in **Table 2**, but both have been widely applied to produce different products and are well-established. The whole-cell transformation of FF and HMF is challenging due to the high toxicity of both compounds towards the cells. Large inoculum sizes increase the tolerance of the cells towards FF and HMF; however, they pose a significant increase in the process cost, and for this reason, they are a crucial parameter to consider. Combining different methodologies leads to hybrid processes, which provide hope for efficient and sustainable biocatalysis by taking the best of each conversion approach [18,84–87]. In this section, the potential of biocatalysis for the preparation of FF and HMF derivatives is discussed.

Table 2. Advantages of the different biocatalytic processes [68,81,88,89].

Whole cells	Enzymes
Inherent presence of different enzymes	Highly selective and efficient
Inherent presence of different cofactors	More control of the reaction
Inexpensive	Easy product recovery
No need for separation and purification steps	Good for fine chemical production
More robust and stable	
Good for bulk chemical production	

5.1. Reduction

5.1.1. Furfuryl alcohol (FA) and 2,5-di(hydroxymethyl)furan (DHMF)

The most recent and relevant biocatalytic approaches for the reduction of FF and HMF to their corresponding alcohol derivatives are summarized in **Table 3**.

Table 3. Biocatalytic reduction of HMF and FF.

Product	Catalyst	[FF/HMF] (mM)	Time (h)	Inoculum size (g/L)	Yield (%)	Ref.
FA	*E. coli* CCZU-K14	200	24	100 (ww)	100	[90]
	E. coli CCZU-A13	300	12	100 (ww)	74	[91]
	B. coagulans NL01	42	3	9 (dw)	97	[92]
	M. guilliermondii SC1103	200	7	50 (ww)	81	[93]
	S. cerevisiae NL22	62	8	14 (dw)	98	[72]
DHMF	*M. guilliermondii* SC1103	100	12	20 (ww)	86	[94]
		200 (FB)	24	20 (ww)	95	
		300	24	50 g/L (ww)	82	[95]
	S. cerevisiae	250	24	60 (ww)	94	[96]
	E. coli CCZU-K14	200	72	100 (ww)	91	[97]
	A. subglaciale F134	180	12	200 (ww)	82	[98]
		500 (FB)	15	200 (ww)	86	
	B.contaminans NJPI-15	100	6	20 (ww)	95	[99]
		700 (FB)	48	20 (ww)	94	

FB: Fed-batch, dw: dry weight, ww: wet weight

The biocatalytic reduction of FF and HMF has been achieved by several whole cell catalysts with encouraging results, most of them described in the last years (2017-2021). It is worth noting that there are two microorganisms, *Meyerozyma guilliermondii* SC1103 and *Escherichia coli* CCZU-

K14, that showed the capability to reduce both compounds [90,93,94,97]. Zhang *et. al.* [93] achieved high FA yields (81 %) within a short time (7 h) starting from 200 mM FF by using whole cells of *M. guilliermondii* SC1103. Li *et al.* (2017) [94] were the first ones to report an efficient reduction of HMF into DHMF by using whole cells of the same strain, achieving quantitative yields for a concentration of substrate of 200 mM in a fed-batch approach (4 cycles). Fed-batch represents an attractive alternative due to the toxic effects of both FF and HMF toward the cells and has also been employed to prepare other derivatives. Later, they acclimatized and immobilized the same strain in calcium alginate beads, which increased the catalytic activities and the HMF-tolerance level of the cells [95]. The alcohol dehydrogenases (ADHs) from *M. guilliermondii* were then heterologously expressed in *S. cerevisiae*, and quantitative yields were obtained for a concentration of HMF of 250 mM [96]. *Escherichia coli* CCZU-K14 has also shown promising results in reducing FF and HMF; however, high inoculum sizes (100 g/L) and expensive co-factors were added to the processes, significantly increasing the cost [97]. Recently, two novel whole-cell biocatalysts, *A. subglaciale* F134 [98] and *B. contaminans* NJPI-15 [99], have been reported achieving the highest concentration of DHMF in the media by using a fed-batch approach. Although promising results have been obtained in the reduction of FF and HMF, the inoculum sizes used are too high to be competitive (from 20 to 200 g/L), adding a high cost to the process. Moreover, most of the processes use high glucose concentrations as co-factor for the efficient regeneration of NAD(P)H. The prices of glucose and DHMF are expected to be of the same order. Therefore, further investigation is needed in the reduction of FF and HMF. Alternatively, the use of lignocellulosic hydrolysates containing high concentrations of FF and HMF may be an exciting approach because it would benefit from both the sugars and the furaldehydes present [20]. Even more interesting would be reducing both compounds with negligible sugar consumption from the lignocellulosic hydrolysates, therefore letting the sugars available for the posterior fermentation into ethanol.

5.2. Oxidation

The most recent and relevant biocatalytic approaches for the oxidation of FF and HMF to their corresponding derivatives are summarized in **Table 4**.

Table 4. Biocatalytic oxidation of FF and HMF.

Product	Catalyst	[FF/HMF] (mM)	Time (h)	Inoculum size (g/L) / Enzyme dosage	Yield (%)	Ref.
FOA	*E. coli* BH	30	200	100 (ww)	100	[76]
	E. coli CtSAPDH	50	5	12.5 (ww)	100	[100]
	P. putida KT2440	200	3	10.5 (dw)	97.5	[101]
DFF	GO	30	72	2 U/mL	2	[102]
	GO + catalase	30	72	2 U/mL (GO), 1700 U/mL (catalase)	23	
	GO + HRP	30	72	2 U/mL (GO), 112 U/mL (HRP)	46	
	GO + catalase + HRP	30	96	4 U/mL U (GO), 1700 U/mL (catalase), 112 U/mL (HRP)	92	
	GO + catalase + HRP	200	168	4 U/mL (GO), 400 U/mL (catalase), 100 U/mL (HRP)	54	[103]
	GO + catalase + HRP[i]	200	168	-	96	
HMFCA	*C. testosteroni* SC1588	160	36	30 (ww)	98	[104]
	D. wulumuqiensis R12	300	36	200 (ww)	90	[105]
		600 (FB)	20	200 (ww)	84	
	P. aeruginosa PC-1	100	6	5 (-)	90	[106]
		800 (FB)	58	5 (-)	90	
FFCA	HMFO	2	5	5 µM	92	[107]
	GO + ADH	100	48	3.2 µM (GO) + 66 µM (ADH)	97	[108]
FDCA	GOM_{3-5}, PaoABC, catalase, HRP	100	6	130 µL GO + 1 µL PaoABC + 33 µL catalase + 70 µL HRP	100	[109]
	AAO + catalase	1.5	144	1.5 µM (AAO) + 2-5 U/mL (catalase)	97	[71]
	R. ornithinolytica BF60 + HmfH	150	72	45 (-)	93	[110]
	E. coli + VDH1 + HmfH	150	30	50 (ww)	96	[111]
	P. putida S12 + HMFO + *HMFH* + *HMFT1*	250	24	20 (OD_{600})	78	[112]

FB: Fed-batch, dw: dry weight, ww: wet weight, GO: Galactose Oxidase, HRP: Horseradish Peroxidase, [i] Immobilized enzymes, HMFCO: 5-Hydroxymethylfurfural Oxidase, ADH: alcohol dehydrogenase, PaoABC: periplasmic aldehyde oxidase, AAO: aryl-alcohol oxidase, HmfH: HMF oxidoreductase, VDH1: vanillin dehydrogenase, HMFT1: HMF transporter.

5.2.1 Furoic acid (FOA)

Several whole-cell catalysts have achieved the oxidation of FF to FOA. Interestingly, Zheng *et al.* (2020) found that the addition of HMF during the exponential growth phase substantially increased the biocatalytic capacity of *P. putida* KT2440, allowing the production of FOA with high yields in a fed-batch approach (3 cycles) for a FF concentration of 200 mM within just 3 h [101]. Moreover, a relatively small inoculum size was used (10.5 g/L), showing encouraging results for FOA production.

5.2.2. 2,5-Diformylfuran (DFF)

The biocatalytic production of DFF is still in its early stage and has only been achieved enzymatically, mainly by a combination of three enzymes: galactose oxidase (GO), catalase, and horseradish peroxidase (HRP). Qin *et al.* (2015) achieved yields >90% through a combination of the three enzymes for a concentration of HMF of 30 mM [102]. Later, Wu *et al.* (2019) achieved yields >95 % using a concentration of HMF of 200 mM through an increase of the enzyme dosage and immobilization of the enzymes in $Cu_3(PO_4)_2$ nanoflowers [103]. However, the productivity achieved was low due to the long reaction times needed (168 h). In transformations like that, where more than one enzyme is needed, whole cells present as an attractive alternative. The whole-cell transformation of HMF into DFF would be theoretically preferable to the enzymatic catalysis thanks to the inherent presence of the different enzymes needed, which would significantly reduce the cost of the process. Moreover, the low productivities reported call for more research.

5.2.3. 5-Hydroxymethyl-2-furancarboxylic acid (HMFCA)

The most promising HMFCA production approaches have been lately achieved by using whole cells. Zhang *et al.* (2017) transformed 160 mM HMF into HMFCA with quantitative yields by adding 20 mM histidine and pH tuning at 7 every 24 h using the *C. testosteroni* SC1588 strain [104]. Cang *et al.* (2019) increased the substrate concentration up to 300 mM and produced HMFCA with a yield of 90% with the tuning of the pH at 7 every 3 h by using the whole cells of *D. wulumuqiensis* R12 [105]. Pan *et al.* (2020) achieved a higher concentration of product in the

media (721 mM) by a fed-batch strategy (8 cycles) using the novel whole-cell biocatalyst *P. aeruginosa* PC-1 with a low inoculum size (5 g/L) [106].

5.2.4. 5-Formyl-2-furancarboxylic acid (FFCA)

FFCA has a highly oxidized but incomplete state, and therefore the synthesis of FFCA with high selectivity is challenging due to the overoxidation into FDCA. For this reason, it is hard to produce with a whole-cell approach, and there are no reports to date. The enzyme 5-hydroxymethyl oxidase (HMFO) showed high efficiency in the oxidation of 2 mM HMF into FFCA [107]. Jia *et al.* (2019) reported a dual-enzyme cascade system to produce FFCA through oxidation of HMF and DFF using GO and an alcohol dehydrogenase (ADH) [108]. A high yield (97%) was obtained starting from 100 mM HMF within 48 h, which may be a promising methodology for FFCA production.

5.2.5. 2,5-Furandicarboxylic acid (FDCA)

The production of FDCA has been approached in several ways: by combining different enzymes in a multistep process, by using a single enzyme that can catalyze the three-step conversion, and by using whole cells. McKenna *et al.* (2017) developed a continuous one-pot reaction using a galactose oxidase mutant (GOM_{3-5}), periplasmic aldehyde oxidase (PaoABC), catalase, and horseradish peroxidase, obtaining a total conversion of 100 mM HMF within 6 h [109]. Serrano *et al.* (2019) described an aryl alcohol oxidase (AAO) able to oxidize HMF to FFCA through DFF [71]. When adding catalase to the reaction, FDCA was produced, indicating that the H_2O_2 produced by the previous oxidations had deleterious effects on FDCA production. As in the case of DFF, whole cells present as an interesting approach for FDCA production for several reasons: i) requirement of different co-factors and enzymes, ii) inherent presence of catalases that catalyze the transformation of the inhibitory H_2O_2 generated by the oxidative process into O_2, and iii) they provide a barrier against aeration and reactive compounds. As opposed to DFF, there are several reports on the whole-cell production of FDCA. Recently, the microorganisms *R. ornithinolytica, E. coli, and P. putida* have been genetically engineered by including or deleting key enzymes to enhance the production of FDCA, allowing the production of FDCA with high yields starting from high concentrations of HMF (150-250 mM) using moderate inoculum sizes (from 20 to 50 g/L).

Objectives of this Doctoral Thesis

The main objective of this Doctoral Thesis is to contribute to the biocatalytic valorization of furfural (FF) and 5-hydroxymethylfurfural (HMF). Specifically:

I. Optimize the enzyme-catalyzed oxidation of HMF to DFF.
   - Evaluate the influence of the different enzymes on the reaction.
   - Assess the interactions between the concentration of substrate, enzyme dosage, and agitation speed.
   - Determine the kinetic parameters.

II. Explore novel whole-cell catalysts with the capability to produce the different derivatives, focusing on the preparation of DFF and DHMF.
   - Find novel whole-cell catalysts with the capability to produce DFF with high selectivities, avoiding its oxidation to FFCA and FDCA.
   - Find novel whole-cell catalysts with the capability to produce DHMF.
   - Optimize the whole-cell oxidation and reduction processes.

III. Find novel biological approaches for the detoxification of lignocellulosic hydrolysates containing high concentrations of FF and HMF in the production of biobased ethanol.
   - Ascertain the feasibility of the whole-cell catalysts found in objective II to detoxify lignocellulosic hydrolysates containing high concentrations of FF and HMF.
   - Establish synergistic interactions between the novel biocatalysts and the ethanol fermenting strains.
   - Improve the ethanol yield and selectivity when using lignocellulosic hydrolysates containing moderate concentrations of FF and HMF.
   - Carry out the fermentation using lignocellulosic hydrolysates in which the fermentation is completely inhibited when the detoxification is not performed.

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CHAPTER 2. GENERAL METHODOLOGY

This Chapter summarizes and represents the general experimental methods used in this Doctoral Thesis, which are further detailed in their corresponding Chapters.

1. Enzymatic reactions

1.1. General procedure

In a standard experiment, HMF was added to an aqueous solution (1 mL deionized H_2O, pH 7) at a specified concentration. Then, specified amounts of catalase, horseradish peroxidase (HRP), and galactose oxidase (GO) were added in this order. The reaction started by the addition of GO, and it was incubated in a 15 mL shake flask at 25 °C and specified agitation speeds (**Figure 1**).

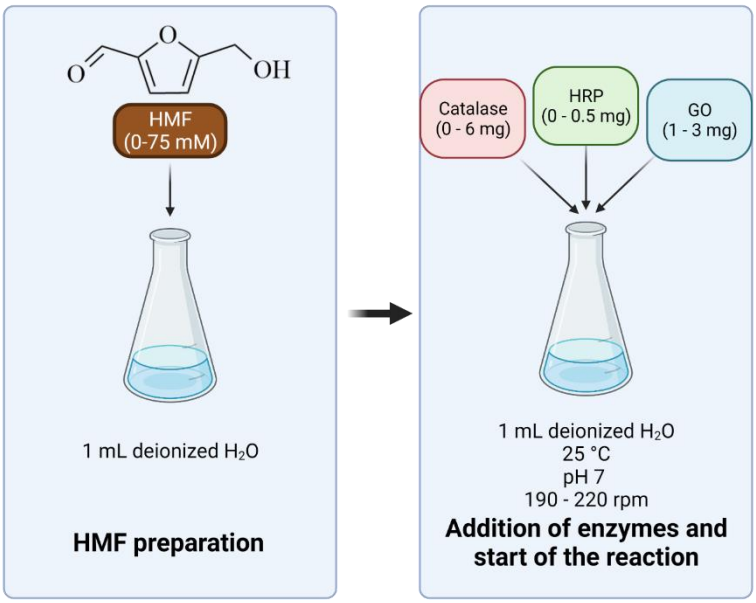

Figure 1. Enzymatic reactions. Created with BioRender.com.

1.2. GC Analysis

At specified reaction times, ethyl acetate was added to the reaction mixture, and the compounds were extracted from the aqueous phase. The GC-FID analyses were performed with an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with an ultra-inert splitless liner containing a piece of glass wool coupled to an FID detector. For the chromatographic separation, a DB-5MS (30 m x 0.25 mm i.d.; 0.25 µm film thickness) column from Agilent was used at a constant flow of 1 mL/min using helium as carrier gas. Injector temperature was 250 °C, and the oven program

was 70 °C (held for 1 min) to 140 °C at 15 °C/min and to 300 °C at 25 °C/min (held for 5 min). Calibration curves were performed periodically for the quantification of the compounds using 5-acetoxymethyl-2-furaldehyde as internal standard.

2. Whole-cell transformations

2.1. General procedure

In a standard experiment, *Fusarium* species were incubated on malt extract agar (MEA: 20 g/L glucose, 20 g/L malt extract, 1 g/L peptone from soybean, 15 g/L agar) for seven days a 28 °C. Then, they were inoculated into flasks containing malt extract by adding either fungal discs (3 or 6) or a solution of spores (1-10 mL, $4*10^6$ spores /mL). The flasks were incubated in a rotatory shaker at 28 °C and 160 rpm. After three days of growth, HMF was added at a specified concentration to the media (**Figure 2**).

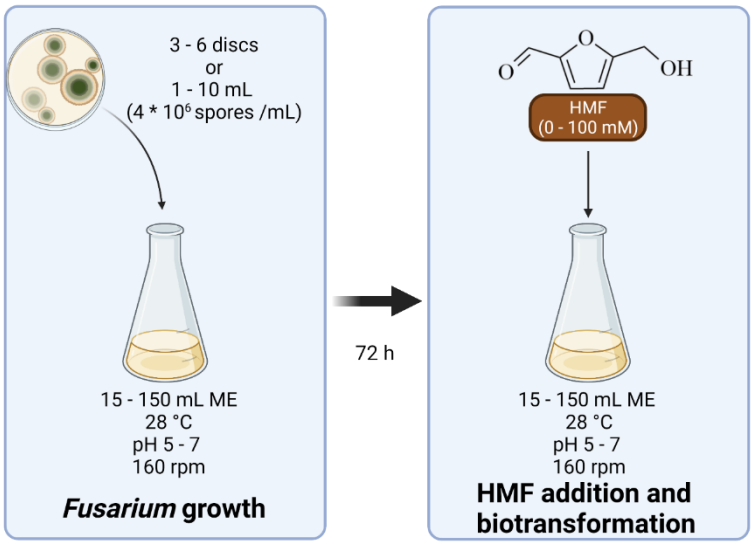

Figure 2. Whole cell transformations. Created with BioRender.com.

2.2. Recovery of the compounds from the reaction media

At the end of the reaction, the media was extracted three times with an equal volume of ethyl acetate. The organic extracts were joined and dried over Na_2SO_4 anh. The mixture was filtrated, and the solvent was evaporated to dryness (**Figure 3**).

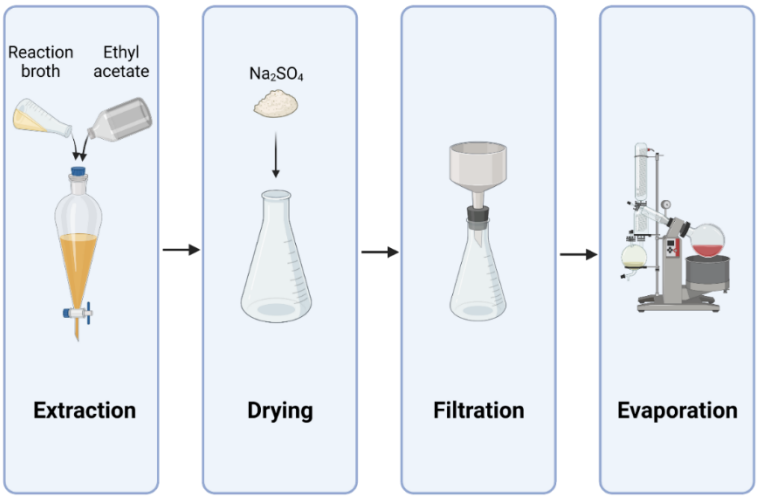

Figure 3. Recovery of the compounds from the reaction media. Created with BioRender.com.

2.3. Bioreactor

F. striatum was inoculated into the bioreactor containing 1.3 L of ME by adding an aqueous suspension of spores (87 mL, 4 * 10^6 spores /mL). The working conditions were 28 °C, 160 rpm, and pH 7. After three days of growth, HMF was added to the media (**Figure 4**).

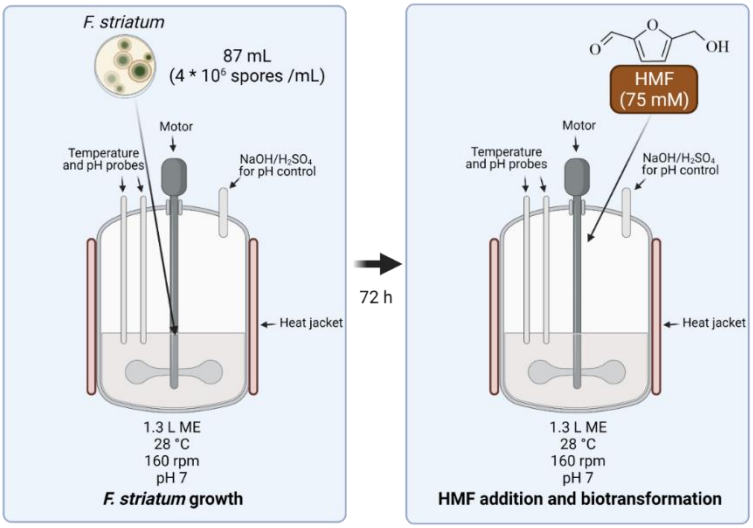

Figure 4. Whole-cell transformations carried out in the bioreactor. Created with BioRender.com.

2.4. GC Analysis

Aqueous aliquots were extracted at selected reaction times. The compounds were extracted from the aqueous aliquots using ethyl acetate. GC-FID analyses were performed with an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with an ultra-inert splitless liner containing a piece of glass wool coupled to an FID detector. For the chromatographic separation, an FFAP (30 m x 0.25 mm i.d.; 0.25 µm film thickness) column from Agilent was used at a constant flow of 1 mL/min using hydrogen as carrier gas. Injector temperature was 230 °C, and the oven program was 100 °C (held for 1 min) to 240 °C at 20 °C/min (held for 5 min). Calibration curves were performed periodically for the quantification of the compounds using 5-acetoxymethyl-2-furaldehyde as internal standard.

3. Simultaneous detoxification and fermentation of lignocellulosic hydrolysates

3.1. General procedure

In a standard experiment, the inoculums of *S. cerevisiae* and *F. striatum* were prepared as follows:

A colony of *S. cerevisiae* CEN.PK XXX grown on an YPX agar plate (yeast extract 10 g/L, peptone from casein 20 g/L, xylose 20 g/L, 15 g/L agar) was incubated in a 250 mL shake flask containing 100 mL of liquid YPX media for 36 h at 30 °C 30 °C and 180 rpm. The cell culture was then propagated to a 250 mL shake flask containing 100 mL of wheat straw hydrolysate (36 g/L glucose, 18 g/L xylose) previously spiked with specified concentrations of FF (0.55 – 2.5 g/L) and HMF (0 – 5 g/L) to reach an initial inoculum size of 0.5 g/L (dry weight).

F. striatum was transferred to 250 mL shake flasks containing 100 mL of liquid ME (20 g/L glucose, 20 g/L malt extract, 1 g/L, peptone from soybean) by the addition of ten 8 mm fungal discs from the agar plates and incubated in a rotatory shaker at 30 °C and 180 rpm for 5 days. The cell culture was centrifuged, the supernatant discarded, and the cells transferred to a 250 mL shake flask containing 100 mL of wheat straw hydrolysate (36 g/L glucose, 18 g/L xylose) previously spiked

with specified concentrations of FF (0.55 – 2.5 g/L) and HMF (0 – 5 g/L) to reach different initial inoculum sizes ranging from 0.22 to 10 g/L (dry weight).

The simultaneous detoxification and fermentation started with the addition of both inoculums to the wheat straw hydrolysate and was incubated at 30 °C and 180 rpm (**Figure 5**).

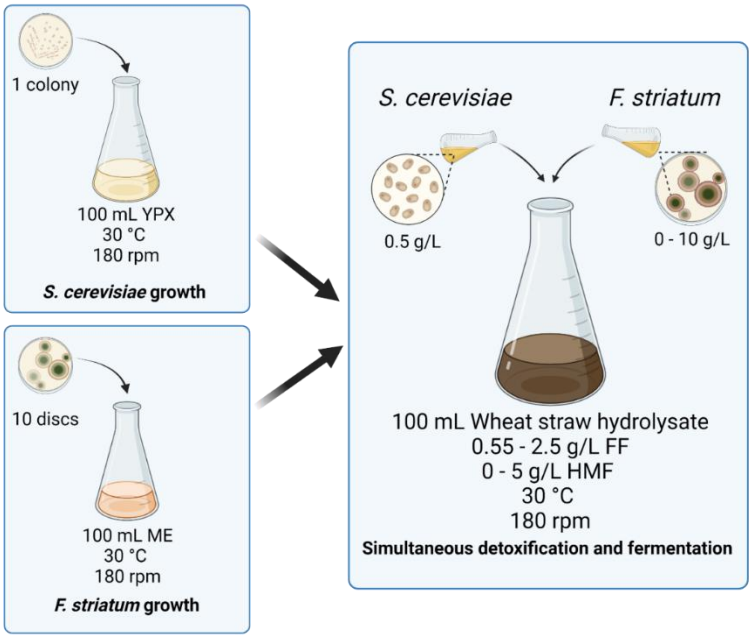

Figure 5. Simultaneous detoxification and fermentation of lignocellulosic hydrolysates. Created with BioRender.com.

3.2. Bioreactor

The simultaneous detoxification and fermentation was carried out in a bioreactor containing 1.5 L of the wheat straw hydrolysate spiked with 3.5 g/L of HMF and 2.5 g/L of FF. *S. cerevisiae* and *F. striatum* were inoculated to the media, and the fermentation was operated at pH 6.0 (using 2 M NaOH and 2 M H_2SO to maintain the required pH), at 30 °C (using a heat jacket and a cooling finger), and at a stirring rate of 450 rpm (using two six-bladed Rushton impellers) (**Figure 6**). A control experiment was performed with the same experimental conditions without the addition of *F. striatum*.

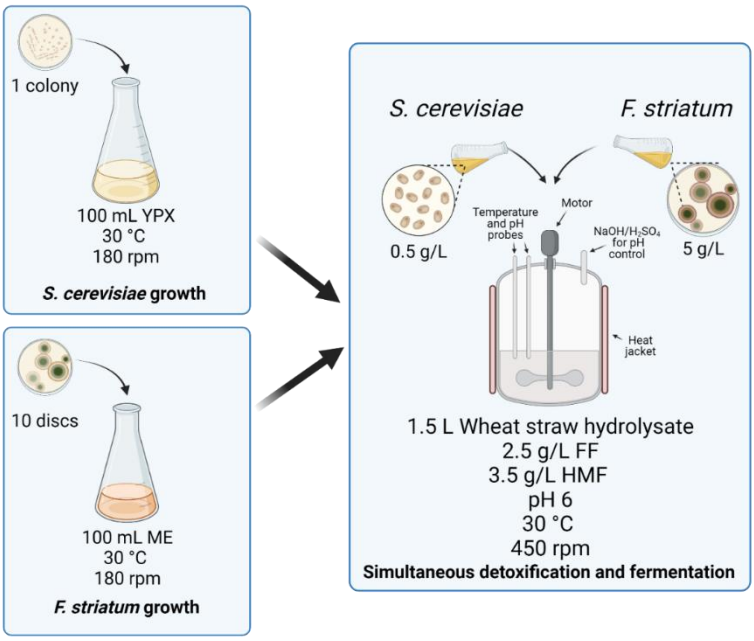

Figure 6. Simultaneous detoxification and fermentation of lignocellulosic hydrolysates carried out in the bioreactor. Created with BioRender.com.

3.3. HPLC / GC Analysis

A sample of 1.5 mL was withdrawn from the shake flasks or the bioreactors at selected fermentation times, filtrated through a 0.20 µm cellulose acetate filter (Labsolute, Renningen, Germany) and stored at -22 °C until analyzed.

The concentrations of glucose, xylose, ethanol, HMF and FF were determined by an Ultimate 3000 HPLC instrument (Thermo Scientific, Massachusets, USA) equipped with an Aminex HPX-87H column (7.8 x 300 mm; 9 µm, BIORAD, California, USA), 4 UV/VIS channels and a refractive index (RI) detector (ERC RefractoMax 520, Prague, Czech Republic). A sample volume of 950 µL was acidified with 50 µL of 5 M H_2SO_4 prior to the injection. The injection volume was 5 µL. The mobile phase consisted of 5 mM H_2SO_4 and the elution was in isocratic mode, with a flow rate of 0.6 mL/min at 50 °C. The method lasted 55 min.

The concentrations of furfuryl alcohol (FA) and 2,5-di(hydroxymethyl)furan (DHMF) were determined by an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with an ultra-inert splitless liner containing a piece of glass wool coupled to an FID detector. For the chromatographic separation, an FFAP (30 m x 0.25 mm i.d.; 0.25 µm film thickness) column from Agilent was used at a constant flow rate of 1 mL/min using hydrogen as carrier gas. Injector temperature was 230 °C, and the oven temperature program was started at 100 °C (held for

1 min), and then increased to 240 °C at a rate of 20 °C/min (held for 5 min). The compounds were extracted from the aqueous samples using ethyl acetate.

4. Statistical analysis

Statistical analyses were assessed using the software JMP Pro 14 (SAS). The results obtained were subjected to analysis of variance (ANOVA). Statistical significance was assessed with the *p*-value in Fisher's test with a 95% confidence level. Means were compared with Tukey HSD test, and significant differences are indicated with different letters. All experiments were conducted at least in duplicate, and the values are expressed as the means ± standard deviations when applicable. The assumption of normality was tested using Shapiro–Wilk normality test.

CHAPTER 3. EXPERIMENTAL, RESULTS, AND DISCUSSION

3.1. Enzymatic oxidation of 5-hydroxymethylfurfural to 2,5-diformylfuran

Enzymatic oxidation of 5-hydroxymethylfurfural to 2,5-diformylfuran

Abstract

The compound 2,5-diformylfuran (DFF), obtained through oxidation of biomass-derived 5-hydroxymethylfurfural (HMF), is a high-value building block of interest for different industries. To date, the efficient biocatalytic preparation of DFF has been mainly achieved by the combination of the enzymes galactose oxidase (GO), catalase, and horseradish peroxidase (HRP). However, the literature is scarce, and the process is still far from being viable due to the high cost of the enzymes and the low productivities obtained. In this work, different reaction parameters, such as the influence of the different enzymes and the ratio among them, were first evaluated. The effects of the HMF concentration, the enzyme dosage, and the agitation speed over the final DFF yields were assessed through Response Surface Methodology using a Box-Behnken design, allowing the estimation of the minimum enzyme dosage needed for a specific concentration of the substrate to obtain high yields. Yields >90 % were achieved without using HRP when the catalase dosage was increased, resulting in a cheaper process. Finally, the kinetic parameters of the enzyme were calculated, and a perfect fit was found for the Michaelis-Menten model with the experimental data obtained.

Keywords: Galactose oxidase, Catalase, Horseradish peroxidase, Biocatalysis, 2,5-Diformylfuran

1. Introduction

The production of fuels and chemicals from biobased renewable feedstocks is of great interest due to the current dependence on fossil resources [1]. The compound 5-hydroxymethylfurfural (HMF) can be obtained from lignocellulosic biomass through dehydration of sugars, and it is considered as one of the "Top 10+4" list of biobased chemicals according to the U.S Department of Energy (DOE) [2]. Several derivatives with a higher market value than that of HMF can be prepared through oxidation/reduction of the hydroxyl and aldehyde groups present in HMF: 2,5-di(hydroxymethyl)furan (DHMF), 2,5-diformylfuran (DFF), 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), 5-formyl-2-furancarboxylic (FFCA) acid, and 2,5-furandicarboxylic acid (FDCA) [3]. DFF is obtained by oxidation of the hydroxyl group of HMF to an aldehyde group, avoiding further oxidation of the aldehyde groups to the corresponding carboxylic acids. This dialdehyde has a wide range of applications as a building block for the production of polymers, pharmaceuticals, fluorescent materials, and polyurethane thermosets, among others [4–7].

Several chemical catalytic methods have been reported for the oxidation of HMF into DFF [2]; however, the biocatalytic synthesis is still in its early stage, and the literature is scarce. Biocatalytic transformations are performed at milder reaction conditions than chemical ones, without the addition of high-cost chemicals, and present higher selectivity. For this reason, they are getting considerable attention in the valorization of HMF and pose an attractive alternative to the current chemical pathways [8,9]. Galactose oxidase (GO) is a copper-containing free radical enzyme described by Cooper *et al*. (1959) that catalyzes the oxidation of primary alcohols to aldehydes [10]. The catalytic reaction consists of oxidative and reductive half-reactions using O_2 as electron acceptor and producing H_2O_2, which inactivates the enzyme [11]. The addition of catalase, which transforms the H_2O_2 into H_2O and O_2, is suggested [12]. Moreover, the oxygen formed could again act as an electron acceptor for the oxidation, improving the biocatalytic activity of GO [13]. Other enzymes reported improving GO activity are peroxidases, such as horseradish peroxidase (HRP), which supposedly oxidize the inactive form of the enzyme into the active radical form [11]. GO has been reported as a good biocatalyst for DFF production in combination with catalase and HRP, obtaining quantitative yields with the combination of the three enzymes starting from 30 mM HMF within 96 h [12]. The co-immobilization of the three enzymes into $Cu_3(PO_4)_2$ nanoflowers has shown encouraging results, and a high yield (96 %) was

obtained in the presence of 200 mM HMF within 168 h [14]. However, the use of the three enzymes adds a high cost, hindering its application on an industrial scale. This and the low productivities obtained are fostering more research to improve the process and better understand the reaction.

Response surface methodology (RSM) is based on mathematical optimization techniques and aims to evaluate the relationship between different variables and one or more responses [15]. It is useful to find the optimum conditions for a process with fewer materials, time, and experiments than traditional techniques such as One Factor at A Time (OFAT) [16,17]. The most common experimental designs are the Central Composite Design (CCD) and the Box Behnken design (BBD). BBD are rotatable or near rotatable designs composed of three equally spaced levels of each factor that allow the fitting of a second-order model and have been widely used in the optimization of several biocatalytic approaches [16–18].

In the present work, the oxidation of HMF into DFF via GO was evaluated. The effect of catalase and HRP on the reaction yields and the optimum enzyme ratio among the enzymes were assessed. Then, the effects of the enzyme dosage, substrate concentration, and agitation speed over the final DFF yields were evaluated through RSM building a BBD. Finally, a kinetic study was performed.

2. Material and methods

2.1 Materials

The enzymes galactose oxidase from *Dactylium dendroides* (lyophilized powder, 3685U/g solid, one unit will produce a ΔA425 of 1.0 per min at pH 6.0 at 25 °C, in a peroxidase and o-tolidine system, reaction volume = 3.4 mL, light path = 1 cm), catalase from bovine liver (lyophilized powder, 2,000-5,000 units/mg protein, one unit will decompose 1.0 µmol of H_2O_2 per min at pH 7.0 at 25 °C, while the H_2O_2 concentration falls from 10.3 to 9.2 mM, measured by the rate of decrease of A240) and horseradish peroxidase (type VI, essentially salt-free, lyophilized powder, ≥250 units/mg solid, one pyrogallol unit will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20 °C) were purchased from Sigma-Aldrich (Missouri, USA). HMF (98%) was

purchased from Fluorochem Ltd. (Hadfield, UK). DFF, 5-acetoxymethyl-2-furaldehyde, and ethyl acetate were purchased from Sigma-Aldrich (Missouri, USA).

2.2 Enzymatic Oxidation

In a typical experiment, the reaction mixture (1 mL deionized H_2O at pH 7 containing HMF at a specified concentration) was prepared adding a specified amount of catalase, HRP, and GO in this order. The reaction started with the addition of GO and was incubated in a 15 mL shake flask at 25 °C and 220 rpm.

2.3 Optimization of the enzyme dosage and ratio

Different amounts of GO (2 or 3 mg) and catalase (2, 4, 5 or 6 mg) were added to the reaction mixture containing 30 mM HMF (**Table 1**), and the results were analyzed 72 h after GO addition.

Table 1. Amount of GO and catalase added.

Experiment	GO (mg)	Catalase (mg)
1	2	2
2	2	4
3	2	5
4	2	6
5	3	2
6	3	4
7	3	5
8	3	6

2.4 Box-Behnken Design

A Box-Behnken design was built to optimize the agitation speed, the concentration of substrate, and enzyme dosage. The experimental conditions of the model are shown in **Table 2**. All runs were performed in duplicate, and the central point was performed in triplicate. The response was the DFF yield obtained within 72 h of reaction.

Table 2. Box-Behnken design.

Factor	Level		
	-1	**0**	**1**
rpm (x_1)	160	190	220
[HMF] (mM) (x_2)	15	37.5	60
E dosage (mg GO-mg catalase) (x_3)	1-2	2-4	3-6

The full second model in terms of the coded variables was:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2$$

Equation 1

Where Y is the parameter to be modeled (DFF Yield), β_0 is a constant coefficient, β_1 , β_2 and β_3 are the regression coefficients for linear effects, β_{11}, β_{22} and β_{33} are the regression coefficients for quadratic effects, β_{12}, β_{13} and β_{23} are the regression coefficients for the interaction effects, and x_1, x_2 and x_3 are the independent coded variables (rpm, [HMF], and E dosage).

2.5 Kinetic Study

Initial reaction rates were calculated for different HMF concentrations ranging from 0 to 75 mM. The reactions were stopped and analyzed 1 h after the addition of GO. The enzyme dosage was 3 mg of GO and 6 mg of catalase. The data was fitted to a Michaelis Menten model with the software JMP Pro 14.

2.6 GC Analysis

At specified reaction times, ethyl acetate was added to the reaction mixture, and the compounds were extracted from the aqueous phase. The GC-FID analyses were performed with an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with an ultra-inert splitless liner containing a piece of glass wool couple to an FID detector. For the chromatographic separation, a DB-5MS (30 m x 0.25 mm i.d.; 0.25 µm film thickness) column from Agilent was used at a constant flow of 1 mL/min using helium as carrier gas. Injector temperature was 250 °C, and the oven program was 70 °C (held for 1 min) to 140 °C at 15 °C/min and to 300 °C at 25 °C/min (held for 5 min).

Calibration curves were performed periodically for the quantification of the compounds using 5-acetoxymethyl-2-furaldehyde as internal standard.

2.7 Statistical Analysis

The statistical analyses were performed using the software JMP Pro 14 (SAS). The results obtained were subjected to analysis of variance (ANOVA). Statistical significance was assessed with the p-value in Fisher's test with a 95% confidence level. Tukey HSD test was performed to discriminate among the means. All experiments were conducted at least in duplicate, and the values are expressed as the means ± standard deviations. The assumption of normality was tested using Shapiro–Wilk normality test.

3. Results and discussion

3.1 Effect of GO, catalase, and HRP on the DFF yields

GO (3 mg, corresponding to 11 U) was first added singly to the reaction mixture containing 30 mM HMF. The reaction yields within 72 h were low (11.3 ± 0.6 %), in agreement with Qin *et al.* (2015), who reported yields of 2 % when adding galactose oxidase singly (4 U) using the same concentration of substrate [12]. Therefore, the addition of catalase was attempted. Yields were significantly improved (29.9 ± 2.4 %) when GO (3 mg) and catalase (2 mg) were added to the reaction mixture, confirming the positive effect of catalase on the reaction. HRP is the third enzyme that exerts positive effects on the HMF oxidation into DFF. However, yields were not improved when HRP (0.5 mg) was added to the reaction mixture, as opposed to the findings by Qin *et al.* (2015), arguably because the amount of HRP added was not enough to observe a significant effect [12]. Although it is reported that HRP activates GO, therefore shortening the conversion time or increasing the final conversions, the mechanism is not fully understood [13]. There is no consensus on whether it must be added when using GO, as studies in literature differ in this aspect [11]. Moreover, its addition contributes to increase the process cost significantly [13], and therefore the addition of HRP was discarded. Yields were not improved with longer reaction times, and no differences were observed between 72 and 96 h of reaction. To elucidate the reason behind the low final yields obtained, a second addition of the enzymes (GO, catalase, or the combination of both enzymes) was performed after 96 h of reaction, and the samples were

analyzed after 96 h (168 h total reaction time). Yields were significantly improved (60 %) when GO was added singly or in combination with catalase, indicating that a similar reaction cycle started. However, yields were not improved when catalase was added alone. These results suggested that the low yields came from the inactivation of the GO as a result of the production of H_2O_2. The inactive GO did not recover its activity with the second addition of catalase, indicating that the inactivation by H_2O_2 may be irreversible. A similar effect was reported by Karra-Chaabouni *et al.* (2003) in the oxidation of hexanol using alcohol oxidase and catalase [19]. Therefore, we hypothesized that final yields could be increased either by increasing the initial amount of GO, having more active enzyme at the beginning of the reaction, or increasing the initial amount of catalase, aiming to avoid the inactivation of GO by transforming all the H_2O_2 produced.

3.2 Optimization of the GO:catalase dosage and ratio

Since catalase is cheaper than GO, an attempt to optimize the enzyme ratio between GO and catalase by increasing the catalase dosage was considered. The optimum enzyme dosage was evaluated by the combination of two amounts of GO (2 or 3 mg) with increasing amounts of catalase (2, 4, 5, or 6 mg) (**Figure 1**).

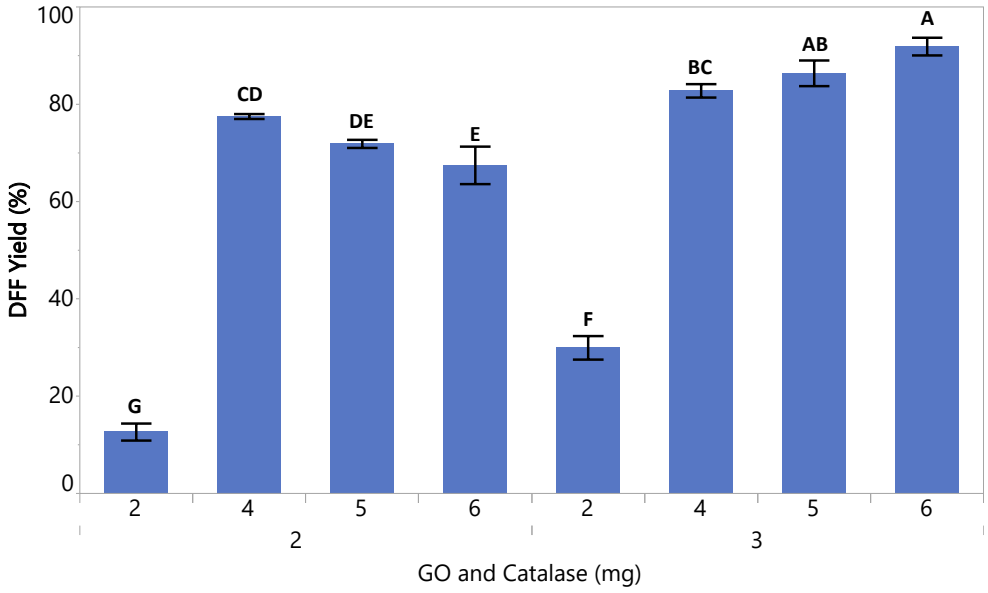

Figure 1. Effect of the GO and catalase amount on the DFF yields. Conditions: 1 mL H_2O, 30 mM HMF, 25 °C, 220 rpm, 72 h, 2 or 3 mg of GO, 2, 4, 5 or 6 mg catalase. Means with different letters are significantly different (Tukey HSD test, $p < 0.05$).

DFF yields were significantly lower when the low amount of GO (2 mg) was added, as expected. Regarding the amount of catalase, an increase from 2 mg to 4 mg exerted a 6-fold increase of the DFF yields in the presence of 2 mg of GO. However, further increases in the amount of catalase had adverse effects on the DFF yields, indicating that they could not be improved within the reaction time studied. Arguably, catalase was in excess when 4 mg were added, transforming all the H_2O_2 generated by the oxidative process, and the low solubility in water may have contributed negatively to the reaction when increasing the amount of enzyme added. The solubility of catalase is higher in phosphate buffer 100 mM, and therefore an attempt to perform the reaction in phosphate buffer was considered. However, yields were significantly lower (12.04 ± 2.5 %), probably due to the formation of $Cu_3(PO_4)_2$ as GO is a copper-containing enzyme [12]. Therefore, the use of phosphate buffer was discarded. In the presence of 3 mg of GO, the increase of the amount of catalase had positive effects until high yields (>90%) were obtained when 6 mg were added. Further increases were not considered because the differences between 5 and 6 mg of catalase were not significant (**Figure 1**). Results suggested that a ratio of 1:2 mg (GO:catalase) was optimum for the reaction, ensuring that catalase was in excess and the inactivation of GO was avoided. Besides protecting GO from the H_2O_2 formed, the presence of high amounts of catalase ensures high availability of O_2 for the reaction, which increases the activity of GO [13]. More importantly, high yields could be obtained without the addition of HRP. Arguably, the positive effects of catalase, when added in high amounts, overcome the need for HRP. However, the mechanism was not fully understood (as it is not the HRP role in the reaction [13]) and requires further work to elucidate the reasons behind these results.

The proportion of catalase used in this work was significantly higher compared to the results in the literature (**Table 3**), but this increase allowed the obtention of quantitative yields without the addition of HRP, resulting in a cheaper process.

Table 3. Comparison of enzyme ratios with literature in the non-immobilized enzymatic oxidation of HMF into DFF.

Unit ratio of GO:Catalase:HRP	Media volume (mL)	[HMF] mM	GO (U)	Time (h)	DFF Yield (%)	Enzyme Cost/mmol DFF (€)*	Ref.
1:475:33[a]	2	30	8	96	92	204	[12]
1:400:25	-	200	20	-	53.8 ± 2.3	-	[14]
1:1886:0	1	30	11	72	91.9 ± 1.8	60	This work

[a] Estimated from mg. * Based on the commercial cost of the enzymes: GO = 0.14 €/U; Catalase = $1.3*10^{-5}$ €/U; HRP = 0.04 €/U.

3.3 Box-Behnken design

The substrate and enzyme concentrations are essential factors to be considered when performing enzymatic assays [20]. Catalase was not completely dissolved in the aqueous media, and for this reason, the effect of the agitation speed was also evaluated. The effects of the rpm, enzyme dosage, and HMF concentration over DFF yields were evaluated with a Box-Behnken design (**Table 4**).

Table 4. Experimental design and responses of the Box-Behnken design. All runs were performed in duplicate, and the central point was performed in triplicate. Results are expressed as the mean.

Run	Coded values			Real values			$Yield_{observed}$ (%)	$Yield_{predicted}$ (%)
	rpm	HMF	E dosage	rpm	[HMF] (mM)	E dosage (mg GO-mg cat)		
1	-1	-1	0	160	15	2-4	78.8	82.6
2	-1	0	-1	160	37.5	1-2	50.0	49.8
3	-1	0	1	160	37.5	3-6	77.3	74.8
4	-1	1	0	160	60	2-4	55.3	52.1
5	0	-1	-1	190	15	1-2	59.8	56.1
6	0	-1	1	190	15	1-2	86.9	83.6
7	0	0	0	190	37.5	2-4	69.6	69.7
8	0	1	-1	190	60	1-2	2.2	4.9
9	0	1	1	190	60	3-6	66.6	70.2
10	1	-1	0	220	15	2-4	77.7	80.2
11	1	0	-1	220	37.5	1-2	23.1	24.3
12	1	0	1	220	37.5	3-6	91.9	92.1
13	1	1	0	220	60	2-4	50.1	46.2

Actual *vs.* predicted yields are shown in **Figure 2**. Analysis of variance (ANOVA) is presented in **Table 5**. R^2 and R^2 adjusted were 0.960 and 0.939, respectively, indicating that the model explained 93.9 % of the variability in the data. The predicted R^2 was 0.902. Therefore, we could expect the model to explain 90.2 % of the variability in predicting new observations. The *p*-value for the model was <0.05, indicating that it was statistically significant, while the lack of fit was statistically insignificant (*p*=0.2112).

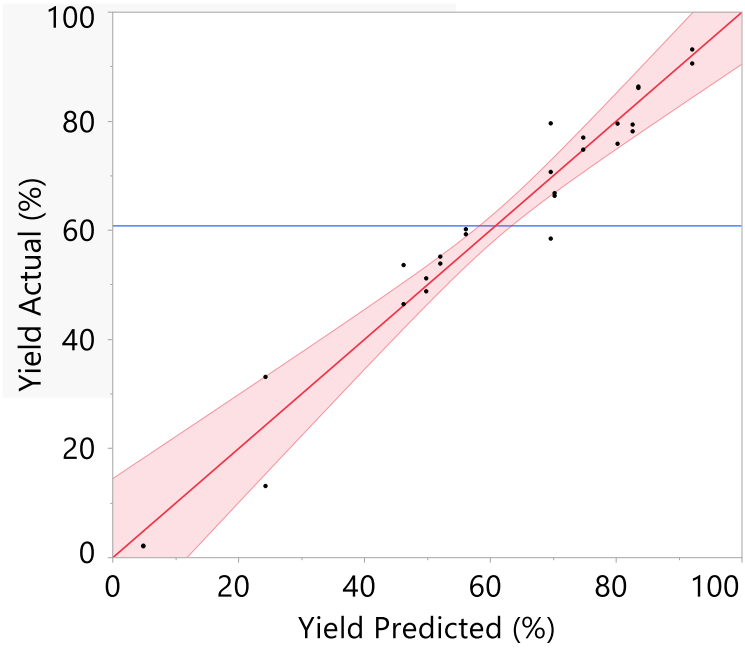

Figure 2. Actual *vs*. predicted yields.

Table 5. ANOVA of the Box-Behnken design.

Source	Sum of Squares	DF	Mean Square	F-ratio	Prob > F
model	15289.492	9	1698.83	45.1529	<0.0001
rpm (x_1)	67.6506	1	67.6506	1.7981	0.1976
[HMF] (x_2)	4169.6078	1	4169.6078	110.8230	<.0001*
E dosaje (x_3)	8606.7368	1	8606.7368	228.7564	<.0001*
x_1x_2	5.8996	1	5.8996	0.1568	0.6970
x_1x_3	918.2755	1	918.2755	24.4066	0.0001*
x_2x_3	720.1013	1	720.1013	19.1394	0.0004*
x_1^2	7.0101	1	7.0101	0.1863	0.6714
x_2^2	167.2448	1	167.2448	4.4452	0.0501
x_3^2	620.7613	1	620.7613	16.4991	0.0008*
Error	639.6084	17			
Lack of fit	171.3331	3	57.1111	1.7074	0.2112
Pure error	468.2752	14	33.4482		
R^2=**0.960**; $R^2_{adjusted}$=**0.939**; $R^2_{predicted}$: **0.902**					

The only non-significant factors were rpm, rpm*[HMF], and rpm*rpm. [HMF]*[HMF] was on the limit of significance (*p*=0.0501), but the linear effect was highly significant. Ideally, the quadratic effects for the variables should be significant in a response surface methodology where the data is fitted to a quadratic model. In this case, neither the linear nor quadratic effects of rpm were significant, although the interaction with the enzyme dosage was highly significant. Therefore, a better model could have been obtained by either modifying the range of the agitation speed values or by including another variable instead. For that, previous process optimization with the path of the steepest ascent [21] or experimental designs such as the Plackett-Burman design [17] would have been helpful. Nonetheless, the model was considered satisfactory as it was highly significant and satisfied all the model adequacy checking. The fitted second-order response function in terms of coded variables was:

$$DFF\ Yield\ (\%) = 60.21 - 2.06x_1 - 16.14x_2 + 23.19x_3 - 0.86x_1x_2 + 10.71x_1x_3 + 9.49x_2x_3 - 1.11x_1^2 - 5.44x_2^2 - 10.48x_3^2$$

Equation 2

Where the negative signs of the regression coefficients for the quadratic terms indicate the existence of the local maximum within the range of study, and the magnitude of the coefficients is proportional to their effects. The response surface plots are shown in **Figure 3**.

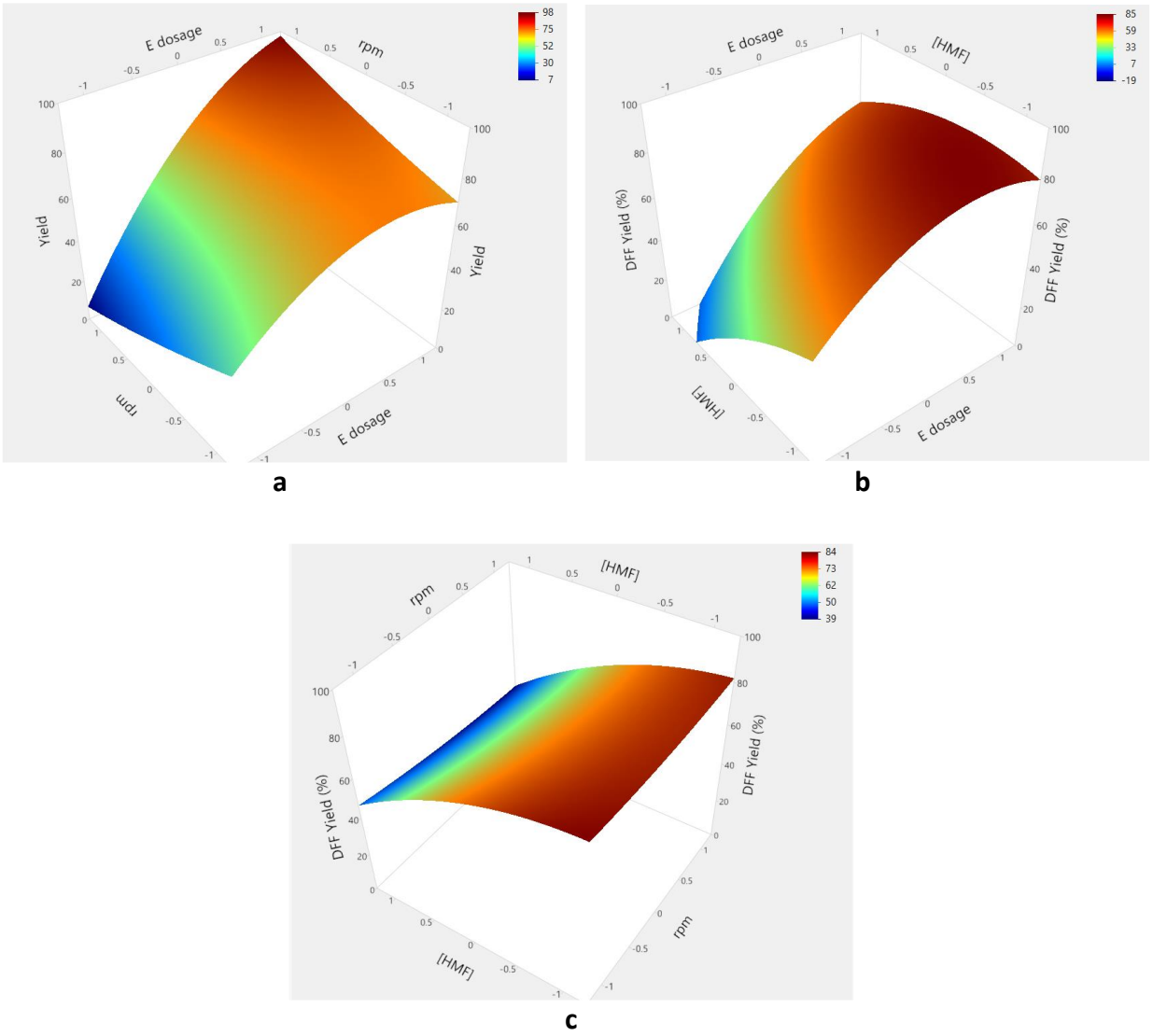

Figure 3. Response surface plots. a) E dosage vs rpm; b) E dosage vs [HMF]; c) rpm vs [HMF]

The model estimated that the optimum conditions were 220 rpm, 22 mM HMF, and 3-6 mg of GO-catalase, with a predicted yield of 94.7 %. As expected, the optimum conditions were found at the high level of the enzyme dosage and close to the low level of the concentration of HMF. Ideally, the optimum conditions should be found within the design space and not at the extremes as in this case [21]. Despite that, the enzyme dosage showed evidence of curvature in the design space as the quadratic effect was significant (**Table 5**). As shown in **Figure 4**, if the HMF

concentration is set at the low level (15 mM), the increase of the enzyme dosage has a positive effect (from yields of 40 to 90 %) until a point where there is no need to increase the enzyme dosage further (around the coded value 0.25, corresponding to 2.25 mg GO and 5.5 mg catalase) because the yields are not significantly improved. Therefore, the model allows the estimation of the minimum enzyme dosage needed to obtain the desired yield for a specific HMF concentration, reducing the process costs.

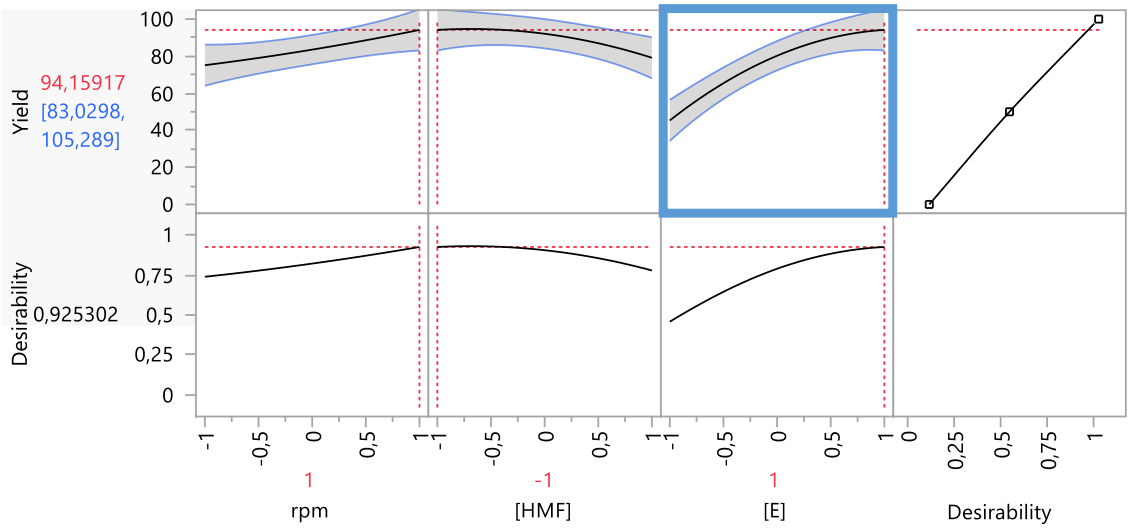

Figure 4. Profiler of the model. Highlighted in blue, effect of the enzyme dosage.

The agitation speed did not significantly affect the reaction, although it had a significant interaction with the enzyme dosage. Significantly better results were obtained for the high level of enzyme dosage (3 mg GO, 6 mg catalase) when the agitation speed was also at the higher level (220 rpm), and therefore the increase of the agitation speed had a positive effect for high amounts of the enzymes. Arguably, the increased agitation speed favors the action of the catalase due to its low solubility in water when added in high amounts. Interestingly, the opposite effect was observed for the low level of enzyme dosage (1 mg of GO and 2 mg of catalase), where significantly better results were obtained for the low level of agitation speed (160 rpm). The effect can be observed in **Figure 3-a**.

3.4 Kinetic study

GO follows the ping-pong bi bi mechanism, in which the product of the first reaction is released before the second substrate binds [22]. In this case, the first substrate (HMF) is oxidized in one half-reaction, and then the enzyme is reoxidized by reducing the second substrate (O_2) into H_2O_2 in the other half-reaction [13] (**Figure 5**).

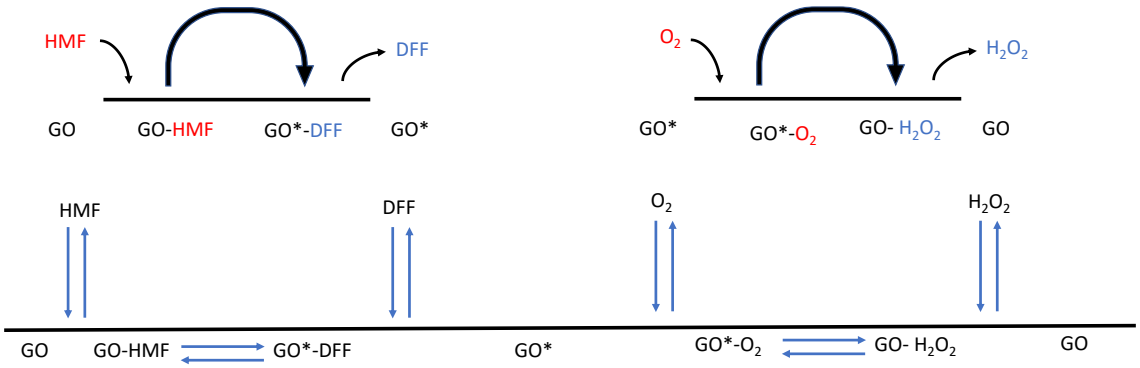

Figure 5. Ping-pong mechanism in the GO-catalyzed oxidation of HMF into DFF. Adapted from [22].

One way to obtain valuable information about the kinetic parameters of reactions involving two substrates is by holding one of the substrates at a constant concentration while increasing the concentration of the second substrate [22]. The literature reporting GO kinetic parameters is scarce, and it is hard to compare results among studies because only the concentration of the alcohol substrate is varied, and therefore the oxygen concentrations may differ. As HMF is more of interest and easier to change and measure its concentration, different concentrations of HMF were assayed while trying to keep the concentration of O_2 in excess. For that, catalase was put in excess (6 g/L) in all the assays as previously studied, ensuring high availability of oxygen.

The data (**Table 6**) was subjected to a Michaelis-Menten fit (**Equation 3**) with the software JMP Pro 14 (**Figure 5**).

Table 6. Initial HMF concentrations and reaction rates.

[HMF] (mM)	V_0 (mM/h)
0.16	0.06
0.31	0.10
0.63	0.22
1.25	0.28
2.5	0.37
5	0.42
10	0.45
15	0.48
30	0.5
45	0.48
60	0.46
75	0.47

$$V = \frac{V_m[S]}{K_m + [S]}$$

Equation 3

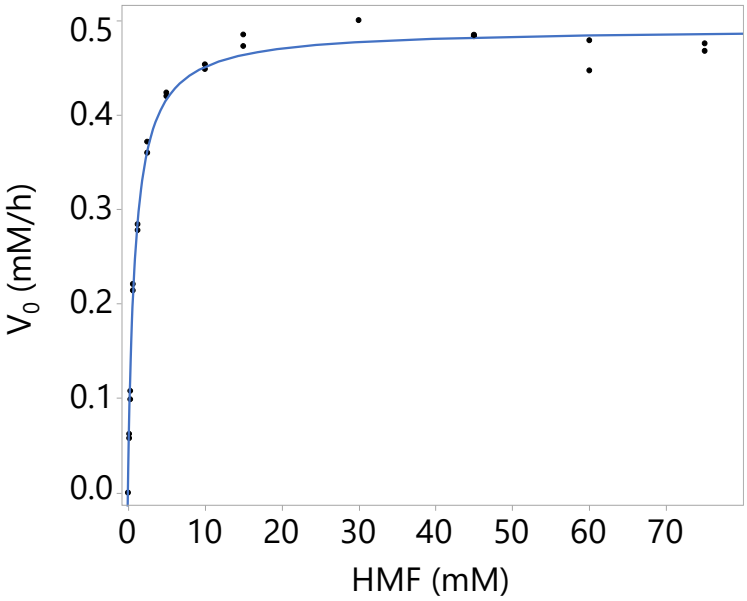

Figure 5. Michaelis-Menten fit.

The R^2 of the fit curve was 0.9918, indicating that the reaction followed the Michaelis-Menten model. The estimated values of K_M and V_m were 0.91 ± 0.05 mM and 0.49 ± 0.01 mM/h, respectively. Therefore, at [HMF] = 0.91 mM, the velocity is half of the V_m. To occupy all the binding sites, a 100-fold K_m is needed, although a 10-fold (9.1 mM) is considered enough due to the practical limitations. Therefore, all the experiments performed in the optimization of the enzymatic reaction were at a saturating concentration of substrate, as indicated [20].

Further work is needed in the biocatalytic oxidation of HMF to DFF. The current enzymatic approaches rely on three different enzymes (GO, catalase, and HRP), which are often produced and purified in different hosts adding a high cost to the process. Although in this work it was demonstrated that quantitative yields can be obtained with the addition of just GO and catalase by optimizing the enzyme ratio, the reaction times were still long, leading to low productivities. The discovery of a whole-cell catalyst with the inherent production of the enzymes needed for the oxidation and, therefore, capable of oxidizing HMF to DFF would be of great interest. However, it is challenging for several reasons: i) the well-known high toxicity of HMF towards microorganisms [8,9], ii) the preference for some microorganisms to reduce HMF into its less toxic derivative DHMF [23–25], iii) the preference for the other oxidative pathway, yielding HMFCA [26–28], and iv) the over-oxidation of DFF to FDCA [29–31].

4. Conclusions

The feasibility of GO to oxidize HMF to DFF has been confirmed. It was found that the addition of HRP to the reaction mixture was not necessary if the amount of catalase added to the reaction mixture was increased up to a 1:1886 U ratio GO:catalase, which allowed the production of DFF with yields >90 %. These results were considered satisfactory since it results in a cheaper process. A Box-Behnken design provided a better understanding of the interaction between the substrate concentration, the enzyme dosage, and the agitation speed, allowing the estimation of the minimum enzyme dosage needed for a specific substrate concentration to obtain the yields desired. Finally, the kinetic parameters of the reaction were studied. GO follows a ping-pong bi bi mechanism and an excellent fit was obtained for the Michaelis-Menten model. The values of K_M and V_m were 0.91 ± 0.05 mM and 0.49 ± 0.01 mM/h, respectively. Further work is needed to better understand the reaction to achieve a viable process at an industrial scale.

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3.2. Biocatalytic transformation of 5-hydroxymethylfurfural into 2,5-di(hydroxymethyl)furan by a newly isolated *Fusarium striatum* strain

After concluding that the enzymatic oxidation of HMF to DFF has some limitations and that whole cells would be a more feasible approach, a screening of different *Fusarium* species, natural producers of the enzyme GO, was considered. It was found that most of the strains had a high capability to reduce HMF to DHMF, while two strains showed the capability to oxidize HMF to DFF with low yields and selectivities.

This Chapter describes the screening results and the optimization of the HMF reduction to DHMF by *F. striatum*, the strain that showed the most promising results.

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Biocatalytic transformation of 5-hydroxymethylfurfural into 2,5-di(hydroxymethyl)furan by a newly isolated *Fusarium striatum* strain

Alberto Millán [1], Núria Sala [2], Mercè Torres [2] and Ramon Canela-Garayoa [1,*]

[1]Chemistry Department, University of Lleida, Alcalde Rovira Roure 191, 25198 Lleida, Spain;

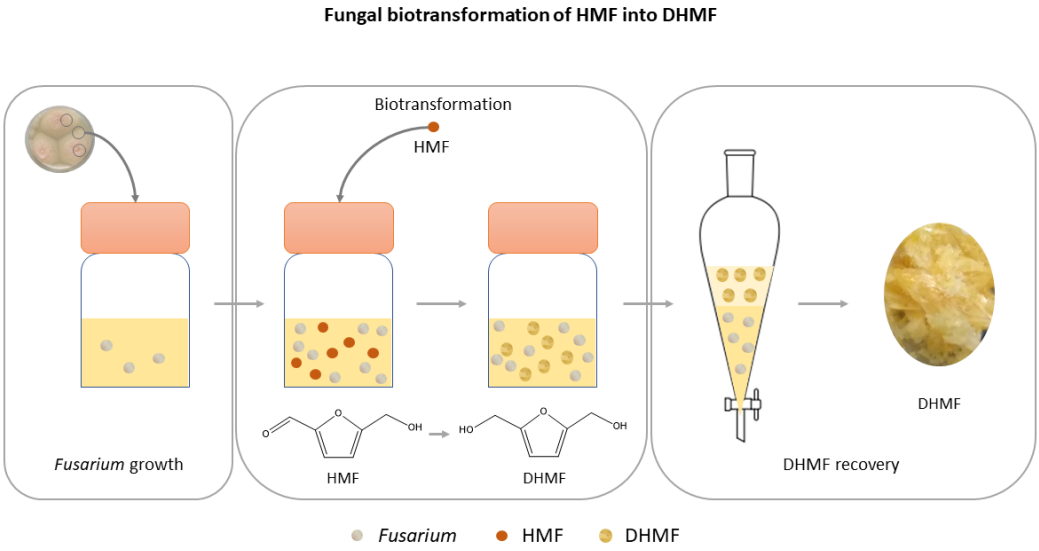
[2]Food Technology Department, University of Lleida, Alcalde Rovira Roure 191, 25198 Lleida, Spain

Abstract

The compound 2,5-di(hydroxymethyl)furan (DHMF) is a high-value chemical block that can be synthesized from 5-hydroxymethylfurfural (HMF), a platform chemical that results from the dehydration of biomass-derived carbohydrates. In this work, the HMF biotransformation capability of different *Fusarium* species was evaluated, and *F. striatum* was selected to produce DHMF. The effects of the inoculum size, glucose concentration, and pH of the media over DHMF production were evaluated by a 2^3 factorial design. A substrate feeding approach was found suitable to overcome the toxicity effect of HMF towards the cells when added at high concentrations (>75 mM). The process was successfully scaled-up at bioreactor scale (1.3 L working volume) with excellent DHMF production yields (95%) and selectivity (98%). DHMF was purified from the reaction media with high recovery and purity by organic solvent extraction with ethyl acetate.

Keywords: 5-hydroxymethylfurfural; biocatalysis; 2,5-di(hydroxymethyl)furan; *Fusarium*; whole cells; biotransformation; platform chemical; biomass; bioreactor

Graphical abstract



1. Introduction

The preparation of compounds that rely on fossil resources, alongside the increasing demand for energy, represent a substantial contribution to climate change that could be averted by finding renewable alternatives. One such alternative is the conversion of biomass, which is available all around the world as high amounts of waste, into platform chemicals that can later be transformed into their corresponding high-value derivatives, in a process of adding value to residues [1,2].

The chemical 5-hydroxymethylfurfural (HMF) results from the dehydration of biomass-derived carbohydrates. It can be transformed into a wide range of high-value derivatives of interest in different industries (2,5-dimethylfuran, 2,5-di(hydroxymethyl)furan, 2,5-diformylfuran, 5-hydroxymethyl-2-furancarboxylic acid, 5-formyl-2-furancarboxylic acid and 2,5-furandicarboxylic acid) due to the presence of both one aldehyde group and one hydroxyl group that can undergo oxidation/reduction reactions. This makes it an interesting intermediate between biomass resources and chemical blocks [3,4]. HMF is also a by-product in the production of bioethanol from lignocellulosic biomass. It can be present in the fermenting broth at concentrations up to 46 mM, depending on the raw material and the treatment applied [5], and it has been identified as an inhibitory compound for sugar-fermenting strains [6,7]. Biodetoxification has been studied as a solution. In this process, HMF is transformed into its less toxic derivative 2,5-di(hydroxymethyl)furan (DHMF) [8–10].

DHMF is a high-value chemical used as an intermediate in the synthesis of resins, fibers, foams, drugs and crown ethers [11]. It has great potential as a substitute for petroleum based aromatic diols during polymerization reactions for the manufacturing of polyurethanes and polyesters thanks to the presence of two double bonds and two hydroxymethyl groups [12,13]. To date, DHMF is synthesized mainly by chemical methods starting from HMF. Although the yields obtained with the catalytic processes are high, they show drawbacks such as the use of high-cost chemicals and extreme conditions [11]. The biocatalytic preparation of DHMF remains an attractive alternative to the current chemical pathways due to its higher selectivity, mild conditions and environmental friendliness [14]. However, the toxicity of HMF towards microorganisms poses a hard challenge for its biotransformation into DHMF with whole cells, and literature is still limited in spite of all the significant advances performed in the last years using yeast and bacteria as biocatalysts [15–20]. This

becomes apparent in literature regarding this biotransformation when performed with filamentous fungi, as it is scarce and focused on the biodetoxification of lignocellulosic material in the bioethanol production process. *Amorphotheca resinae* ZN1 and *Pleurotus ostreatus* have been proposed as a solution, tolerating concentrations of HMF ≤ 30 mM [8–10]. Nevertheless, low or undescribed selectivity, poor HMF tolerance at high substrate concentrations and long reaction times make them not suitable for DHMF production.

Filamentous fungi have great potential for biodegradation, bioremediation, and biotransformation purposes. Among them, *Fusarium* species are well-known for their capacity for extracellular protein production and their ability to break down and degrade complex compounds such as chemical pollutants and lignocellulosic biomass, and for this reason they are emerging as promising biocatalysts for industrial applications [21–23]. Different polysaccharides with antioxidant and immunomodulatory activities have been prepared from *Fusarium* [24]. Their recovery after the biotransformation processes would assist in the transition to a circular economy. Finally, products derived from the biotransformation with *Fusarium* can be labeled as "natural" [23].

In this work, we report for the first time the HMF biotransformation capability of different *Fusarium* species. A new *F. striatum* strain isolated by our group was selected to produce DHMF, and the HMF-tolerance level was studied. The effects of the inoculum size, glucose concentration and pH over the DHMF production were evaluated by a 2^3-factorial design. A substrate-feeding approach was studied in order to increase the final product concentration in the media. Besides, a scale-up of the process in a bioreactor (1.3 L) and subsequent recovery of the product from the reaction media were performed.

2. Materials and Methods

2.1. Materials

The strain of *F. striatum* was isolated by our laboratory from food waste. The isolation was performed as follows: 10 g of food waste were added to 90 mL of saline peptone water and homogenized for 2 min with a paddle blender. Then 100 µL were plated in Petri dishes containing potato dextrose agar. After five days of growth at 28 °C, the fungi grown in the Petri dishes were isolated by plating them separately in new plates with the same medium. *F. culmorum* was obtained from the Spanish Type Culture Collection (CECT2148). *F. sporotrichioides* (B3), *F. tricinctum* (T263), *F. poae* (G1), *F. chlamydosporium* (T773) and *F. sambucinum* (B6) belong to the culture collection of the Food Technology department (University of Lleida). HMF (98%) was purchased from Fluorochem Ltd. (Hadfield, UK). DHMF (97%) was purchased from Apollo Scientific (Stockport, UK). 5-Acetoxymethyl-2-furaldehyde and 5-methylfurfural were purchased from Sigma-Aldrich (Misuri, USA). Ethyl acetate was purchased from Honeywell (Morristown, USA).

2.2. Cultivation of Fusarium Cells

Fusarium strains were maintained by replications on malt extract agar (MEA: 20 g/L glucose, 20 g/L malt extract, 1 g/L peptones from soybean, 15 g/L agar) at 4 °C. Before biotransformation experiments, they were activated in MEA for seven days at 28^{o}C.

2.3. Screening

The activated strains of the *Fusarium* species were inoculated into flasks containing 15 mL of malt extract media (ME: 20 g/L glucose, 20 g/L malt extract, 1 g/L peptones from soybean) by the addition of three discs of 8 mm from the Petri dishes containing MEA. pH was previously adjusted at 7 with 1 M NaOH. The flasks were incubated in a rotatory shaker at 28 °C and 160 rpm. After three days, HMF was added to the media in order to obtain the desired concentration of the substrate (50 mM).

2.4. Biotransformation Experiments

In a standard experiment, the activated strain of *F. striatum* was inoculated into flasks containing 15 mL of ME either by the addition of fungal discs of 8 mm or by the addition of 1 mL of an aqueous suspension of spores (4.0×10^6 spores/mL). pH was previously adjusted at 7 with 1 M NaOH. The flasks were incubated in a rotatory shaker at 28 °C and 160 rpm. After three days HMF was added to the media in order to obtain the desired concentration of the substrate (50–100 mM).

2.5. Effect of Inoculum Size, Glucose Concentration, and pH

A 2^3 factorial design was used to assess the effects of the inoculum size, the pH and the initial concentration of glucose in the media over the DHMF production. Each factor was run at two levels and the design was replicated twice. The response variable was the mmol of DHMF produced and it was analyzed within 24 and 72 h after the first HMF addition. HMF was added sequentially three times in time intervals of 24 h.

2.6. Sporulation of F. Striatum

F. striatum was incubated in three different malt extract media (ME: 20 g/L glucose, 20 g/L malt extract, 1 g/L peptones from soybean, 15 g/L agar; ME-: 20 g/L glucose, 20 g/L malt extract, 15 g/L agar; and ME--: 20 g/L glucose, 10 g/L malt extract, 15 g/L agar) in either presence or absence of visible light for 10 days. Spores were counted with a Neubauer chamber.

2.7. Scale-up in the Shake Flasks

The activated strain of *F. striatum* was inoculated into flasks containing 75 or 150 mL of ME by the addition of 5 or 10 mL of an aqueous suspension of spores (4.0×10^6 spores/mL), respectively. pH was previously adjusted at 7 with 1 M NaOH. The flasks were incubated in a rotatory shaker at 28 °C and 160 rpm. After three days, HMF was added to the media in order to obtain the desired concentration of substrate (75 mM).

2.8. Scale-up in the Bioreactor

The activated strain of *F. striatum* was inoculated into the bioreactor containing 1.3 L of ME by the addition of 87 mL of an aqueous suspension of spores (4.0×10^6 spores/mL). The working conditions were 28 °C, 160 rpm, pH 7, minimum aeration. After three days of growth, HMF was added to the media in order to obtain the desired concentration of substrate (75 mM).

2.9. Recovery of DHMF from the Reaction Media

The biotransformation broth resulting from the 5-fold scale-up assay was extracted three times with an equal volume of ethyl acetate. The organic extracts were joined and dried over Na_2SO_4 anh. The mixture was filtrated, and the solvent was evaporated to dryness yielding a yellow solid. Finally, the crude reaction product was analyzed by NMR to assess its purity.

2.10. GC-FID Analysis

The compounds were extracted from the aqueous aliquots using ethyl acetate. GC-FID analyses were performed with an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with an ultra-inert splitless liner containing a piece of glass wool coupled to an FID detector. For the chromatographic separation, an FFAP (30 m × 0.25 mm i.d.; 0.25 µm film thickness) column from Agilent was used at a constant flow of 1 mL/min using hydrogen as carrier gas. Injector temperature was 230 °C, and the oven program was 100 °C (held for 1 min) to 240 °C at 20 °C/min (held for 5 min). Calibration curves were performed periodically for the quantification of the compounds using 5-acetoxymethyl-2-furaldehyde as internal standard.

2.11. Statistical Analysis

The statistical analyses were performed using the software JMP Pro 14 (SAS). The results obtained were subjected to analysis of variance (ANOVA). Statistical significance was assessed with the *p*-value in Fisher's test with a 95% confidence level. Tukey HSD test was performed to discriminate among the means. All experiments were conducted at least in duplicate, and the values are expressed as the means ± standard deviations. The assumption of normality was tested using Shapiro–Wilks normality test.

3. Results and discussion

3.1. Fusarium Screening

All *Fusarium* species studied were able to transform 50 mM of HMF to some extent. *F. striatum* metabolized 100% of the HMF within 24 h, while *F. sporotrichioides*, *F. poae* and *F. tricinctum* metabolized 95% of the HMF added within the same time (**Figure 1a**). Moreover, *F. striatum*, *F. sporotrichioides*, *F. poae, F. chlamydosporium and F. tricinctum* yielded DHMF with high selectivities (>95%) (**Figure 1b**). The by-product 5-methylfurfural was identified and quantified in the biotransformation media in small concentrations and was considered for the calculation of the selectivity towards DHMF production. The products resulting from the biotransformation with *F. sambucinum* and *F. culmorum* are currently being studied.

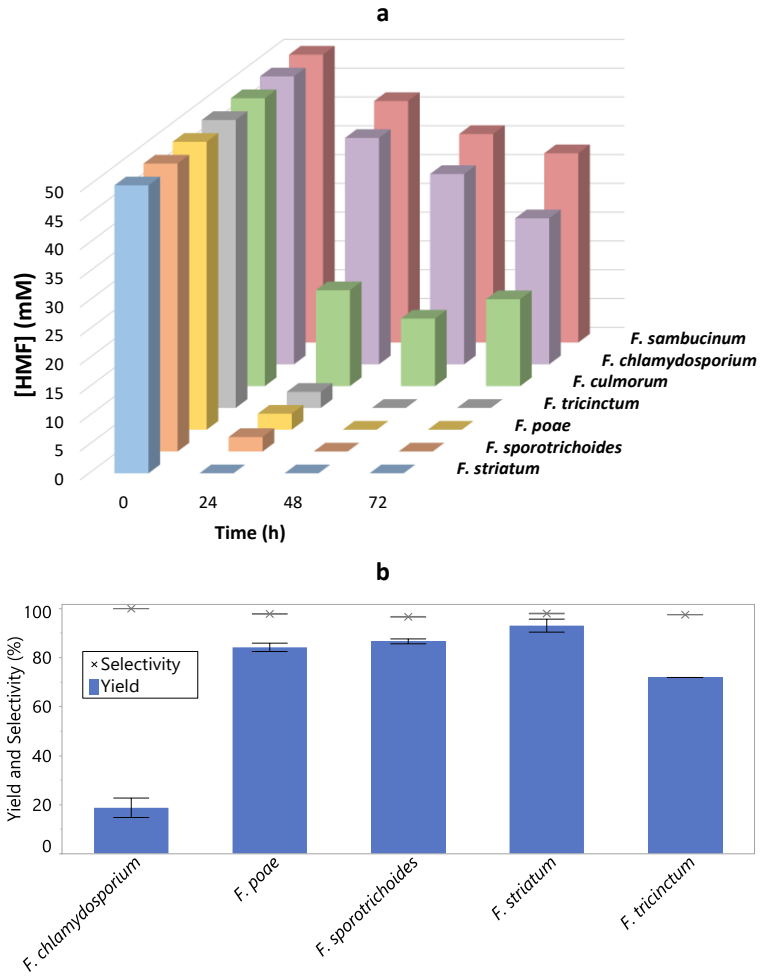


Figure 1. Biocatalytic synthesis of DHMF by different *Fusarium* species. Conditions: 50 mM HMF, 15 mL ME medium, three discs biocatalyst, pH 7, 160 rpm, 28 °C; (a) Concentration of HMF; (b) Yield and selectivity towards DHMF production.

F. striatum was selected to produce DHMF due to its high yield and selectivity towards DHMF production. Moreover, the culture of *F. striatum* under the conditions studied resulted in a broth without the formation of large filamentous mycelia and a yeast-like growth was observed (**Figure S1** Supplementary material). It was preferred against the excessive growth of mycelia of the other species for its advantages regarding manipulation and process control [25].

3.2. Effect of the Concentration of HMF

The concentration of HMF is one of the critical points in the biocatalytic production of DHMF with whole cells due to the HMF toxicity towards microorganisms. Therefore, when working with whole cells, efficient biotransformation of HMF into DHMF is usually limited by the HMF-tolerance level of the microorganisms employed. Consequently, the tolerance of *F. striatum* was studied by increasing the concentration of HMF added (**Figure 2**). Yields towards DHMF production were quantitative (>95%) for concentrations of HMF equal or lower than 75 mM under the conditions studied. However, lower yields were observed when the initial concentration of HMF was higher than 75 mM. The initial reaction rates (V_0) decreased when the initial concentration of substrate was increased. Therefore, there was an inhibitory effect of HMF, confirming its toxic effect towards the cells when added at high concentrations.

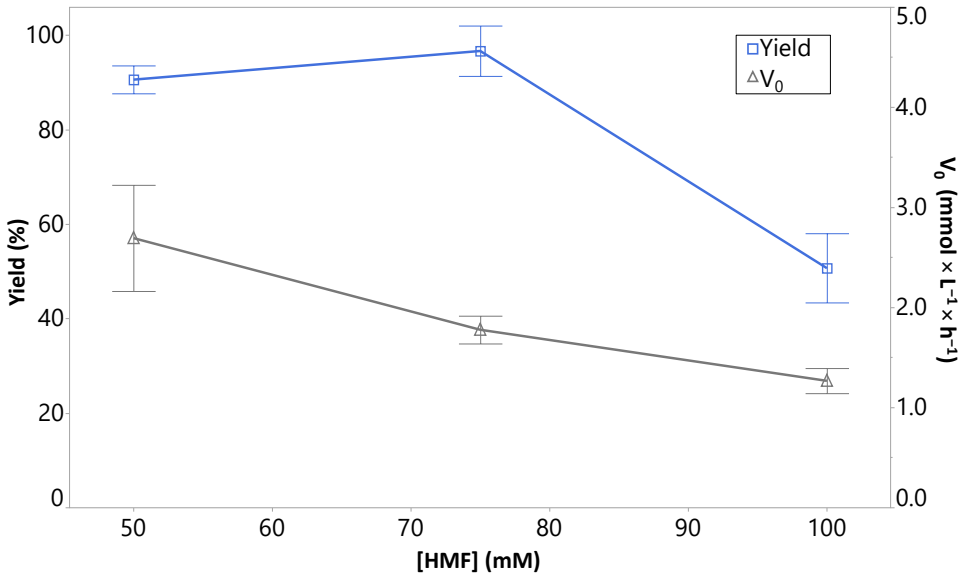


Figure 2. Effect of HMF concentrations on DHMF yields and initial reaction rates. Conditions: increasing concentrations of HMF: 50, 75 and 100 mM, 15 mL ME medium, three discs biocatalyst, pH 7, 160 rpm, 28 °C.

3.3. Effect of the Inoculum Size, Glucose Concentration, and pH

The Design of Experiments (DOE) offers many advantages compared to One-Factor-at-a-Time (OFAT) approach in biotransformation optimization. For instance, it allows the estimation of the interactions of different variables, if any, as well as yielding more information from fewer experiments [26]. The effects of some conditions that may influence the DHMF production capability of *F. striatum* were evaluated by a 2^3 factorial design. The inoculum size, concentration of the carbon source and pH were selected among the factors influencing the biotransformation process. The increase of the glucose concentration and inoculum size can have positive effects over the growth rate of the cells and the reaction rates of the HMF reduction, respectively [8,17]. Filamentous fungi are typically grown at pH 5 tolerating a pH range from 4 to 9. However, maximum growth of most of them is observed near neutral pH [27]. Therefore, the values selected were 20 or 40 g/L of glucose, three or six discs of inoculum size and pH values of 5 or 7. The sequential addition of substrate, known as substrate-feeding, is an approach that can overcome the toxicity effects of substrates like HMF when added at high concentrations. Consequently, the effect of these factors was studied within one (t = 24 h) and three (t = 72 h) consecutive additions of substrate. Reduced models were performed by the removal of selected nonsignificant terms to produce more effective models. **Table 1** shows the data corresponding to the 2^3 full factorial design.

Table 1. 2^3 full factorial design.

Run	Coded Levels			Real Values			Responses	
	X_1	X_2	X_3	Inoculum Size (Discs)	pH	[Glucose] (g/L)	mmol DHMF (24 h)	mmol DHMF (72 h)
1	−1	−1	−1	3	5	20	0.81 ± 0.08	1.41 ± 0.13
2	1	−1	−1	6	5	20	0.96 ± 0.07	1.31 ± 0.21
3	−1	1	−1	3	7	20	0.85 ± 0.04	2.12 ± 0.31
4	1	1	−1	6	7	20	1.02 ± 0.03	2.14 [1]
5	−1	−1	1	3	5	40	0.78 ± 0.07	1.18 ± 0.26
6	1	−1	1	6	5	40	0.95 ± 0.02	1.31 ± 0.00
7	−1	1	1	3	7	40	0.80 ± 0.09	1.58 ± 0.32
8	1	1	1	6	7	40	0.95 ± 0.06	1.90 ± 0.37

[1] Sample loss

In the analysis performed within 24 h of the first addition of HMF only the inoculum size showed a significant effect on DHMF production ($p < 0.05$). With the addition of six discs of biocatalyst, 0.97 ± 0.04 mmol of DHMF were produced; while with the addition of 3 discs of biocatalyst 0.81 ± 0.04 mmol of DHMF were produced. The glucose consumed within this time was significantly higher when the inoculum size consisted of six discs instead of 3. Glucose can act as a co-substrate and provide the reduced form of the cofactors needed for the reduction process (NAD(P)H) [16]. Moreover, glucose can increase the growth rate of the cells [8], which may explain why results were better as the inoculum size increased. The effect of the initial concentration of glucose of the media was not significant, probably because it was not completely metabolized even when it was added at the lowest concentration (20 g/L), and therefore there is no need to increase its concentration. The effect of pH and the interactions among parameters were not significant either.

Interestingly, in the analysis performed within three additions of substrate (72 h) only pH showed a significant effect on the DHMF production ($p < 0.05$). When the pH of the media was initially adjusted at 7, 2.04 ± 0.24 mmol of DHMF were produced; while when the pH was initially adjusted at 5, 1.42 ± 0.22 mmol of DHMF were produced. pH of the media experienced a continuous decrease attributable to the production of organic acids, although this is not the only possible reason [28]. Therefore, having an initial pH of 7 can delay the acidification of the media and provide a more suitable environment for the reduction reaction after long reaction times and three consecutive additions of substrate. The effect of the initial concentration of glucose was not significant, as it was not completely consumed when 20 g/L were added (there were 4 g/L remaining). Therefore, 20 g/L of glucose are enough under these conditions and the process is less expensive if glucose is added at lower concentrations. Moreover, there were no significant differences in the glucose consumed between both levels of inoculum size at this time, which can explain the lack of significance of this factor after three additions of substrate. To evaluate whether glucose is needed for the process, an assay was performed in a glucose-free malt extract media under the optimized conditions. Yields of 22.85 ± 2.54% were obtained within 24 h, indicating that the presence of glucose is essential for the biotransformation under these conditions.

Based on the statistical analyses, 20 g/L of glucose and pH 7 were selected as the optimal conditions, and the inoculum size and type were further optimized.

3.4. Inoculation with Spores

The inoculation of *F. striatum* from a suspension of spores was evaluated in order to optimize the inoculum size and type in the DHMF production. The addition of a known concentration of spores allows higher reproducibility of the results and greater control over the amount of biocatalyst added when compared with the addition of fungal discs. Moreover, fungal morphology in the submerged culture and the course of the biotransformation are highly influenced by the type of the inoculum [27].

Nutrient limitation and light presence are two of the most common mechanisms used to induce sporulation in filamentous fungi. Therefore, the effect of these two variables was assayed with a two-way ANOVA. There was a significant effect of the visible light, media and interaction of both variables, indicating complex mechanisms undergoing in the sporulation of *F. striatum* (**Figure 3**). The highest concentration of spores was obtained in the ME media in the presence of visible light, which means that nutrient depletion does not induce the sporulation of *F. striatum* under the conditions assayed. This should not be surprising, as the mechanisms by which fungal sporulation is induced by nutrient limitation and light remain unknown and depend on the microorganism studied [29].

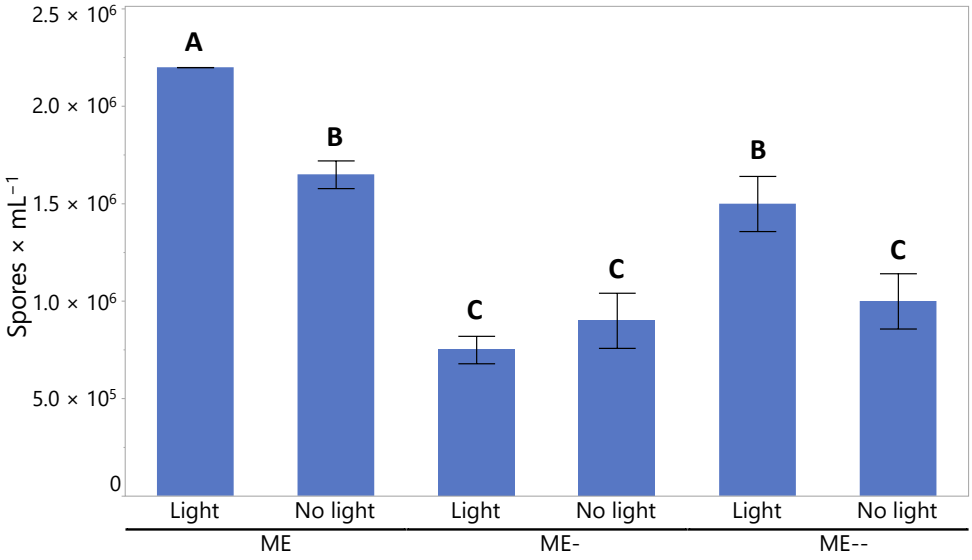


Figure 3. Effect of nutrient depletion and visible light presence on sporulation of *F. striatum*. Conditions: 28 °C, 10 days, presence or absence of light. Media: ME (20 g/L glucose, 20 g/L malt extract, 1 g/L peptones from soybean, 15 g/L agar), ME- (20 g/L glucose, 20 g/L malt extract, 15 g/L agar), ME-- (20 g/L glucose, 10 g/L malt extract, 15 g/L agar). Light: presence of visible light. No light: absence of visible light. Means with different letters are significantly different (Tukey HSD test, $p < 0.05$).

The inoculation with spores did not have any effect on the growth of *F. striatum* in the submerged culture, and therefore a homogeneous culture without the formation of large filamentous mycelia was observed as in the previous experiments performed by the addition of fungal discs. The addition of 1 mL of a suspension of $4*10^6$ spores/mL led to higher yields (92.8 ± 5.6%) than the ones obtained with the addition of six discs within 24 h (71.8 ± 2.3%). On average, $7.5×10^5$ spores were obtained from each disc. This indicates that the better results obtained were most likely due to the change of the type of the inoculum, which may influence the metabolite and enzyme production of the cells [27]. Therefore, the inoculation with spores was selected for further experiments. Results could not be improved by further increases of the concentration of spores, probably due to nutrient limitation. Results may be improved by adding a higher inoculum size, but that would probably require higher concentration of nutrients in the media. Glucose was not completely consumed once HMF was metabolized, and therefore nitrogen may be the limiting nutrient. Peptones are added at a low concentration in the process (1 g/L) because they are some of the most expensive components in culture media [30]; therefore, the increase of peptone concentration was not considered in this study. Another alternative is the use of one media for the growth of the biocatalyst and another media for the biotransformation of HMF, allowing more freedom and control over the inoculum size [31]. For this purpose, a hydrolysate

naturally containing both glucose and HMF could be used as media for the biotransformation, thus adding value to the waste [14]. HMF is present in pretreated lignocellulosic hydrolysates at concentrations up to 46 mM, while the glucose concentration in these hydrolysates can be up to 70 g/L [5]. To evaluate the performance of *F. striatum* under high concentrations of glucose, the biotransformation was performed by adding an initial concentration of HMF of 75 mM and initial concentrations of glucose up to 80 g/L to the media. There were no significant differences on the yields or reaction rates in the range of glucose concentration from 20 to 80 g/L, indicating that *F. striatum* may be a perfect candidate to perform the biotransformation of HMF into DHMF using lignocellulosic hydrolysates as reaction media. Moreover, the biotransformation of high concentrations of HMF present in lignocellulosic hydrolysates by *F. striatum* may be of interest in the lignocellulosic ethanol industry, as DHMF showed to be less toxic and does not inhibit the ethanol fermentation (work in progress) [8–10].

3.5. Substrate Feeding Approach

A substrate-feeding approach was performed in order to overcome the toxicity effect of HMF when added at concentrations higher than 75 mM, and therefore to increase the final concentration of DHMF in the media. The concentrations of HMF and DHMF were monitored and HMF was added again after it was completely metabolized. A new cycle of reaction started within the second addition of substrate and similar reaction rates were observed. A yield of 96.80 ± 4.05% was obtained within 60 h of biotransformation using a total concentration of 150 mM. Moreover, high selectivities (>98%) were obtained through all the reaction (**Figure 4a**).

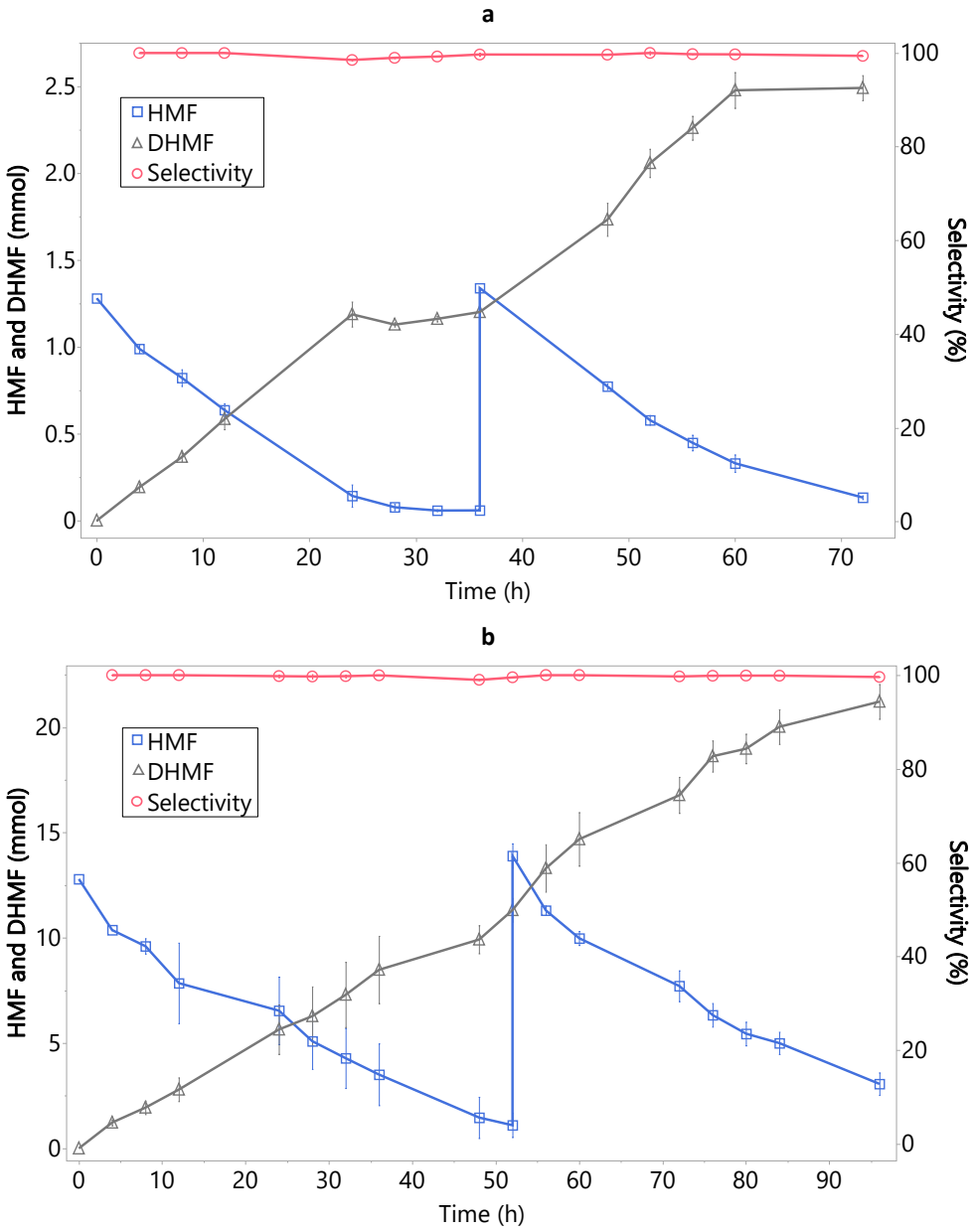


Figure 4. Substrate feeding approach. Conditions: 75 mM HMF, 1 or 10 mL of an aqueous suspension of spores ($4.0*10^6$ spores/mL). 160 rpm, 28 °C. After HMF was almost consumed, 75 mM were added; (a) 15 mL ME medium; (b) 150 mL ME medium.

3.6. Scale-up of the Reaction

The scale-up is one of the major challenges in any biotechnological process due to the decrease in the process performance [32] and no data is reported for the biocatalytic production of DHMF with working volumes higher than 20 mL to the best of our knowledge.

Two scale-ups (5 and 10-fold) were performed in the shake flasks. The concentrations of DHMF produced were fitted to a linear regression with $R^2 > 0.95$ for the different samples (**Figure 5**), indicating a constant rate of DHMF production during the biotransformation regardless of the media volume.

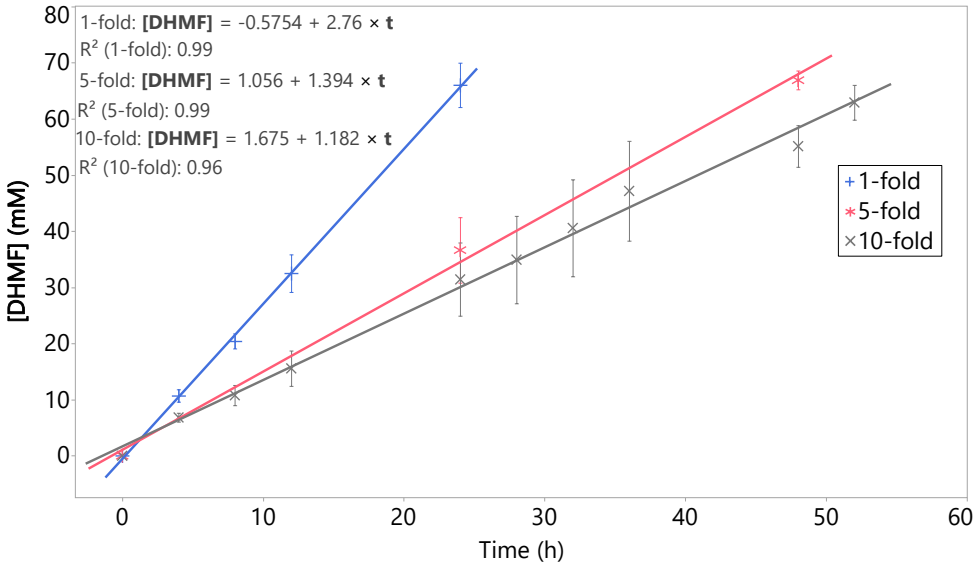


Figure 5. Effect of the scale-up of the reaction on reaction rates. Concentrations of HMF were fitted to a linear regression. Conditions: 75 mM HMF, 15, 75, or 150 mL ME medium, 1, 5 or 10 mL of an aqueous suspension of spores ($4.0*10^6$ spores/mL), pH 7, 160 rpm, 28 °C.

The reaction rates were significantly slower when increasing the volume of media, which is expected in any scale-up process, as oxygen transfer and mixing may be negatively affected. However, final yields were well reproducible regardless of the working volume, and yields >90% were obtained in all cases within 24, 48 and 52 h, respectively. Once the practicality of the process was confirmed, the substrate feeding approach was scaled up (10-fold). HMF was added within 52 h of biotransformation and a second cycle of reaction started, confirming that the substrate-feeding is a feasible approach in this process even in a scale-up performed in shake flasks. The selectivity was high (>98%) through all the reaction (**Figure 4b**).

3.7. Bioreactor

The growth of filamentous fungi in bioreactors presents difficulties due to the formation of mycelium, which causes different problems such as wrapping around the impellers, blockages and spread into nutrient and sampling feed lines, and an increase in broth viscosity [25]. However,

due to the absence of formation of large filamentous mycelia by *F. striatum* under the conditions studied, none of the problems mentioned above was observed even with a low agitation rate of 160 rpm. The reaction rates were faster when compared with the previous scale-ups performed in shake flasks. Therefore, working in a bioreactor provided a much better environment for the biotransformation, which could be attributable to better oxygen transfer and mixing. Yields of 95% and a selectivity of a 98% were achieved within 40 h (**Figure 6**). The maximum productivity was 5.7 g/L/d. Although still modest, the process has potential for further development and improvement [14]. To the best of our knowledge, this is the first time that the productivities of the HMF reduction into DHMF are described at bench scale in a bioreactor. Therefore, we have demonstrated that the process can be scaled-up, and further optimization of the conditions in the bioreactor might improve the process performance.

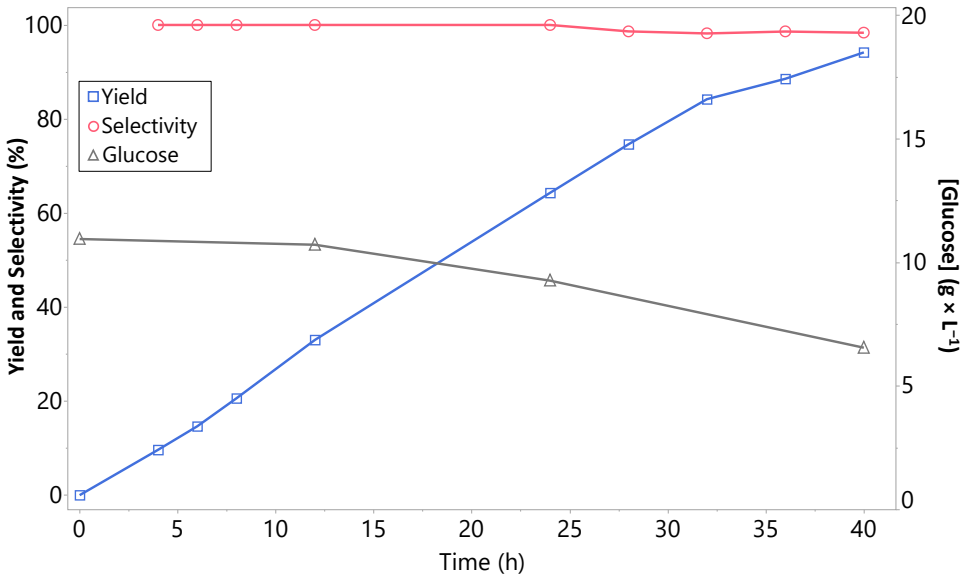


Figure 6. Biocatalytic production of DHMF at bioreactor scale. Conditions: 75 mM HMF, 1.3 L ME medium, 87 mL of an aqueous suspension of spores ($4.0*10^6$ spores/mL), pH 7, 160 rpm, minimum aeration, 28 °C.

3.8. Recovery of the DHMF from the Reaction Broth

DHMF was recovered at gram scale from the reaction broth using ethyl acetate. The recovery yield was 85% and the yellow solid isolated had a purity over 90%, measured by NMR.

4. Conclusions

In the present study, we report the biocatalytic upgrade of HMF by different *Fusarium* species at concentrations of substrate ≥50 mM for the first time to the best of our knowledge. A new *F. striatum* strain isolated from food waste has shown to be a good biocatalyst for the biotransformation of HMF to DHMF, transforming 75 mM of HMF with quantitative yields and a high selectivity within 24 h. A substrate-feeding approach allowed a higher final concentration of product in the media, which is highly desirable in any biocatalytic process and one of the harder challenges in the biocatalytic upgrade of toxic substrates. Finally, the growth of *F. striatum* in the media, under the conditions studied, allowed a scale-up of the process at bioreactor scale (1.3 L) with a high yield (95%) and selectivity (98%). The successful recovery of DHMF with a high purity from the reaction broth by organic solvent extraction closed an efficient process.

Furthermore, results obtained indicate that *F. striatum* may be a perfect candidate to perform the biotransformation of HMF using lignocellulosic hydrolysates as biotransformation media, benefiting from both HMF and sugars present. Its application would add value to the residues by metabolizing the HMF present, toxic for the sugar fermenting strains, and simultaneously producing high concentrations of DHMF. This approach is currently being studied.

Acknowledgments

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Supplementary materials

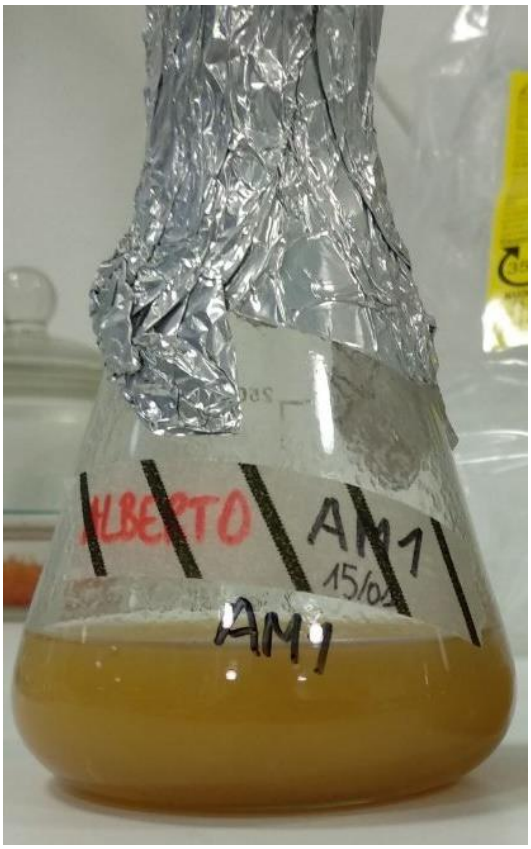


Figure S1. *F. striatum* growth in malt extract broth.

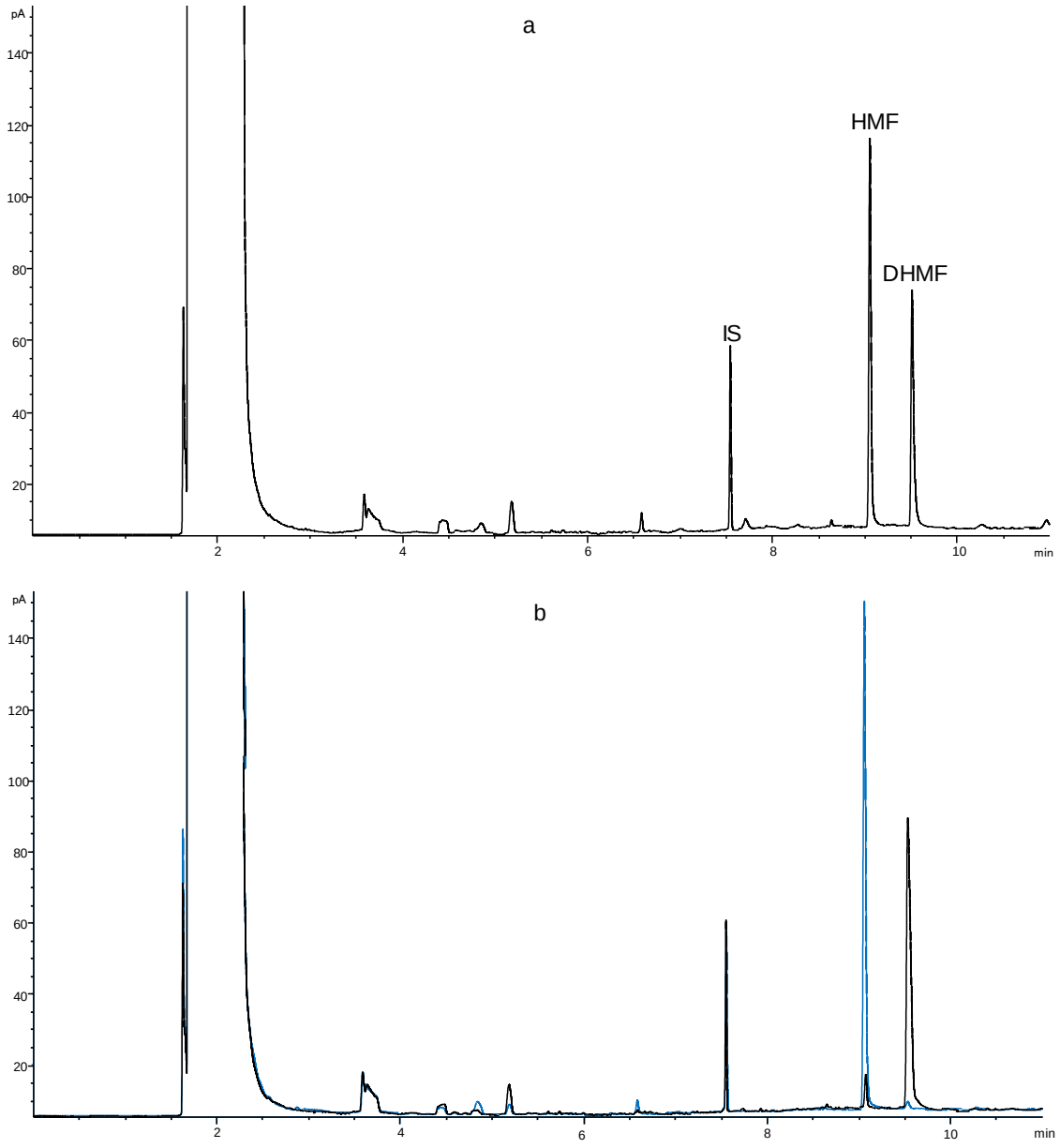


Figure S2. Chromatograms from the GC-FID measurements: a) Commercial standards; b) Biotransformation results. In blue, sample at t=0 h after HMF addition. In black, sample from a completed biotransformation.

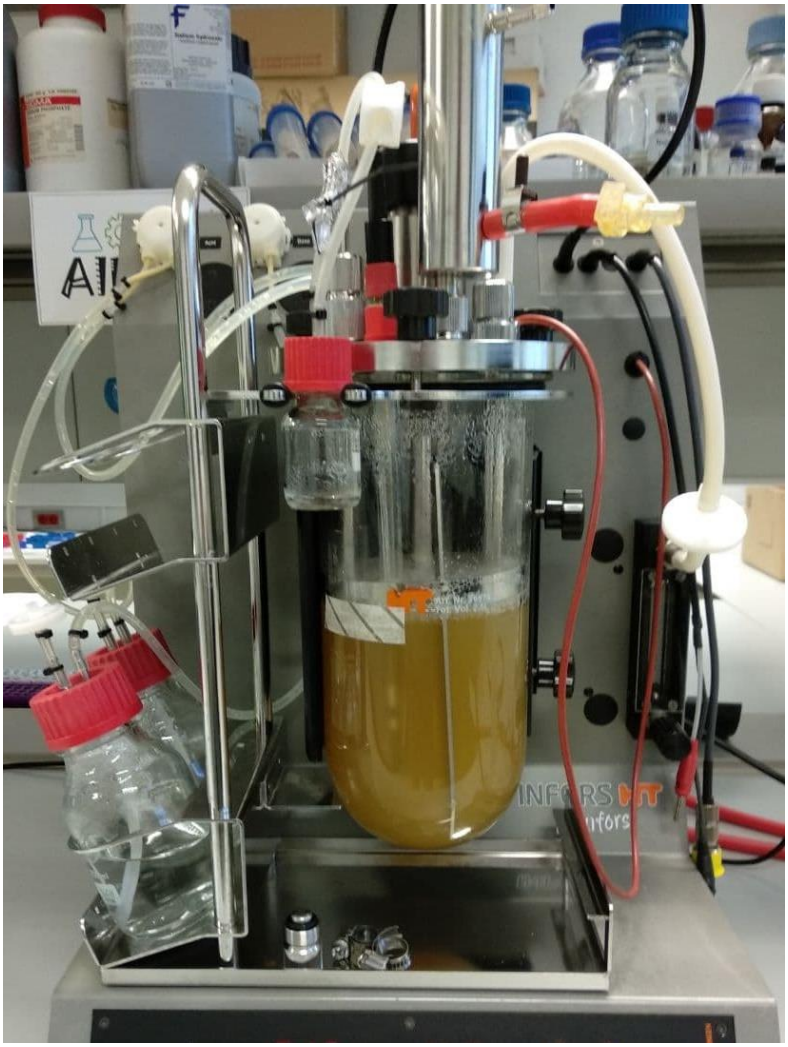


Figure S3. *F. striatum* growth in the bioreactor

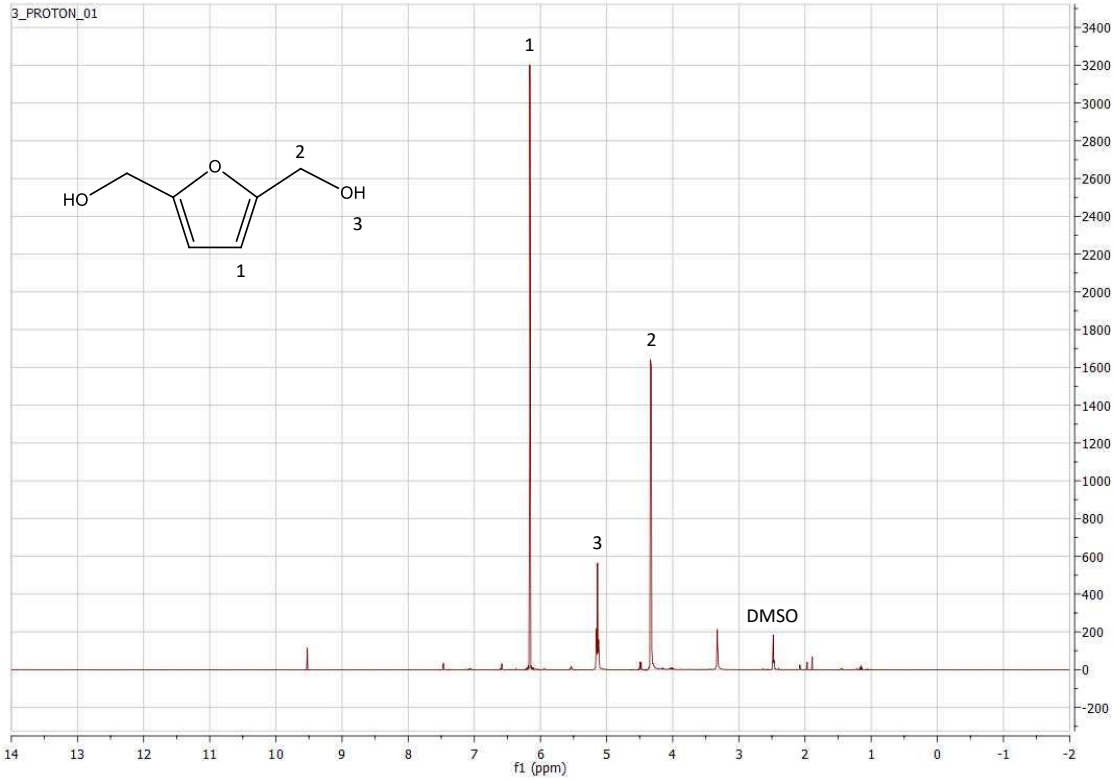


Figure S4. ^{1}H NMR in $DMSO_{d6}$ of the DHMF recovered as a yellow solid

3.3. A novel and efficient biotechnological approach to produce 2,5-diformylfuran from biomass-derived 5-hydroxymethylfurfural

F. sambucinum and *F. culmorum* showed the capability to oxidize HMF to DFF in the screening described in Chapter 3.2. However, the DFF yields and selectivities were low for both strains. After some preliminary assays, *F. culmorum* showed a higher capability to perform the oxidation, and therefore it was selected for further optimization of the process, which is described in the present Chapter.

The results of this Chapter have been published in *ACS Sustainable Chemistry & Engineering*. 9, 43 (2021) 14550-14558.

A novel and efficient biotechnological approach to produce 2,5-diformylfuran from biomass-derived 5-hydroxymethylfurfual

Alberto Millán Acosta*†, Clara Cuesta Turull†, Diana Cosovanu†, Núria Sala Martí‡, Ramon Canela-Garayoa*†

†Chemistry Department, University of Lleida, Alcalde Rovira Roure 191, 25198 Lleida, Spain;

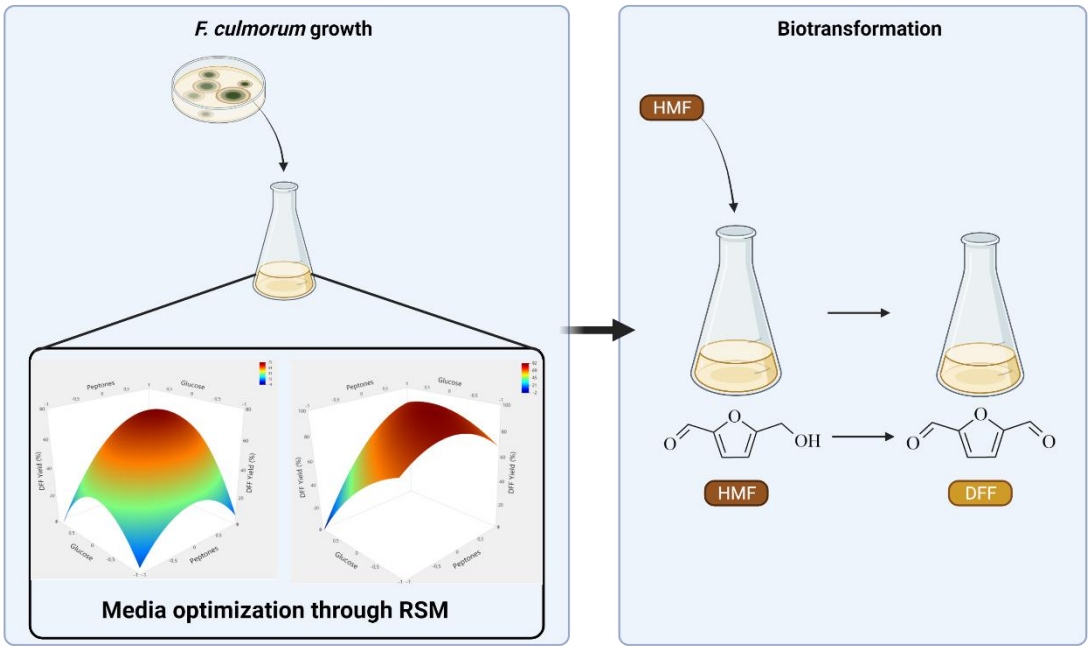
‡Food Technology Department, University of Lleida, Alcalde Rovira Roure 191, 25198 Lleida, Spain;

Abstract

The preparation of compounds of interest from biomass-derived 5-hydroxymethylfurfural (HMF) has attracted considerable attention. One such compound is 2,5-diformylfuran (DFF), obtained through oxidation of the hydroxyl group of HMF. Herein, we describe for the first time the whole-cell oxidation of HMF to DFF by *Fusarium culmorum* EAN 51. Although the chemocatalytic transformation of HMF into DFF has been widely studied, biocatalytic processes have been scarcely reported and are limited to enzymatic synthesis using the combination of several enzymes. The whole-cell transformation of HMF into DFF is preferable thanks to the inherent presence of the different enzymes, significantly reducing the cost of the process. *F. culmorum* showed a high capability to reduce to 2,5-di(hydroxymethyl)furan (DHMF) and oxidize to DFF the substrate with high yields depending on the nitrogen source and the concentration of peptone and glucose in the media, which highly affected the redox capability of this strain. After careful optimization of the concentration of both nutrients through Response Surface Methodology, 50 mM HMF were transformed into DFF with a high yield (92 %) and selectivity (94 %). These results open a new line of investigation in the sustainable production of DFF from renewable biobased resources.

Keywords: Biotransformation, RSM, *Fusarium culmorum*, Biocatalysis, HMF, DFF

Graphical abstract



1. Introduction

The dependence on fossil resources to prepare chemical building blocks is a concern that manifests the need for greener chemistry based on new sustainable production pathways from renewable biobased resources. Lignocellulosic biomass, which is abundant as waste, can be transformed into several compounds of commercial interest[1]. One such compound is 5-hydroxymethylfurfural (HMF), a platform chemical obtained through dehydration of sugars present in lignocellulosic material. It is considered one of the "Top 10+4" list of biobased chemicals according to the US Department of Energy (DOE)[2]. The presence of one aldehyde group and one hydroxyl group allows the preparation of different added-value derivatives such as 2,5-di(hydroxymethyl)furan (DHMF), 2,5-diformylfuran (DFF), 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), 5-formyl-2-furancarboxylic (FFCA) acid, and 2,5-furandicarboxylic acid (FDCA) through oxidation/reduction reactions. The market value of these compounds is higher than that of HMF, and for this reason it is considered as an excellent intermediate between biomass waste and biochemicals[3]. DFF results from the oxidation of the hydroxyl group present in HMF, and therefore it contains two symmetrical aldehyde groups. It is a precursor with applications in the synthesis of polymers, fluorescent materials, and therapeutics, among others[4–8], and it may be an interesting building block for the preparation of biobased polyurethane thermosets[9].

The synthesis of DFF has been carried out using chemical catalysis[2,10]. Yan *et al.* (2017) catalyzed the selective oxidation of HMF to DFF at 130 °C and 3 MPa O_2 with high conversion (93.7 %) and selectivity (94.5 %) using nanobelt-arrayed vanadium oxide hierarchical microspheres[11]. Later, a DFF yield of 99 % was achieved at 120 °C and 20 mL/min O_2 starting from HMF using highly active and robust vanadium dioxide embedded mesoporous carbon spheres[12]. The chemocatalytic synthesis of DFF has also been studied starting from fructose by dehydration to HMF and selective oxidation to DFF. Zhao *et al.* (2017) achieved a DFF yield of 77 % at 150 °C and 20 mL/min O_2 using a series of protonated molybdenum trioxide and nitrogen-doped carbon bifunctional catalysts[13]. Further, the catalyst could be recovered and reused without significant loss of activity. Recently, carbon nanoplates synthesized by a molten-salt method showed a DFF yield of 70.3 % in a one-pot and one-step conversion of fructose to DFF performed at 140 °C using oxygen as the only oxidant[14].

Although the recent results obtained by chemocatalytic methods are promising and encouraging, biocatalytic preparation is an attractive alternative thanks to its advantages such as milder reaction conditions, no need for high-cost chemicals as many of the chemical processes, and higher selectivity[8]. Nonetheless, reports in the literature are scarce. The enzymatic oxidation of HMF to DFF via galactose oxidase (GO), catalase, and horseradish peroxidase (HRP) has been reported with almost quantitative yields within 96 h for a concentration of HMF of 30 mM[15]. The main drawbacks of this study were that the use of these three enzymes added a high cost to the process, and the DFF productivity was low, considering the long reaction time needed. Later, Wu *et al.* (2019) immobilized the same three enzymes into $Cu_3(PO_4)_2$ nanoflowers, which allowed a higher concentration of substrate (200 mM) while keeping high yields, although the reaction times were still long (168 h), leading to a low productivity[16]. Recently, the fungal enzymes glyoxal oxidase (MtGLOx)[17] and aryl alcohol oxidase (CgrAAO)[18] have shown good preliminary results in the catalytic synthesis of DFF from HMF starting from low concentrations of substrate (≤ 20 mM). Although promising results have been obtained by enzymatic catalysis, the low concentrations of substrate and productivities obtained call for more research. Furthermore, the whole-cell transformation of HMF into DFF has not been described. Whole cells offer advantages in the biocatalytic upgrade of HMF because they are low-priced, more stable, provide a protective environment to enzymes, and there is no need for separation and purification of the enzymes[4,19,20]. Specifically, in the HMF oxidation to DFF, the whole-cell transformation is of great interest due to the need to combine different enzymes for efficient oxidation. The use of a single whole-cell catalyst would reduce the cost of the process thanks to the inherent presence of all the enzymes required. Moreover, whole cells allow the implementation of hybrid processes combining microbial and enzyme catalysis, which may be promising in the valorization of compounds such HMF[21].

The biggest challenge for the whole-cell transformation of HMF is its high toxicity towards microorganisms, and more research is needed to advance towards the valorization of HMF in this regard[4,8]. *Fusarium* species have proven to be good biocatalysts for the transformation of HMF due to their high tolerance towards high HMF concentrations using a low inoculum size[22], and some *Fusarium* species are natural producers of the enzyme GO[23,24], the catalyst of the oxidation of HMF to DFF, as mentioned above. Therefore, *Fusarium* species present as promising candidates for the whole-cell oxidation of HMF to DFF. Moreover, the recovery of

polysaccharides with immunomodulatory and antioxidant properties from *Fusarium* after the biotransformation would assist in integrating the process in the circular economy[25]. Furthermore, products obtained through biotransformation using *Fusarium* can be labeled as "natural"[26].

Response surface methodology is an experimental technique widely used to find the optimum conditions for a process. It consists of the use of mathematical optimization techniques to evaluate the relationship between a set of variables and one or more responses, and it is based on the sequential design and analysis of experiments[27,28]. Commonly, a first-order design (such as a factorial design) is first assessed, and a linear model is fit. It is possible to use widespread data points at this stage to get an overview of the design space[29]. When evidence of curvature is found (with techniques like the path of the steepest ascent or the addition of central points to the factorial design[30]), a second-order design (such as a Central Composite Design (CCD)) can be built from the initial or a new factorial design by the addition of axial points. This allows the fit of a quadratic model that, ideally, will locate a region of interest within the design space where the process is improved[29–31]. However, this is not the only possibility, and different experimental approaches are described, such as performing a new CCD experiment in each optimization step by discarding parts of the design space that give suboptimal responses (Adaptive RSM) or centrally building a new design space around each successive optimum (Successive RSM)[27]. RSM presents several advantages in medium optimization compared to traditional methodologies such as One Factor At a Time (OFAT), which is based on performing changes of one variable at a time while keeping others constant at fixed values. For instance, more information (such as interactions among the variables) can be obtained with fewer experiments, time, and material[28,32].

The capability of *F. culmorum* EAN51 to biotransform HMF into an unknown compound was previously reported in a screening of different *Fusarium* species[22]. This compound was further identified as DFF, however, the yield and selectivity obtained were low and far from optimal. In this work, the capability of this fungus to produce DFF is assessed by evaluating the effect of different nitrogen sources and through optimization of the concentration of peptone and glucose in the culture medium through RSM.

2. Experimental section

2.1. Materials

F. culmorum EAN 51 was obtained from the Spanish Type Culture Collection (CECT2148). HMF (98%) was purchased from Fluorochem Ltd. (Hadfield, UK). DHMF (97%) was purchased from Apollo Scientific (Stockport, UK). DFF, ethyl acetate, casein hydrolysate, and glucose were purchased from Sigma-Aldrich (Missouri, USA). Malt extract was purchased from Condalab (Madrid, Spain). Peptone digest of meat was purchased from Biokar (Barcelona, Spain). Peptone from soybean was purchased from Acros Organics (Pittsburgh, Pennsylvania).

2.2. Cultivation of F. culmorum

F. culmorum was maintained by replications on malt extract agar (MEA: 20 g/L malt extract, 20 g/L glucose, 1 g/L peptone from soybean, 15 g/L agar) at 4 °C. Before biotransformation experiments, it was activated in MEA for seven days in the dark at 28 °C.

2.3. Selection of the nitrogen source

The activated strain of *F. culmorum* was inoculated into flasks containing 15 mL of MES (20 g/L malt extract, 20 g/L glucose, 9 g/L peptone from soybean), MEM (20 g/L malt extract, 20 g/L glucose, 9 g/L peptone from meat), or MEC (20 g/L malt extract, 20 g/L glucose, 9 g/L peptone from casein) by the addition of three fungal discs of 8 mm taken from the solid MEA medium. The pH of the media was 5.25. The flasks were incubated in a rotatory shaker in the dark at 28 °C and 160 rpm. After three days, HMF was added to the media at a concentration of 50 mM. Analyses were performed 72 h after the HMF addition.

2.4. Optimization of the glucose and peptone concentrations

The activated strain of *F. culmorum* was inoculated into flasks containing 15 mL of MEC containing different concentrations of glucose and peptones by adding three fungal discs of 8 mm taken from the solid MEA medium. The pH of the media was 5.25. The flasks were incubated in a rotatory shaker in the dark at 28 °C and 160 rpm. After three days, HMF was added to the media at a concentration of 50 mM.

2.5. Response surface methodology

Two successive Central Composite Designs were built for the optimization of the glucose and peptones concentration in the media. The experimental conditions of both models are shown in **Table 1**. The two factor Central Composite Designs were developed starting from 2^2 factorial points, adding a central point performed in quintuplicate, and finally adding 4 axial points (α = 1.41). The response for both models was the maximum DFF yield obtained within 48 h of reaction.

Table 1. First and second Central Composite Designs.

Factors	Levels									
	First CCD					Second CCD				
	-α (-1.41)	**-1**	**0**	**1**	**α (1.41)**	**-α (-1.41)**	**-1**	**0**	**1**	**α (1.41)**
[Peptone] g/L	0.5	3	9	15	17.5	6.3	7.5	10.5	13.5	14.7
[Glucose] g/L	0.20	6	20	34	39.8	14.9	17	22	27	29.1

The full second order model in terms of the coded variables was:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2$$

where Y represents the parameter to be modeled (DFF Yield), β_0 is a constant coefficient, β_1 and β_2 are the regression coefficients for linear effects, β_{11} and β_{22} are the regression coefficients for quadratic effects and β_{12} is the regression coefficient for the interaction effect. x_1 and x_2 are the independent coded variables ([peptone] and [glucose], respectively).

The model was validated by running independent assays at the optimum conditions in triplicate and at different points within the design space in duplicate.

2.6. GC-FID Analysis

Aqueous aliquots were withdrawn from the reaction broth at selected reaction times. The compounds were extracted from the aqueous aliquots using ethyl acetate. GC-FID analyses were performed with an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with an ultra-inert splitless liner containing a piece of glass wool coupled to an FID detector. For the chromatographic separation, an FFAP (30 m × 0.25 mm i.d.; 0.25 µm film thickness) column from Agilent was used at a constant flow of 1 mL/min using hydrogen as carrier gas. Injector

temperature was 230 °C, and the oven program was from 100 °C (held for 1 min) to 240 °C at 20 °C/min (held for 5 min). Calibration curves were performed periodically for the quantification of the compounds.

2.7. Determination of the amino acid profile by HPLC

The amino acid content of samples was determined on freeze-dried and pulverized tissue. Acidic hydrolysis of the sample (50 mg) was carried out using 5 mL of 6N HCl (110 °C, overnight, under N_2) [33,34]. Hydrolysis tubes were cooled and centrifuged at 3,000 *g* for 30 min to remove particulate matter. Aliquots of 25 µL of hydrolysate were evaporated using a SpeedVac and reconstituted in 500 µL of water:acetonitrile (20:80, v/v). Samples were filtered through a 0.22 µm hydrophilic polytetrafluoroethylene (PTFE) membrane before injection. The injection volume was 5 µL. Quantification of individual amino acids was performed using a method described by Guo *et al.* (2013) with modifications[35]. UHPLC was performed using a Waters Acquity system equipped with a BEH Amide column (2.1 x 150 mm; 1.7 µm). The mobile phase consisted of solvent A (10 mM ammonium formate in water with 0.15% formic acid) and solvent B (ammonium formate - saturated acetonitrile with 0.15% formic acid). The gradient elution followed was 15% A and 85% B maintained for 3 min at 0.5 mL/min. Then, from 15% to 20% A in 3 min; from 20% to 24% A in 1.5 min; from 24% to 60% A at 0.6 mL/min in 1.5 min and maintained for 3 min. Finally, initial conditions were regained in 2 min. The flow rate of the mobile phase was 0.5 mL/min, and the column temperature was maintained at 30 °C. The column was cleaned with weak (20% acetonitrile) and strong (80% acetonitrile) washing solvents between injections. Detection and quantitation of amino acids in the hydrolysate were performed by using a multiple reaction monitoring method (MRM) in a triple quadrupole detector (TQD) mass spectrometer. The system was equipped with an electrospray ionization (ESI) source operated in positive ion mode. Parameters in the source were set as described in the bibliography [35].

2.8. Statistical Analysis

The statistical analyses were assessed using the software JMP Pro 14 (SAS). The results obtained were subjected to analysis of variance (ANOVA). Statistical significance was assessed with the p-value in Fisher's test with a 95% confidence level. The assumption of normality was tested using Shapiro–Wilk normality test.

3. Results and discussion

3.1 Effect of the nitrogen source

The selection of adequate nitrogen and carbon sources and the concentration of both nutrients in the media can significantly improve the yields and productivities of biocatalytic processes when working with whole cells[32]. Filamentous fungi require organic compounds as a carbon and energy source due to their heterotrophic nature. Sugars are the preferred carbon source for the growth of the cells because they are easily incorporated into the microorganism metabolism. Among them, glucose is one of the most common ingredients of microbial media[36], it is crucial for the biotransformation of HMF by *Fusarium* species[22], and has proven to be a good carbon source for the production of GO by *Fusarium* species[37]. Therefore, it was selected as the carbon source. However, the preferred nitrogen source is not as evident and highly depends on the microorganism and the process studied. Nitrogen may be added to the media as inorganic compounds such as ammonia or nitrate or as organic compounds such as peptones or free amino acids, and it plays a crucial role in the metabolite and enzyme production of the cells[38]. The effect of the nitrogen source was assessed by studying the influence of three different peptones (soybean, meat, and casein) over the HMF transformation (**Figure 1**).

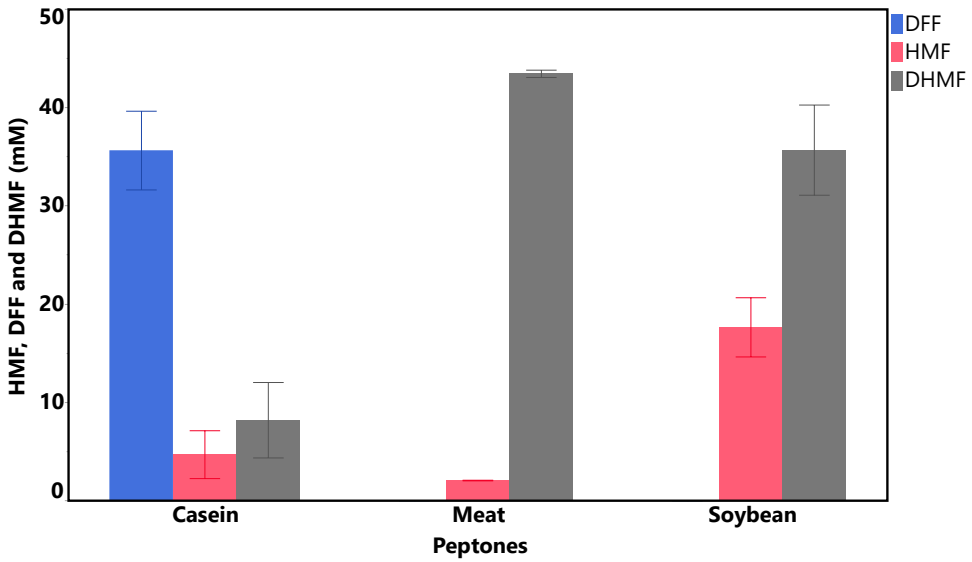


Figure 1. Effect of the nitrogen source on the HMF transformation. Conditions: 50 mM HMF, 15 mL MEM, MES or MEC medium, three discs biocatalyst, pH 5.25, 160 rpm, 28 °C, 72 h.

When soybean or meat peptones were added to the media, HMF was exclusively reduced to 2,5-di(hydroxymethyl)furan (DHMF), a process reported using whole cells as biocatalysts[22,39–41]. However, when peptone from casein was added to the media, both DHMF and DFF were produced and identified in the reaction broth. This indicated that the oxidative process was favored when peptone from casein was used as a nitrogen source under the conditions studied. However, the DFF yield (70 %) and selectivity (80 %) were not optimal. Moreover, HMF was not wholly transformed. For these reasons, peptone from casein was selected for further optimization.

The production, secretion, and maturation of GO, the enzyme likely involved in the oxidation of HMF to DFF by *F. culmorum*, is a complex process that requires several steps and cofactors[23]. The effect of the different peptones on the redox capability of *F. culmorum* may be attributed to a different amino acid profile, peptides of different types and sizes, and the presence of microelements. The amino acid profile of the three peptones was determined by HPLC (Supplementary Information) and revealed significant differences in the amino acid content, which might influence the metabolism of the fungus. Moreover, the total percentage of amino acids in each peptone was different (84%, 94%, and 66% for meat, casein, and soybean peptones, respectively. Further, not only the amino acid profile and total content but the form of the amino acids (free amino acids or different peptides) determine the biological value of a particular peptone, directly impacting the protein synthesis of the microorganism[42]. Finally, the presence of certain microelements is crucial for the activity of some enzymes. For example, GO activity can be significantly improved in the presence of Cu^{2+}, Mg^{2+}, and Mn^{2+} [37], and therefore their presence at different concentrations on the protein hydrolysates may also influence the redox capability of the strain. For these reasons, it is difficult to establish a relationship between a nitrogen source and the synthesis of certain enzymes. Further studies on a genetic level may provide valuable information[43].

3.2. Optimization of the glucose and peptone concentration in the media through Response Surface Methodology

Glucose and peptone concentrations were selected as variables due to their strong influence on the metabolism of filamentous fungi[36]. The selection of the variable ranges is one of the most critical points in any optimization study and highly depends on the process studied. Due to the

capability of *F. culmorum* to reduce and oxidize HMF, widely spread points within the common values of both nutrients in microbiological media were first selected to get an overview of the design space. It is worth mentioning that the strategy employed in the sequential development of any model highly depends on the nature of the response system, and it should be adapted to every situation. Therefore, a 2^2 factorial design was first performed to study the effects of glucose and peptone concentration over DFF production (**Table 2**, runs 1-4).

There was DFF production only for the high level of both variables, with a yield of 52 %. DHMF was also produced with a lower yield (26 %). The other combinations of the variables yielded DHMF exclusively with high yields (Supporting Information). This indicated that peptone from casein only induced the oxidation into DFF under certain conditions. Further increases of the concentration of both nutrients above the higher levels (15 g/L peptones and 34 g/L glucose) were not considered due to the high cost implied and the unrealistic approach it would pose. Instead, a study within the ranges selected was evaluated, taking into account that the objective is not only to maximize the DFF yield but to do it at the lowest cost possible. One of the limitations of two-level factorial designs is the assumption of linearity in the factor effects. Adding a central point to the 2^2 design can overcome that by allowing the estimation of curvature from second-order effects[30]. Therefore, the design was augmented by including the central point performed in quintuplicate, as shown in **Table 2** (runs 5-9).

Table 2. Full factorial design augmented with the central point.

Run	Coded levels		Real values		Response
	Peptones	Glucose	[Peptone] (g/L)	[Glucose] (g/L)	DFF Yield (%)
1	1	1	15	34	51.69
2	1	-1	15	6	0
3	-1	1	3	34	0
4	-1	-1	3	6	0
5	0	0	9	20	67.06
6	0	0	9	20	71.76
7	0	0	9	20	75.04
8	0	0	9	20	60.65
9	0	0	9	20	81.82

After fitting a first-order model to the data, the lack of fit was highly significant (p<0.001), indicating that something was missing in the model. Furthermore, the data suggested strong evidence for curvature in the region studied. One way to assess the presence of quadratic curvature is by calculating the difference between Y_f and Y_c, being Y_f the average of the runs at the factorial points (**Table 2**, runs 1-4), and Y_c the average of the center point runs (**Table 2**, runs 5-9). The largest this value is the more evidence for quadratic curvature in the model[30]. In this case, Y_f - Y_c = -58.35, a value large enough to suggest the presence of quadratic effects. This indicated that the optimum was somewhere within the region considered in the first linear model, and there was no need to increase the concentration of both nutrients further.

3.2.1. First Central Composite Design

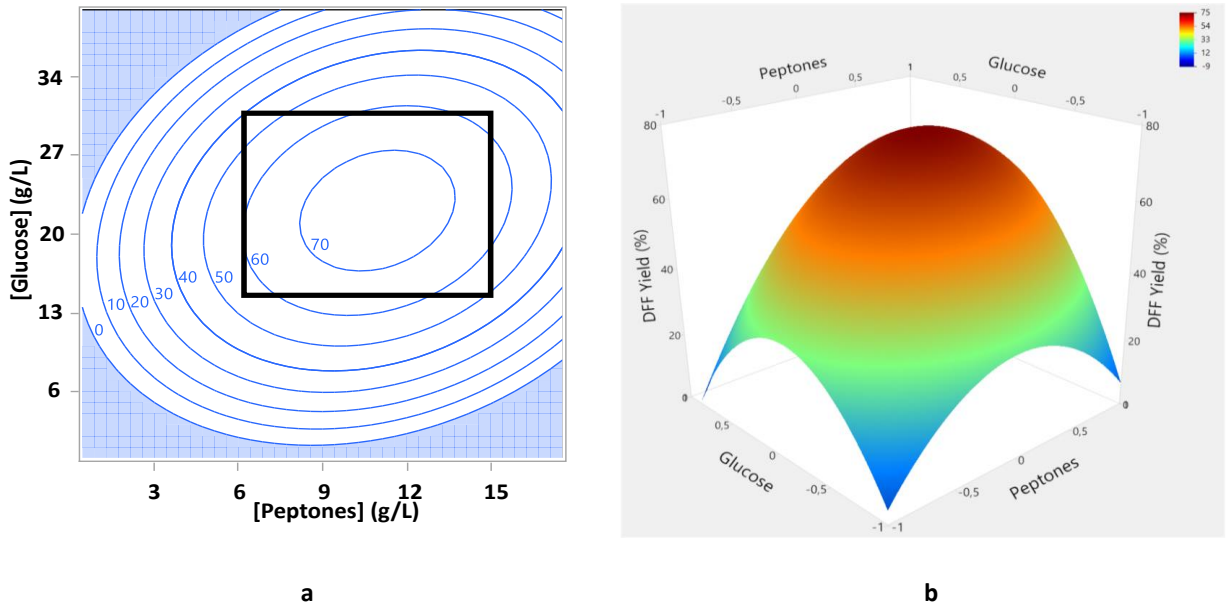
The findings suggested that augmentation of the design to allow a complete second-order model fitting would be useful. Therefore, the model was augmented by adding axial runs (α = 1.41), transforming it into a rotatable central composite design (**Table 3**). This allowed the fitting of a complete second-order model and efficient estimation of pure quadratic terms. The information about the model can be found in the Supporting Information. Overall, it satisfied all the model adequacy checking while being statistically significant.

Table 3. Experimental design and responses of the first Central Composite Design.

Run	Coded levels		Real values		Response	
	Peptone	Glucose	[Peptone] (g/L)	[Glucose] (g/L)	DFF $Yield_{observed}$ (%)	DFF $Yield_{predicted}$ (%)
1	1	1	15	34	51.69	45.28
2	1	-1	15	6	0	6.49
3	-1	1	3	34	0	-8.79
4	-1	-1	3	6	0	4.11
5	0	0	9	20	67.06	71.26
6	0	0	9	20	71.76	71.26
7	0	0	9	20	75.04	71.26
8	0	0	9	20	60.65	71.26
9	0	0	9	20	81.82	71.26
10	1.41	0	17.5	20	43.24	42.70
11	-1.41	0	0.5	20	0	2.84
12	0	1.41	9	39.8	0	10.28
13	0	-1.41	9	0.2	0	-7.99

Several conditions gave a response value of 0, suggesting that the design space may have been too ample, and therefore most of the factorial and axial points resided far away from the optimum. Despite that, the model allowed a visual interpretation of the data that gave an overview of the oxidation process within the wide region studied (**Figure 2**), identifying a region of interest containing the optimum where the subsequent experimentation should take place (**Figure 2-a**).

Results indicated that the oxidation capability of *F. culmorum* was highly affected by the concentration of glucose and peptone in the growth medium. Both nutrients must be at specific concentrations for the oxidation to occur with high yields. The reductive pathway was favored above or below these concentrations, and DHMF was produced (Supporting Information), while DFF yields decreased drastically. This indicated strong evidence of curvature within the region studied, which was in concordance with the excellent fit of the quadratic model.



a **b**

Figure 2. Contour plot (a) and response surface (b) of the first CCD. The region of interest selected for further experimentation is squared.

The model was good at estimating the curvature showed by both variables and locating the region of interest, but it provided little information about the optimum. The ranges selected for both variables were too widespread, and extreme responses were observed (note that only three of the nine conditions assayed yielded DFF). Moreover, the maximum yields predicted by the

model were not optimal (74.1 ± 9.7 %). The results manifested the need for further optimization to determine how sensitive the response was when moving within the region of interest containing the estimated optimum, therefore obtaining more information about the process and, hopefully, a better estimation of the optimum conditions. Due to the evidence of curvature provided, a second quadratic model was built around the region of interest, highlighted in **Figure 2-a**, establishing a smaller design space within the one used in the first model.

3.2.2. Second Central Composite Design

A randomized rotatable central composite design was performed. The central point and the ranges of the variables were selected according to the information obtained from the first model. Therefore, the parts of the design space that corresponded to suboptimal responses were discarded, and the new design space was built around the optimum previously found. The maximum DFF yield was generally observed at 48 h for the different conditions (Supporting information), and therefore the analysis was performed at that time. **Table 4** shows the experimental design, the responses observed, and the responses predicted by the model.

Table 4. Experimental design and responses of the second Central Composite Design.

Run	Coded levels		Real values		Response	
	Peptone	Glucose	[Peptone] (g/L)	[Glucose] (g/L)	DFF $Yield_{observed}$ (%)	DFF $Yield_{predicted}$ (%)
1	0	0	10.5	22	80.28	86.32
2	0	-1.41	10.5	14.9	82.48	84.17
3	0	1.41	10.5	29.1	42.14	43.15
4	1	1	13.5	27	86.78	86.90
5	0	0	10.5	22	90.84	86.32
6	-1.41	0	6.3	22	17.94	21.02
7	0	0	10.5	22	86.96	86.32
8	1	-1	13.5	17	67.92	67.55
9	-1	-1	7.5	17	77.85	75.04
10	-1	1	7.5	27	0	-2.33
11	1.41	0	14.74	22	79.22	78.83
12	0	0	10.5	22	87.40	86.32
13	0	0	10.5	22	86.10	86.32

Analysis of variance (ANOVA) is presented in **Table 5**. The model explained 98.57 % of the variability in the data, and we could expect the model to explain 97.25 % of the variability in predicting new observations.

Table 5. ANOVA of the second Central Composite Design.

Source	Sum of Squares	DF	Mean Square	F-ratio	Prob > F
Model	10233.995	5	2046.80	167.5672	<0.0001
[Peptone] (x_1)	3341.5097	1	3341.5097	273.5625	<0.0001
[Glucose] (x_2)	1682.9611	1	1682.9611	137.7805	<0.0001
x_1*x_2	2338.4825	1	2338.4825	191.4467	<0.0001
x_1*x_1	2303.3001	1	2303.3001	188.5664	<0.0001
x_2* x_2	893.0352	1	893.0352	73.1109	<0.0001
Error	85.504	7	12.21		
Lack of fit	26.9934	3	8.9978	0.6151	0.6405
Pure error	58.5101	4	14.6275		
R^2=0.9917; $R^2_{adjusted}$=0.9857; $R^2_{predicted}$: 0.9725					

The *p*-value for the model was <0.05, indicating that it was statistically significant, while the lack of fit was statistically insignificant (*p*=0.6405). The concentration of peptone and glucose, the interaction between both variables, and the quadratic effects were highly significant (*p*<0.0001). Therefore, both the concentration of peptone and glucose showed curvature evidence in the response within the design space. The fitted second-order response function in terms of coded variables was:

$$DFF\ Yield\ (\%) = 86.32 + 20.44x_1 - 14.50x_2 - 18.20{x_1}^2 - 11.33{x_2}^2 + 24.18x_1x_2$$

Where the negative signs of the regression coefficients for the quadratic terms indicate the existence of a local maximum within the design space, and the magnitude of the coefficients is proportional to their effects.

Results displayed in **Figure 3** confirm that the oxidation capability of *F. culmorum* was highly affected by the concentration of glucose and peptone in the media. It is worth noting that the contour plot of the second model (**Figure 3-a**) differs from the same region within the first model (**Figure 2-a**). This reinforced the previous hypothesis that the first model provided little

information about the optimum, although it successfully located the region of interest. Interestingly, the visual results suggest that high yields come from a certain ratio between glucose and peptone concentration. Peptone and glucose are known to be some of the most expensive ingredients of culture media. Therefore, the results obtained were useful because besides estimating the optimum conditions, they estimate the minimum amount of both nutrients that gives satisfactory enough yields, reducing the cost of the process.

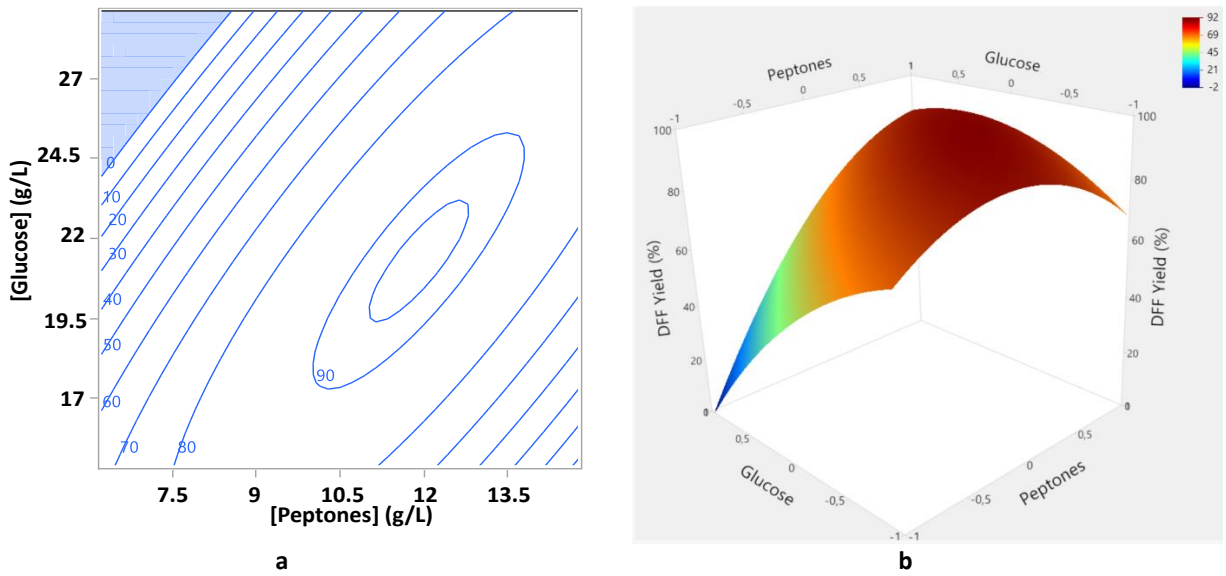


Figure 3. Contour plot (a) and response surface (b) of the second CCD.

3.3. Validation of the model

Although RSM models are usually validated only at the optimum conditions, the accuracy of the model's predictions within different regions of the design space was considered of interest. Therefore, the model was validated by running independent assays at the optimum point (**Table 6**, run 1), at three selected points within the design space where the predicted DFF yields were higher than 80 % (**Table 6**, runs 2-3-4) and at one point where the predicted yield was ≤0 (**Table 6**, run 5). In addition, the central point from the first model, which was within the design space, was also considered (**Table 6**, run 6).

Table 6. Validation of the second CCD. Conditions: 50 mM HMF, three discs biocatalyst, pH 5.25, 160 rpm, 28 °C.

Run	Coded levels		Real values		Predicted yield (%)	Observed yield (%)
	Peptone	Glucose	[Peptone] (g/L)	[Glucose] (g/L)		
1[a]	0.47	-0.14	11.9	21.3	92.12	92.08± 4.30
2	0.5	0.5	12	24.5	87.95	90.04 ± 0.21
3	0	-1	10.5	17	89.49	86.63 ± 0.50
4	-0.2	-0.2	9.9	21	84.92	86.40*
5	-1.35	-1	6.3	27	≤0	0
6	-0.5	-0.4	9	29	80.38	71.27 ± 8.00

* Sample loss, [a] Optimum conditions

The predictions made by the model were highly accurate for the optimum (**Table 6**, run 1) and most of the other conditions evaluated (**Table 6**, runs 2-5), with errors <5%. For run 6 the yields were slightly overestimated, arguably due to the high variability observed among the repetitions. Nevertheless, the validation was satisfactory considering the innate variability between independent assays when working with whole cells. Moreover, it confirmed that there was a wide range of conditions in which high yields (>85 %) were obtained. **Figure 4** shows the concentration of the different compounds during the reaction under the optimum conditions. Interestingly, there was some DHMF production within the first 6 h of the reaction that remained at a low concentration until high concentrations of DFF were quantified within 31 h, leading to a high yield (92 %), selectivity (94 %), and productivity (4.4 g/L/d). Moreover, DFF was stable in time as no significant differences were observed at 48 h. This indicated that DFF was no further oxidized to the corresponding acids by *F. culmorum*, making it an interesting biocatalyst for DFF production.

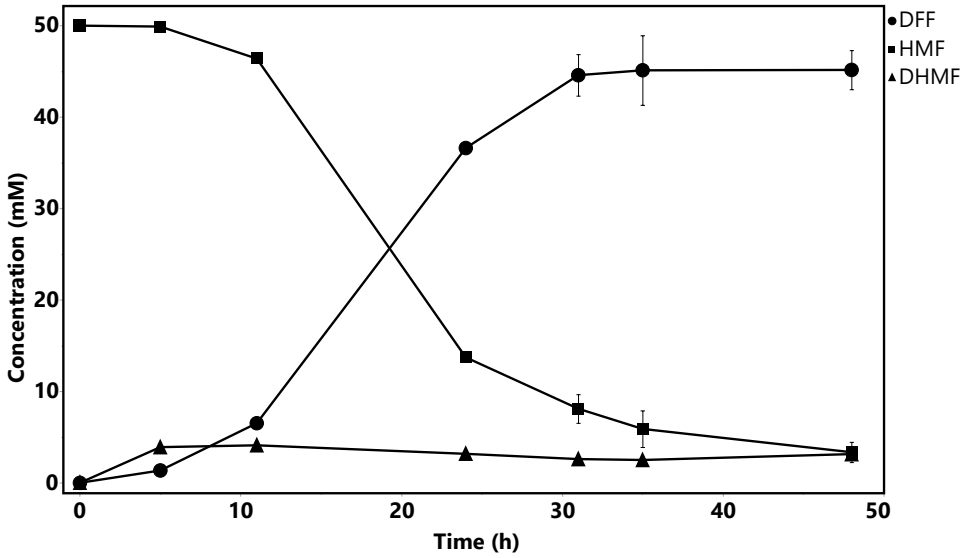


Figure 4. Reaction profile under the optimum conditions. Conditions: 50 mM HMF, 15 mL medium (20 g/L malt extract, 21.3 g/L glucose, 11.9 g/L peptones), three discs biocatalyst, pH 5.25, 160 rpm, 28 °C.

3.4. Toxicity of HMF and DFF towards the cells

The toxicity level of HMF towards the cells was assessed by increasing the initial concentration of HMF added to the media up to 100 mM (**Figure 5**). There was a decrease in the DFF yield when increasing the initial concentration of HMF, achieving a DFF yield of 70 and 40 % within 48 h for concentrations of HMF of 75 and 100 mM, respectively. A fed-batch approach was considered to overcome the toxic effect, in which 50 mM HMF were added again once the substrate was metabolized entirely (**Figure 6**). However, there was no further transformation of the HMF added in the second cycle, suggesting that the toxicity level of DFF towards the cells is also around 50 mM under the conditions studied. Different approaches can be taken to overcome the toxic effect, such as increasing the inoculum size of the cells by adding more discs or the inoculation in the form of spores[22]. To that end, the inoculation of the fungus from a suspension of spores was assessed. However, there was HMF reduction to DHMF with high yields (data not shown), indicating that the way of inoculation of the fungus has a significant effect on the HMF biotransformation. Another possibility that is currently being considered is to perform a biphasic system with a solvent that separates the DFF produced from the media. Further work considering these alternatives would improve the process performance and efficiency.

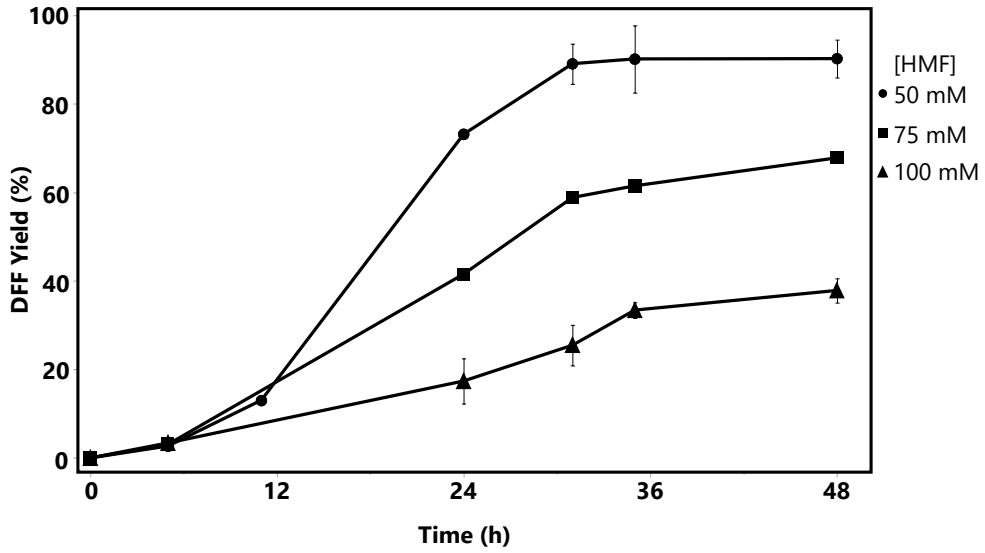


Figure 5. Effect of the initial concentration of HMF on the DFF yield. Conditions: 50, 75, or 100 mM HMF, 15 mL medium (20 g/L malt extract, 21.3 g/L glucose, 11.9 g/L peptones), three discs biocatalyst, pH 5.25, 160 rpm, 28 °C.

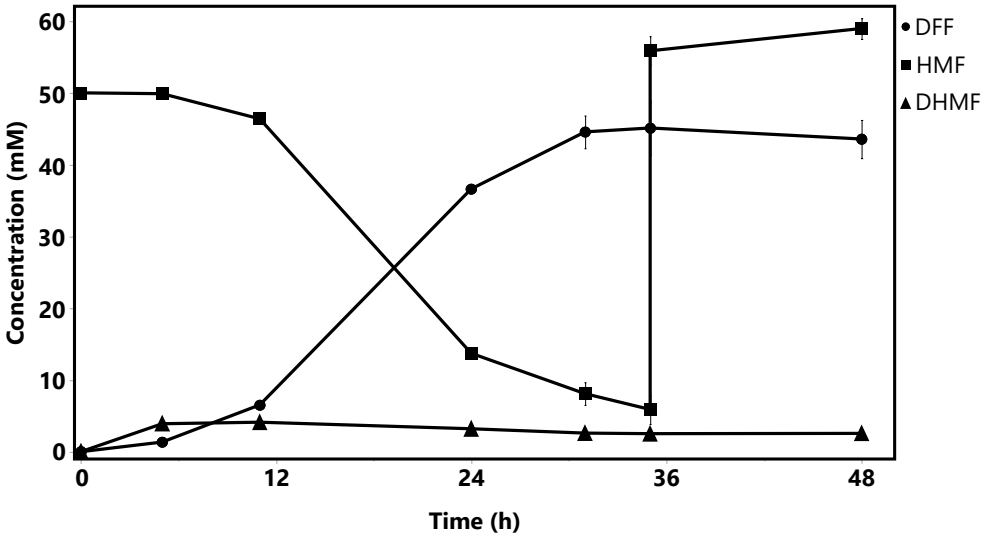


Figure 6. Fed-batch approach. Conditions: 50 mM HMF, 15 mL medium (20 g/L malt extract, 21.3 g/L glucose, 11.9 g/L peptones) three discs biocatalyst, pH 5.25, 160 rpm, 28 °C.

Although several results have been reported in the enzymatic synthesis of DFF from HMF, the low productivities obtained manifest the need for further investigation. Moreover, the combination of different enzymes, which are usually produced and purified separately in different hosts, is needed to achieve a high yield and selectivity[15,16,44], adding a high cost to the process. *F. culmorum* shows the capability to reduce to DHMF and oxidize to DFF high concentrations of HMF, depending on media conditions. The enzymes that catalyze the reduction of HMF to DHMF when using whole cells are described in various microorganisms. For instance, Martins *et al.* (2020) and Ran *et al.* (2014) hypothesized that an aldehyde oxidoreductase is

responsible for the reduction in the filamentous fungi *Aspergillus nidulans* and the fungi *Pleurotus ostreatus*, respectively. However, the whole-cell oxidation of HMF to DFF has not been described to the best of our knowledge. *Fusarium* species are natural producers of the enzyme GO[23,24], which catalyzes the oxidation of HMF to DFF with quantitative yields in combination with catalase and HRP[15,16]. Arguably, *F. culmorum* can produce the enzymes needed for the efficient oxidation of HMF to DFF, but their expression is highly dependent on the nitrogen source and the peptone and glucose concentrations in the media. Hybrid processes, understood as the combination of different biocatalytic approaches (fermentation, microbial catalysis, enzyme catalysis…), provide hope in the biocatalytic sustainable transformation of substrates like HMF[21]. Therefore, the novel process presented could also be used as an intermediate step in the synthesis of other HMF derivatives (such as FDCA) produced through further oxidation of DFF.

4. Conclusions

Herein, we describe the whole-cell oxidation of HMF to DFF for the first time to the best of our knowledge. The use of a single whole-cell catalyst to produce DFF represents a significant advance thanks to the inherent presence of all the enzymes required, reducing the cost of the process. The nitrogen source and the concentration of peptone and glucose in the reaction media highly influenced the transformation of HMF by the strain of *F. culmorum* EAN 51. The concentration of both nutrients was optimized through RSM, allowing DFF production with a high yield (92%) and selectivity (94%) starting from 50 mM HMF under the estimated optimum conditions. Moreover, the RSM study provided a better understanding of the conditions needed to efficiently oxidize HMF, which could be of interest for further optimization of the reaction. The knowledge acquired may not be limited to the biocatalyst and process used. Furthermore, the process described meets several principles of green chemistry. There is no use or generation of toxic substances, the solvents and auxiliaries used are safe, it is conducted at ambient temperature and pressure, the substrate and the biocatalyst come from renewable feedstocks, there is no use of derivatives, the biocatalyst is highly selective and biodegradable, and it is performed under mild and safe conditions. These results open a new line of investigation in the sustainable production of DFF from renewable biobased resources.

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Supplementary materials

Table S1. Amino acid profile of the different peptones.

Concentration (mg amino acid / g sample)			
Amino acid	**Meat**	**Casein**	**Soybean**
Ala	28.6 ± 0.8[a]	18.6 ± 0.3[b]	15.1 ± 0.2[c]
Arg	61.3 ± 2.8[a]	41.3 ± 2.0[c]	53.0 ± 3.2[b]
Asx	69.6 ± 0.6[ab]	68.2 ± 2.4[b]	72.2 ± 0.8[a]
Cys	6.2 ± 0.5[a]	1.2 ± 0.5[c]	3.4 ± 0.6[b]
Glx	125.8 ± 5.8[b]	149.6 ± 7.1[a]	118.6 ± 4.8[b]
Gly	70.7 ± 5.5[a]	27.3 ± 3.9[c]	57.1 ± 5.3[b]
Hyp	10.5 ± 0.2[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
His	23.5 ± 0.7[b]	31.2 ± 1.3[a]	17.7 ± 0.7[c]
Ile	36.4 ± 1.0[b]	56.1 ± 0.8[a]	20.2 ± 1.3[c]
Leu	76.6 ± 1.9[b]	90.5 ± 5.8[a]	64.3 ± 1.2[c]
Lys	82.2 ± 4.2[b]	98.3 ± 2.8[a]	50.1 ± 2.1[c]
Met	30.7 ± 0.5[b]	37.2 ± 2.3[a]	7.8 ± 0.1[c]
Phe	20.7 ± 0.4[b]	26.7 ± 1.0[a]	18.1 ± 0.9[c]
Pro	66.4 ± 2.9[b]	90.7 ± 4.5[a]	31.7 ± 0.9[c]
Ser	46.4 ± 2.0[b]	62.1 ± 0.8[a]	41.5 ± 0.7[c]
Thr	36.3 ± 1.6[a]	37.7 ± 1.0[a]	30.7 ± 0.7[b]
Tyr	26.3 ± 0.7[b]	47.8 ± 0.4[a]	24.0 ± 1.0[c]
Val	53.8 ± 1.9[b]	77.7 ± 2.7[a]	35.5 ± 0.8[c]

Means with different letters are significantly different (Tukey HSD test, $p < 0.05$)

Table S2. DFF and DHMF yields of the first Central Composite Design.

Run	[Peptone] (g/L)	[Glucose] (g/L)	DFF Yield (%)	DHMF Yield (%)
1	15	34	51.69	25.86
2	15	6	0	>90
3	3	34	0	>90
4	3	6	0	>90
5	9	20	67.06	21.16
6	9	20	71.76	16.46
7	9	20	75.04	14.37
8	9	20	60.65	25.08
9	9	20	81.82	0
10	17.49	20	43.24	22.99
11	0.51	20	0	>90
12	9	39.8	0	>90
13	9	0.2	0	>90

Table S3. ANOVA of the first Central Composite Design.

Source	Sum of Squares	DF	Mean Square	F-ratio	Prob > F
Model	13916.457	5	2783.29	31.8552	0.0001
[Peptones] (x_1)	1591.3965	1	1591.3965	18.2137	0.0037
[Glucose] (x_2)	334.4616	1	334.4616	3.8280	0.0913
$x_1 * x_2$	667.8769	1	667.8769	7.6439	0.0279
$x_1 * x_1$	4096.9381	1	4096.9381	46.89	0.0002
$x_2 * x_2$	8564.6704	1	8564.6704	98.0238	<0.0001
Error	611.613	7	87.37		
Lack of fit	355.38260	3	118.461	1.8493	0.2787
Pure error	256.23083	4	64.058		
R^2=0.958; $R^2_{adjusted}$=0.928; $R^2_{Pedicted}$: 0.799					

Table S4. DFF and DHMF yields of the second Central Composite Design.

Run	[Peptone] (g/L)	[Glucose] (g/L)	DFF Yield (%)	DHMF Yield (%)
1	10.5	22	80.28	7.53
2	10.5	14.93	82.48	0
3	10.5	29.07	42.14	24.28
4	13.5	27	86.78	6.40
5	10.5	22	90.84	0
6	6.26	22	17.94	11.95
7	10.5	22	86.96	0
8	13.5	17	67.92	10.23
9	7.5	17	77.85	7.36
10	7.5	27	0	25.14
11	14.74	22	79.22	0
12	10.5	22	87.40	0
13	10.5	22	86.10	0

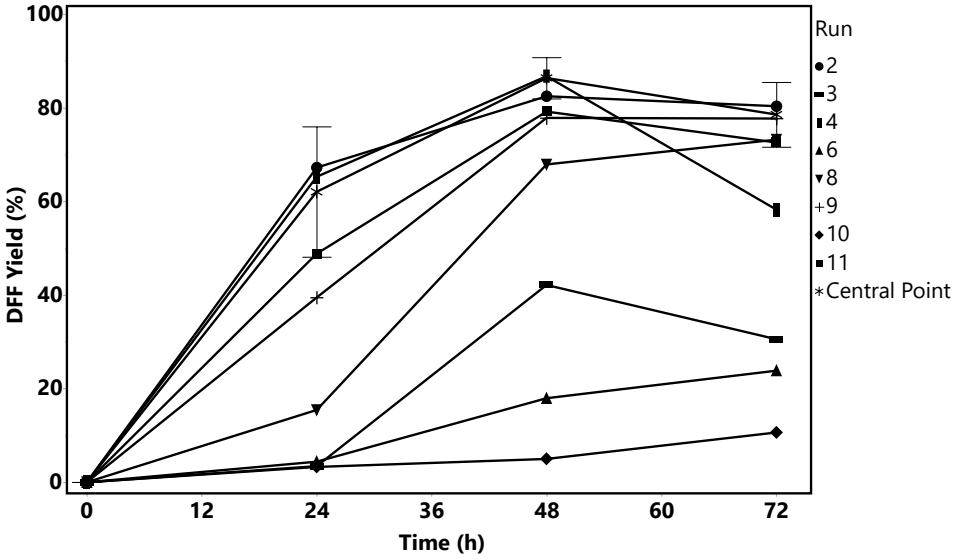


Figure S1. DFF yields for the different conditions of the second Central Composite Design. Conditions: 50 mM HMF, three discs biocatalyst, pH 5.25, 160 rpm, 28 °C.

3.4. Co-cultivation of a novel *Fusarium striatum* strain and a xylose consuming *Saccharomyces Cerevisiae* yields an efficient process for simultaneous detoxification and fermentation of lignocellulosic hydrolysates

Due to the high capability of *F. striatum* to reduce HMF into its less toxic alcohol derivative DHMF (Chapter 3.2), the application of this strain as a biological detoxification method in the lignocellulosic ethanol industry was considered of interest.

The results showed in the present Chapter were carried out in the Department of Chemical and Biochemical Engineering (Technical University of Denmark) under the supervision of Krist V. Gernaey. A collaboration with Sune Tjalfe Thomsen, from the Department of Geosciences and Natural Resource Management (University of Copenhagen), was established.

The results of this Chapter have been published in *Chemical Engineering Journal*. 426 (2021) 131575.

Co-cultivation of a novel *Fusarium striatum* strain and a xylose consuming *Saccharomyces cerevisiae* yields an efficient process for simultaneous detoxification and fermentation of lignocellulosic hydrolysates

Alberto Millán Acosta[1*], Diana Cosovanu[1], Pau Cabañeros López[2], Sune Tjalfe Thomsen[3], Krist V. Gernaey[2*], Ramon Canela-Garayoa[1*]

[1] Chemistry Department, University of Lleida-DBA Center, Alcalde Rovira Roure 191, 25198 Lleida, Spain;

[2] Process and Systems Engineering Center (PROSYS), Department of Chemical and Biochemical Engineering, Technical University of Denmark, Building 228 A, 2800 Kgs. Lyngby, Denmark

[3] Department of Geosciences and Natural Resource Management, University of Copenhagen, Frederiksberg, Denmark

Keywords: *Fusarium striatum*, Furfural, 5-Hydroxymethylfurfural, Co-culture, Ethanol, Detoxification

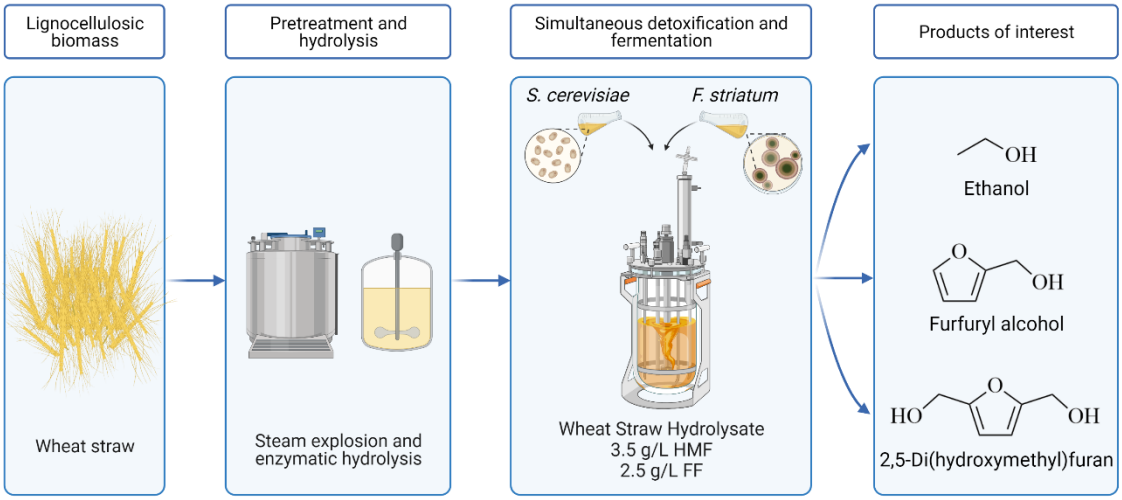
Abstract

Furfural (FF) and 5-hydroxymethylfurfural (HMF) are furan derivatives commonly generated during the pretreatment of lignocellulosic biomass and often considered among the most inhibitory compounds towards the sugar fermenting strains due to their acute toxicity and high concentrations. The present study describes the simultaneous detoxification and fermentation of lignocellulosic hydrolysates containing high concentrations of FF and HMF by a co-culture of a novel *Fusarium striatum* strain and a xylose consuming *Saccharomyces cerevisiae* strain. The process demonstrates a superior performance than those previously described in the literature, as FF and HMF were efficiently transformed into their less toxic added-value alcohol derivatives by *F. striatum* with high yields (99 % and 86 %, respectively) and the higher detoxification rates reported (0.56 g/L/h and 0.13 g/L/h, respectively). There was no sugar consumption by the filamentous fungus during the detoxification process, rendering it available for ethanol fermentation by *S. cerevisiae*, which started immediately after the detoxification of the inhibitors. Ethanol productivities were significantly higher when increasing the inoculum size of

F. striatum, confirming its potential for the detoxification of the lignocellulosic hydrolysate. High ethanol yields (0.4 g/g) and productivities (0.46 g/L/h) were obtained in a bench-scale bioreactor (1.5 L) in the presence of 3.5 g/L HMF and 2.5 g/L FF, a concentration of furan derivatives that completely inhibited the fermentation process in the absence of *F. striatum*. The presented process allows access to lignocellulosic materials and pretreatment methods that result in high concentrations of FF and HMF that are currently not feasible, representing a significant advance for the lignocellulosic ethanol industry.

Keywords: *Fusarium striatum*, Furfural, 5-Hydroxymethylfurfural, Co-culture, Ethanol, Detoxification

Graphical abstract



1. Introduction

Lignocellulosic material, often derived from agricultural or forestry wastes, has received significant attention as a potential alternative to fossil fuels due to its high sugar content. This has promoted intensive research in the biological transformation of different lignocellulosic feedstocks into more valuable products such as ethanol, produced after fermenting the sugars using yeasts such as *Saccharomyces cerevisiae* [1]. However, despite the significant advances achieved during the last thirty years, the recalcitrant structure of lignocellulosic material still hinders its utilization at a commercial scale. Making the sugars accessible for the fermentation requires a pretreatment step where the lignocellulosic material is exposed to high temperatures and/or extreme (acid or alkaline) pH [2,3]. This promotes the generation of various compounds that inhibit yeast metabolism and reduce the productivity of lignocellulosic ethanol processes [4]. These inhibitors are often classified according to their chemical structure as furan derivatives (mainly furfural (FF) and 5-hydroxymethylfurfural (HMF)), weak acids (such as acetic-, formic- or levulinic acids), and phenolic compounds (such as vanillin or p-hydroxybenzoic acid) [4,5]. FF and HMF are sugar dehydration products considered among the most inhibitory compounds due to their acute toxicity (both inhibit the central carbon metabolism of *S. cerevisiae* [6,7]) and the high concentrations (reaching up to 6 g/L of HMF [8] and 7 g/L of FF [9] depending on the feedstock and the pretreatment method). Their toxicity towards the cells is increased when both compounds are present simultaneously due to their combined inhibitory effects [10]. Upon contact with the inhibitors, *S. cerevisiae* attempts to reduce their concentration by converting them into less inhibitory compounds. This detoxification phase extends the length of the fermentation and reduces the volumetric productivity. However, if the concentration of inhibitors exceeds the detoxification capabilities of the cell culture, it can result in severe stress and loss of cell culture viability [11].

Previous studies have found that lignocellulosic hydrolysates with a total concentration of HMF and FF above 2.5 g/L can reduce the ethanol production rate by an order of magnitude [11]. This results in some biomass not being sufficiently pretreated to balance the severity towards the inhibitor content, thereby reducing the sugar availability. Therefore, there is limited accessibility to lignocellulosic feedstocks and pretreatment methods, as only those yielding lower HMF and FF concentrations can be efficiently used to produce ethanol. In order to increase the fraction of

accessible feedstocks, various methods to reduce the concentration of inhibitors prior to the fermentation have been proposed in the literature [12]. The different detoxification approaches are often classified as physical, chemical, or biological methods [13]. Physical detoxification requires the addition of chemical compounds to extract the inhibitors (often using precipitation, liquid-liquid, or solid-liquid extraction [12,13]), while chemical detoxification takes advantage of the instability of the inhibitors at extreme conditions such as pH [13]. Even though these methods can efficiently reduce the concentration of inhibitors, the costs associated with their implementation are still too high to be competitive in the ethanol market [12,14]. Some of these methods often result in partial removal of the sugars together with the inhibitors, reducing the availability of fermentable sugars [13]. Moreover, high chemical use is associated with fossil inputs, making them less sustainable. Biological detoxification methods use specific microbes able to handle and remove high concentrations of inhibitors. In contrast to the physical or chemical methods, biological detoxification offers significant advantages, such as milder reaction conditions, no need for chemical addition, fewer side-reactions, and lower energy requirements [12]. The potential detoxification of lignocellulosic hydrolysates using monocultures of different species of fungi, such as *Trichoderma reesi* [14], *Amorphotheca resinae ZN1* [15], *Coniochaeta ligniaria* [16], or *Pleurotus Ostreatus* [17], has been previously studied due to their high tolerance towards high concentrations of inhibitors (0.3 – 3.8 g/L). However, one of the main drawbacks of biological detoxification is the low conversion rates of the inhibitors. An attractive possibility is the co-culture of two different microorganisms to achieve a synergistic effect between the different organisms to detoxify the media and produce ethanol. Fungal co-cultures have been previously studied in the lignocellulosic ethanol industry mainly to produce lignocellulolytic enzymes [18]. However, their use in the detoxification of inhibitors has received little attention. Yu *et al.* [19] showed that *A. nidulans* FLZ10 was able to remove 0.38 g/L of HMF and 0.02 g/L of FF within 72 h from steam-exploded corn stover in a co-culture with *S. cerevisiae*, obtaining productivities 3.25 times higher than that without detoxification. Zhu *et al.* [20] showed that a co-culture of two strains *of S. cerevisiae,* a xylose-consuming, and an inhibitor-tolerant strain, degraded 0.7 g/L of FF and 0.4 g/L of HMF within 6 and 48 h, respectively. Even though these results are encouraging, the tested concentrations of inhibitors and the detoxification rates were rather low to represent a significant advantage for the lignocellulosic ethanol industry.

The novel strain of *F. striatum* UdL-TA-3.335 is an attractive organism as it displays a high tolerance towards high concentrations of HMF (up to 9.5 g/L) while retaining high detoxification rates (0.4 g/L/h). Moreover, it can be efficiently grown in bioreactors, making it a promising biocatalyst for industrial applications [21]. *S. cerevisiae* is the standard microorganism used for ethanol fermentation [22,23]. The xylose-fermenting *S. cerevisiae* CEN.PK XXX strain has the capability to ferment both glucose and xylose, which is valuable in the lignocellulosic ethanol industry due to the presence of both sugars in lignocellulosic hydrolysates [24]. The objective of the present work is to study the potential of a co-culture between *F. striatum* UdL-TA-3.335 and *S. cerevisiae* CEN.PK XXX to promote the simultaneous detoxification of HMF and FF and the fermentation of lignocellulosic hydrolysates. The capabilities of *F. striatum* to efficiently detoxify different mixtures of HMF and FF and to establish beneficial synergies with *S. cerevisiae* are assessed at various scales using wheat straw hydrolysate spiked with different concentrations of the inhibitors.

2. Materials and methods

2.1. Preparation of the wheat straw hydrolysate

Dried winter wheat straw composed of cellulose (34–40%), hemicellulose (20–25%), and lignin (20%) [25–27] provided by TK Energy ApS (Denmark) was pretreated using steam explosion and enzymatically hydrolyzed at the previously described pilot plant facilities in the Department of Chemical Engineering at Lund University (Sweden) [28–30].

2.1.1. Steam explosion

Prior to pretreatment, the wheat straw was soaked to secure uniform steam penetration. A total of 10 kg of wheat straw were soaked in water for 1 h at a biomass-water ratio of 1:10 prior to press filtration at 200 bar for 3 - 5 min (obtaining a dry matter (DM) content of 45 %). The pretreatment was done at 200 °C, and 16.8 bar on 1.5 kg of moist washed wheat straw with a retention time of 10 min, terminated with a rapid pressure release enabling explosive decomposition of the biomass structure. This procedure was repeated 14 times, producing a total of 41.4 kg of pretreated wheat straw at 20 %DM, which was stored at 4 °C until use.

2.1.2. Enzymatic hydrolysis

Prior to the enzymatic hydrolysis, the pretreated material was diluted with milliQ water to 10 %DM to facilitate stirring, and the pH was adjusted to 4.8 using 50 % NaOH. The enzymatic hydrolysis was done in 150 L reactors with a working volume of 75 L at 50 °C for 72 h, with stirring at 300 rpm and with 5000 U of Cellic CTec2 (Novozymes A/S, Bagsværd, Denmark) per kg of slurry (corresponding to an enzyme/cellulose mass ratio of 6%). The resulting slurry was stored in 5 L containers at -20 °C until use. The slurry contained 36 g/L glucose, 18 g/L xylose, 0.55 g/L FF and 0 g/L HMF.

2.2. Microorganisms

A volume of 1 mL of glycerol stock of *S. cerevisiae* CEN.PK XXX (background *S. cerevisiae* CEN.PK 122 MDS with the overexpression of XKS1 and the insertion of *XYL1* and *XYL2* from *Scheffersomyces stipites* to promote the consumption of xylose [24]) (kindly provided by Prof. Carl Johan Franzén from the Chalmers University of Technology, Sweden) was spread onto a YPX agar plate (yeast extract 10 g/L, peptone from casein 20 g/L, xylose 20 g/L, 15 g/L agar) and incubated for 48 h at 30 °C and 180 rpm, and stored in the fridge at 4 °C for one month.

F. striatum (UdL-TA-3.335) was previously isolated from food waste by our group [21]. It was maintained by replications on malt extract agar (MEA: 20 g/L glucose, 20 g/L malt extract, 1 g/L, peptone from soybean, 15 g/L agar) at 4 °C. Before fermentation experiments, it was activated in MEA for seven days at 28 °C.

2.2.1 Preparation of the inoculums

A colony of *S. cerevisiae* CEN.PK XXX grown on a YPX agar plate was incubated in a 250 mL shake flask containing 100 mL of liquid YPX media for 36 h at 30 °C and 180 rpm. The cell culture was then propagated to a 250 mL shake flask containing 100 mL of wheat straw hydrolysate supplemented with 5 g/L of yeast extract and 10 g/L of peptone from casein to reach an initial inoculum size of 0.5 g/L (dry weight).

F. striatum was transferred to 250 mL shake flasks containing 100 mL of liquid ME by the addition of ten 8 mm fungal discs from the agar plates and incubated in a rotatory shaker at 30 °C and 180 rpm for 5 days. The cell culture was centrifuged, the supernatant discarded, and the cells

transferred to a 250 mL shake flask containing 100 mL of wheat straw hydrolysate to reach different inoculum sizes ranging from 0.22 to 10 g/L (dry weight).

2.3. Competition for fermentable sugars

S. cerevisiae (0.5 g/L) was inoculated into 100 mL of wheat straw hydrolysate containing 0.55 g/L of furfural singly or as a co-culture with *F. striatum* (0.22 g/L). The furfural and ethanol concentrations were monitored.

To compare the glucose uptake by both microorganisms, *S. cerevisiae* (0.5 g/L) and *F. striatum* (0.22 g/L) were incubated separately as monocultures into the wheat straw hydrolysate containing 0.55 g/L of furfural and the glucose concentration was monitored.

2.4. Simultaneous detoxification and fermentation

A total of 22 different shake flask experiments were done with different concentrations of *S. cerevisiae*, *F. striatum,* FF, and HMF (**Table 1**). All shake flasks contained 100 mL of wheat straw hydrolysate with the monoculture of *S. cerevisiae* or the co-culture with *F. striatum* and were incubated in a rotatory shaker at 30 °C and 160 rpm. HMF and FF were spiked to the media at different concentrations ranging from 0 - 5 g/L (HMF) and 0.55 - 2.5 g/L (FF) prior to the inoculation of the cells (**Table 1**).

Table 1. Experimental conditions of the shake flask experiments performed in 100 mL of wheat straw hydrolysate.

Experiment	Inoculum size (g/L)		[Glucose] (g/L)	[Xylose] (g/L)	[Inhibitors] (g/L)	
	S. cerevisiae	*F. striatum*			HMF	FF
1	0.5	0.22	36	18	2	1
2	0.5	0.22			3.5	0.3
3	0.5	0.22			2	2
4	0.5	0.22			3.5	2.5
5	0.5	0.22			5	1.5
6	0.5	0	36	18	5	1.5
7	0.5	0.22				
8	0.5	0.67				
9	0.5	1.25				
10	0.5	2.02				
11	0.5	2.30				
12	0.5	2.69				
13	0.5	4.40				
14	0.5	0	36	18	3.5	2.5
15	0.5	0.22				
16	0.5	0.67				
17	0.5	1.25				
18	0.5	2.02				
19	0.5	2.30				
20	0.5	2.69				
21	0.5	5				
22	0.5	10				

2.5. Bioreactor

Bioreactor experiments were carried out in a 3 L bioreactor (Applikon, Delft, The Netherlands). A volume of 1.5 L of the wheat straw hydrolysate containing 36 g/L glucose and 18 g/L xylose and spiked with 3.5 g/L of HMF and 2.5 g/L of FF, was inoculated with a co-culture of *S. cerevisiae* (0.5 g/L) and *F. striatum* (5 g/L). The fermentations were operated at pH 6.0 using 2 M NaOH and 2 M H_2SO_4 for maintaining a constant pH of 6, at 30 °C using a heat jacket and a cooling finger, and at a stirring rate of 450 rpm using two six-bladed Rushton impellers. A control experiment was done with the same experimental conditions without the addition of *F. striatum* (**Figure 1**).

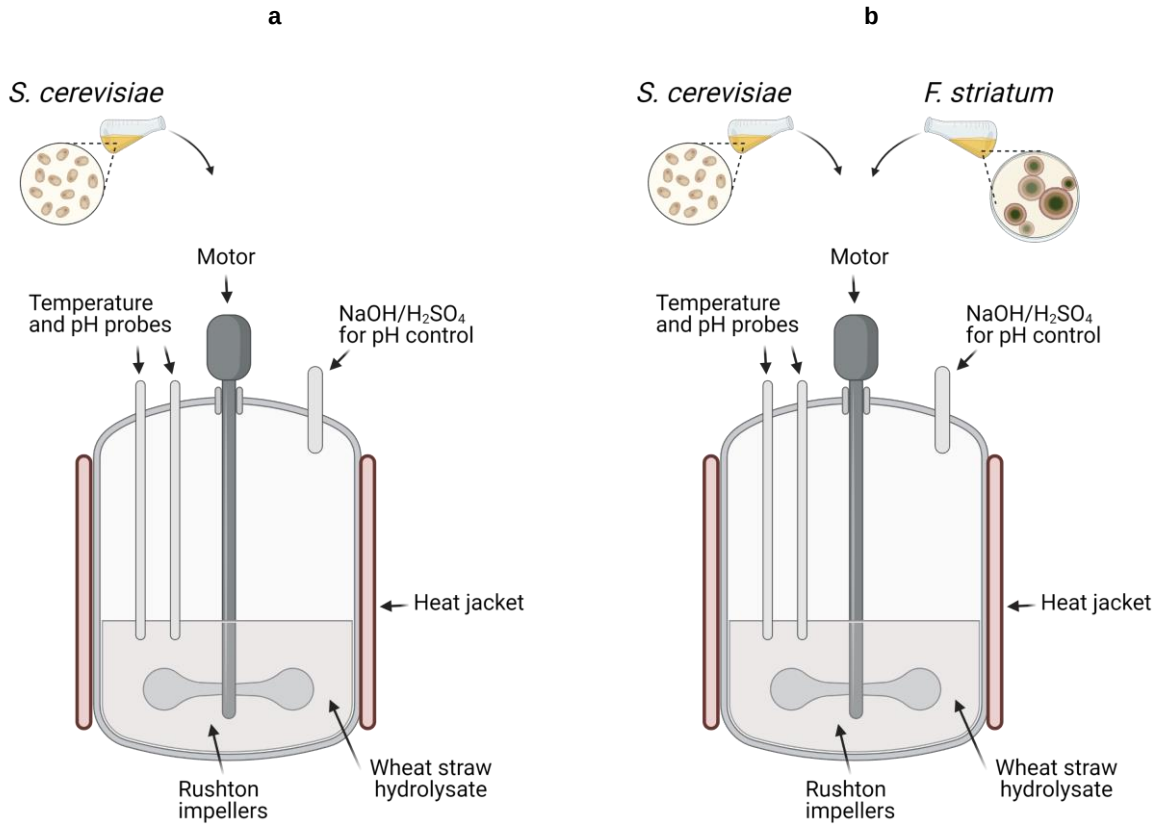


Figure 1. Diagram of the bioreactors. **a)** Control, **b)** Co-culture.

2.6.Chromatography analysis

A sample of 1.5 mL was withdrawn from the shake flasks or the bioreactors at selected fermentation times, filtrated through a 0.20 µm cellulose acetate filter (Labsolute, Renningen, Germany), and stored at -22 °C until analyzed.

The concentrations of glucose, xylose, ethanol, HMF, and FF were determined by an Ultimate 3000 HPLC instrument (Thermo Scientific, Massachusets, USA) equipped with an Aminex HPX-87H column (7.8 mm x300; 9 µm, BIORAD, California, USA), 4 UV/VIS channels and a refractive index (RI) detector (ERC RefractoMax 520, Prague, Czech Republic). A sample volume of 950 µL was acidified with 50 µL of 5 M H_2SO_4 prior to the injection. The mobile phase consisted of 5 mM H_2SO_4 and the elution was in isocratic mode, with a flow rate of 0.6 mL/min at 50 °C. The method lasted 55 min.

The concentrations of furfuryl alcohol (FA) and 2,5-di(hydroxymethyl)furan (DHMF) were determined by an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with an ultra-inert splitless liner containing a piece of glass wool coupled to an FID detector. For the

chromatographic separation, an FFAP (30 m x 0.25 mm i.d.; 0.25 µm film thickness) column from Agilent was used at a constant flow rate of 1 mL/min using hydrogen as carrier gas. Injector temperature was 230 °C, and the oven temperature program was started at 100 °C (held for 1 min), and then increased to 240 °C at a rate of 20 °C/min (held for 5 min). The compounds were extracted from the aqueous samples using ethyl acetate.

2.7. Determination of fermentation parameters

Ethanol yield was calculated as the ratio between the maximum ethanol concentration and the initial concentration of fermentable sugars (glucose + xylose). The ethanol productivity was calculated as the ratio between final ethanol concentration and total fermentation time. FF and HMF degradation rates were defined as the quantity of inhibitor consumed within the first 3 h.

2.8.Statistical analysis

The statistical analyses were assessed using the software JMP Pro 14 (SAS). The results obtained were subjected to analysis of variance (ANOVA). Statistical significance was assessed with the *p*-value in Fisher's test with a 95% confidence level. Means were compared with the Tukey HSD test, and significant differences are indicated with different letters. All experiments were conducted at least in duplicate, and the values are expressed as the means ± standard deviations when applicable.

3. Results and discussion

3.1. Competition for fermentable sugars

The potential competition for the carbon source between *S. cerevisiae* and *F. striatum* was assessed by comparing the fermentation profiles of a monoculture of *S. cerevisiae* (used as a control) and the co-culture of *F. striatum* and *S. cerevisiae* (**Figure 2.a**).

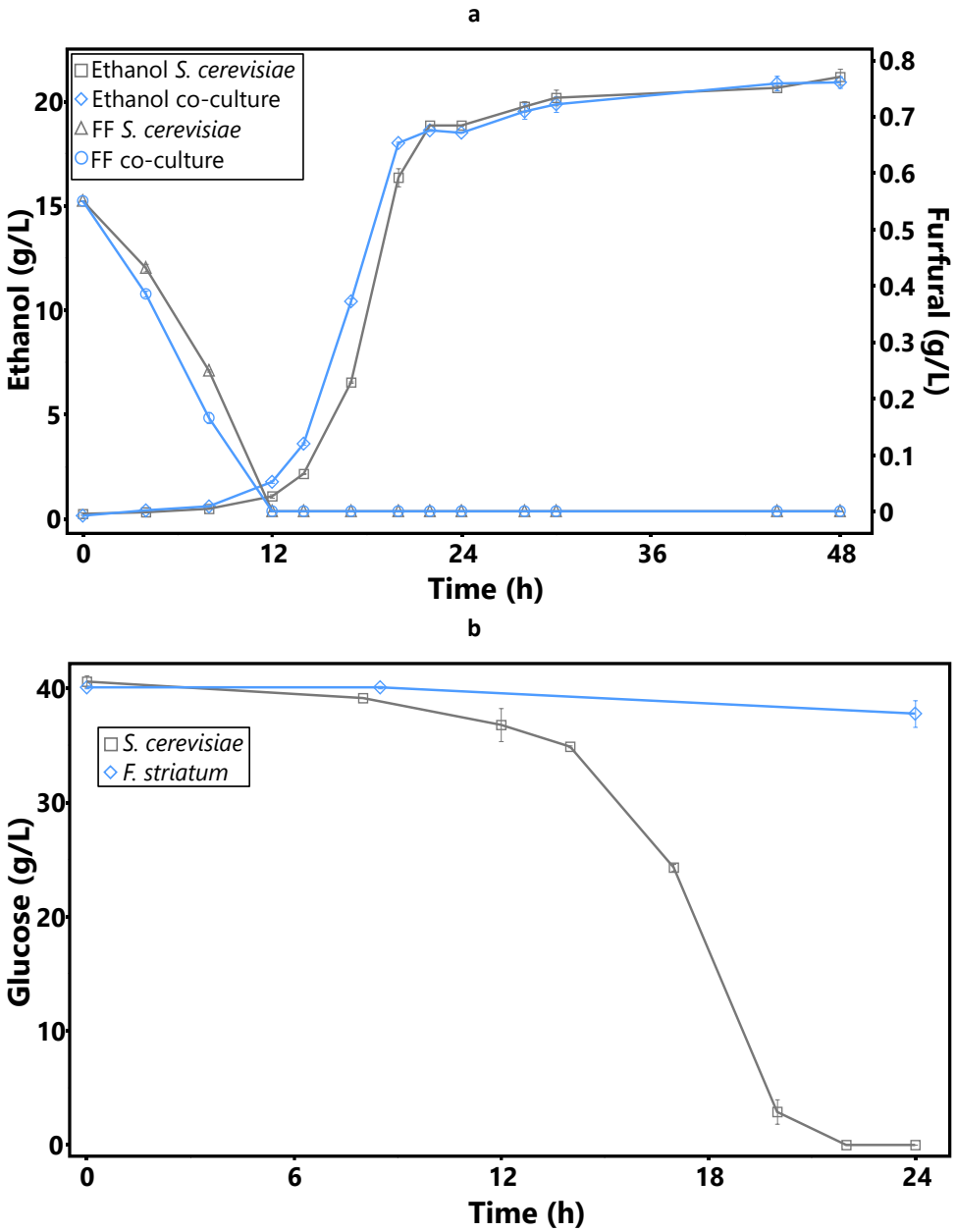


Figure 2. Competition for fermentable sugars. **a)** Ethanol fermentation and furfural concentration in wheat straw hydrolysate by the monoculture of *S.* cerevisiae and the co-culture, **b)** Glucose consumption in wheat straw hydrolysate by the monocultures of both microorganisms.

The results show that the final ethanol concentration in the co-culture and the control experiments was very similar and no significant differences were detected between them (**Figure 2.a**). The yields and productivities obtained were 0.38 g/g and 0.47 g/L/h in both cases, suggesting that no competition for the carbon source occurred. Moreover, in both experiments the fermentation only started after FF was completely metabolized. This occurred slightly faster in the co-culture than in the control experiment, arguably due to the increased detoxification capabilities of *F. striatum.* To further confirm these results, the consumption of glucose by monocultures of both microorganisms in the same wheat straw hydrolysate was studied **(Figure 2.b**). The results show that *S. cerevisiae* takes up glucose at a much faster rate than *F. striatum*: while *S. cerevisiae* depleted all the glucose within the first 24 h of the fermentation, *F. striatum* only consumed 5% of the initial glucose within that time. A similar trend was observed for xylose in a 48 h time frame. This indicates a synergistic effect of the co-culture in which *F. striatum* detoxifies FF but does not compete significantly with yeast for fermentable sugars due to the different uptake rates between both microorganisms. Similar synergistic behaviors have previously been described for a co-culture of *S. cerevisiae* and *A. nidulans* FLZ10 [19]. Therefore, a facultative mutualism was observed between both species under these conditions, as both benefited from each other's presence but could also perform their tasks separately.

3.2.Simultaneous detoxification of high concentrations of inhibitors and fermentation

To study the capabilities of the co-culture to detoxify media with high concentrations of HMF and FF, a series of five fermentations (**Table 1** experiments 1-5) of wheat straw hydrolysate spiked with different amounts of both inhibitors was conducted. The results of these five experiments are shown in **Table 2**. In all cases, the total concentration of HMF and FF exceeded the 2.5 g/L previously identified as the threshold in limiting the ethanol production rate [11]. The results showed that both HMF and FF were efficiently metabolized when their concentration together was equal to or lower than 4 g/L (**Table 2** experiments 1-3) with an ethanol yield around 0.4 g/g. However, the productivities decreased with increasing concentrations of furan derivatives due to the longer detoxification times (**Table 2** experiments 1-3**)**. No growth was detected when the total inhibitor concentration was above 5 g/L (**Table 2,** experiments 4-5), arguably due to the high toxicity of the inhibitors.

Table 2. Ethanol yields and productivities of the co-culture at different inhibitor concentrations.

Experiment	[Glucose] (g/L)	[Xylose] (g/L)	[HMF] (g/L)	[FF] (g/L)	Yield (g/g)	Productivity (g/L/h)
1			2	1	0.41 ± 0.03	0.40 ± 0.02
2			3.5	0.3	0.42 ± 0.01	0.31 ± 0.00
3	36	18	2	2	0.40*	0.29*
4			3.5	2.5	0	0
5			5	1.5	0	0

* One replicate

Then, the effect of the inoculum size of *F. striatum* (ranging from 0 to 10 g/L) on the detoxification capability of the co-culture was studied in the wheat straw hydrolysate spiked with two different combinations of HMF and FF in high concentrations (**Figure 3**). The first combination of inhibitors contained 5 g/L of HMF and 1.5 g/L of FF (experiments 6-13 in **Table 1**), and the second combination contained 3.5 g/L of HMF and 2.5 g/L of FF (experiments 14-22 in **Table 1**). Note that both cases resulted in no growth when the inoculum size was 0.22 g/L (**Table 2** experiments 4 and 5).

The results of this study showed that *S. cerevisiae* alone was not able to completely detoxify any of the tested inhibitor concentrations, and no growth was detected after 120 h of incubation. A partial FF detoxification was observed, although ethanol was not produced (**Figure 3**, and **Table 3** experiments 6 and 14). On the other hand, the inoculum size of *F. striatum* had a clear positive effect on the detoxification of HMF and FF. The addition of 4.4 g/L of *F. striatum* resulted in complete detoxification of both inhibitors within the first 21 h of fermentation for the first combination of inhibitors (**Figure 3.a**, and **Table 3** experiment 13), while the second combination of inhibitors required an initial inoculum size of 10 g/L to be metabolized within the initial 25 h (**Figure 3.b,** and **Table 3** experiment 22).

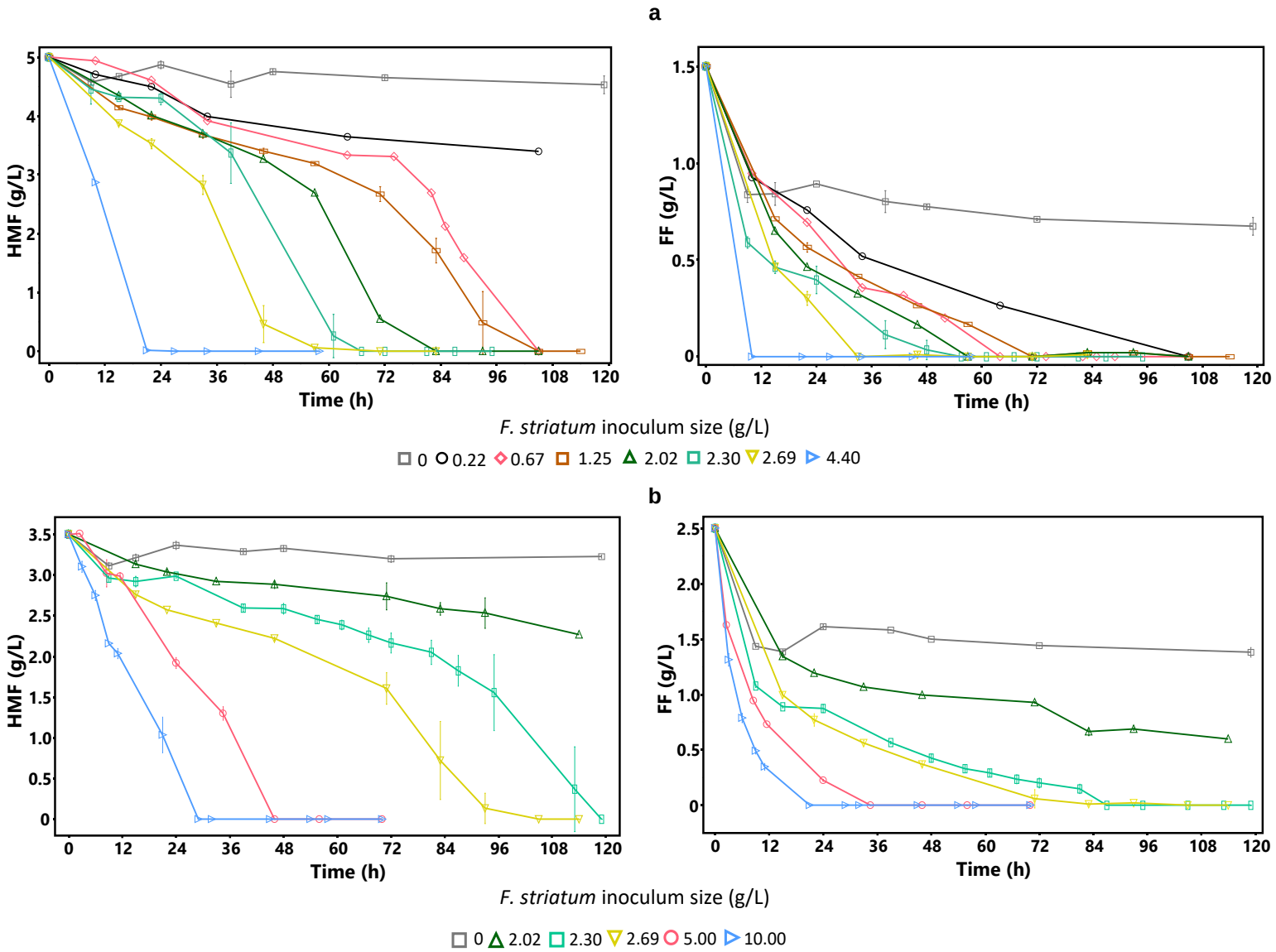


Figure 3. Effect of the inoculum size of *F. striatum* on the metabolization of inhibitors. **a)** Initial concentration of inhibitors: 5 g/L HMF + 1.5 g/L FF, **b)** Initial concentration of inhibitors 3.5 g/L HMF + 2.5 g/L FF

Glucose and xylose were efficiently fermented once HMF and FF were completely metabolized (**Figure 4**). Final yields and productivities for the different *F. striatum* inoculum sizes and inhibitor concentrations are shown in **Table 3**.

Table 3. Ethanol yields and productivities at different inhibitor concentrations and *F. striatum* inoculum sizes.

Experiment	[HMF] (g/L)	[FF] (g/L)	Inoculum size (g/L)	Yield (g/g)	Productivity (g/L/h)
6	5	1.5	0	0	0
7			0.22	0	0
8			0.67	0	0
9			1.25	0.36*	0.17*
10			2.02	0.39 ± 0.00[a]	0.20 ±0.00[d]
11			2.30	0.41 ± 0.01[a]	0.28 ± 0.01[c]
12			2.69	0.39 ± 0.00[a]	0.31 ± 0.00[b]
13			4.40	0.37 ± 0.01[a]	0.42 ± 0.01[a]
14	3.5	2.5	0	0	0
15			0.22	0	0
16			0.66	0	0
17			1.25	0	0
18			2.02	0	0
19			2.30	0.39 ± 0.01[a]	0.16 ± 0.00[c]
20			2.69	0.38 ± 0.01[a]	0.19 ± 0.01[c]
21			5	0.41 ± 0.01[a]	0.32 ± 0.01[b]
22			10	0.37 ± 0.01[a]	0.35 ± 0.01[a]

* One replicate

Means with different letters are significantly different (Tukey HSD test, $p < 0.05$)

A minimum *F. striatum* inoculum size of 1.25 g/L was needed to detoxify the concentration of HMF and FF present in the first combination of inhibitors (**Table 3** experiments 6-13) while a minimum *F. striatum* inoculum size of 2.30 g/L was needed for the second combination of inhibitors **(Table 3** experiments 14-22). Both furan derivatives were co-metabolized, although there was a preference for FF metabolization and an increase of the HMF detoxification rate once FF was completely consumed. These findings suggest a stronger toxic effect coming from FF, which has been pointed out previously [10].

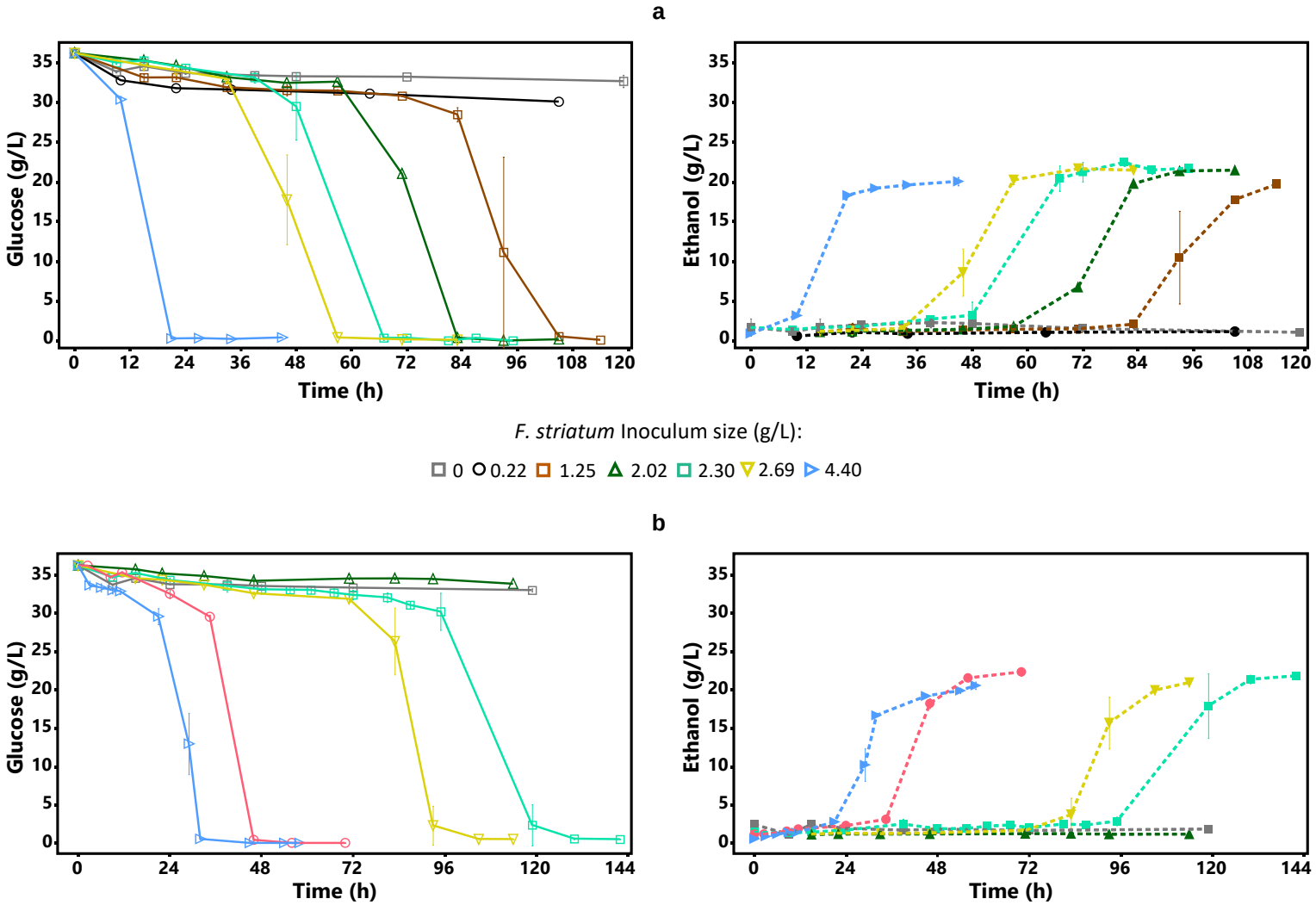


Figure 4. Effect of the inoculum size of *F. striatum* on glucose depletion and ethanol production. **a)** Initial concentration of inhibitors: 5 g/L HMF + 1.5 g/L FF, **b)** Initial concentration of inhibitors 3.5 g/L HMF + 2.5 g/L FF. Xylose is not included for clarity purposes.

The fermentation of sugars was only possible after detoxifying both inhibitors, which happened only in the presence of *F. striatum*. The ethanol yields were not significantly different when increasing the inoculum size (**Table 3**) and they were very similar to the ones obtained without the spiking of inhibitors (0.38 g/g), indicating that there was no competition for the sugars even when increasing the inoculum of *F. striatum*. The detoxification of furan derivatives with negligible glucose consumption by filamentous fungi was also observed for *A. nidulans* FZL10 and *A. resinae* ZN1 under lower concentrations of both inhibitors [15,19], arguably due to the inhibition of glycolysis enzymes in the presence of FF and HMF [10,31–33]. Moreover, there was a significant increase in productivity when the inoculum size was increased, confirming the synergistic effect of the co-culture and the potential of *F. striatum* for the detoxification of HMF and FF in lignocellulosic hydrolysates. Therefore, an obligatory mutualism was observed between both species under conditions with high concentrations of HMF and FF, as *S. cerevisiae* could not metabolize the inhibitors and ferment the sugars when incubated alone under the same conditions.

3.3. Bioreactor

The simultaneous detoxification of furan derivatives and ethanol fermentation was scaled up to a bench-scale bioreactor with 1.5 L of working volume. The media was spiked with the most toxic combination of inhibitors previously assayed, which corresponded to an HMF and FF concentration of 3.5 and 2.5 g/L, respectively (**Table 4**).

Table 4. Ethanol yields and productivities in the bioreactor.

Experiment	[HMF] (g/L)	[FF] (g/L)	Inoculum size (g/L)		Yield (g/g)	Productivity (g/L/h)
			S. cerevisiae	*F. striatum*		
Control	3.5	2.5	0.5	0	0	0
Co-culture			0.5	5	0.40	0.46

3.3.1. Ethanol fermentation and sugar consumption

Figure 5 shows the profile of the fermentation for both the monoculture of *S. cerevisiae* and the co-culture. In the absence of *F. striatum*, *S. cerevisiae* was not able to perform the fermentation due to the toxicity effect of both HMF and FF, and high concentrations of sugars were present after 72 h of incubation. When both microorganisms were inoculated together in the media, the

ethanol yield achieved was 0.4 g/g, similar to the yield obtained in shake flasks. Based on a theoretical ethanol yield of 0.51 g ethanol/g of sugars, an ethanol yield of 78.4 % was obtained. It is worth noting that the maximum ethanol yields on xylose are usually lower than the theoretical (around 0.40 g ethanol/g xylose) [34,35], which can also be observed in **Figure 5**. This leads to lower ethanol yields when considering xylose in the calculation.

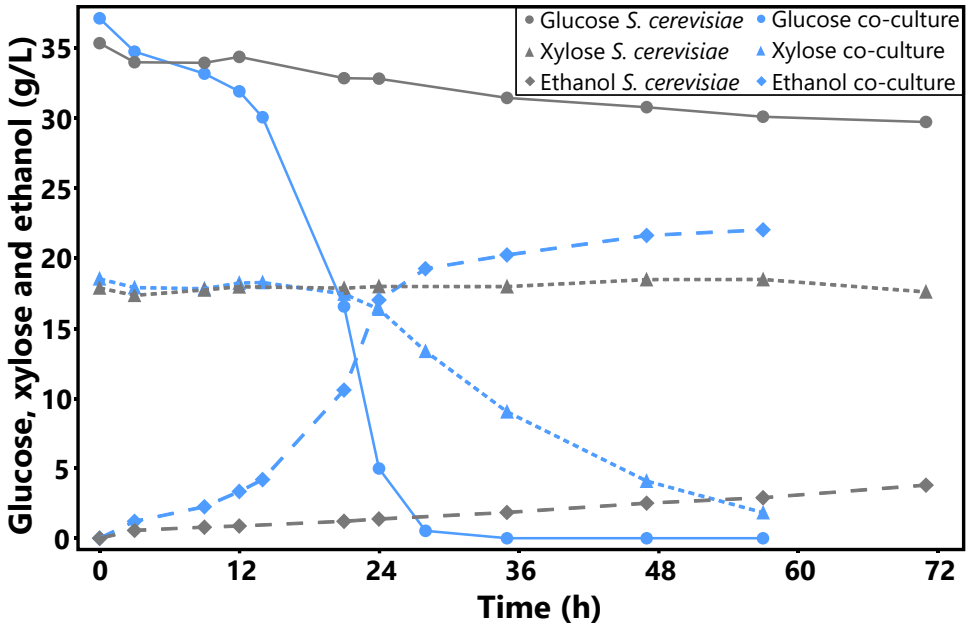


Figure 5. Fermentation profile in the bioreactors.

The ethanol productivity obtained at 47 h was 0.46 g/L/h, which is well comparable with other reported results in the literature considering the higher concentration of inhibitors assayed in this work (**Table 5**). Ethanol productivities are greatly affected by the presence of FF and HMF in the lignocellulosic hydrolysates [8]. Therefore, the high productivity obtained under high concentrations of FF and HMF confirms the feasibility of this process. Moreover, both glucose and xylose were fermented and considered to calculate the productivity. As shown in **Figure 5**, xylose is consumed at a much slower rate than glucose by this strain, leading to lower ethanol productivities when xylose is considered for the calculations. The productivity obtained in the bioreactor was higher than the one obtained in shake flasks for the same inoculum size and concentration of inhibitors (0.32 g/L/h, **Table 3**). This indicates that the co-culture had no problems growing in the bioreactor and that the bioreactor provided a better environment for simultaneous detoxification and fermentation. The improved fermentation performance in the bioreactor can arguably be due to the better mixing obtained in the bioreactor, the anaerobic

growth of the cells (which indicates that *F. striatum* is able to detoxify the inhibitors growing anaerobically), and the control of the pH throughout the fermentations (which was kept at 6). Weak acids typically present in lignocellulosic hydrolysates, like acetic or formic acid, have inhibitory effects when they are in their undissociated form. This can be alleviated by keeping the pH above the pK_a value of the weak acids [36].

Table 5. Comparison of FF and HMF degradation rates in lignocellulosic hydrolysates with literature results.

Microorganism	Total detoxification time (h)	Concentration (g/L)		Degradation rate (g/L/h)		Ethanol productivity (g/L/h)[c]	Simultaneous detoxification and fermentation	Ref.
		FF	HMF	FF	HMF			
C. ligniaria NRRL30616	17	1.26	0.30[a]	0.074	0.015	-	No	[37]
	22	1.31[a]	0.35[a]	0.048	0.007	-	No	[38]
	24	3.22[a]	0.42[a]	0.130	0.009	-	No	[16]
Bordetella sp. BTIITR	16	0.42	1.03	0.026[b]	0.064[b]	-	No	[10]
	20	0.60	1.30	0.063[b]	0.088[a,b]	-	No	[39]
Enterobacter sp. FDS8	3	1.70[a]	0.40[a]	0.540	0.12	-	No	[3]
A. nidulans FLZ10	72	0.02	0.38	0.0002	0.005	0.35[b]	Yes	[19]
S. cerevisiae	48	0.70	0.40	0.12[b]	0.025[b]	0.42[b]	Yes	[20]
S. cerevisiae	24	1.50	0.40	0.15	0.017	0.51	Yes	[40]
S. cerevisiae PE-2	16	1.20	0.30	0.24[b]	0.013[a,b]	0.56[b]	Yes	[41]
F. striatum	21	2.50	3.50	0.56	0.13	0.46	Yes	This work (Bioreactor)
	21	1.50	5	0.50	0.220	0.42	Yes	This work (Exp. 13)

[a] Not completely metabolized within the total detoxification time showed
[b] Estimated from a figure
[c] Final productivity

3.3.2. Detoxification of the inhibitors

The detoxification of the inhibitors was significantly faster in the bioreactor inoculated with the co-culture. *S. cerevisiae* alone could not metabolize the inhibitors, while in the presence of *F. striatum* FF was completely metabolized within 9 h and HMF within 21 h (**Figure 6**), with degradation rates of 0.56 g/L/h for FF and 0.13 g/L/h for HMF. The highest degradation rates reported in the literature of FF and HMF present in lignocellulosic hydrolysates are 0.54 g/L/h and 0.12 g/L/h, respectively (**Table 5**). However, in that study, the concentrations of FF and HMF evaluated were 1.7 and 0.4 g/L, almost 3 times lower than the concentration evaluated in this work [3]. Moreover, the detoxification and fermentation steps were performed separately [3]. The degradation rates are significantly lower in other studies found in literature, while also using lower concentrations of inhibitors (**Table 5**). Therefore, *F. striatum* gave the highest degradation rates yet reported (to the best of our knowledge), considering the higher concentration of inhibitors evaluated, and with the advantage that the detoxification and fermentation were performed simultaneously. Note that the HMF degradation rates are higher in the presence of 5 g/L HMF and 1.5 g/L FF (**Table 5**), which confirms the higher toxicity of FF towards the cells.

Additionally, furfuryl alcohol (FA) and 2,5-di(hydroxymethyl)furan (DHMF), the added value alcohol derivatives of both inhibitors [42–44], were quantified in high concentrations in the fermentation broth (**Figure 6**). Their presence did not interfere with the fermentation process, confirming their lower toxicity compared with their precursors [32,45]. The production of these compounds with high yields (99.4% for FA and 86.0% for DHMF) is an advantage compared with the complete metabolization of the inhibitors, as they can be recovered from the fermentation broth [21,46,47]. This would potentially add value to the lignocellulosic hydrolysates by benefiting from both the furan derivatives and the sugars present [42], transforming the problem of the presence of excessive quantities of inhibitors into an advantage.

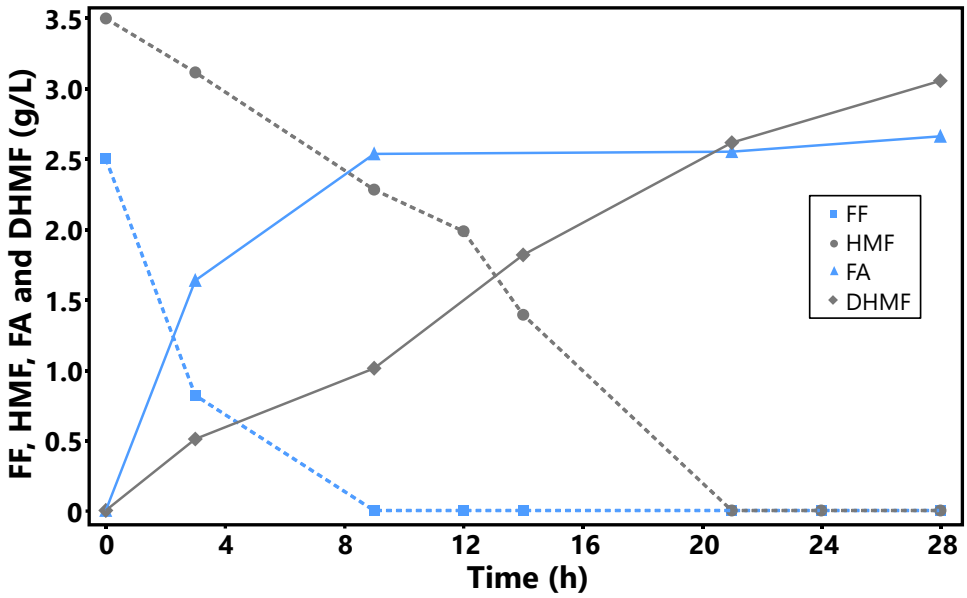


Figure 6. Transformation of the inhibitors into their less toxic derivatives by the co-culture in the bioreactor.

The biodetoxification of inhibitors in lignocellulosic hydrolysates by microorganisms can be approached in two different ways. In the first one, the inhibitors are removed from the solid pretreated lignocellulose in a solid-state fermentation before the hydrolysis step; in the second one, they are removed from the diluted pretreated hydrolysate. Significant advances have been made in the last years in the detoxification of the solid pretreated material [48,49], achieving complete conversions of 5.5 mg/g DM of FF and 2.3 mg/g DM of HMF in 36 h using a heterozygous diploid structure of *A. resinae* ZN1 [50]. However, little progress has been achieved in the detoxification of inhibitors in lignocellulosic hydrolysates. As discussed above, the reported biodetoxification approaches suffer from being able to handle only low inhibitor concentrations, show low degradation rates, require separate biodetoxification and fermentation steps, result in incomplete conversion of the inhibitors, and/or consumption of the sugars in the biodetoxification process. Therefore, the process described in this work is superior to those reported in the literature. Moreover, it meets the requirements needed for a feasible and efficient microbial detoxification of lignocellulosic hydrolysates, as it is performed at pH 6, 30 °C, within 21 h, and with an inoculum size ≤ 5 g/L [51].

4. Conclusions

The co-culture of *F. striatum* and *S. cerevisiae* has proven to be a feasible solution for substrates containing high concentrations of HMF and FF for several reasons: i) there is no competition for sugars, and therefore the sugars are completely available for ethanol fermentation by *S. cerevisiae*; ii) there is a synergistic effect of the co-culture in which *F. striatum* enhances the degradation rates of the inhibitors and *S. cerevisiae* excels in the fermentation; iii) the simultaneous detoxification and fermentation shortens the process, increasing the ethanol productivity; iv) added-value derivatives are formed with high yields from HMF and FF and could be recovered from the fermenting broth, adding more value to the lignocellulosic hydrolysate; and v) the process can be efficiently scaled-up in a bioreactor. The co-culture allowed ethanol production with a high yield and productivity in the presence of 3.5 g/L HMF and 2.5 g/L FF, a concentration of furan derivatives that resulted in the loss of cell culture viability in the absence of *F. striatum*. Moreover, the presented process demonstrates a superior performance than those previously reported in the literature, as it is able to handle significantly higher inhibitor concentrations with higher degradation rates. This allows access to unprecedented lignocellulosic materials and pretreatment methods that result in high quantities of FF and HMF. Although a moderate inoculum size (5 g/L) was needed for the efficient detoxification of high concentrations of inhibitors, this approach can also be used in substrates containing a lower concentration of inhibitors, in which the inoculum size can be significantly decreased while still achieving excellent ethanol productivities. Further, the production of FA and DHMF with high yields during the detoxification represents a significant novel advance in the valorization of the lignocellulosic waste. Finally, this process meets the requirements needed for a feasible and efficient microbial detoxification of lignocellulosic hydrolysates, as it is performed at pH 6, 30 °C, within 21 h, and with an inoculum size ≤ 5 g/L.

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Supplementary materials

Figure S1. Bioreactor set up for the control and the co-culture experiments

CHAPTER 4. GENERAL DISCUSSION

As stated in Chapter 1, the main objective of this thesis was to contribute to the biocatalytic valorization of FF and HMF. After an initial introduction to set the state-of-art, the experimental work consisted of four Chapters. First, the optimization of the enzymatic oxidation of HMF to DFF was attempted. The biocatalytic oxidation of HMF to DFF is still in its early stage, and the literature is scarce and limited to enzymatic synthesis. The most efficient reported methods use the combination of three enzymes (galactose oxidase (GO), catalase, and horseradish peroxidase (HRP) [1,2]), which adds a high cost to the process and results in low productivities. When the work presented in Chapter 3.1 was started, there was only one study describing this reaction with these enzymes [1]. In this Chapter, the catalytic performance of the three enzymes was assessed by evaluating different parameters of the reaction. It was found that the addition of HRP was not necessary if the dosage of catalase used was increased. This resulted in a cheaper process than those reported, which may help in the industrial viability of the process. A Box-Behnken design was built to evaluate the interactions among the concentration of HMF, the enzyme dosage, and the agitation speed, allowing the estimation of the enzyme dosage required for a specific HMF concentration to obtain high yields. Finally, the kinetic parameters of the enzyme were calculated, and a perfect fit for the Michaelis-Menten model was found. The research carried out determined an optimum ratio between GO:catalase that allowed DFF production with a high yield (>90%). However, it was concluded that the enzymatic oxidation of HMF into DFF via GO and catalase still had several limitations, such as the high cost of the enzymes and the low productivities obtained. For this reason, the whole-cell transformation was considered of interest, due to the inherent presence of the different enzymes needed for the reaction. Moreover, the whole-cell transformation of HMF into DFF was not described in the literature. *Fusarium* species are natural producers of GO [3,4]. Therefore, the first approach consisted of a screening of different *Fusarium* species to study their capability to biotransform HMF (Chapter 3.2), since the biotransformation of HMF by *Fusarium* species was not described in the literature. Six different *Fusarium* strains belonging to the collection of the Food Technology department were selected together with one strain that was isolated from food waste.

Surprisingly, most of the strains evaluated in the screening had a high capability to transform HMF into DHMF, a high-value HMF derivative of interest in different industries [5]. This compound results from the reduction of the aldehyde group of HMF. Bibliographic research on this biotransformation indicated that the literature was limited to one yeast, *M. guilliermondii*

SC1103 [6]. Therefore, the study of the feasibility of *Fusarium* species to transform HMF into DHMF was considered of interest. While the work presented in Chapter 3.2 was performed, new biocatalysts were reported for the HMF reduction to DHMF. However, although encouraging, the processes described in the literature require high amounts of inoculum size (from 20 to 200 g/L), high concentrations of glucose as a cofactor, and/or the addition of other expensive cofactors to the reaction media, therefore adding a high cost [6–11]. For these reasons, the process needs further investigation and development to become industrially viable. Results in Chapter 3.2 show that *F. striatum* has proven to be a good biocatalyst for the transformation of HMF into DHMF. Although the productivities reported are slightly lower than in other studies, the inoculum size used (in the form of spores) was also lower, and therefore the process was cheaper in comparison. Moreover, the addition of expensive cofactors to the reaction media was not needed. Furthermore, the process was successfully scaled up in a bench-scale bioreactor, obtaining a quantitative DHMF yield (95 %) and selectivity (98 %), showing promising results for further optimization.

In the *Fusarium* screening, *F. sambucinum* and *F. culmorum* showed DFF production when HMF was added to the media. However, the DFF yields and selectivities of these two strains were low and far from being optimal. After some preliminary assays, it was found that *F. culmorum* was a more promising whole-cell biocatalyst for DFF production, and the work related to this process is shown in Chapter 3.3. The capability of *F. culmorum* EAN 51 to transform HMF into DFF was assessed and optimized through Response Surface Methodology, building two successive Central Composite Designs that allowed the production of DFF with a high yield (92 %) and selectivity (94 %) through optimization of the concentration of peptones and glucose in the reaction media, which highly affected the redox capability of the strain. The results presented are groundbreaking because it is the first time that the whole-cell oxidation of HMF to DFF is achieved. In addition, it has advantages in contrast to enzymatic synthesis, such as being inexpensive, more stable, and with no need for separation and purification steps to isolate the enzymes [12–14]. Specifically, in the HMF oxidation to DFF, the whole-cell transformation is of great interest due to the need for several enzymes to carry out the transformation. Therefore, using a single whole-cell catalyst for DFF production represents a significant advance thanks to the inherent presence of all the enzymes required, significantly reducing the cost of the process. For this reason, these results open a new line of investigation in the production of DFF.

Finally, Chapter 3.4 focused on integrating the novel biocatalytic pathways discovered as detoxification methods in lignocellulosic ethanol production. HMF and FF are considered among the most inhibitory compounds derived from lignocellulosic material in biobased ethanol production. Currently, the fraction of accessible feedstocks and pretreatment methods are limited to those yielding lower concentrations of HMF and FF during the pretreatment [15,16]. Due to the capability of *F. striatum* to transform high concentrations of HMF into its less toxic derivative DHMF (Chapter 3.2), the application of this strain in the biological detoxification of lignocellulosic hydrolysates was considered of interest. A co-culture of *Fusarium striatum* and a xylose-consuming *Saccharomyces cerevisiae* has proven to be a feasible solution for the simultaneous detoxification and fermentation of lignocellulosic hydrolysates containing high concentrations of HMF and FF, as it overcomes the common drawbacks of biological detoxification: i) it can handle significantly higher inhibitor concentrations with higher degradation rates, ii) the detoxification and fermentation steps are performed simultaneously, iii) there is complete detoxification of the inhibitors, and iv) there is no consumption of sugars during the detoxification process. For these reasons, the process is clearly superior to others reported in the literature. Due to the high capability of *F. striatum* to reduce furans, the added-value alcohol derivatives of FF and HMF (FA and DHMF, respectively) were produced with high yields, adding more value to the lignocellulosic waste and transforming the problem of the high concentrations of furans into an advantage. Moreover, the novel process meets the requirements needed for a feasible and efficient microbial detoxification of lignocellulosic hydrolysates [17] and allows access to lignocellulosic hydrolysates that are currently not feasible due to the high concentration of furaldehydes that result from their pretreatment.

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CHAPTER 5. GENERAL CONCLUSIONS

Objective I. Optimize the enzyme-catalyzed oxidation of HMF to DFF.

1. The enzymatic oxidation of HMF to DFF was achieved with a high yield (>90 %) through optimization of the ratio between galactose oxidase and catalase, omitting the addition of horseradish peroxidase.
2. The Box-Behnken design allowed the estimation of the minimum enzyme dosage needed to obtain quantitative yields for a specific concentration of HMF, optimizing the cost of the process.
3. An excellent fit was found for the Michaelis-Menten model in the oxidation of HMF to DFF via galactose oxidase and catalase.
4. The process still had some limitations, and further work is needed to achieve a viable and efficient enzymatic oxidation of HMF to DFF.

Objective II. Explore novel whole-cell catalysts with the capability to produce the different derivatives, focusing on the preparation of DFF and DHMF.

5. *Fusarium* species have shown the capability to reduce HMF to DHMF and oxidize HMF to DFF. *F. striatum* showed the higher DHMF yield and selectivity, while *F. culmorum* showed the most promising preliminary results for DFF production.
6. *F. striatum* has proven to be a promising biocatalyst to produce DHMF, transforming 75 mM within 24 h using small inoculum sizes in the form of spores.
7. A substrate-feeding approach allowed a higher concentration of DHMF in the media, overcoming the toxicity effect of HMF toward the cells.
8. Thanks to the yeast-like growth of *F. striatum*, the process was successfully scaled-up in a bench-scale bioreactor (1.3 L), obtaining a high DHMF yield (95 %) and selectivity (98 %).
9. *F. striatum* may be a promising candidate for the detoxification of lignocellulosic hydrolysates containing high concentrations of furfural (FF) and 5-hydroxymethylfurfural (HMF).
10. The nitrogen source and the concentration of glucose and peptones in the media highly influenced the redox capability of *F. culmorum*, showing the capability to reduce and oxidize HMF.

11. A high DFF yield (92 %) and selectivity (94 %) were obtained after careful optimization of the concentration of both nutrients through Response Surface Methodology starting from 50 mM HMF.
12. The use of whole cells (*F. culmorum*) to oxidize HMF to DFF has been described for the first time, opening a new line of investigation.

Objective III. Find novel biological approaches for the detoxification of lignocellulosic hydrolysates containing high concentrations of FF and HMF in the production of biobased ethanol.

13. The co-culture of *F. striatum* and a xylose-consuming *S. cerevisiae* is a novel process that showed better performance than other biological detoxification methods previously reported in the literature and overcame the main drawbacks of biological detoxification: it was able to handle significantly higher inhibitor concentrations with higher degradation rates, the detoxification and fermentation steps were performed simultaneously, there was a complete transformation of the inhibitors, and there was no consumption of sugars during the detoxification process.
14. An increase in the inoculum size of *F. striatum* resulted in higher degradation rates of FF and HMF and significantly higher ethanol productivities.
15. FA and DHMF, the added-value alcohol derivatives of FF and HMF, were produced with high yields (99 % and 86 %, respectively). This adds more value to the lignocellulosic hydrolysate, increasing the feasibility of the process and transforming the problem of the high concentration of inhibitors into an advantage.
16. The co-culture allows access to lignocellulosic materials and pretreatment methods that result in high concentrations of FF and HMF that are currently not feasible, representing a significant advance for the lignocellulosic ethanol industry.