

Article

Biocatalytic Transformation of 5-Hydroxymethylfurfural into 2,5-di(hydroxymethyl)furan by a Newly Isolated *Fusarium striatum* Strain

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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 2³ 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



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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 biodegradation of lignocellulosic material in the bioethanol production process. *Amorphoteca 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. Results

2.1. *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.

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].

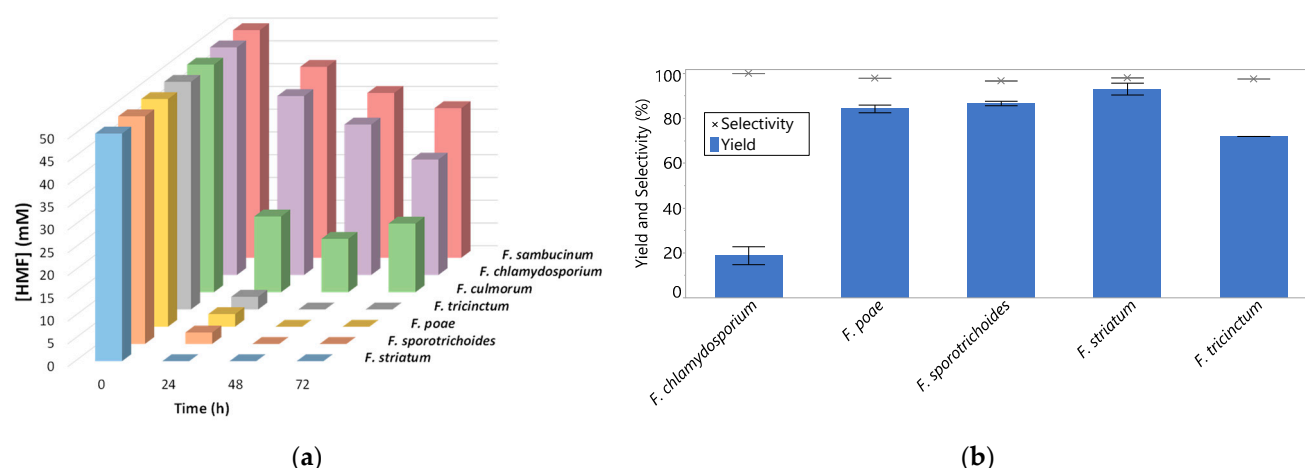


Figure 1. Biocatalytic synthesis of DHMF by different *Fusarium* species. Conditions: 50 mM HMF, 15 mL ME medium, three discs biocatalyst, pH 7, 160 rpm, 28 °C; (a) Concentration of HMF; (b) Yield and selectivity towards DHMF production.

2.2. 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.

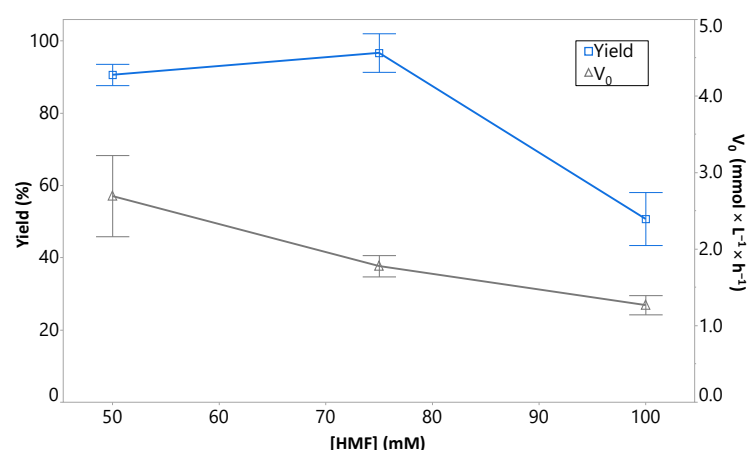


Figure 2. Effect of HMF concentrations on DHMF yields and initial reaction rates. Conditions: increasing concentrations of HMF: 50, 75 and 100 mM, 15 mL ME medium, three discs biocatalyst, pH 7, 160 rpm, 28 °C.

2.3. 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 2³ 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. All runs were performed by duplicate and the responses are expressed as means \pm standard deviations.

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

¹ $n = 1$.

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 ($20 \text{ g} \times \text{L}^{-1}$), 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 $20 \text{ g} \times \text{L}^{-1}$ were added (there were $4 \text{ g} \times \text{L}^{-1}$ remaining). Therefore, $20 \text{ g} \times \text{L}^{-1}$ 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 $22.85 \pm 2.54\%$ were obtained within 24 h, indicating that the presence of glucose is essential for the biotransformation under these conditions.

Based on the statistical analyses, $20 \text{ g} \times \text{L}^{-1}$ of glucose and pH 7 were selected as the optimal conditions, and the inoculum size and type were further optimized.

2.4. 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].

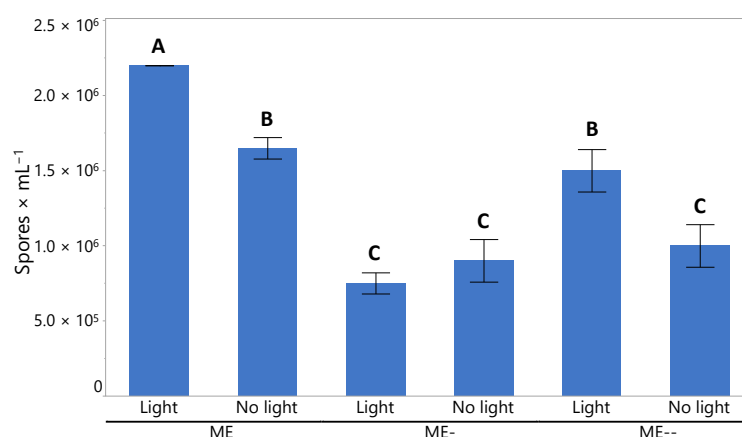


Figure 3. Effect of nutrient depletion and visible light presence on sporulation of *F. striatum*. Conditions: 28 °C, 10 days, presence or absence of light. Media: ME ($20 \text{ g} \times \text{L}^{-1}$ glucose, $20 \text{ g} \times \text{L}^{-1}$ malt extract, $1 \text{ g} \times \text{L}^{-1}$ peptones from soybean, $15 \text{ g} \times \text{L}^{-1}$ agar), ME- ($20 \text{ g} \times \text{L}^{-1}$ glucose, $20 \text{ g} \times \text{L}^{-1}$ malt extract, $15 \text{ g} \times \text{L}^{-1}$ agar), ME- - ($20 \text{ g} \times \text{L}^{-1}$ glucose, $10 \text{ g} \times \text{L}^{-1}$ malt extract, $15 \text{ g} \times \text{L}^{-1}$ 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 4.0×10^6 spores $\times \text{mL}^{-1}$ led to higher yields ($92.8 \pm 5.6\%$) than the ones obtained with the addition of six discs within 24 h ($71.8 \pm 2.3\%$). 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 ($1 \text{ g} \times \text{L}^{-1}$) 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 $70 \text{ g} \times \text{L}^{-1}$ [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 $80 \text{ g} \times \text{L}^{-1}$ to the media. There were no significant differences on the yields or reaction rates in the range of glucose concentration from 20 to $80 \text{ g} \times \text{L}^{-1}$, 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].

2.5. 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 $96.80 \pm 4.05\%$ 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).

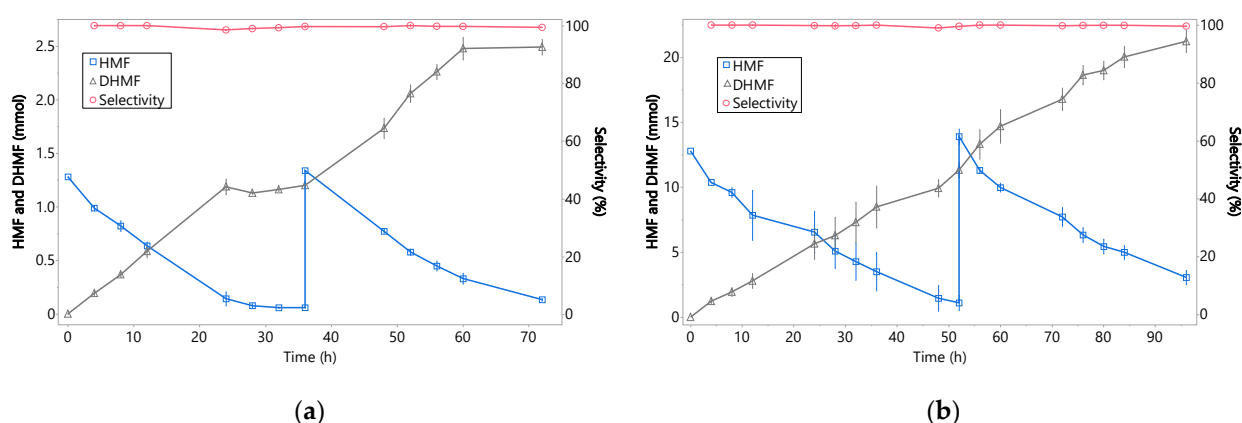


Figure 4. Substrate feeding approach. Conditions: 75 mM HMF, 1 or 10 mL of an aqueous suspension of spores (4.0×10^6 spores $\times \text{mL}^{-1}$). 160 rpm, 28 °C. After HMF was almost consumed, 75 mM were added; (a) 15 mL ME medium; (b) 150 mL ME medium.

2.6. 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.

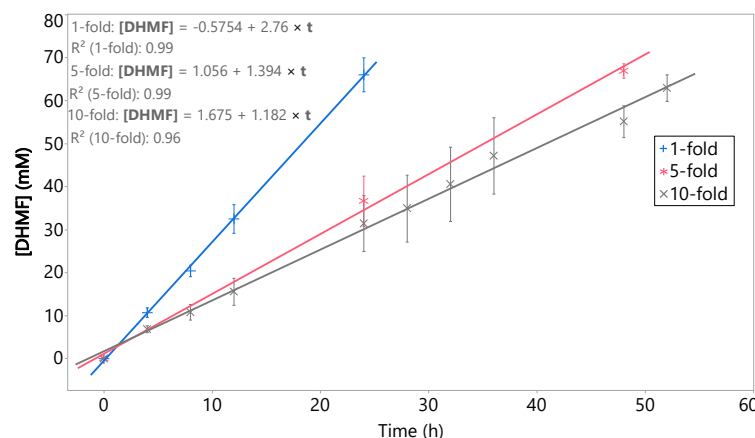


Figure 5. Effect of the scale-up of the reaction on reaction rates. Concentrations of HMF were fitted to a linear regression. Conditions: 75 mM HMF, 15, 75 or 150 mL ME medium, 1, 5 or 10 mL of an aqueous suspension of spores (4.0×10^6 spores \times mL $^{-1}$), pH 7, 160 rpm, 28 °C.

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).

2.7. 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 $5.7 \text{ g} \times \text{dm}^{-3} \times \text{d}^{-1}$. 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.

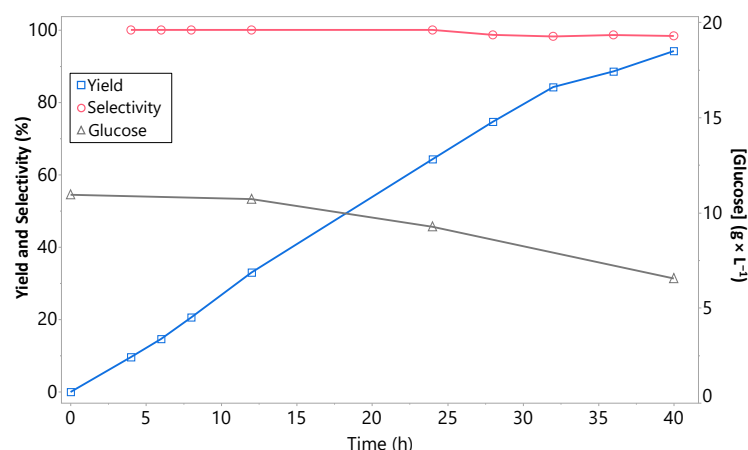


Figure 6. Biocatalytic production of DHMF at bioreactor scale. Conditions: 75 mM HMF, 1.3 L ME medium, 87 mL of an aqueous suspension of spores (4.0×10^6 spores \times mL $^{-1}$), pH 7, 160 rpm, minimum aeration, 28 °C.

2.8. 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.

3. Materials and Methods

3.1. 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 μ L 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).

3.2. Cultivation of *Fusarium* Cells

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

3.3. Screening

The activated strains of the *Fusarium* species were inoculated into flasks containing 15 mL of malt extract media (ME: 20 g \times L $^{-1}$ glucose, 20 g \times L $^{-1}$ malt extract, 1 g \times L $^{-1}$ 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).

3.4. 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 \times mL⁻¹). 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).

3.5. Effect of Inoculum Size, Glucose Concentration and pH

A 2³ 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.

3.6. Sporulation of *F. Striatum*

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

3.7. 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).

3.8. 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).

3.9. 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 Na₂SO₄ 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.

3.10. 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 \times 0.25 mm i.d.; 0.25 μ m 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.

3.11. 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 \pm standard deviations. The assumption of normality was tested using Shapiro–Wilks normality test.

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.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2073-4344/11/2/216/s1>, Figure S1: *F. striatum* growth in malt extract broth, Figure S2: Chromatograms from the GC-FID measurements, Figure S3: *F. striatum* growth in the bioreactor, Figure S4: ^1H NMR in DMSO-d_6 of the DHMF recovered as a yellow solid.

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