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Challenges in ethanol production with *Fusarium oxysporum* through consolidated bioprocessing

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Fusarium oxysporum has been reported as being able to both produce the enzymes necessary to degrade lignocellulosic biomass to sugars and also ferment the monosaccharides to ethanol under anaerobic or microaerobic conditions. However, in order to become an economically feasible alternative to other ethanol-producing microorganisms, a better understanding of its physiology, metabolic pathways, and bottlenecks is required, together with an improvement in its efficiency and robustness. In this report, we describe the challenges for the future and give additional justification for our recent publication.

Keywords: *Fusarium oxysporum*, lignocellulose, biomass, ethanol

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Fusarium oxysporum is probably more commonly known as a plant pathogen, as the pathogenic strains are responsible for vascular wilt disease in more than 120 different species.[1] Even so, *F. oxysporum* is a ubiquitous microorganism, whose global distribution reveals a wider ecological influence and a recognized ability to survive even without any pathogenic activity.[2] This can be partly attributed to the fact that this microorganism has the ability to produce a wide range of biomass-degrading enzymes and can generally use both hexozes and pentoses.[3,4]

The ability of *F. oxysporum* to ferment ethanol was discovered almost a century ago, with the first, still traceable, report—that of White and Willman.[5] In the early reports, the fermentation performance of the fungus was compared with that of *Saccharomyces cerevisiae*, leading to the first conclusions that both microorganisms were alike regarding the ratio of CO_2 to ethanol produced, when hexoses were used. But *S. cerevisiae* does not ferment pentoses and *F. oxysporum* consumes the ethanol it produces, and its rate of fermentation is so low that it takes days to complete—as opposed to hours by the yeast. Since the first discovery, our understanding of metabolism has improved and *F. oxysporum* has been studied thoroughly, but the fundamental challenges in using such a filamentous fungus in consolidated bioprocessing for ethanol production still remain.

Considering that *S. cerevisiae* has been selected through thousands of years of selective evolution for its ability to grow on different types of glucose-rich hydrolysates for the production of different types of alcoholic beverages, the probability of success in finding a new organism that can compete with yeast in the fermentation efficiency is quite low. Even so, in order for filamentous fungi, such as *F. oxysporum*, to become advantageous in a realistic sense, a number of improvements would have to be made, either through genetic modification or evolutionary engineering, in combination with process development and optimization.

Christakopoulos et al.[3] screened three (then) newly isolated strains for their ability to ferment cellulose to ethanol and they selected one based on the cellulolytic enzymes released: *F. oxysporum* F3. A number of studies on this strain have been published. This has improved our knowledge of its biomass-degrading toolbox[6] and our understanding of its metabolic processes during growth and during fermentation of glucose to ethanol.[7,8] Improvement in its efficiency of ethanol production has even been shown, upon

overexpression of a native xylanase.[9] *F. oxysporum* F3 was also the parental strain used in our study.

One major disadvantage of using *F. oxysporum* as an ethanol-producing organism is its slow growth and consequent requirement for long cultivation. This might be the most difficult problem to tackle, as growth depends not only on the supply of monosaccharides from the polymeric substrate, but also on the inherent metabolic rate of the microorganism. A few efforts have been made in this aspect. For example, in two very similar studies, Fan et al. showed that heterologous overexpression of a transaldolase resulted in lower biomass yields than when using the parental strain.[10,11] On the other hand, our homologous overexpression of both phosphoglucomutase and transaldolase resulted in an increase in the maximum specific growth rate and a biomass yield similar to that of the parental strain, when using glucose as the sole carbon source. With xylose, the same strain had much higher biomass yields and a slightly higher maximum specific growth rate.[12] Despite the differences in the experimental layout (e.g., our culture was performed in bioreactors where aeration can be more efficient than in flasks), these differences could also be the result of heterogeneity between different strains, in the same way that different *F. oxysporum* strains have varying efficiency in fermenting glucose or cellulose to ethanol.[3,13] What is perhaps more important is that we showed that the maximum specific growth rate of *F. oxysporum* could be improved, and there might still be room for further improvement, considering that even higher maximum specific growth rates using glucose have been reported in other filamentous fungi.

The growth rate will affect how quickly the microorganism can reach the critical mass required before being transferred to anaerobic conditions for the fermentation. The biomass yield, i.e., the amount of fungal biomass per mass of carbon source, should be as high as possible. Lower values have also been reported in the past, and they have been attributed to extensive formation of by-product.[14] The increase in biomass yield in our work during growth in xylose may be associated with the lower relative amounts of most intracellular metabolites detected in the transformed strain.

Ethanol production and ethanol yield are other important considerations for the feasibility of the filamentous fungus-driven bioethanol production. These values vary significantly depending on the strain, the nature of the substrate used, and the experimental set-up. For example, Christakopoulos et al. reported a maximum ethanol concentration of 8.2 g L^{-1}, or 80% of the theoretical, with glucose as sole carbon source and using the same (parental) strain that we used in our study. When cellulose was used as substrate, 89.2% of the theoretical yield was achieved.[3] For wheat straw, the ethanol concentration and yield varied between 3 g L^{-1} and 60% and 8 g L^{-1} and 30%, respectively, depending on the concentration of straw in the medium.[15] For untreated wheat bran, an ethanol yield of 34% of the theoretical was achieved, which was increased to 56% with the constitutive expression of a xylanase in *F. oxysporum* F3.[9] In another study with brewer's spent grain, *F. oxysporum* never produced more than 9 g L^{-1} ethanol. In terms of yield based on the total glucose and xylose content of the substrate, it gave 109 g ethanol per kg of dry substrate or 60% of the theoretical.[16] In general, the ethanol concentrations achieved have been low compared with *S. cerevisiae*, which in wine fermentation can surpass 100 g L^{-1}. It is, however, evident that the fungus can be more efficient in the fermentation of lignocellulosic material, where hydrolysis and fermentation of both hexoses and pentoses can be achieved in the same vessel. It seems that with genetic modifications and process engineering, the efficiency of filamentous fungi such as *F. oxysporum* could be improved. Our preliminary data, from the anaerobic fermentation of glucose have shown almost a doubling of ethanol concentration from the overexpression of phosphoglucomutase and transaldolase, up to 20 g L^{-1} ethanol from glucose under the experimental conditions used.

Although acetate can be consumed by *F. oxysporum*, it is also the main by-product of the sugar fermentation and of course, from a bioprocessing point of view, it is not a desirable product when ethanol is the compound of interest. Our preliminary results have shown that overexpression of phosphoglucomutase and transaldolase reduces the amount of acetate produced in glucose fermentation. No effect was observed on acetate yield, when xylose was fermented. The efficiency of *F. oxysporum* in growing on and fermenting lignocellulosic material is also related to the inhibitors present in this material. In contrast to acid hydrolysis where even more inhibitory compounds are formed, consolidated bioprocessing should provide a more efficient and natural process for ethanol production, especially when coupled with mild pretreatment methods. In this respect, it looks promising that the fungus is capable of degrading and fermenting a wide variety of different substrates under anaerobic or limited oxygen conditions in the presence of inhibitory compounds such as furan derivatives, phenolic compounds, and weak acids.[17]

Lastly, we should also discuss the effects of ethanol as an inhibitor of the growth and survival of *F. oxysporum*. The fungus is generally considered to be tolerant to ethanol,[4] although the effects of ethanol and the level of ethanol tolerance are unclear. We have shown that under aerobic conditions, both the maximum mycelium production and the specific growth rate are affected by the presence of ethanol, although to different extents. The maximum allowable ethanol concentration above which cells would not grow was predicted to be 72 g L^{-1}. Under limited aeration conditions, the ethanol-producing ability of the cells was completely inhibited at 45 g L^{-1} ethanol. Finally, when the ethanol produced was partially removed from the fermentation system of *F. oxysporum* in a stepwise manner, the final ethanol production was found to be 38.4 g L^{-1} (Paschos et al., submitted), which is very close to the crucial ethanol concentration of 4–5% (w/v) in the broth that is considered to be a minimum prerequisite for a feasible large-scale distillation process.[18]

Interestingly, the lignocellulolytic secretome of *F. oxysporum*, combined with its ability to ferment xylose, has

significantly improved ethanol production from lignocellulosic substrates when it is present in co-culture with *S. cerevisiae*.[19]

Although the background knowledge that we have acquired about this microorganism cannot compete with the huge amount of information available on *S. cerevisiae*, it is evident that, despite its potential, *F. oxysporum* must be improved—both in terms of ethanol production and ethanol tolerance. This is likely to open a new round of studies on this multifaceted issue, with screening for strains of *F. oxysporum* that tolerate and produce high concentrations of ethanol, with improved tolerance and production from genetic modifications and evolutionary engineering, and with the development of novel bioprocessing set-ups that would enable the production of ethanol above the limit of 4–5% (w/v) from lignocellulose.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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