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Innovative microscale workflow from fungi cultures to Cell Wall-Degrading Enzyme screening

Roxane Raulo, Egon Heuson, Ali Siah, Vincent Phalip and Renato Froidevaux

Summary
This study aimed at developing a complete miniaturized high-throughput screening workflow for the evaluation of the Cell Wall-Degrading Enzyme (CWDE) activities produced by any fungal strain directly cultivated on raw feedstock in a submerged manner. In this study, wheat straw was selected as model substrate as it represents an important carbon source but yet poorly valorised to yield high added value products. Fungi were grown in a micro-bioreactor in a high-throughput (HT) way to replace the fastidious shaking flask cultivations. Both approaches were compared in order to validate our new methodology. The range of CWDE activities produced from the cultures was assayed using AZO-dyed and pNP-linked substrates in an SBS plate format using a Biomek FXp pipetting platform. As highlighted in this study, it was shown that the CWDE activities gathered from the micro-bioreactor cultivations were similar or higher to those obtained from shake flasks cultures, with a lower standard deviation on the measured values, making this new method much faster than the traditional one and suitable for HT CWDE production thanks to its pipetting platform compatibility. Also, the results showed that the enzymatic activities measured were the same when doing the assay manually or using the automated method.

Introduction
Enzymes secreted by filamentous fungi have a key role in the degradation of the most abundant biopolymers found in nature, that is cellulose and hemicelluloses. These enzymes (Cell Wall-Degrading Enzymes, CWDE) are of great interest in the industrial conversion of lignocellulosic substrates into component sugars, which then serve as substrates for the synthesis of biofuels and other highly valuable platform molecules. However, the industrial conversion of lignocellulosic biomasses to such monomers faces two major bottlenecks: (i) the plant cell wall is highly recalcitrant to enzymatic degradation due to the presence of lignin which reduces enzyme accessibility to cellulose and hemicelluloses and (ii) a lack of high-throughput methods to rapidly screen for the enzymatic activities associated to these degradation capabilities in plant-degrading strains.

Fungi are the predominant source of enzymes currently being used on an industrial scale for the conversion of lignocellulosic biomass to platform molecules (Archer, 2000; Sims et al., 2010; Gusakov, 2011). Improving the efficiency of enzymatic saccharification has been an active area of research during the last decade, with efforts dedicated towards the discovery and characterization of novel saccharolytic enzymes (Phalip et al., 2005; Carapito et al., 2009; Banerjee et al., 2010), to be used in biorefineries. A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power and value-added chemicals from biomass. As refineries, biorefineries can provide multiplex chemicals by fractioning the lignocellulosic biomass into multiple intermediates that can be further converted into value-added products (Menon and Rao, 2012). The past few years have seen a strong increase in research topics aiming at the isolation and development of novel fungal enzyme mixtures, which can be tailored to...
efficiently degrade recalcitrant lignocellulose into monomeric sugars with the help of mild or no thermochemical pretreatment (Berrin et al., 2012; Couturier et al., 2012). Currently, functional screening of fungi is associated with low throughput and prohibitive costs, which severely limits the discovery of novel enzymatic activities and better CWDE producing strains. Fermentation experiments in shake flasks, bench-scale bioreactors, or a combination of both, are the conventional methods for both fungi screening and fermentation optimization. However, the screening use of these two methods is limited to a small number of strains that can be tested in parallel, as well as the large amount of substrate (feedstock), cultivation medium, sterilization steps, etc. that require such manipulations. Of course, several HT small scale cultivation approaches have already been designed and have proven their efficiency to seek enzyme activities produced by microorganisms. Among them, the recently released microbioreactor device from M2PLabs was extensively used for such application, helping to greatly speed up the cultivation phase. However, when it comes to raw feedstocks as substrates, with filamentous fungi in submerged fermentation, no use of such device has been reported. Developing a screening methodology that would allow for a larger number of cultivations to be performed in parallel directly on raw feedstock, while retaining conditions that can be easily controlled upon scale-up, would consequently greatly reduce the development time and costs of CWDE cocktails from filamentous fungi, that could later be used to improve the biorefineries’ saccharification processes.

In this study, we present a novel and original approach to screen and select CWDE cocktails from two different fungal strains in a semi-automated workflow (Fig. 1). The selected strains are Penicillium chrysogenum, a saprophytic species that has not been extensively studied for feedstock degradation, and...
Zymoseptoria tritici, the causal agent of wheat blotch.

The approach is based on the use of two HT robotic equipment, a Biomek FXp pipetting platform from Beckman Coulter, and the microbioreactor from M2PLabs. This study is the first report of the use of the microbioreactor for the implementation of high-throughput filamentous fungi cultures on complex biomasses followed by the high-throughput screening of enzyme activities. This approach is greatly speeding up the screening of fungal CWDE activities by scaling down the whole process to less than 2 ml fermentations. Therefore, with the workflow, we expect to increase the likelihood of discovering interesting strains and enzymes for industrial applications that can directly be used on raw feedstocks in biorefineries.

Results and discussion

Automated enzymatic assaying validation

The automated enzymatic assay results were validated by comparing the relative activity measured when plates were prepared and read manually with the values obtained after the semi-automated method. It is noteworthy to precise that, for the semi-automated method, all the pipetting action were performed by the Biomek platform. The only manual step was the centrifugation step.
Both methods showed similar results with $13.8 \pm 0.48 \times 10^{-3}$ mUI ml$^{-1}$ and $13.1 \pm 0.42 \times 10^{-3}$ mUI ml$^{-1}$, respectively, measured with the pNP-glucopyranoside assay when performed manually and with the pipetting station, using wheat straw as substrate and P. chrysogenum as fungal strain after 7 days of incubation (Fig. 2).
Cell Wall-Degrading Enzyme activities gathered from fungi

The capability of the strains used in this study to degrade wheat straw was assayed using a microbioreactor for the implementation of high-throughput filamentous fungi cultures on complex biomasses followed by the high-throughput screening of enzyme activities (Fig. 3).

Enzymatic activities were assayed after both 4 and 7 days of cultures on wheat straw of *P. chrysogenum* and *Z. tritici*. For all the enzymatic assays tested, an increase in enzymatic activities was observed between the D4 and D7 time points (Fig. 4). Using this semi-automated method, the tests assayed (Table 1) were able to discriminate for specific activities for each fungus. The AZO-cellulose test showed higher activities after 7 days of cultures in shake flasks and in the microbioreactor for *Zymoseptoria tritici*. Enzyme activity (A) on AZO-xylan, (B) on AZO-cellulose, (C) on AZO-rhamnogalacturonan, (D) on AZO-barley glucan, (E) on pNP-glucopyranoside and (F) on pNP-xylopyranoside. For cultures of *P. chrysogenum* and *Z. tritici* in shake flasks, spores were washed with 0.01% (v/v) Tween-80 solution and 5.10^6 spores per ml in suspension were inoculated into 250 ml sterile Erlenmeyer flasks containing 100 ml M3 medium (Mitchell et al., 1997), supplemented with 1% milled wheat straw (w/v), and incubated for 7 days at 25°C on a shaker (Infors HT Multitron standard, Switzerland) set to 150 r.p.m. Media were autoclaved at 110°C for 30 min instead of 121°C to limit the Maillard reaction. Supernatants were manually collected at day 0, day 4 and day 7 after inoculation for enzymatic analysis. Feedstock remaining debris and fungi cells were removed from the supernatant through a filtration step using a 0.22 μm syringe filter. All experiments were conducted in biological triplicates. The enzymes activities were measured according to the protocol described previously. A t-test was performed for statistical analysis. * indicates a significant difference between the activity measured in the shake flask and in the microbioreactor (*P < 0.05).

Fig. 5. Enzyme activities measured using AZO-substrates and pNP-linked substrates after 7 days of cultures in shake flask cultures and in the microbioreactor for *Zymoseptoria tritici*. Enzyme activity (A) on AZO-xylan, (B) on AZO-cellulose, (C) on AZO-rhamnogalacturonan, (D) on AZO-barley glucan, (E) on pNP-glucopyranoside and (F) on pNP-xylopyranoside. For cultures of *P. chrysogenum* and *Z. tritici* in shake flasks, spores were washed with 0.01% (v/v) Tween-80 solution and 5.10^6 spores per ml in suspension were inoculated into 250 ml sterile Erlenmeyer flasks containing 100 ml M3 medium (Mitchell et al., 1997), supplemented with 1% milled wheat straw (w/v), and incubated for 7 days at 25°C on a shaker (Infors HT Multitron standard, Switzerland) set to 150 r.p.m. Media were autoclaved at 110°C for 30 min instead of 121°C to limit the Maillard reaction. Supernatants were manually collected at day 0, day 4 and day 7 after inoculation for enzymatic analysis. Feedstock remaining debris and fungi cells were removed from the supernatant through a filtration step using a 0.22 μm syringe filter. All experiments were conducted in biological triplicates. The enzymes activities were measured according to the protocol described previously. A t-test was performed for statistical analysis. * indicates a significant difference between the activity measured in the shake flask and in the microbioreactor (*P < 0.05).
measurements for Z. tritici while the other tests showed that P. chrysogenum was more efficient at degrading these substrates. In addition, the method allowed the measurement of low enzyme activities, the lowest being 0.18 $10^{-3}$ mUI ml$^{-1}$ for P. chrysogenum on the pNP-xylopyranoside substrate. These results, and the sensitivity of the assays, demonstrated the potential for the developed method to be used for HT screening of CWDEs on complex substrates.

Performance comparison between the microbioreactor and the flask cultures

The comparison in enzymatic activities measured in shake flask cultures compared to the one obtained using the microbioreactor was performed with the Z. tritici strain over a time course of 7 days. When comparing the data obtained from both the cultures in shake flasks and in the microbioreactor, the trend in CWDE activities measured was the same with the 5 AZO-substrates, all along the culture. For instance, at day 7, when considering the AZO-xylan substrate, the same levels of enzyme activities were measured for two types of culture, with 1.06 $10^{-3}$ mUI ml$^{-1}$ and 1.37 $10^{-3}$ mUI ml$^{-1}$ measured in the shake flask cultures and in the microbioreactor respectively (Fig. 5). The most significant results were obtained for the pNP-linked substrates, with an increase in the enzyme activities measured for the two types of pNP-substrates in the microbioreactor which may be due to a better oxygen transfer rate in the microbioreactor (Fig. 5). These results highlight the potential of the described method for HT screening as the cultures volume was reduced by a factor 70 while allowing for differential enzyme activities measurement. In addition, HT screening of the enzyme activities was also achieved with the use of the Beckman Coulter robot.

Conclusion

When designing an industrial fermentation, a large number of experiments are required to respectively select a strain, improve the cultivation medium and optimize the fermentation procedure, measure the enzymes activities and finally select the most efficient strain for the degradation of a specific biomass. The limitations of shake flasks cultures like the inability to control parameters, other than agitation rate and temperature, usually means that the scale-up to the bioreactor scale is rather complicated as it is neglecting, for instance, the effect of oxygen supply on growth and the metabolite production during fermentation. For the first time, these results highlighted the potential of the microbioreactor to screen filamentous fungi species on raw feedstocks since similar and higher CWDE activities were observed from the microbioreactor cultures compared to the shake flask ones. These cultivations were done directly using shredded wheat straw as raw substrate in a submerged manner, proving the ability of the equipment to run accurate cultivations on such complex material with some advantages compared to the current flask cultures method (i.e. more conditions screened, less amount of substrate needed, better activities as shown in Fig. 4). In addition, the scale-up from small scale to large scale bioreactor is often more predictable than from shake flasks to bioreactor (Rohe et al., 2012). Overall, the differences in activities measured in this work were strong enough to discriminate between the screened strains with different responses obtained depending on the substrate assayed. Using this tool, an increased number of strains and parameters will be tested in a close future, setting this workflow as an efficient starting point for the discovery of fungal CWDE cocktails that could later be scaled up to an industrial production level for biorefineries purpose. This method is currently being upgraded as a fully automated process with the sampling of the supernatants done directly in the wells using a Beckman Coulter robot (Biomek NX® with a BioLector Pro® integrated to the deck).

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Fungal strains cultivation in the BioLector.