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Small scale screening of yeast strains enables high-throughput evaluation of performance in lignocellulose hydrolysates

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Abstract

Second generation biorefineries demand efficient lignocellulosic hydrolysis fermenting strains and recent advances in strain isolation and engineering have progressed the bottleneck in developing production hosts from generation of strains into testing these under relevant conditions. In this paper, we introduce a methodology for high-throughput analysis of yeast strains directly in lignocellulosic hydrolysates. The Biolector platform was used to assess aerobic and anaerobic growth of 12 *Saccharomyces cerevisiae* strains and their ΔPdr12 mutants in wheat straw hydrolysat. The strains evaluated included lab, industrial and wild type strains and the screening could capture significant differences in growth and ethanol production among the strains. The methodology was also demonstrated with corn stover hydrolysat and the results were in line with shake flask cultures. Our study demonstrates that growth in lignocellulosic hydrolysates could be rapidly monitored using 1 ml cultures and that measuring growth and product formation under relevant conditions are crucial for evaluating strain performance.

Keywords: Bioethanol; Strain evaluation; *Saccharomyces cerevisiae*; Wheat straw hydrolysate; Adaptation; Bioethanol

1. Introduction

Substituting fossil raw materials with biological resources is an indispensable component of a forward-looking climate change policy. Second generation (2G) bioethanol and other biofuels, produced from lignocellulosic materials; biomass unsuited for food and feed applications, will continue to play an important role towards the bioeconomy where renewable biological resources are used to produce food, energy and industrial goods. In order for biofuels to become a competitive alternative to fossil based fuels, the production needs to be significantly more efficient and cost competitive. The selection or development of efficient production strains for biocommodities is one way to improve the competitiveness of biorefineries.

Synthetic biology, automation, and affordable DNA synthesis has substantially decreased the time needed for strain construction and allows for multiple strain variants to be made simultaneously. Metabolic engineering of production strains has recently seen great progress through the CRISPR/Cas9 genome editing technology (Stovicek et al., 2017). CRISPR/Cas9 technologies have been developed for different industrial strains, allowing simultaneous disruption of two alleles of a gene or several genes simultaneously (Stovicek et al., 2015). Various CRISPR/Cas9 systems have been developed for industrial biotechnology applications and this is expected to increase the number of chemicals and products that can be produced by microorganisms and broaden the diversity of strains suitable for industrial production (as reviewed by Donohoue et al., 2018). We have recently established the CRISPR interference technology in industrial yeast (Cámara et al., 2020). This technology for modulating endogenous gene expression without promoter engineering simplifies the manipulation of strains for bioproduction (as reviewed by Donohoue et al., 2018). Therefore, the bottleneck in developing production hosts is moving forward from generation of strain variants into testing these under relevant conditions.

In order to accelerate testing of large numbers of strains or clones under different conditions, a number of microbioreactor systems have been developed and commercialized. As one example, the Biolector® system, allows quantitative detection of biomass concentrations via scattered light. This platform has been used for evaluating growth of bacteria and yeast (Back et al., 2016; Knesy et al., 2009; Toerrock et al., 2015) and even filamentous fungi (Mózsik et al., 2019). Biomass
concentrations of up to 50 g l\(^{-1}\) cell dry weight could be linearly correlated to scattered light intensities (Kensy et al., 2009). Still, the microbiorereactor cultivations reported on so far have been conducted in defined laboratory media and not with industrially relevant substrates such as lignocellulosic hydrolysates. A common challenge in translating research results into application is that research on tolerance is most often conducted in defined medium with added inhibitors. These conditions will, however, significantly differ from industrial processes where ethanol is produced from a complex hydrolysate.

A substantial amount of work has been done to isolate novel, more robust yeast strains as well as to increase the tolerance of strains through adaptive laboratory evolution and/or metabolic engineering. Previous research has shown that the tolerance level of individual strains and natural isolates varied significantly (Almeida et al., 2009; da Conceição et al., 2015). While some industrial yeast strains currently used for bioethanol production have been developed through extensive strain improvement, other studies have demonstrated that wild types isolated from harsh environments could produce bioethanol and withstand inhibitors comparably to industrial strains (da Conceição et al., 2015). The presence of inhibitory by-products, resulting from pre-treatment of the lignocellulosic biomass, forms a great challenge for development of 2G bioethanol production processes (as reviewed by Robak and Balcerék, 2018). The composition of the hydrolysate is dependent on the biomass source as well as the pre-treatment and hydrolysis conditions used (Galbe and Zacchi, 2007). Weak acids, furan derivatives and phenolic compounds that are formed or released during hydrolysis of biomass are inhibitory for the cells, resulting in sub-optimal ethanol yield and productivity during fermentation (as reviewed by Martin and Jönsson, 2003). Thus, production of lignocellulose derived bioethanol requires not only a microorganisms that is able to ferment all sugars in the hydrolysates, but also exhibits tolerance to the inhibiting compounds. Therefore, we set out to develop a method for using the Biolector as a microbioreactor screening platform for development of yeast cultivations in media containing lignocellulosic hydrolysate. To that end, growth and fermentation capacity were evaluated for various yeast strains, including laboratory and industrial strains as well as wild type isolates and genetic variants thereof.

### 2. Materials and methods

#### 2.1. Strains and media

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1; in addition to the parental strains, strains with all copies of *PDR12* deleted were analyzed. The xylose-utilizing diploid industrial strain KE6–12, derived from TMB3400 with *XR* and *XDH* from *Pichia stipitis* integrated into the genome (Albers et al., unpublished) that our lab uses for studying second generation bioethanol processes, was used for development of the screening method. KE6–12 and CR01, an industrial strain derived from TMB3400, was used for validating the use of the Biolector with corn stover hydrolysate (CSH). In addition, commercial bioethanol yeast strains PE-2 (Fermentec, Brazil), Ethanol Red (Fermentis, USA) and DGI 342 (Danisco Distillers, Denmark), two commonly used laboratory strains; CEN.PK113-7D (Entian and Kötter, 2007) and S288C (Mortimer and Johnston, 1986) and a selection of wild yeast strains from the LCBM collection (da Conceição et al., 2015) of the Federal University of Ouro Preto (propp at ufop.br) were used to validate the screening method. The LCBM collection includes 138 strains isolated from cachaça distilleries located in Brazil (Supplementary Table S1). Here, we selected a few strains performing well at pH 4 and in the presence of acetic acid (LCBM103, LCBM109, LCBM110, LCBM126) for validation. LCBM67 and LCBM97 were chosen due to demonstrated high ethanol production and aluminium tolerance (da Conceição et al., 2015). The strains were maintained in yeast extract peptone dextrose (YPD) medium containing 10 g l\(^{-1}\) yeast extract, 20 g l\(^{-1}\) peptone, and 20 g l\(^{-1}\) glucose, supplemented with 20 g l\(^{-1}\)

| Strain Description | Aerobic lag phase (h) | Anaerobic growth a | Anaerobic ethanol produced by parent (g l\(^{-1}\)) | Anaerobic ethanol produced by PDR12 mutant (g l\(^{-1}\)) | Ethanol produced compared to parent a |
|--------------------|----------------------|--------------------|-----------------------------------------------|-----------------------------------------------|-------------------------------------|
| KE6–12 Haploid lab strain | 16.8 | 0.10 | 27.6 ± 0 | 30.6 ± 0.3 | + |
| S288C Haploid lab strain | 16.0 | 0.16 | 28 ± 0 | 28 ± 0.4 | 0 |
| Ethanol Red diploid industrial | 5.1 | 0.21 | 33 ± 0.7 | 31.1 ± 1 | + |
| DGI 342 diploid industrial | 5.1 | 0.17 | 33.1 ± 1 | 31.2 ± 1 | + |
| POI 342 strain | 5.1 | 0.21 | 29 ± 0 | 30.9 ± 0.5 | + |
| KE 6–12 wild type isolate | 10.1 | 0.09 | 27.6 ± 0 | 27.3 ± 0.3 | + |
| KE 6–12 wild type isolate | 10.1 | 0.10 | 26.3 ± 0 | 28 ± 0.4 | + |
| PE 2 wild type isolate | 20.1 | 0.11 | 26 ± 0 | 29 ± 0.3 | + |

**Legend:** + indicates no significant difference between parent and mutant, *−* indicates the parent performed better than the mutant.
agar for preparation of solid medium.

The parental strains were compared with strains with all copies of *PDR12* deleted using marker-free CRISPR/Cas9-based cloning. Construction of the ΔPdr12 strains was done as described in Cámara et al. (2020), using Cas9 expressed from a plasmid, together with an sgRNA cassette containing the GAAATGATGTCTAAGTATAA protospacer sequence. The *PDR12* gene was disrupted using a double-stranded dDNA oligo (TGTACAAGGTGATTCTCTATGTGGTCTGGA CCAAAGGggcagcggttagttaAGGTACGTUATTCTAGGCGAGCT TGATTTCCATTTC) introducing a stop codon in the beginning of the gene, and mutants were verified using colony PCR for confirming correct integration of the dDNA. Only mutants with all *PDR12* copies deleted were analyzed.

The xylose and glucose concentrations were adjusted to be similar in

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**Fig. 1.** Cultivation of *S. cerevisiae* KE6–12 (A) in microbioreactors at varying OD values, varying wheat straw hydrolysate (WSH) concentrations and with or without salts added to the medium, under aerobic conditions; (B) in flasks shaken at 200 rpm, at OD 1.0 and containing medium with different hydrolysate concentrations, with or without added salts, under aerobic conditions; (C) in microbioreactors, in medium containing 80% WSH, under aerobic conditions; (D) in microbioreactors, in medium containing 80% WSH, under anaerobic conditions. (E) Cultivation of *S. cerevisiae* CR01 in microbioreactors, in medium containing 80% WSH or 70% corn stover hydrolysate (CSH) or, under anaerobic conditions.
all experiments, corresponding to the concentrations found in 90% wheat straw hydrolysate medium (WSH; 68.8 g l\(^{-1}\) glucose, 36.4 g l\(^{-1}\) xylose). For hydrolysate adaptation and screening, minimal medium (Verduyn et al., 1992) with varying concentrations of wheat straw or corn stover hydrolysate was used. Additional salts (2.3 g l\(^{-1}\) Urea, 3.1 g l\(^{-1}\) KH\(_2\)PO\(_4\) and 0.5 g l\(^{-1}\) MgSO\(_4\)) were supplemented to cultures unless indicated. All hydrolysates were produced by steam explosion (10 min incubation, 190 °C for wheat straw and 200 °C for corn stover) after being impregnated with a 0.2% (w/w) solution of sulfuric acid overnight. The WSH batch used in the development of the screening method had a composition of 68.8 g l\(^{-1}\) glucose, 36.4 g l\(^{-1}\) xylose, 1.2 g l\(^{-1}\) formic acid, 4.7 g l\(^{-1}\) acetic acid, 0.6 g l\(^{-1}\) HMF, and 3.0 g l\(^{-1}\) furfural (van Dijk et al., 2019). The corn stover hydrolysate (CSH) had a composition of 69.0 g l\(^{-1}\) glucose, 23.9 g l\(^{-1}\) xylose, 2.0 g l\(^{-1}\) formic acid, 5.3 g l\(^{-1}\) acetic acid, 0.4 g l\(^{-1}\) HMF, and 3.1 g l\(^{-1}\) furfural. The WSH batch used in the screening of the ΔPdr12 strains had a composition of 80 g l\(^{-1}\) glucose, 30.6 g l\(^{-1}\) xylose, 0.8 g l\(^{-1}\) formic acid, 7.7 g l\(^{-1}\) acetic acid, 0.3 g l\(^{-1}\) HMF, and 4.2 g l\(^{-1}\) furfural. All hydrolysates were kindly provided by Dr. Mats Galbe at Lund University (Sweden).

2.2. Culture conditions and HPLC analysis

Yeast pre-cultures were grown overnight in 50 ml YPD medium in 250 ml shaking flasks. For adapted pre-cultures appropriate amount of hydrolysate, as indicated when results are presented, was supplemented. Strains were evaluated for growth in hydrolysate using microbioreactors, in the Biolector platform (m2p-Laboratories GmbH, Germany) with 1 ml of growth medium in FlowerPlates sealed with a gas-permeable sealing foil with evaporation reduction layer. The cultivation was set at 1200 rpm, 30 °C and humidity of 85% using an anaerobic chamber continuously purged with N\(_2\) gas was used to ensure anaerobic conditions throughout the entire microbioreactor experiment. The liquid medium was not deoxygenized. The growth of the strains was verified in 250 or 100 ml shaking flasks with 50 or 20 ml growth medium, shaking at 200 rpm and 30 °C. Samples were taken frequently during the flask cultivation for OD measurement and HPLC analysis as described previously (van Dijk et al., 2019). The compositions of the broth from the micro-\nbioreactor cultures were also analyzed at the end of the cultivation for substrate consumption and product formation. All cultivations were done with at least two repetitions.

3. Results and discussion

Aerobic and anaerobic growth of different strains in wheat straw hydrolysate (WSH) was monitored in microbioreactors. The use of plates with flower-shaped wells allows for better aeration than in standard micro-titre plates which enables the use of viscous cultivation medium containing high amounts of hydrolysates. The scattered light-based detection system of the Biolector enables measuring growth in highly coloured medium.

3.1. Monitoring growth in microbioreactors

A starting OD of 1 was well suited for following growth in hydrolysates in the microbioreactors (Fig. 1a-e). Notably, the inoculum needed for growth in WSH was significantly higher compared to when cells were grown in YPD medium, where no difference was seen between cultures starting from an OD of 0.2, 0.5 or 1 (Fig. 1a). The higher hydrolysate concentration led to a longer lag phase, similarly to what has been reported before (Almeida et al., 2009). Addition of urea or salts (KH\(_2\)PO\(_4\) and MgSO\(_4\)) was needed in order to maintain a high specific growth rate, but these additions did not influence the length of the lag phase (Fig. 1a).

Growth in medium with acetic acid, one of the major inhibitors in lignocellulosic hydrolysates has been shown to lead to a drastic loss in cell viability (Nygård et al., 2014) which may explain the need for a greater inoculum when cells are exposed to a toxic environment. Xiros and Olsson (2014) reasoned that with a higher inoculum the amount of inhibitors per cell decreases. Another plausible explanation is that a larger inoculum causes faster detoxification of the medium.

Growth of KE6–12 was also measured in 100 ml shaking flasks (Fig. 1b). In the microbioreactor cultures there was a great difference in the lag phase of the cultures with 70% WSH compared to the cultures with 80% WSH (Fig. 1a), whereas this was not seen in the shake flasks (Fig. 1b). It may be that better aeration in the shake flasks allowed for better adaptation and detoxification of the medium. Still, the growth profiles and ranking of the conditions was similar both in the microbioreactor and the shake flask cultures, suggesting the growth in lignocellulosic hydrolysates could be monitored using the microbioreactor platform. More thorough characterization of strain performance should be done at a larger scale, preferably under controlled conditions.

3.2. Monitoring adaptation and fermentation in microbioreactors

Short-term adaptation of yeast has been shown to improve tolerance and fermentation of lignocellulosic hydrolysates (van Dijk et al., 2019). Here, we set out to establish conditions for studying short-time adaptation in microbioreactors. Short-term adaptation using 10% WSH lead to a 40 h reduction in lag phase when cells of cultures started from OD 1 were grown aerobically in medium containing 80% WSH (Fig. 1c). Notably, when the starting inoculum was increased to OD 2 or OD 4, the KE6–12 cells had a 20 or 35 h shorter lag phase and short-term adaptation to growth in hydrolysate did not influence the growth of the cells (Fig. 1c).

In order to study the impact of short-term adaptation in anaerobic conditions, cells were pre-grown in 0, 14 or 40% WSH, after which medium with 80% WSH was inoculated at a starting OD of 1. Non-adapted cells displayed a slower growth than adapted cells which started growing after 16 or 6 h, respectively (Fig. 1d). Similarly to what was previously reported (Nielsen et al., 2015; Tomás-Pejó et al., 2010; Tomás-Pejó and Olsson, 2015), a higher amount of hydrolysate in the pre-culture allowed for better subsequent fermentation capacity. After 24 h, the cultures adapted at 0, 14 or 40% WSH had produced 4.5, 22 or 39 g l\(^{-1}\) ethanol, respectively. The ethanol production measured was similar to what we previously measured from fermentations performed in 200 ml screw-top shake flask cultures (van Dijk et al., 2019) further demonstrating that screenings done in the microbioreactors mimic what can be seen in larger cultures. Similarly to what was seen when short-term adaptation was performed in fed-batch (van Dijk et al., 2019), the final ethanol yield (g ethanol/g substrate) was not affected by the adaptation. The anaerobic growth of CR01 was evaluated in both WSH and corn stover hydrolysate (CSH), a hydrolysate that was darker and more inhibitory to the cells than WSH (Fig. 1e). CR01 cells short-term adapted to the respective hydrolysates could clearly be distinguished from non-adapted cells, whereas the effect of adaptation was more pronounced in CSH compared to WSH (Fig. 1e). In previous work we compared the performance of CR01 and KE6–12 and found short-term adaptation to be highly strain dependent (van Dijk et al., 2019). This observation from 200 ml shake flask cultures was reproduced in this study using 1 ml microbioreactor cultures. In short, our study shows that the effect of adaptation can be studied in microbioreactors allowing for high-throughput investigation also of physiological phenomena.

3.3. Evaluating different strains in lignocellulosic hydrolysate

Growth in 70% WSH was evaluated for two of the most commonly
The Biolector platform was successfully used to assess aerobic and anaerobic growth of 12 *Saccharomyces cerevisiae* strains and their respective ΔPdr12 mutants in wheat straw hydrolysate. The methodology developed was also shown to enable monitoring growth in corn stover hydrolysate. The results collected in this paper show that using relevant conditions and measuring not only growth, but also product formation is crucial to perform a good screen of strain performance, especially when working with lignocellulosic hydrolysates.

### 4. Conclusions

The Biolector platform was successfully used to assess aerobic and anaerobic growth of 12 *Saccharomyces cerevisiae* strains and their respective ΔPdr12 mutants in wheat straw hydrolysate. The methodology developed was also shown to enable monitoring growth in corn stover hydrolysate. The results collected in this paper show that using relevant conditions and measuring not only growth, but also product formation is crucial to perform a good screen of strain performance, especially when working with lignocellulosic hydrolysates.
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