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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 hydrolysate 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 Δ*Pdr12* mutants in wheat straw hydrolysate. 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 hydrolysate 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.

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 novel, more 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; Kensy et al., 2009; Toeroek et al., 2015) and even filamentous fungi (Mózsik et al., 2019). Biomass

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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 microbioreactor 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 Balcerek, 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 Martín and Jönsson, 2003). Thus, production of lignocellulose derived bioethanol requires not only a microrganisms that is able to ferment all sugars in the hydrolysates, but also exhibits tolerance to the inihibiting compounds. Therefore, we set out to develop a method for using the Biolector as a microbioreactor screening platform suitable for 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@ufop.br) were used to validate the screening method. The LBCM 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 (LBCM103, LBCM109, LBCM110, LBCM126) for validation. LBCM67 and LBCM97 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}

Table 1
Influence of *PDR12* deletion in different strain backgrounds on aerobic and anaerobic growth and ethanol production during anaerobic fermentation of medium containing 70% wheat straw hydrolysate (WSH).

Strain	Description	Aerobic lag phase (h)	Aerobic specific growth rate (h^{-1})	Aerobic growth[a]	Anaerobic growth[a]	Anaerobic ethanol produced by parent (g l^{-1})	Anaerobic ethanol produced by *ΔPdr12* mutant (g l^{-1})	Ethanol produced *ΔPdr12* mutant compared to parent[b]
CEN.PK113-7D	Haploid lab strain	16.8	0.10	+	0	27.6 ± 0.3	29.8 ± 2	0
S288C	Haploid lab strain	n.a.	n.a.	0	–	28 ± 1	28.2 ± 04	0
Ethanol Red	Diploid industrial strain	3.4	0.16	–	0	28.4 ± 1.1	30.7 ± 0.5	+
DGI 342	Diploid industrial strain	5.1	0.21	0	0	29 ± 0.3	30.9 ± 1.1	+
PE-2	Diploid industrial strain	21.8	0.04	0	+	33 ± 0.7	31.1 ± 2.1	0
KE 6–12	Diploid industrial strain	8.4	0.13	–	+	33.1 ± 0.2	38.3 ± 1	+
LBCM67	Wild type isolate	8.4	0.08	0	+	31.3 ± 2.4	28.3 ± 1.8	0
LBCM97	Wild type isolate	6.8	0.13	+	0	29.5 ± 2	29.9 ± 1	0
LBCM103	Wild type isolate	8.4	0.12	–	–	28.4 ± 0.9	28 ± 0.4	0
LBCM109	Wild type isolate	10.1	0.11	0	+	27.3 ± 0	27.6 ± 0	+
LBCM110	Wild type isolate	20.1	0.09	0	–	26.9 ± 0.6	26.9 ± 1	0
LBCM126	Wild type isolate	10.1	0.07	–	+	26 ± 0	26.3 ± 0.1	+

Legend: "0" indicates no significant difference between parent and mutant, "+" indicates the mutant performed better than the parent, "–" indicates the parent performed better than the mutant.
[a] A student's *t*-test was used to determine whether growth curves were significantly different. For each strain background, the parental strain was compared to the mutant and the difference was deemed significant at a p-value < 0.05.
[b] Ethanol values were considered different when the difference between two values was greater than 1 standard deviation.

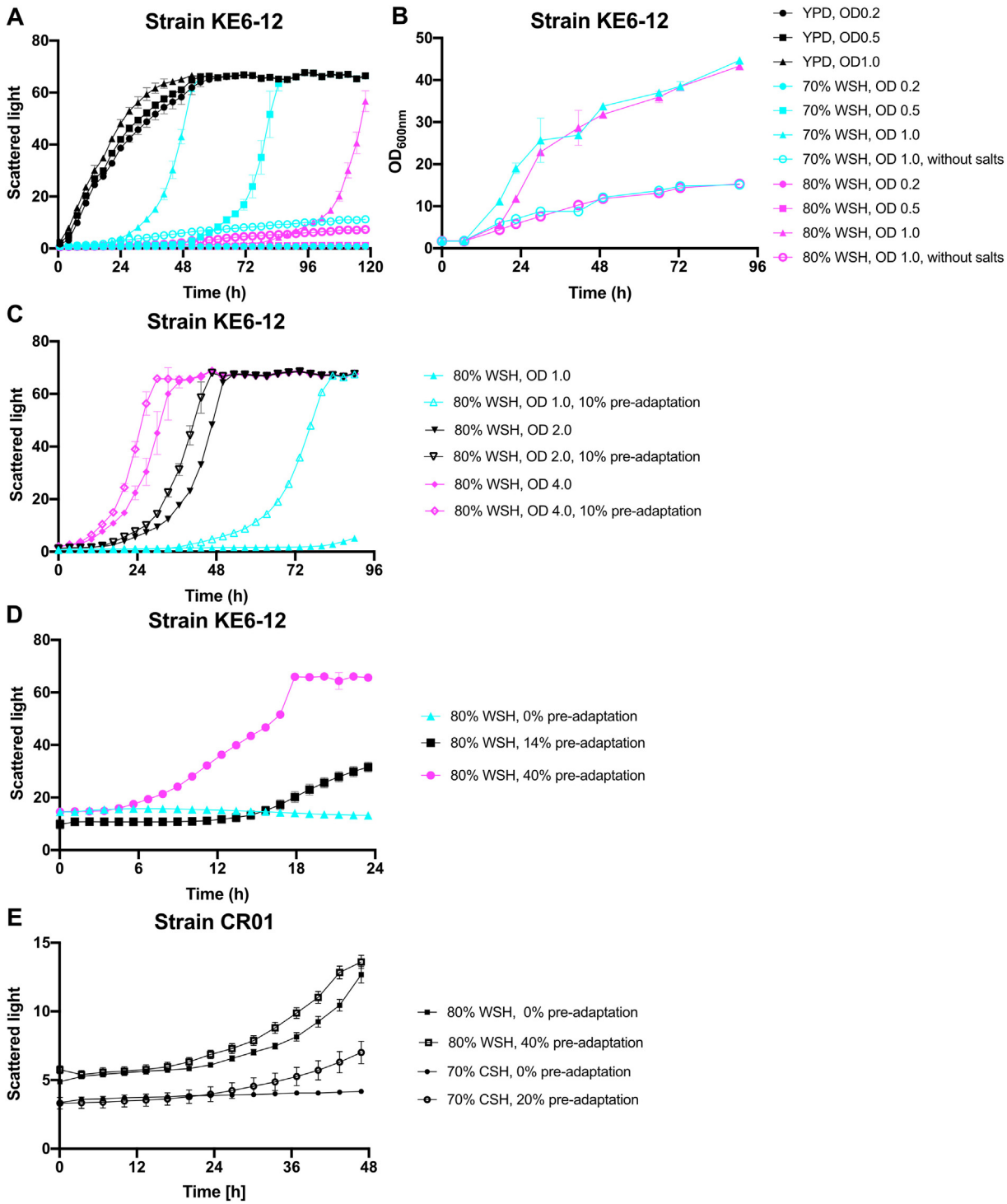

Fig. 1. Cultivation of *S. cerevisiae* KE6–12 (A) in microbioreactors at varying OD values, varying wheat straw hydrolysate (WSH) concentrations and with or without salts added to the medium, under aerobic conditions; (B) in flasks shaken at 200 rpm, at OD 1.0 and containing medium with different hydrolysate 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.

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 Δ*Pdr12* 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 (TGTACAAGGTGAATTCTCCTATGATGGTCTGGACCAAAGCggccaggcggtaattaggtaAGGTTACGTTATTTACTGTCCCGAGCTTGATTTCCATTTC) 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

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 g l^{-1} KH_2PO_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 Δ*Pdr12* 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 amount of inoculum that is specified for each set of experiments. Online parameters were measured in 20 min intervals, using an excitation and emission filter at 600 nm for scattered light. For anaerobic fermentation experiments, 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 microbioreactor 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_2PO_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

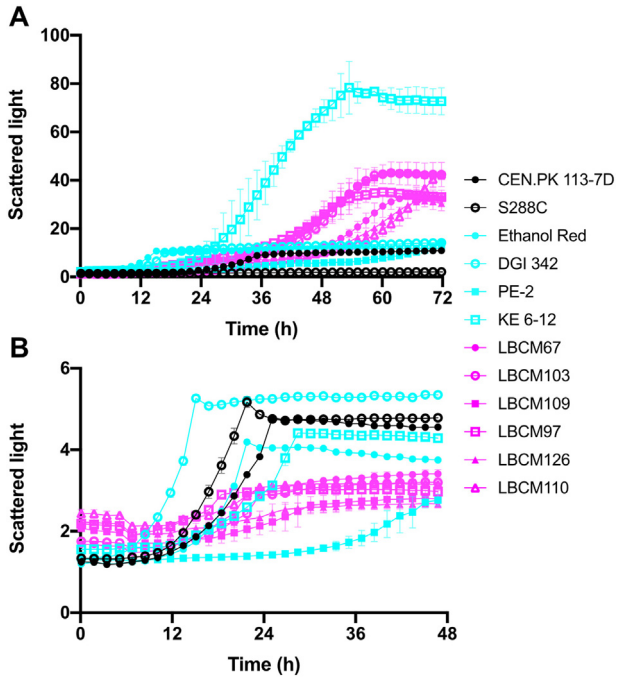

Fig. 2. (A) Aerobic and (B) anaerobic cultivation of strains in medium containing 70% wheat straw hydrolysate (WSH). Lab strains are shown in black, industrial strains are shown in cyan and wild type isolates are shown in magenta. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

studied laboratory strains, four industrial strains and six wild type isolates from the LBCM collection (Table 1). Under aerobic conditions, the lag phase of the evaluated strains varied from 3 to 22 h, after which the growth rates of the strains ranged from 0.04 to 0.21 h h^{-1} (Fig. 2a). KE6–12, harbouring the xylose consumption pathway, was able to consume all xylose and glucose found in the medium whereas other strains did not utilize xylose or only converted it to xylitol (Supplementary Fig. S1). Notably, the LBCM strains were able to convert a larger amount of xylose into xylitol than commonly used industrial strains Ethanol Red, DGI 342 and PE-2. At the end of the cultivation, ethanol was detected in the media of the laboratory and most industrial strains, while KE6–12 and the LBCM strains had consumed all the ethanol produced (Supplementary Fig. S1). Under aerobic conditions all LBCM strains grew to much higher cell densities than Ethanol Red, DGI 342 and PE-2 (Fig. 2a), indicating that these have a great potential for use in 2G biorefineries.

In order to demonstrate the power of the developed methodology, aerobic growth of strains in which the *PDR12* gene was deleted were compared to their parental strains using the Biolector (Table 1, Supplementary Fig. S2). Deletion of *PDR12* in laboratory strain CEN.PK113-7D has previously been shown to improve tolerance towards acetic and formic acid (Nygård et al., 2014) as well as lignocellulosic hydrolysates (Nygård et al., 2015). Indeed, improved aerobic growth of the CEN.PK113-7D*ΔPdr12* strain compared to the parental strain was observed in WSH medium. However, this improvement was only observed in part of the other strains evaluated (Table 1, Supplementary Fig. S2). These results highlight that our methodology allows for investigation of the effect that strain background has on a specific genetic alteration in a high-throughput fashion in hydrolysate containing medium.

The Biolector platform also allows for monitoring growth under anaerobic conditions. As expected, only one or a few doublings in cell density were observed for anaerobic cultivation of the tested strains (Fig. 2b) because *S. cerevisiae* is auxotrophic for ergosterol in the absence of oxygen (Valachovič et al., 2001). The growth observed in our study was likely due to residual metabolites present from the aerobic pre-cultures. Notably, the LBCM strains and PE-2 hardly showed any residual growth under anaerobicity while the other strains accumulated higher levels of biomass (Fig. 2b). Although cell growth can be easily, continuously monitored using the developed methodology, ultimately, product formation capability is the main motivation for such screenings. In order to test whether cell growth can be used to determine which strains are better producers, the ethanol concentration in the microwells was measured after 48 h fermentation (Table 1). We observed no clear correlation between aerobic and anaerobic growth or between growth and ethanol titres measured after 48 h of anaerobic fermentation (Table 1, Supplementary Figs. S4 and S5). In order to rank the strains accurately in terms of ethanol production, earlier measurements of ethanol production would be required.

4. Conclusions

The Biolector platform was successfully used to assess aerobic and anaerobic growth of 12 *Saccharomyces cerevisiae* strains and their respective Δ*Pdr12* 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.

CRediT authorship contribution statement

Marlous van Dijk: Investigation, Validation, Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **Ignis Trollmann:** Investigation, Validation, Writing - review & editing. **Margarete Alice Fontes Saraiva:** Investigation, Writing - review & editing. **Rogelio Lopes Brandão:** Writing - review & editing, Funding acquisition. **Lisbeth Olsson:** Writing - review & editing, Supervision, Funding acquisition. **Yvonne Nygård:** Conceptualization, Methodology, Supervision, Project administration, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2020.100532.

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