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Effect of diluted hydrolysate as yeast propagation medium on ethanol production

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ABSTRACT

Yeast propagation using 50% diluted hydrolysate in water was utilized for the fermentation of hydrolysate derived from pre-treated ensiled sweet sorghum. The purpose was to condition the yeast to the inhibitors generated during the ensiling of sweet sorghum. The conditioned seed cultures exhibited similar fermentation performance and superior kinetics than the inoculum prepared in YPD medium. Furthermore, the conditioned yeast showed increased tolerance to the increased levels of these inhibitors, including ethanol, acetic and lactic acids, demonstrating an effective way to increase the robustness of yeast fermentation for ethanol production.

1. Introduction

World primary energy consumption is estimated to climb 46–124% from 2015 to 2100 due to an increasing world population and increasing energy consumption per capita (International Energy Agency, 2017; Mearns, 2018). Transport currently accounts for 28% of global energy consumption (International Energy Agency, 2017) and oil derivatives constitute 93% of the energy consumed in this sector (International Energy Agency, 2017). In the US, the transportation sector is one of the largest contributors to greenhouse gas emissions, but liquid biofuels offer attractive alternatives with the potential to decarbonize this sector. Ethanol remains the most prevalent commercial biofuel, comprising 72% of biofuels produced globally in energy terms (REN21, 2017).

Sorghum bicolor (sweet sorghum) has limited uses in the global food industry but offers potential as a dedicated crop for bioethanol production, demonstrating superior drought- and heat-stress tolerance and ability to grow on agriculturally marginal lands when compared to sugarcane and sugar beet (Hill et al., 2006; Barcelos et al., 2016). Sweet sorghum can be ensiled with *Saccharomyces cerevisiae* to produce ethanol from free sugars in a primary fermentation (Gallagher et al., 2018). The cellulosic fraction that remains after ensiling can be then pre-treated and hydrolyzed prior to a secondary fermentation to produce cellulosic ethanol.

Developing a robust fermentation process is critical to the success of commercial cellulosic ethanol production. The conditions of the upstream operations including primary ensiling, pre-treatment and hydrolytic saccharification (or hydrolysis) may considerably alter the concentrations of metabolic inhibitors produced from the biomass and thus negatively affect the secondary fermentation. For example, the contamination of heterofermentative lactic acid bacteria during primary ensiling steps could significantly increase lactate concentrations and the inclusion of air during ensiling may also increase acetic acid concentration prior to the secondary cellulosic ethanol fermentation (Gallagher et al., 2018). Ethanol produced during the primary fermentation during ensiling can also have a negative effect on the secondary fermentation.

Researchers have developed several strategies to overcome the inhibitory effects of hydrolysate, including vitamin feeding (Alfenore et al., 2002), increasing aeration (Alfenore et al., 2004) and supplementation of zinc in the culture medium (Zhao et al., 2009). However, although these methods could improve fermentation performance, they may not be economically appealing.

Evolutionary engineering, which aims to adaptively evolve microbial strains that are resistant to specific inhibitors, has been well studied (Tomás-Pejó and Olsson, 2015; Nielsen et al., 2015; Smith et al., 2014; Koppram et al., 2012). Improved fermentation performance with sugarcane bagasse hydrolysate containing high content of microbial inhibitors has been demonstrated using evolved microbial strains (Martin et al., 2007). However, depending on feedstock and pre-treatment conditions, the biocatalysts that tolerate the inhibitors generated in sugarcane bagasse may not be directly transferable to alternative sources of pre-treated biomass. Hence biocatalysts may require acclimation to different biomasses and, potentially, to the outputs from

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different pre-treatment processes (Jönsson and Martín, 2016).

We therefore investigated the use of ensiled sweet sorghum hydrolysate as a medium for yeast propagation to increase tolerance of the microbial seed cultures to the most prevalent metabolic inhibitors produced with that pre-treated biomass: acetic acid, lactic acid and ethanol. We then assessed the effectiveness of these cultures for ethanol production and kinetics during cellulosic fermentation, compared to native seed cultures.

2. Materials and methods

2.1. Microorganisms

A commercial, recombinant strain of *Saccharomyces cerevisiae* was purchased from Lallemand and used in this investigation. Before the strain was commercially available in SLY (stabilized liquid yeast), strains were first provided in petri dishes and grew in YPD medium for initial evaluation as in Section 3.1. After the strain was selected for further work and the SLY of the strain became commercially available, the SLY was directly pitched into fermentation or seed propagation. The percentage of dry cell weight (DCW) in the SLY was 23–25%. Due to the high viscosity of the SLY suspension, it was diluted with sterilized water (30–50% dilution based on volume) before inoculation.

2.2. Ensiling, pretreatment and hydrolysis

Sorghum bicolor (sweet sorghum) *var.* SugarT was ensiled to produce ethanol near Hereford, TX in May 2015 as described previously (Gallagher et al., 2018). After ensiling for 1–2 months, the biomass was treated by Reverse Acid Pre-Treatment (RAPT) at 9% undissolved solids (UDS), and 3.75% α-hydroxyethane sulfonic acid (HESA), at 125 °C, for 1 h (Patent WO 2012/061596 A1). After pre-treatment, the pH of the biomass was adjusted to 5.3 using NH_4OH. Hydrolysis was then conducted in a 5 L reactor for ~ 4 days, at 53 °C with approximately 14% total solids (TS) and 5–10% CTec3 (Novozymes) enzyme loading, based on cellulose content. The resulting "RAPT hydrolysate" was stored at 4 °C prior to use.

2.3. Preparation of filtered hydrolysate, seed propagation and fermentations

The pH of the hydrolysate was adjusted to 5.8 using NH_4OH. The hydrolysate was clarified by centrifugation and the supernatant filtered under vacuum through glass microfibre paper GF/B (1.0 µm pore; Whatman®) to remove solids and particulates. The solution was then filter-sterilised (0.45 µm Nalgene™ Rapid-Flow™ polyethersulfone (PES) filters (Fisher)), and stored at 4 °C.

Where appropriate, additional ethanol, lactic or acetic acids were added to the hydrolysate to the required levels and the pH adjusted to 5.80.

The filtered hydrolysate was diluted to 50% and 75% in sterile, distilled water or in YPD medium (20 g L^{-1} peptone, 20 g L^{-1} dextrose, 10 g L^{-1} yeast extract) and these media used for propagation and acclimation of the seed cultures. The SLY was diluted with sterilized water and added to 25 mL of the medium with an initial cell density of 0.5 g L^{-1}. Seed cultures were incubated in 125 mL, vented, baffled Erlenmeyer flasks, at 32 °C with orbital shaking (160 rpm) for 17–24 h, depending on the media.

The pH of the unfiltered RAPT hydrolysate was adjusted to 5.80 and used as the fermentation base-medium. Seed cultures were centrifuged and re-suspended to 0.5 g L^{-1} in the different fermentation media. To determine the effects of initial ethanol concentration, 10% volumetric mass transfer was used instead for the inoculation to simulate more accurately practical operations. Fermentations were either performed in one-way vented 50 mL Falcon tubes, incubated at 32 °C and with shaking at 200 rpm, or in 250 mL Duran® bottles containing 180 mL hydrolysate hooked to an alcohol fermentation monitor (AFM), at 32 °C, with stirring at 200 rpm. The AFM system monitors the amount of CO_2 that evolves from the fermentation broth by measuring the off-gas flow rate. The CO_2 production rate (mL/min) and total CO_2 production (mL) could then be obtained from the automated AFM.

2.4. Sampling and analysis

Samples from the fermentation flasks were analysed using High-Performance Liquid Chromatography (HPLC) to determine the proportion of glucose, xylose, arabinose, acetic acid, lactic acid, glycerol, and ethanol therein. Samples were clarified using Spin-X® Centrifuge Tube Filters with a 0.22 µm nylon membrane, according to the manufacturer's instructions. Samples were diluted 10-fold in 10 mM sulfuric acid in distilled water. HPLC was performed using a Biorad HPX 87H column equipped with guard column at 65 °C with the mobile phase of 0.005% sulfuric acid in distilled water at a flow rate of 0.6 mL min^{-1}. The injection volume was 20 µL. RI (refractive index) detector (Shodex RI-101) was used with internal temperature at 50 °C. Concentrations of glucose, xylose, arabinose, acetic acid, lactic acid, glycerol and ethanol were determined against an external calibration curve of known standards. Ethanol yield is calculated as the percentage of produced ethanol of the theoretical maximum based on consumed sugar respectively, assuming a theoretical conversion of glucose and xylose to ethanol of 0.51 g/g.

3. Results and discussion

3.1. Comparison of different seed culture media

YPD medium is the most used medium for yeast cell propagation but, due to its high cost, its use is not suitable for most industrial applications, particularly fermentations for the production of biofuels or bulk-chemicals. In this investigation, we evaluated the use of RAPT hydrolysate in the culture medium to both reduce propagation and maintenance costs and maintain a microbial seed culture that may be more adapted to the industrial fermentative medium in which it will be eventually used. 50% RAPT hydrolysate was chosen for the initial experiment. Two experiments were conducted, i.e. (1) displacing 50% YPD with hydrolysate to understand the effect of hydrolysate on propagation; (2) removing YPD from the culture medium to understand if

Table 1
Results of seed culture in different media.

Exp.	Cases	Ini. glucose (g L^{-1})	Ini. xylose (g L^{-1})	Ini. ethanol (g L^{-1})	End. glucose (g L^{-1})	End. xylose (g L^{-1})	End. ethanol (g L^{-1})	Pro. ethanol (g L^{-1})	OD600	$Y_{x/s}$	$Y_{p/s}$
1	YPD	19.18	0.00	0.00	0.00	0.00	7.78 ± 0.06	7.78 ± 0.06	3.74 ± 0.11	0.134 ± 0.004	0.405 ± 0.003
1	YPD/Hyd	30.67	15.32	9.75	0.77 ± 0.17	1.75 ± 0.00	25.67 ± 0.11	15.93 ± 0.11	7.95 ± 0.14	0.126 ± 0.001	0.368 ± 0.002
2	H2O/Hyd	26.69	11.87	11.33	0.83 ± 2.36	0.34 ± 0.00	22.64 ± 0.48	11.30 ± 0.48	6.76 ± 0.03	0.116 ± 0.007	0.303 ± 0.006
2	YPD/Hyd	36.27	11.94	11.36	0.00	3.35	28.49	17.13	6.68	0.101	0.38

Note: $Y_{x/s}$ and $Y_{p/s}$ represent the yield for biomass and ethanol calculated as the cell growth in CDW and produced ethanol over the consumed sugar concentration, respectively.

50% hydrolysate alone could provide all the nutritional need by the yeast during propagation.

Table 1 summarizes the result of the seed culture in different media, in shake flasks. In experiment 1, the cell culture in YPD reached a lower final OD_{600nm} of 3.74 with a higher biomass yield (based on consumed sugar) of 0.134. This is probably due to the lower mount of sugar (19 g L^{-1} glucose), higher content of complex, nitrogenous compounds (20 g L^{-1} peptone and 10 g L^{-1} yeast extract) and no inhibitors present in the culture medium. When 50% YPD was substituted by hydrolysate, although the biomass yield was lower, the final OD_{600nm} was 7.95, much higher than YPD alone and ascribed to the higher initial sugar content in the culture medium. For downstream applications, this high OD_{600nm} is preferred during propagation. In experiment 2, we compared the propagation medium of 50% YPD + 50% Hydrolysate (YPD/Hyd) with 50% water + 50% Hydrolysate (H_2O/Hyd). The cells cultured in 50% hydrolysate with water consumed almost all the sugars in ≈18 h and produced similar OD_{600nm} and biomass yield as those in 50% hydrolysate with YPD (Table 1). Hence, 50% hydrolysate in water (H_2O/Hyd) supported sufficient cell growth during propagation than the more complex and costly alternatives. However, significant amounts of ethanol were produced in all cases due to Crabtree effect. With the high initial glucose concentration, even in the presence of air, yeasts undergo fermentative metabolism and produce ethanol (Walker, 1998). Also, this seed propagation was carried out in shake flask, without sufficient aeration supply. Thus, we observed this low biomass yield (> 0.14 g/g) with relatively high ethanol yield (> 0.30 g/g). To evaluate the performance of the yeast propagated in H_2O/Hyd during fermentation, the seeds cultured in different medium were then transferred into the hydrolysate for subsequent fermentation with the same starting CDW of 1 g L^{-1}.

Fig. 1 summarizes 24-h and 48-h fermentation results using the cells from different seed cultures. In all cases, after 24 h, glucose was almost completely consumed. Conversely, xylose consumption was different depending on the seed culture used. Fermentations using seeds cultured in YPD medium showed slower xylose consumption of 45% compared to the others which had been pre-adapted with 50% RAPT hydrolysate with > 80% consumption. Accordingly, a lower 24-h ethanol production was observed for fermentations using seeds from YPD. The seeds cultured from different medium showed similar ethanol yield. Therefore, after 2-day fermentation with all the sugars consumed, similar ethanol productions were achieved from the fermentations. The CO_2 production profiles, proxies for ethanol production, in Fig. 2B and C further illustrate the differences in the fermentative performance of the seed cultures. Fig. 2A depicts the predicted ethanol production calculated from CO_2 profiles versus ethanol measured by HPLC. The high regression coefficient R^2 of 0.95 demonstrated that the CO_2 profiles measured by AFM could well be applied to represent the real-time ethanol production. Compared to the seeds cultured from YPD, the seeds cultured in YPD/Hyd showed a shorter lag phase (< 2h) at the beginning of the fermentation and higher CO_2 production rate (0.14 mmol min^{-1}). For the seeds cultured in YPD/Hyd, the fermentation was complete after approximately 30 h, as indicated by the absence of CO_2 production, due to the depletion of the sugars. For seed cultures in YPD, the fermentation concluded at approximately 40 h, i.e. 30% more slowly than for seeds cultured in YPD/Hyd. The longer lag phase and extended fermentation time for YPD derived culture could be due to the extra time required for the yeast to (1) adapt to the relative toxicity of hydrolysate and (2) switch its metabolism on for xylose utilization. Overall, these results demonstrate that yeast seed cultures in 50% hydrolysate with water showed similar fermentation performance and even better kinetics than the seeds in YPD.

Using hydrolysate at different water dilution levels was further tested to determine the optimal hydrolysate concentration for seed propagation. In this study, only higher hydrolysate percentages (50%/75%/100%) were tested. The hypothesis was that the closer to the eventual fermentation medium the seed culture conditions are, the better they might perform during fermentation.

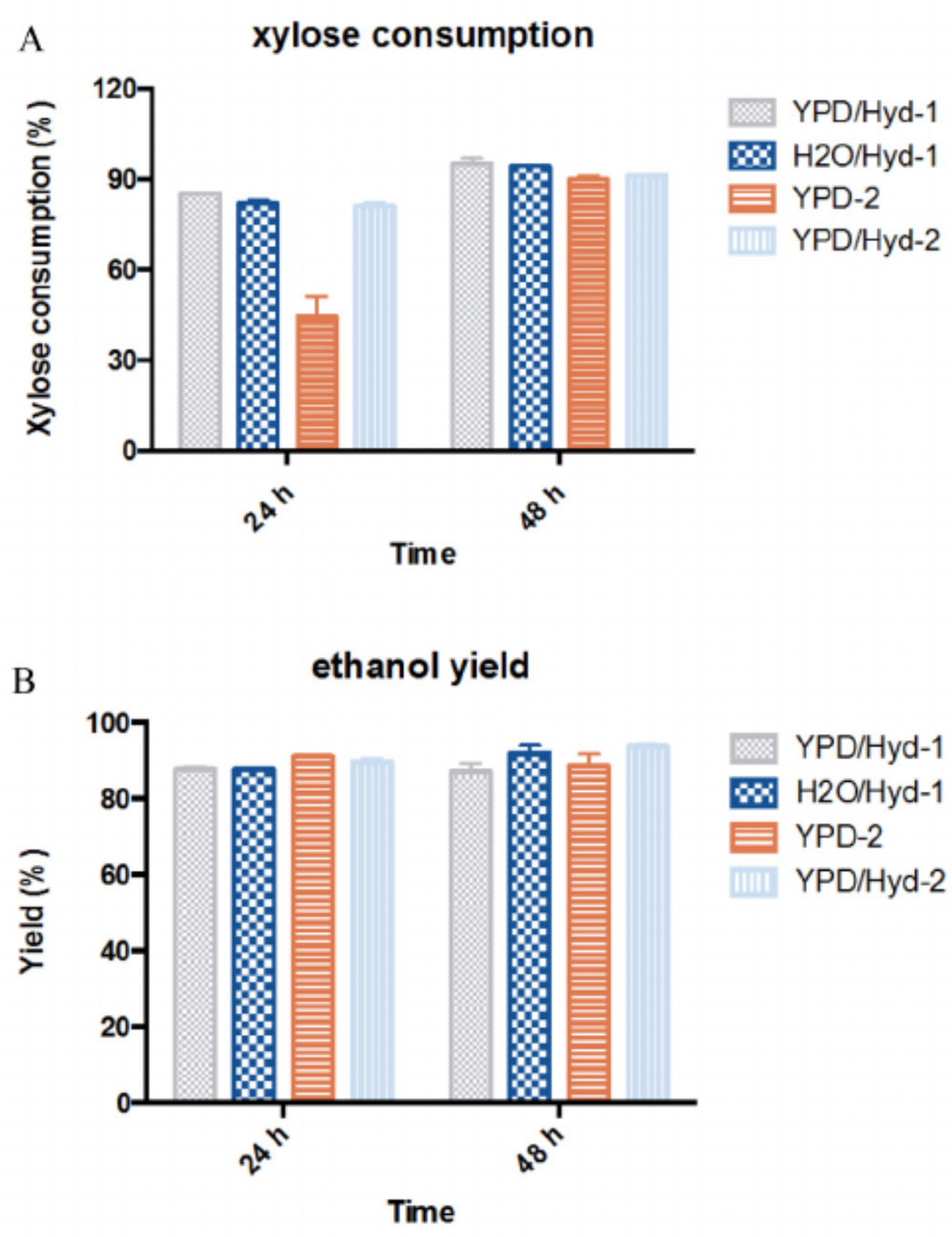

Fig. 1. 24-h and 48-h fermentation results (A. xylose consumption; B. ethanol yield) using the seeds cultured from different medium. Note: YPD/Hyd-1, H2O/Hyd-1 represent the fermentations in Experiment 1 using the seeds cultured in 50% hydrolysate diluted by YPD (YPD/Hyd) and water (H2O/Hyd), respectively; YPD-2, YPD/Hyd-2 represent the fermentations in Experiment 2 using the seeds cultured in YPD and in YPD/Hyd, respectively. The percentage of yield was calculated as the produced ethanol titer divided by consumed sugar concentration (including glucose, xylose, galactose) over the theoretical value of 0.51 (g ethanol/g sugar). The xylose consumption percentage was calculated as the consumed xylose over the initial concentration. All the data are the average of the two replicates with error bars of standard deviation.

The propagations were performed following a staggered procedure over 24 h, with an initial OD_{600nm} of 0.52 ± 0.02. Fig. 3 shows the seed culture profiles for cell growth, xylose utilization and ethanol and glycerol production. Table 2 shows the data analysis of 18-h seed culture. Increasing hydrolysate percentage in the seed culture medium from 50% to 100% increased the inhibition of seed propagation due to increased initial concentrations of sugar, acids and ethanol. With 50% hydrolysate, sugar utilization started at ~4 h after inoculation, with all glucose consumed in 12 h and 90% xylose consumed after 18 h. When the hydrolysate percentage increased to 75% and 100%, the onset of sugar utilization was delayed to 6 h and 10 h, respectively. After 18 h seed culture, 70% xylose was left in the 75% hydrolysate, whereas almost no xylose was used for 100% hydrolysate. Similarly, the delayed cell growth and ethanol production were also observed with 75% and 100% hydrolysate. Cells in 50% and 75% hydrolysate entered stationary phase after approximately 18 h, achieving OD_{600nm} of 7.16 and 6.71, respectively; whereas yeast cultured in 100% hydrolysate showed slowed growth with a lower OD_{600nm} of 5.75 after 22 h.

As observed before, high amounts of ethanol were produced during seed propagation using diluted hydrolysate. More ethanol was produced in 75% hydrolysate than in 50% hydrolysate (22.1 g L^{-1} compared to 18.4 g L^{-1}) after 18 h seed culture, most probably due to the higher initial sugar concentration in the concentrated hydrolysate

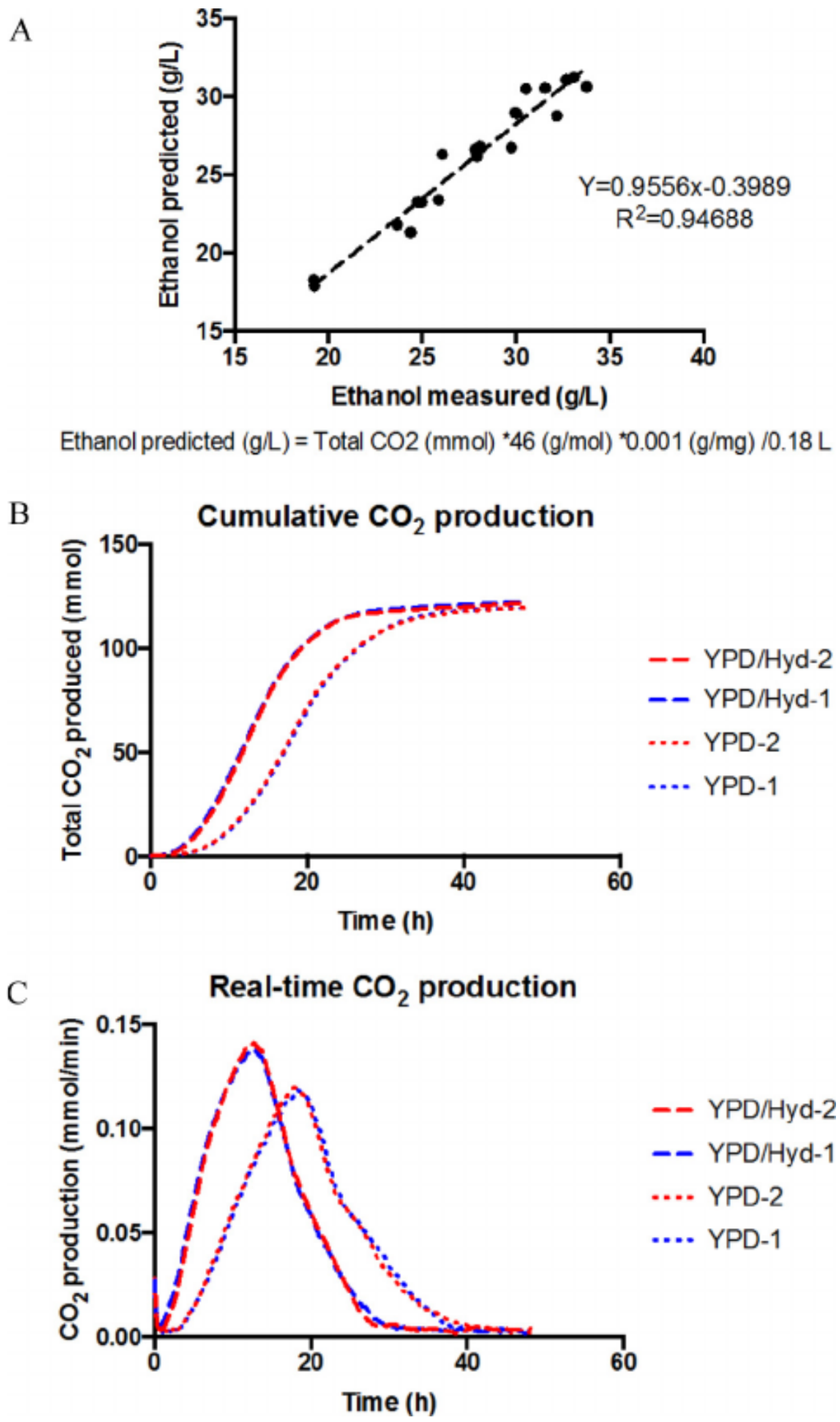

Fig. 2. Predicted ethanol production from CO_2 profiles vs. ethanol measured by HPLC and CO_2 production profiles of the yeast fermentation in AFM with the seeds cultured under different conditions. Note. A. total produced CO_2 (mmol); B. CO_2 production rate (mmol/min). The predicted ethanol production from CO_2 profiles was calculated as the produced cumulative CO_2 in mole times ethanol molecular weight divided by the volume of the fermentation broth (0.18 L).

solution. Surprisingly, only 10.6 g L^{-1} ethanol was produced in 100% hydrolysate with much less sugar consumption. The yield for 18 h ethanol and biomass production were similar for these three cases (Table 2). However, the yield for glycerol production increased with the increase of hydrolysate percentage in propagation medium suggesting cells were more stressed in the cases of higher concentration of hydrolysate. Based on these results 50% hydrolysate in water (H2O/Hyd) was chosen for the following studies.

3.2. Effects of initial ethanol concentrations in hydrolysate on fermentation

As the ethanol concentrations could vary significantly in the ensiling process, it is important to understand its impact on downstream fermentation, and if the yeast propagated in 50% hydrolysate could cope with this variability in ethanol concentration. From an unpublished model and data on the ensiling and separation process prior to pre-treatment, the initial ethanol concentrations in fermentation medium could range from 22 to 32 g L^{-1}. Therefore, in this study, hydrolysate with different ethanol concentrations (22, 27 and 32 g L^{-1}) were tested for fermentation to understand the effect of that ethanol range on fermentation performance. The yeast seed-culture used was propagated in H2O/Hyd. As the control, SLY without seed propagation was directly pitched into the fermentation with the initial CDW of 0.5 g L^{-1}. Direct pitch of SLY was used as a control in these experiments because preliminary experiments demonstrated similar fermentation performance as YPD cultured seed (results not shown). Also, the use of commercial SLY was more practical and has been widely used as the starting inoculum in the industrial process.

Fig. 4 shows the effects of initial ethanol concentrations on fermentations with conditioned yeast and directly pitched SLY. The direct-pitched fermentation showed no xylose consumption and less than 10 g L^{-1} ethanol produced after one day of fermentation. Xylose consumption and ethanol production in these cultures was negatively correlated with initial ethanol concentrations. Conversely, the fermentations with conditioned seed cultures showed faster xylose consumption and higher ethanol production and increased tolerance to variations in initial ethanol concentration. For example, similar ethanol production and xylose consumption was observed with initial ethanol concentrations of 22 and 27 g L^{-1} with only a 6% drop in produced ethanol at 32 g L^{-1} ethanol after 2 days fermentation. Moreover, the production caught up eventually as demonstrated by the data acquired following 5 days fermentation. In addition, the initial ethanol concentrations seemed to have little impact on the ethanol and glycerol yield for conditioned cells, whereas the glycerol yield for SLY appeared to be higher than those for conditioned cells. However, with large error bars, the differences were not significant. Glycerol is typically produced when yeast experience stress. In the case of SLY, higher initial ethanol concentrations in the fermentation medium generated stressful conditions for the yeast, resulting in overall poorer performance. Conversely, the conditioned cells showed improved tolerance to the different initial concentrations of ethanol, and therefore comparatively greater growth and faster production.

A number of studies have been performed to investigate the inhibitory effects of ethanol on yeast fermentation including the mechanisms of inhibition, influencing factors, cell stress responses, and strategies to increase tolerance (Stanley et al., 2010; Deparis et al., 2017). In general, ethanol inhibits yeast growth even at relatively low concentrations, reducing cell specific growth rates and inhibiting cell division. High ethanol concentration may cause reduced cell vitality and increased cell death (Birch and Walker, 2000). Ethanol also impacts cell metabolism and macromolecular biosynthesis through inducing heat shock-like proteins, lowering RNA and protein accumulation rates, denaturing intracellular proteins and glycolytic enzymes and reducing their activity (Hu et al., 2007; Stanley et al., 2010). Athmanathan et al. (2011) studied the product ethanol inhibition on ethanol fermentation by recombinant yeast. The yeast ceased fermentation when the initially-added ethanol reached 90 g L^{-1}, whereas the maximum ethanol production reached 110 g L^{-1} when ethanol was produced *in situ* from glucose. This demonstrated that yeasts are more tolerant to produced ethanol than artificially added ethanol in the medium. Conversely, we observed the inhibition effect on directly pitched fermentation by adding 22 g L^{-1} initial ethanol. The high ethanol tolerance in Athmanathan et al.'s (2011) study could be attributed to the rich fermentation medium containing yeast extract and peptone, whereas in our study no additional nitrogen sources was added. In addition, the hydrolysate used in our study also contained acetic and lactic acid, which further elevated the inhibition effect. In general, as mentioned in other research, the effects of ethanol are strain dependent, and the degree of inhibition is related to environmental factors (Alfenore et al., 2002). Nevertheless, high initial levels of ethanol present in cellulosic fermentation is unique due to our upstream ensiling process which produced ethanol from the free sugars available in sorghum. Therefore, not much work has been done to address such issues. Consequently, propagating the yeast in 50% hydrolysate is a viable approach to at

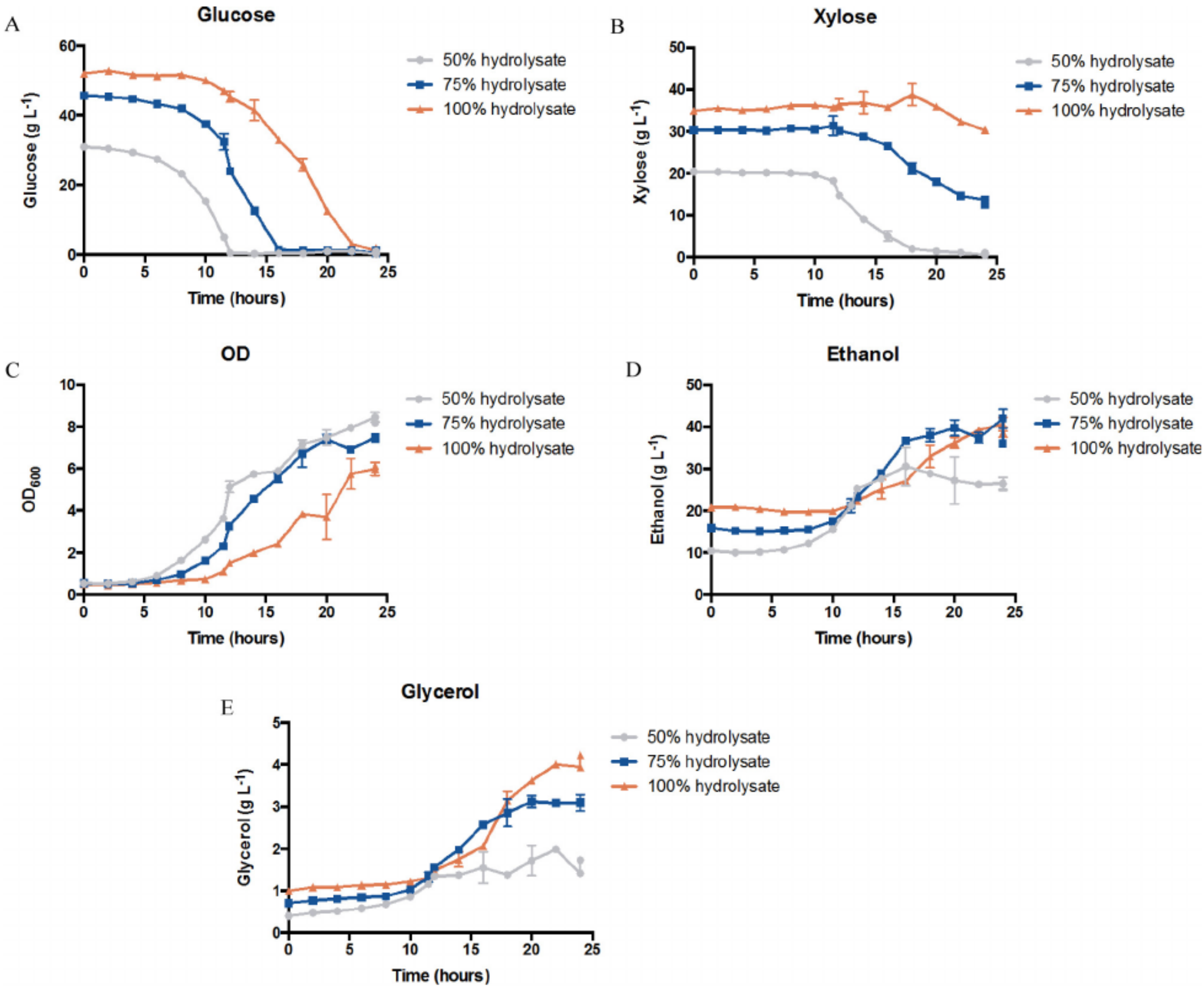

Fig. 3. Seed propagation profiles using diluted hydrolysate as the culture medium. (A. glucose; B. xylose; C. cell growth in OD; D. ethanol; E. glycerol). Note: The average of triplicates was plotted with error bars of standard deviations.

Table 2
Seed culture results using diluted hydrolysate.

	50% Hyd	75% Hyd	100% Hyd
18-h glucose consumed (%)	100.00 ± 0.00	100.00 ± 0.00	52.27 ± 0.44
18-h xylose consumed (%)	90.38 ± 0.33	30.03 ± 4.35	0.00 ± 0.00
18-h ethanol produced (g L^{-1})	18.41 ± 0.79	22.08 ± 1.53	10.60 ± 0.55
18-h ethanol yield	72.6% ± 3.2%	83.1% ± 8.0%	83.6% ± 7.3%
18-h glycerol produced (g L^{-1})	1.37 ± 0.05	2.85 ± 0.32	3.03 ± 0.08
18-h glycerol yield (g glycerol/g consumed sugar)	0.02 ± 0.00	0.04 ± 0.01	0.08 ± 0.01
18-h cell growth OD600	7.16 ± 0.21	6.71 ± 0.64	3.85 ± 0.13
18-h biomass yield (g CDW/g consumed sugar)	0.13 ± 0.00	0.11 ± 0.01	0.13 ± 0.00

Note: The yield for glycerol and biomass were calculated as the produced glycerol and biomass in cell dry weight (CDW) over the consumed sugar concentration.

least mitigate the ethanol inhibition effect and generate more lignocellulosic ethanol at a faster rate that would be obtained using directly pitched SLY.

3.3. Effects of initial acetic acid and lactic acid concentrations

The concentrations of acetic and lactic acids in ensiled biomass could be high enough to prevent adequate growth or metabolic activity of the yeast if inclusion of air or contamination with *Lactobacillus* occurs during ensiling, which may negatively affect ethanol yields from the cellulosic fermentation process. Therefore, the effects of initial concentrations of acetic and lactic acids on fermentation were investigated. To prepare the fermentation medium, certain amounts of acetic and lactic acids were added into the same batch of hydrolysate with the pH adjusted to the same level of 5.8. The hydrolysates with different acetic and lactic acids were used to prepare the 50% filtered hydrolysate for the seed propagation. Directly pitched SLY was used as the control with the initial CDW of 0.5 g L^{-1}. The initial ethanol concentration for all the cases was ~20 g L^{-1}.

Both initial acetic acid and lactic acid negatively impacted on ethanol production when SLY were used in the fermentation (Fig. 5). For the effects of acetic acid, the 24-h ethanol production at 9 g L^{-1} initial acetic acid was much lower than that at 6 g L^{-1} due to little xylose consumption, but after 48 h fermentation similar productions were reached at both 6 and 9 g L^{-1} acetic acid; whereas 14 g L^{-1} acetic acid or above totally inhibited the fermentation with no sugar consumption and zero ethanol production. The ethanol yields for both cases at 6 and 9 g L^{-1} acetic acid were similar, but it appeared that

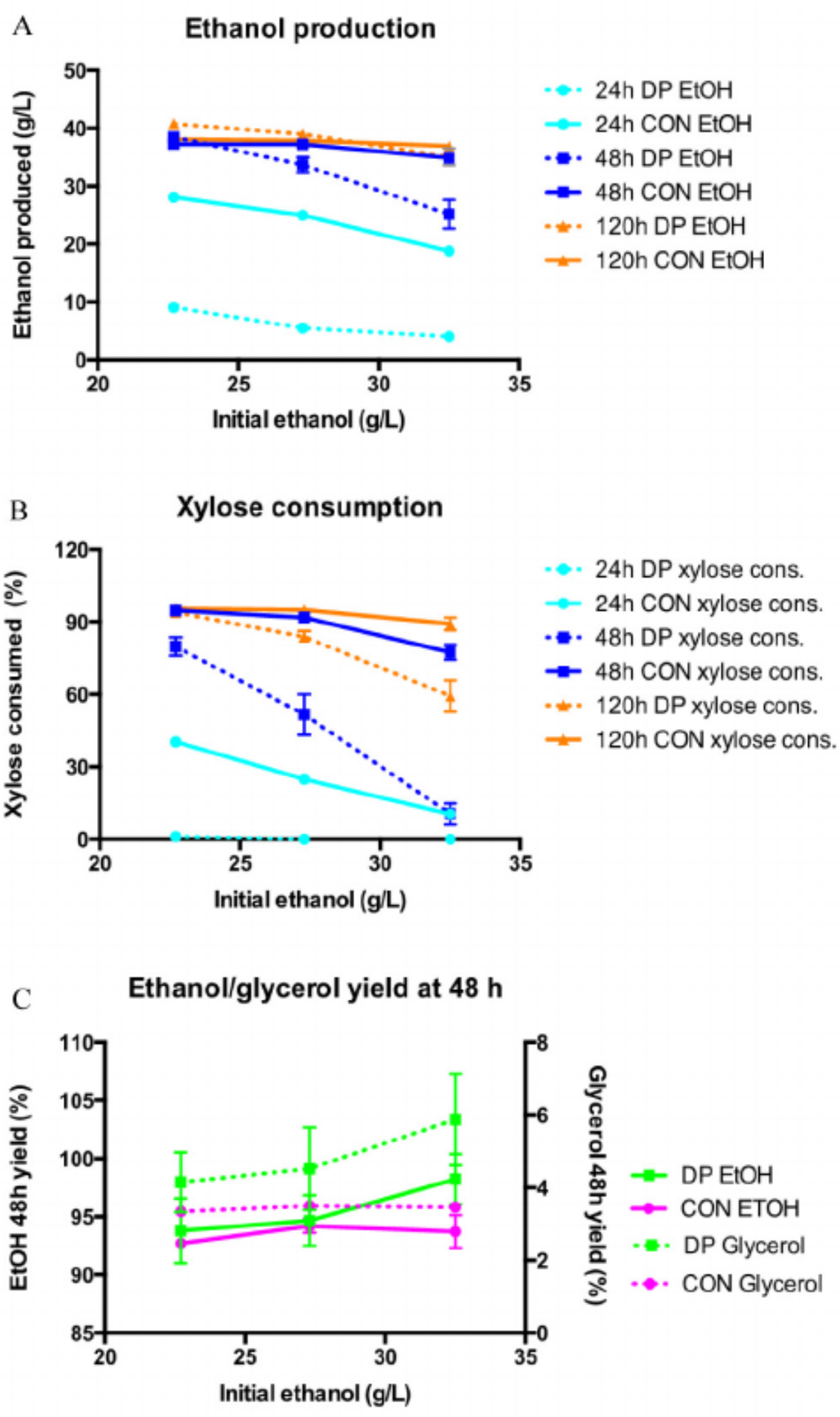

Fig. 4. Fermentation results (A produced ethanol concentrations; B xylose consumption percentage; C 48 h ethanol and glycerol selectivity) using the seeds cultured from 50% hydrolysate with different initial ethanol concentrations. Note: CON and DP in Fig. 4 represent the fermentations with conditioned yeast by 50% filtered hydrolysate in water and with directly pitched SLY. 24 h/48 h/120 h DP EtOH in Fig. 4A represent ethanol production after 24 h/48 h/120 h fermentation using directly pitched SLY, similar to the cases with CON. The effects of initial ethanol were carried out with initial acetic acid of 9 g/L and initial lactic acid of 6 g/L. The data are the average of the duplicates. The error bar reflects ± standard deviations.

more glycerol was produced with higher initial acetic acid concentration. Due to little sugar consumption, the ethanol and glycerol yields were not calculated for the cases with $> 9\,g\,L^{-1}$ acetic acid. For the effects of lactic acid, increasing lactic acid concentrations reduced 24 h ethanol production due to decreased xylose consumption, suggesting the slowdown of fermentation kinetics; however, similar ethanol productions were reached after 48 h fermentation with lactic acid concentration up to $12\,g\,L^{-1}$. Further increasing lactic acid concentration to $16\,g\,L^{-1}$ significantly dropped the ethanol production after 48 h, with no xylose consumption. The ethanol yields for the cases with different initial lactic acid were roughly similar, but the glycerol yield increased along with the increase of lactic acid concentration, indicating increased stress on cell growth.

Conditioning with H2O/Hyd significantly increased the tolerance of yeast to increased levels of acidity in the hydrolysate compared to direct pitch controls. Different from the fermentation with directly pitched SLY, increasing acetic acid from $6\,g\,L^{-1}$ to $14\,g\,L^{-1}$ (with $6\,g\,L^{-1}$ lactic acid) dropped the xylose consumption from 96% to 78% and ethanol production from $37.1\,g\,L^{-1}$ to $33.0\,g\,L^{-1}$ after 24 h fermentation. However, after 48 h fermentation, the levels of xylose consumed and ethanol produced were not significantly different to $6\,g\,L^{-1}$ acetic acid. Results showed that the conditioned yeast could tolerate up to $18\,g\,L^{-1}$ acetic acid with only a slightly lower 48 h ethanol production. For the effects of lactic acid, there was only a small drop in 24-h ethanol production at $18\,g\,L^{-1}$ lactic acid due to less xylose consumption, but 48 h fermentation showed similar ethanol production. For the effects of acids on ethanol and glycerol yields, the ethanol yields using conditioned yeasts under various acetic and lactic acid concentrations were similar. However, the glycerol yields increased with the increase of acetic or lactic acid concentrations, showing the increased cellular stress. Nevertheless, the glycerol yield of conditioned yeasts was significantly less than that of SLY under the same acetic or lactic acid concentrations, suggesting the increased tolerance of conditioned yeasts to acetic acid and lactic acid.

When both acetic and lactic acids were at higher levels, $10\,g\,L^{-1}$ of both reduced 24-h xylose consumption and ethanol production by 7.2% and 7.6%, but reached similar ethanol production after 48 h; whereas $14\,g\,L^{-1}$ of both lactate and acetate considerably dropped 24-h ethanol production and xylose consumption, and extending fermentation time to 48 h increased the production but still less than other treatments. Conditioned yeast therefore demonstrated improved tolerance to acids level up to $18\,g\,L^{-1}$ of acetic or lactic (with initial ethanol concentration of 21 g/L), if the other acid was maintained low. However, when both acids were high ($\sim 14\,g\,L^{-1}$) fermentation performance was negatively affected even after a conditioning step. Also, the comparison of yeast tolerance to different acid concentrations showed that acetic acid is more toxic to the yeast than lactic acid.

Acetic acid and lactic acid are well-documented inhibitors of yeast fermentation. A common observation based on previous studies was that acetic acid resulted in decreased biomass yield and ethanol production rate (Phowchinda et al., 1995; Taherzadeh et al., 1997, Casey et al., 2010). The decrease in the fermentation rate was explained by the decrease in intracellular pH. Acetic acid enters the cell only in the non-dissociated form by simple diffusion. Once inside the cell, acetic acid dissociates and if the extracellular pH is lower than the intracellular pH this will lead to the accumulation of acetate and to the acidification of the intracellular environment (Liu, 2011; Thomas et al., 2002). The acidification of the cytoplasm, in turn, results in diverse effects including inhibition of amino acid uptake and carbohydrate metabolism. In this study, as indicated by 24-h xylose consumption and ethanol production, the decrease of fermentation rate was observed with addition of acetic acid, especially for directly pitched fermentation. Similar observations were made in previous studies that xylose consumption rates were negatively affected by acetic acid (Helle et al., 2003; Bellissimi et al., 2009; Casey et al., 2010).

The inhibitory effects of acetic and lactic acids and the levels at which yeast can tolerate could be modulated by the yeast itself, the methods and media used during seed culture propagation and the fermentation conditions. Casey et al. (2010) showed that increasing media pH could effectively mitigate the inhibitory effect of acetic acid by reducing the concentrations of un-dissociated acetic acid. Nevertheless, the inhibitory effect was still observed with high acetic acid concentration of $15\,g\,L^{-1}$ at pH of 6. Large portions of xylose ($\sim 42\%$) was left after 48 h fermentation. However, in our study, we observed enhanced tolerance to acids by conditioning the yeast with hydrolysate during seed propagation. The conditioned yeast could tolerate up to $18\,g\,L^{-1}$ acetic acid with only a slightly lower 48 h ethanol yield (3% less), let alone that the hydrolysate also contained $6\,g\,L^{-1}$ lactic acid and $21\,g\,L^{-1}$ ethanol. The addition of lactic acids and ethanol could potentiate the inhibition effect of acetic acid (Pampulha and Loureiro-Dias (1989).

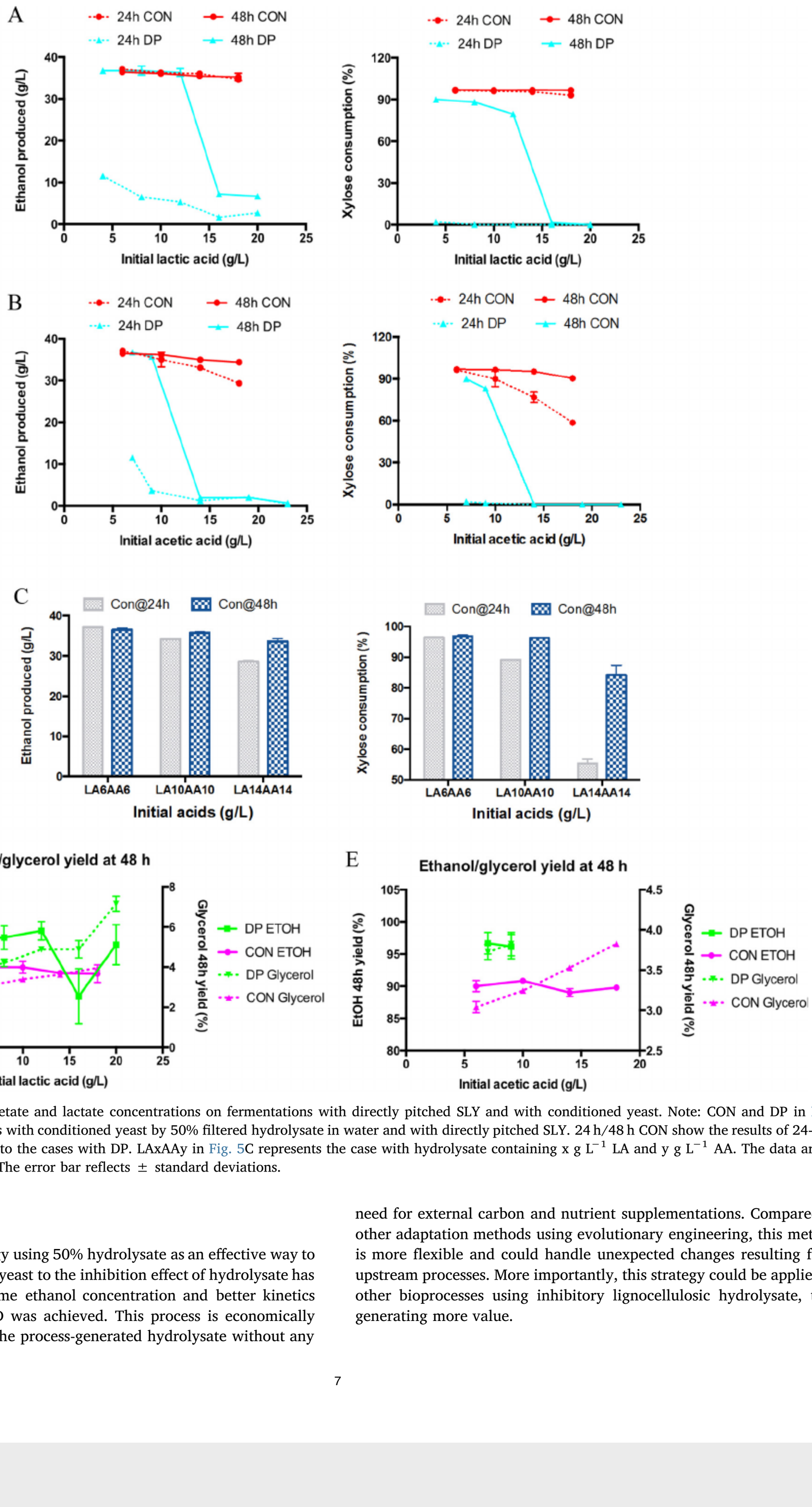

Fig. 5. Effects of initial acetate and lactate concentrations on fermentations with directly pitched SLY and with conditioned yeast. Note: CON and DP in Fig. 4 represent the fermentations with conditioned yeast by 50% filtered hydrolysate in water and with directly pitched SLY. 24 h/48 h CON show the results of 24-h and 48-h fermentation, similar to the cases with DP. LAxAAy in Fig. 5C represents the case with hydrolysate containing x g L^{-1} LA and y g L^{-1} AA. The data are the average of the duplicates. The error bar reflects ± standard deviations.

4. Conclusions

A propagation strategy using 50% hydrolysate as an effective way to increase tolerance of the yeast to the inhibition effect of hydrolysate has been developed. The same ethanol concentration and better kinetics than cells grown in YPD was achieved. This process is economically appealing since we use the process-generated hydrolysate without any need for external carbon and nutrient supplementations. Compared to other adaptation methods using evolutionary engineering, this method is more flexible and could handle unexpected changes resulting from upstream processes. More importantly, this strategy could be applied to other bioprocesses using inhibitory lignocellulosic hydrolysate, thus generating more value.

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