Succinic acid production from lignocellulosic hydrolysate by *Basfia succiniciproducens*

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Abstract: The production of chemicals alongside fuels will be essential to enhance the feasibility of lignocellulosic biorefineries. Succinic acid (SA), a naturally occurring C4-diacid, is a primary intermediate of the tricarboxylic acid cycle and a promising building block chemical that has received significant industrial attention. *Basfia succiniciproducens* is a relatively unexplored SA-producing bacterium with advantageous features such as broad substrate utilization, genetic tractability, and facultative anaerobic metabolism. Here *B. succiniciproducens* is evaluated in high xylose-content hydrolysates from corn stover and different synthetic media in batch fermentation. SA titers in hydrolysate at an initial sugar concentration of 60 g/L reached up to 30 g/L, with metabolic yields of 0.69 g/g, and an overall productivity of 0.43 g/L/h. These results demonstrate that *B. succiniciproducens* may be an attractive platform organism for bio-SA production from biomass hydrolysates.

Keywords: biorefinery, biochemical, corn stover, xylose, *Actinobacillus succinogenes*

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Abbreviations: AA= acetic acid, DDAPH= deacetylated, dilute-acid pretreated hydrolysate, FA= formic acid, HMF= 5-hydroxymethylfurfural, HMF alcohol= 5-(hydroxymethyl)furfuryl alcohol, LA= lactic acid, OD= optical density, SA=succinic acid.

1. Introduction

Lignocellulose will likely make a significant contribution to a balanced renewable energy portfolio, especially for the sustainable production of transportation fuels and chemicals (Chundawat et al., 2011). Given the high capital and operating cost of making fuels from lignocellulose combined with the current low prices in fossil fuel markets, the production of chemicals from biomass is significantly more appealing given the higher selling prices of commodity and specialty chemicals (Biddy et al. 2016). In particular, succinic acid (SA) is a promising intermediate to manufacture from biomass-derived sugars and other renewable carbon sources. Significant work has been conducted to date for SA production at bench-scale from glucose and, more recently, from a variety of feedstocks (Akhtar et al., 2014). Furthermore, bio-based SA is also currently manufactured industrially in companies such as Reverdia, Myriant, BioAmber, and BASF (Cok et al., 2014) from starch-based sugars and glycerol utilizing host organisms such as recombinant *Escherichia coli*, native SA-producers, and several engineered yeast, but SA production from lignocellulosic hydrolysate has yet to be realized at commercial scale.

Of the natural SA producing bacteria, *B. succiniciproducens* CCUG 57335, is of particular interest. This microbe was isolated in 2008 from bovine rumen (Scholten & Dägele, 2008). *B. succiniciproducens* is a member of the *Pasteurellaceae* family and is characterized as non-pathogenic, gram-negative, facultative anaerobic, and capnophilic (Kuhnert et al., 2010). *B. succiniciproducens* produces SA natively via both the reductive

and oxidative TCA cycle branches (Fig. 1a) (Becker et al., 2013) similarly to another native SA producer, *Mannheimia succiniciproducens* (Beauprez et al., 2010), but differently to the well characterized microbe *Actinobacillus succinogenes*, which only produces SA via the reductive branch of the TCA cycle (McKinlay et al., 2010). Likely due to the relatively recent isolation of *B. succiniciproducens*, literature on this microbe is limited. The first publication characterizing this species appeared in 2010 (Kuhnert et al., 2010), although a prior study in 2008 described the performance of a SA-producing bacteria, dubbed DD1, which was later classified as *B. succiniciproducens* (Scholten & Dägele, 2008). The bacterium generates SA, lactic acid (LA), formic acid (FA), and acetic acid (AA) from various carbon sources such as glycerol, sucrose, glucose, fructose, xylose, arabinose, galactose, and mannose (Scholten & Dägele, 2008). Two subsequent publications regarding this organism demonstrated improved SA production via fermentation (Scholten et al., 2009) and metabolic engineering (Becker et al., 2013). In the former, *B. succiniciproducens* was cultivated in continuous fermentation using glycerol as a substrate, but SA productivities and titers were low for commercial purposes (Scholten et al., 2009). In the metabolic engineering study, metabolic fluxes of native and engineered strains were carefully detailed (Becker et al., 2013). Therein, the authors demonstrated SA titers of 20 g/L (from glucose) in the wild-type strain and improved SA yields from 0.48 to 0.71 g/g in the genetically modified strain (pyruvate formate lyase (*Pfl*D) and lactate dehydrogenase (*Ldh*A) knockouts).

Despite the promising features of *B. succiniciproducens* and the genetic tools developed to enhance SA production, to our knowledge, no work to date has reported its fermentation performance in biomass hydrolysates. In fact, several recent reviews regarding

microbial SA production do not even discuss this bacterium (Akhtar et al., 2014; Cheng et al., 2012; Tan et al., 2014; Yi et al., 2013). Several native and engineered bacteria have been already tested in biomass hydrolysates such as *A. succinogenes* (Akhtar et al., 2014; Bradfield et al., 2015; Salvachúa et al., 2015), *Anaerobiospirillum succiniciproducens* (Lee et al., 2003), *M. succiniciproducens* (Kim et al., 2004), *E. coli* (Tan et al., 2014; Wang et al., 2011), and *Corynebacterium glutamicum* (Xu et al., 2015a; Xu et al., 2015b). The selection of both an appropriate substrate and host to produce bio-based SA is critical to industrial manufacturing. For instance, *E. coli* is very efficient for producing SA from pure sugars; however, this model microbe can typically be quite sensitive to inhibitors present in hydrolysates such as acetate, furans, or phenolic compounds (Franden et al., 2013). Similar to engineered *E. coli*, recombinant *C. glutamicum* can produce very high titers of SA from glucose (Okino et al., 2008), but similar titers have not yet been reported from biomass hydrolysate. Conversely, *A. succiniciproducens* is an opportunist pathogen and strictly anaerobic (Beauprez et al., 2010), rendering it inappropriate for industrial purposes. Similarly, *M. succiniciproducens,* apart from not having a clear classification in terms of pathogenicity, exhibits a high number of auxotrophies (Song et al., 2008b). *A. succinogenes*, one of the most efficient native species producing SA, has not been yet successfully genetically modified to improve SA yields, although current efforts are being conducted to that end (McKinlay et al., 2010; Zheng et al., 2013).

In view of these considerations, *B. succiniciproducens* is an attractive candidate microbe to produce SA industrially from diverse mixtures of sugars; however, it is essential to first evaluate this bacterium in biomass hydrolysates. To that end, the aim of the current work was to perform a comprehensive analysis of *B. succiniciproducens* growing in diverse

carbon sources such as pure sugars (in the presence and the absence of potential inhibitors that can be found in realistic hydrolysates) and a high xylose-content hydrolysate from corn stover, at different carbon concentrations and in batch fermentation, to compare the bacterial performance producing SA. In addition, some insights of the metabolism of this bacterium during fermentation are also presented.

2. Materials and methods

2.1. Hydrolysate preparation

The biomass-derived substrate used in the current study is a deacetylated, dilute-acid pretreated hydrolysate (DDAPH) from a pilot-scale pretreatment process with corn stover, using an identical setup to our previous efforts with *A. succinogenes* (Bradfield et al., 2015; Salvachúa et al., 2015). Fig. 1b shows the process flow for obtaining DDAPH. In detail, corn stover was provided by Idaho National Laboratory (INL Lot #5), and was knife-milled and sieved through a ¾" screen. Deacetylation was performed at 8% (w/w) total solids (TS) concentration with 1,500 kg total mass at 80°C for 2 h at a 0.4% NaOH (w/w) loading in a Dynamic Impregnator vessel with 15 rpm mixing. After deacetylation, the spent caustic liquor was drained from the vessel, leaving the remaining solids at 12% TS. The remaining solids were rinsed with 950 kg of water, which was drained from the vessel and discarded. The solids were then subjected to dilute acid pretreatment with H_2SO_4 at a concentration of 8 g H_2SO_4/kg of biomass) at 160°C for 10 min with the residence time based on the assumption of plug flow in a 1 ton/d continuous horizontal reactor. Pretreated, de-acetylated solids were pressed to obtain the hydrolysate. The hydrolysate pH was around 1.9 after dilute acid pretreatment and was neutralized by NaOH (10 N) as needed for the

fermentations. The hydrolysate was filter-sterilized and stored at 4°C prior to further processing.

2.2. Microorganism

Native *B. succiniciproducens* CCUG 57335 (also categorized as DSM 22022 and JF4016) was purchased from the Culture Collection of the University of Goteborg (CCUG), Sweden. The bacterium was revived in 50 mL of autoclaved Brain Heart Infusion (BHI) (Sigma-Aldrich) at a concentration of 37 g/L plus 1% glucose in 150 mL serum bottles. Cells were incubated at 37°C and 180 rpm in the presence of CO_2 (60 mL) overnight. Bacterial broths were mixed with glycerol (20% final concentration) in cryovials, stored at -80°C, and defrosted prior to revival in the same media.

2.3. Seed culture preparation and fermentation conditions

Revived bacteria in BHI were inoculated at an initial optical density (OD) of 0.1 at 600 nm in 0.5 L BioStat-Q Plus fermenters (Sartorius) with 300 mL of tryptic soy broth (TSB) (Fisher Scientific) media plus 2.5% glucose in order to produce large inoculum volumes for further fermentations. The pH was maintained at 6.8 via supplementation of 4 N NaOH. The temperature was controlled at 37°C, and the agitation was fixed at 300 rpm. CO_2 was sparged at 0.1 vvm to maintain anaerobic conditions. Seed fermentations were run overnight (~17 h).

2.4. Experimental design for batch fermentation assays

The base media selected to examine bacterial performance on different sugars and/or hydrolysate consisted of 3 g/L K_2HPO_4, 1 g/L NaCl, 0.2 g/L $MgCl_2$ x $6H_2O$, 0.2 g/L $CaCl_2$ x $2H_2O$, 6 g/L yeast extract, 1 g/L $(NH_4)_2SO_4$ and 10 g/L corn steep liquor (CSL) (Sigma-Aldrich). CSL was prepared at a concentration of 200 g/L (20X) and then boiled at 105°C for 15 minutes (Bradfield & Nicol, 2014). After cooling, solids were separated and the supernatant was autoclaved at 121°C for 30 min and used as a nutrient source. The carbon source was examined as a primary variable of interest. First, glucose was supplemented at concentrations of 60, 80, and 100 g/L to evaluate inhibition due to initial substrate concentration. Then, "mock sugars" and a "mock DDAPH" substrate were utilized at an initial 60 and 80 g/L of sugars. Glucose, xylose, arabinose, and galactose were added to both media to mimic the sugar concentrations in DDAPH (Table 1). In addition, some potential inhibitors (AA, furfural (≥99% purity, Sigma Aldrich, St. Louis, MO), and 5-hydroxymethylfurfural (HMF) (≥98% purity, Santa Cruz Biotechnology, Dallas, TX) were also added in "mock DDAPH". The concentration of sugars and inhibitors in both media are detailed in Table 1. Lastly, DDAPH was evaluated with two controls in parallel, "mock DDAPH" and pure xylose, all at an initial concentration of 60 g/L. Because the lag phase for bacterial growth and acid production was considerably longer in DDAPH, another experiment was run at an initial sugar concentration of 40 g/L in the hydrolysate. All these fermentations were carried out in bioreactors in anaerobic conditions, as detailed in the previous section, and started at an initial OD_{600} of 0.1 from the seed fermenters. Fermentations were run in duplicate excluding the fermentation with "mock sugars" at 80 g/L. Samples (~2 mL) from the fermentations were taken in aseptic conditions at various

time points to follow bacterial growth, sugar consumption, acid production (e.g. SA, FA, AA, and LA), ethanol production, pyruvate and inhibitors concentrations (furfural, HMF).

2.5. Analytical methods

Bacterial growth was tracked with optical density at 600 nm (OD_{600}) in a spectrophotometer, as a measurement of cells in suspension in the fermentation broth. To measure OD_{600} from hydrolysates, 1 mL of fermentation broth was centrifuged (12,000 rpm, 3 min) and then the bacterial pellet resuspended in 1 mL of water to eliminate color interferences. Samples were then filtered through a 0.2 µm syringe filter before placing them in high pressure liquid chromatography (HPLC) vials to analyze carbohydrates (glucose, xylose, arabinose, and galactose), furfural, HMF, organic acids (SA, FA, AA, LA), ethanol, and pyruvate. Carbohydrate HPLC analysis was performed by injecting 6.0 µL of 0.2 µm filtered culture supernatant onto an Agilent 1100 series system equipped with a Phenomenex® Shodex SUGAR 7u SP0810 20A Column 300mm x 8mm plus an anion guard column and cation guard column (Bio-Rad Laboratories) at 85°C run using a mobile phase of NanopureTM water at a flow rate of 0.6 mL/min and a refractive index detector for detection. HPLC analyses for ethanol and all organic acids, except for pyruvic acid, were performed by injecting 6.0 µL of 0.2 µm filtered culture supernatant onto an Agilent 1100 series system equipped with a Bio-Rad HPLC Organic Acid Analysis Column, Aminex® HPX-87H Ion Exclusion Column 300mm x 7.8mm and a cation H+ guard column (Bio-Rad Laboratories) at 55°C using a mobile phase of 0.01 N sulfuric acid at a flow rate of 0.6 mL/min and a refractive index detector for detection. Pyruvic acid HPLC analysis was performed by injecting 6.0 µL of 0.2 µm filtered culture supernatant onto an

Agilent 1100 series system equipped with a Phenomenex® Rezex™ RFQ-Fast Acid H+ (8%) Column 100mm x 7.8mm and a cation guard cartridge (Bio-Rad Laboratories) at 85°C using a mobile phase of 0.01 N sulfuric acid at a flow rate of 1.0 mL/min and a diode array detector at 315 nm for detection. Analytes were identified by comparing retention times and spectral profiles with pure standards.

Furfuryl alcohol, 5-(hydroxymethyl)furfuryl alcohol (HMF alcohol) - but also furfural and HMF- were analyzed by HPLC Diode Array Detector and Electrospray Ionization-Tandem Mass Spectrometry to study the conversion of the aldehydes to their corresponding alcohols. Individual chemical standards of furfural, 5-(hydroxymethyl)furfural (≥99% purity, Sigma Aldrich, St. Louis, MO), furfuryl alcohol and 5-HMF (≥98% purity, Santa Cruz Biotechnology, Dallas, TX) were used to fully characterize bioconversions over fermentations time. Analysis of samples was performed on an Agilent 1100 LC system equipped with a G1315B diode array detector (DAD) and an Ion Trap SL (Agilent Technologies, Palo Alto, CA) mass spectrometer (MS) with in-line electrospray ionization (ESI). Each sample was injected diluted 10-fold at a volume of 25 µL into the LC/MS system. Primary degradation compounds were separated using reverse-phase chromatography on a Develosil C30 RPaqueous, 5µm, 4.6 x 250 mm column (Phenomenex, Torrance, CA) at an oven temperature of 30°C. The HPLC method consisted of a solvent regime using eluents of A) water modified with 0.03% formic acid, and eluent B) 9:1 acetonitrile and water also modified with 0.03% formic acid. At a flow rate of 0.7 mL min-1, the gradient chromatography was as follows: starting at 0% B to 7% B in 13 min; 18 min, 7% B; 21 min, 8.5% B; 31 min, 10% B; for a total run time of 35 min including equilibrium. Flow from the HPLC-DAD was directly routed to the ESI-MS ion

trap. The DAD was used to monitor chromatography at 210 nm for a direct comparison to MS data. MS and MS/MS tuned parameters are as follows: smart parameter setting with target mass set to 165 Da, compound stability 70%, trap drive 50%, capillary at 3500 V, fragmentation amplitude of 0.75 V with a 30 to 200 % ramped voltage implemented for 50 msec, and an isolation width of 2 m/z (He collision gas). The ESI nebulizer gas was set to 60 psi, with dry gas flow of 11 L min-1 held at 350°C. MS scans and precursor isolation-fragmentation scans were performed across the range of 40-350 Da in positive-ion mode.

2.6. Calculation of SA yields and productivity

Reported metabolic yields were calculated as the SA production (g) divided by sugar consumption (g) at the end of the fermentation. The dilution from base addition was considered to correct final SA and sugar concentrations. Instantaneous productivity (g/L/h) was calculated as SA production (g/L) between the time (h) of fermentation at each sample point.

2.7. Calculation of lumped reactions and net NADH generation

The stoichiometric model of *B. succiniciproducens* from Becker et al. (Becker et al., 2013) was extended to include xylose uptake by including ATP-dependent xylose uptake (EC 3.6.3.17), xylose isomerase (EC 5.3.1.5), and xylulose isomerase (EC 2.7.1.17). An illustration of the altered model is provided in Figure 1A. With this model, elementary flux modes were calculated (Terzer & Stelling, 2008) assuming the free exchange of ATP, ADP, NAD+, and NADH with the environment. Lumped reactions were then found by considering only the boundary species and scaling the elementary modes to 1 mol of

glucose or 1.2 mol of xylose. Expected net NADH generation was calculated by summing the expected NADH production for each fermentation product.

∑3. Results and Discussion

3.1. Evaluation of B. succiniciproducens *performance at different initial glucose concentrations*

Biomass hydrolysates from biorefinery streams consist typically of high glucose or high xylose concentrations, depending on the fractionation strategy employed. *B. succiniciproducens* has been reported to naturally produce SA from different carbon sources (Kuhnert et al., 2010); however, due to the scarce information about this organism, a systematic study using different carbon sources and in batch fermentation conditions was first conducted. In this section, different initial glucose concentrations were utilized to test the bacterial performance producing SA and to identify potential substrate inhibition.

The first parameter evaluated was bacterial growth. *B. succiniciproducens* formed aggregates and subsequently a biofilm during the fermentation. This effect is clearly observed as a decrease of the cells in suspension in the fermentation broth (Fig. 2a). Bacterial aggregation has been already reported for this microbe in continuous fermentation but at significantly longer times (18 days) (Scholten et al., 2009) than the detected in the current study (24 h). Biofilm formation has been already described in other SA-producing organisms from the same family (Pasteurellaceae), such as *A. succinogenes*, as result of a possible stress response to high acid concentration (Corona-González et al., 2008; Corona-Gonzalez et al., 2010; Salvachúa et al., 2015). Despite the formation of bacterial aggregates (after ~24 h), glucose consumption (Fig. 2b) and SA production (Fig. 2c) did not cease,

highlighting the fact that cells are still metabolically active as previously reported for *A. succinogenes* (van Heerden & Nicol, 2013). In terms of glucose utilization, calculated rates at 24 h were similar at the three initial sugar concentrations (~2.4 g/L/h) (Fig. 2b), but utilization was incomplete at the initial 100 g/L of glucose (after 5 days of incubation), which also slowed considerably after 48 h. SA titers and production rates were also slightly lower with an initial 100 g/L of glucose (Fig. 2c). Consequently, although sugar inhibition was not very significant at these glucose levels, to reach complete utilization of sugars and higher SA titers, we selected 60 and 80 g/L as the initial sugar content to prepare mock media in the subsequent fermentations.

B. succiniciproducens also produces a variety of acids besides SA, namely LA, FA, and AA (Table 2). LA was the second most abundant acid produced, followed by AA and FA. Total acids production (considering SA, LA, FA, and AA) was slightly higher in fermentations at initial 80 g/L (51.4 ± 1.0 g/L) than at 60 (47.9 g/L ± 1.87 g/L) and 100 g/L (44.3 ± 0.94 g/L). Considering that glucose utilization was complete at 80 g/L (Fig. 2b), it is likely that more carbon is directed to the production of biomass and/or other byproducts at this concentration than at an initial 60 g/L of sugars. Ethanol and pyruvate have also been described as possible byproducts in native and recombinant *B. succiniciproducens* (Becker et al., 2013). However, the concentrations of these two metabolites did not exceed 1 g/L in any case (data not shown). It is also noteworthy that CO_2 was sparged during the cultivation, as CO_2 can be fixed by *B. succiniciproducens*. Consequently, this is another factor that may affect the carbon balance besides acids and biomass production. Deeper

analysis will be required to close mass balances; however, a complete metabolic profiling is not in the scope of the current study and will be pursued in a future study.

3.2. Evaluation of B. succiniciproducens *performance in "mock sugars" and "mock DDAPH"*

The corn stover pretreatment process used in the current study utilizes high temperatures in the presence of H_2SO_4. These conditions often result in the formation of inhibitors such as acetates, furfural, and HMF that can negatively affect microbial growth (Franden et al., 2013). Thus, two different substrates, "mock sugars" (containing mixed sugars) and "mock DDAPH" (containing mixed sugars plus potential inhibitors) were evaluated at two different initial sugar concentrations (60 and 80 g/L). The amount of each substrate component is detailed in Table 1. Bacterial growth at the beginning of the fermentation (7 h), dubbed "cells in suspension", was first analyzed (Fig. 3a). The initial sugar concentration seemed to not influence initial growth when using "mock sugars", yet more dramatic differences were found when using "mock DDAPH", which exhibited 4.5-fold higher ODs at 60 g/L compared to 80 g/L after 7 h of incubation. In addition, bacterial behavior (in terms of SA production (Fig. 3b) and sugars utilization (Fig. 3c-f)) at an initial sugar concentration of 60 g/L in the presence and the absence of inhibitors was also very similar (Fig. 3a). This result suggests that the inhibitor concentration at that dilution (43% DDAPH, Table 1) does not adversely affect bacterial performance. However, a considerable initial lag and lower SA titers were observed at an initial sugar concentration of 80 g/L in "mock DDAPH" compared to the other treatments. These results could be due

to the increase of inhibitor concentration in the media but also from the higher initial sugar concentrations.

Furfuryl alcohol and HMF alcohol were also tracked by LC-MS/MS in this experiment. It was observed that both furfural and HMF were completely reduced to their corresponding alcohols between 24 and 29 hours in "mock DDAPH" at initial sugar concentration of 80 g/L. This result demonstrates that this organism can detoxify these aldehydes from the broth, similarly to *A. succinogenes* (Salvachúa et al., 2015). Based on these results, 60 g/L was the sugar concentration selected for fermentations in DDAPH hydrolysate to avoid an initial lag and thus increase the productivity in batch fermentations.

SA titers obtained in pure glucose (Fig. 2c) and mock sugar fermentations were also compared. SA production from mock sugars was ~5 g/L lower than from glucose at 72 h of fermentation (around 25 g/L instead of 30 g/L). One of the main reasons is that sugars - in particular xylose, arabinose, and galactose - were not completely consumed at that time (Fig. 3c,d,f), which differs from the behavior observed in pure glucose utilizing same initial sugar concentrations (Fig. 2b). This result unsurprisingly indicates that *B. succiniciproducens* varies in its efficiency utilizing different sugars. Xylose, which is the main sugar in the mock media, and glucose exhibited the highest utilization rates around 0.8-0.9 g/L/h during the first 7 h of fermentation, followed by arabinose (0.1 g/L/h) and galactose (0.06 g/L/h). This order of sugar preference, at the sugar concentrations utilized, is also similar to the one reported for *A. succinogenes* (Salvachúa et al., 2015).

3.3. Evaluation of B. succiniciproducens *performance in lignocellulosic hydrolysate, DDAPH*

Fermentation performance on DDAPH, "mock DDAPH", and pure xylose, all at an initial sugar concentration of 60 g/L, was compared. In addition, DDAPH was also evaluated at an initial sugar concentration of 40 g/L after observing a considerable initial lag (~24 h) for growth (Fig. 4a), SA production (Fig. 4b), and sugar utilization (Fig. 4c) in DDAPH at 60 g/L. Hydrolysates can contain inhibitors apart from the three previously mentioned (acetate, furfural, HMF) such as phenolic compounds derived from lignin (Palmqvist & Hahn-Hägerdal, 2000) or xylo-oligosaccharides (Franden et al., 2013), both of which can increase lag. As a result of the decrease in DDAPH concentration, the lag for SA production was substantially reduced from 24 h to ~8 h. Previous studies have demonstrated that the lag phase is also correlated with the reduction of furfural to furfuryl alcohol and sugar utilization begins when that conversion is nearly complete (Salvachúa et al., 2015); similar results are found in the current study. Furfural in DDAPH (60 g/L) is metabolized at approximately 24 h (Fig. 5a), corresponding to the initial lag phase time (Fig. 4a-f). These events seem, as described above, to form a primary detoxification step by the organism.

Despite the lag phase, the SA titer in DDAPH (60 g/L) at 72 h of incubation was up to 30 g/L, similar to that obtained from pure glucose fermentations, indicating that the performance of the bacterium was not hindered in the hydrolysate once detoxified. Moreover, xylose, glucose, and arabinose were almost totally consumed and only 1 g/L of galactose remained. *A. succinogenes* was also tested in similar hydrolysates at slightly higher initial sugar concentrations (Salvachúa et al., 2015). Its lag phase for xylose

utilization was longer than observed for *B. succiniciproducens*, and the utilization of xylose was completed after 5 days of incubation instead of 3 days (Fig. 4c). These results suggest that *B. succiniciproducens* seems to detoxify the hydrolysate more rapidly and perform better in terms of sugar utilization in DDAPH than *A. succinogenes.* However, SA titers were higher in the latter (42 g/L), due to lower yields of non-target acid byproducts (Salvachúa et al., 2015).

3.4. Analysis of fermentation product distributions

While the molar ratios in products were largely similar between each of the fermentation conditions tested (Table 2), some differences in acid production were observed. In order to understand the potential causes and consequences of these differences in product formation, we adapted a stoichiometric model of *B. succiniciproducens* from Becker et al. (Becker et al., 2013). Using this model, we calculated lumped reactions for the formation of each major fermentation product (Table 3). These reactions reveal that maximum ATP production is achieved by coupling the reductive succinate fermentation pathway with the oxidative acetate/formate fermentation pathway. Additionally, since xylose requires more ATP to import on a per-carbon basis, ATP yields on xylose are lower than that of glucose.

Glucose media resulted in nearly equimolar production of formate and acetate, while fermentation on xylose-containing media produced acetate to formate in about a 2:1 ratio. As the ratio of formate to acetate affects the redox state of the cell, we calculated the expected net NAD+ generation for each fermentation run using the reactions identified in Table 3. These results indicate that while each condition generates a net positive amount of NAD+, fermentations on glucose alone resulted in much higher levels of unexplained

reduction (Fig. 6a). This could be due to an active oxidative pentose phosphate flux, which might be higher in glucose-only growth due to the high flux through G6P. Interestingly, FA production in DDAPH (Fig. 5c, d) is also different to that reported for other native SA-producer organisms. For instance, *A. succinogenes* is able to fully convert FA to CO_2 and H_2O when biofilm formation begins (Salvachúa et al., 2015). However, this is not the case of *B. succiniciproducens*, where FA (Fig. 5c) increases until it reaches a plateau similar to the other acids.

Another difference in the fermentation products between media is the usage of the redox-neutral lactic acid production pathway. In the current study, lactic acid is the main byproduct in most of the fermentations, which differs from the data reported by Becker *et al.* (Becker et al., 2013), where the production of LA is significantly lower than AA and FA. As this pathway is not as ATP-efficient as the equimolar production of succinate, formate, and acetate, high fluxes to lactate may indicate the cell is not starved for energy. This hypothesis seems consistent with experimental observations in the current work, since lactic acid production is highest in glucose-only media and lowest in hydrolysate. These results also highlight the inherent differences between mock and real hydrolysates. Although both media consist of the same sugars and main inhibitors, DDAPH likely includes other minor compounds that affect the metabolism of the bacterium.

3.5. SA yields and productivity comparisons

It is well known that yields, productivity, and titers are often the main drivers in fermentation for cost effective production of a target chemical. Titers have already been presented for the current work. As a comparison, the highest SA titer reported to date to our

knowledge from biomass is included in a patent which claims values up to 70.6 g/L (Guettler et al., 1996) on corn fiber hydrolysates with *A. succinogenes*. Apart from these data, titers are typically around 50 g/L or below when using lignocellulosic substrates (Akhtar et al., 2014). In the current study, we have obtained 30 g/L (without accounting for bioreactor dilution due to base addition) which could likely be enhanced by utilizing engineered strains to reduce byproduct formation and increase SA yields (Becker et al., 2013). Concerning SA metabolic yields, the highest values reported in the current study were found in both hydrolysates (at initial 40 and 60 g/L of sugars), reaching values up to 0.69 g SA/g sugars (Fig. 6b). As detailed above, this is mainly due to a decrease of LA production in the biomass hydrolysates. As the maximum theoretical yield is 1.71 mol/mol (1.12 g/g, Table 3), 0.69 g/g is 62% of theoretical yield. Equal yields were reported when using a double knockout (*ldh*A and *pfl*D) of *B. succiniciproducens* using glucose as carbon source (Becker et al., 2013) and a engineered strain of *C. glutamicum* from xylose-enriched corn cob hydrolysates (Wang et al., 2014). These data highlight the importance of substrate and media composition to alter byproduct generation with native species. Compared to other native organisms in similar hydrolysates and batch fermentation, *B. succiniciproducens* exhibits slightly lower yields than *A. succinogenes* (66% in DDAPH (Salvachúa et al., 2015)) but higher than *M. succiniciproducens* (56%) from wood hydrolysates (Kim et al., 2004). The total acids yield (SA plus AA, LA, and FA) are also reported in Fig. 6b. The maximum acid yields are again observed in DDAPH, up to 0.99 g acids/g sugars and the lowest in the non-optimized media at initial 100 g/L of glucose (0.84 g acids/g sugars).

Regarding SA productivity, the results obtained in mock media and DDAPH are presented in Fig. 6c. Similar overall productivities were found in the presented cases, up to 0.425 g/L/h at the end of the fermentation. However, there is a notable decrease and a delay on the "maximum instantaneous productivity" when using DDAPH (approximately 0.5 g/L/h) instead of "mock DDAPH" (1.30 g/L/h) (Fig. 6c), mainly due to the initial lag phase for SA production in the former. Similar differences have been reported when growing *A. succinogenes* in mock media and biomass hydrolysates (Salvachúa et al., 2015). In parallel, to compare the efficiency of the organism producing SA in hydrolysate and "mock DDAPH", the 24 h lag found in DDAPH was omitted and the productivity was also calculated from that point. In this case, both productivity profiles were similar (Fig. 6c) indicating that the capacity of the bacterium producing SA is not reduced in hydrolysate once the hydrolysate is biologically detoxified. It is also evident that productivity decays dramatically after reaching a maximum production rate, likely due to product inhibition. Inhibition due to high acid concentration has been previously reported among SA-producing organisms. For instance, *M. succiniciproducens* ceased the growth when total acids exceeded 17.2 g/L (Song et al., 2008a), and *A. succinogenes* at 25–30 g/L, apart from forming biofilm around these concentration values, also exhibits a significant decrease in productivity (Salvachúa et al., 2015). In the current study, *B. succiniciproducens,* seemed to present a similar trend. For instance, productivity dramatically dropped in "mock DDAPH" after 12 h (Fig. 6b), in parallel to the formation of bacterial aggregates/biofilm (Fig. 4a). At that point, the acids concentration (SA, LA, AA, and FA) was approximately 26 g/L (Fig. 4b, Fig. 5b,c,d). Taken together, although carbon sources and fermentation modes were different, SA productivities obtained in the current study are significantly higher than that

reported for the same bacterial species in continuous fermentation using glycerol as carbon source (0.094 g/L/h) (Scholten et al., 2009), but lower than those reached in serum bottles using glycerol (1.5 g/L/h) (Scholten & Dägele, 2008). Compared to other organisms, the overall productivity in *B. succiniciproducens* was higher than in *A. succinogenes* when using the same hydrolysate in batch fermentation (0.32 g/L/h for the latter) (Salvachúa et al., 2015) but lower than obtained in *M. succiniciproducens* (1.17 g/L/h) (Kim et al., 2004).

Conclusions

The current work has demonstrated for the first time that the native bacterium *B. succiniciproducens* is able to grow and naturally produce high titers of SA from a lignocellulosic xylose-rich hydrolysate. In fact, reported SA yields are also slightly higher in biomass hydrolysate than pure sugar cultivations. Despite promising results, the three primary fermentation metrics, titer, yield, and productivity, could be improved for further industrial viability. To enhance SA titers and yields, engineered *B. succiniciproducens* strains such as the *Pfl* or *Ldh*A knockouts (Becker et al., 2013) (to decrease and/or avoid the formation of the byproducts formic and lactic acid, respectively) could be evaluated in biomass hydrolysates. Additionally, to improve productivity, continuous fermentation is suggested since the microbe would not suffer from product inhibition (as occurs in batch or fed-batch fermentation strategies). Indeed, continuous fermentation has been already demonstrated to be quite effective in other biofilm-forming SA-producing organisms from biomass hydrolysates (Bradfield et al., 2015).

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Figure captions

Fig. 1. (A) Metabolic network of native *B. succiniciproducens* adapted from the stoichiometric model in Becker et al. (Becker et al. 2013). Xylose utilization reactions (green) were added. Arrow thicknesses correspond to the predicted reaction fluxes for optimal growth on glucose: thicker arrows indicate higher flux, while dashed arrows indicate zero flux. In anaerobic conditions, the reductive branch of the TCA is the principal route that leads to SA production. (B) Diagram of the process configuration to produce SA by *B. succiniciproducens* in C5-enriched liquor from corn stover in the current study. High xylose-content hydrolysate from corn stover (DDAPH) was produced through a deacetylation step followed by dilute-acid pretreatment (DDAP). We note that enzymatic hydrolysis, biological upgrading of the C6-liquor, separations, and catalytic upgrading were not performed in the current work, but rather are shown to demonstrate the potential for an integrated process.

13DPG=3-Phospho-D-glyceroyl phosphate; 2PG=D-Glycerate 2-phosphate; 3PG=3-Phospho-D-glycerate; 6PGC=6-Phospho-D-gluconate; AC=Acetate; ACALD=Acetaldehyde; ACCOA=Acetyl-CoAACTP=Acetyl phosphate; ADP=Adenosine diphosphate; AKG=2-Oxoglutarate; ATP=Adenosine triphosphate; CIT=Citrate; CO2=Carbon dioxide; COA=Coenzyme; ADHAP=Dihydroxyacetone phosphate; E4P=D-Erythrose 4-phosphate; ETOH=Ethanol; F6P=D-Fructose 6-phosphate; FDP=D-Fructose 1,6-bisphosphate; FOR=Formate; FUM=Fumarate; G3P=Glyceraldehyde 3-phosphate; G6P=D-Glucose 6-phosphate; GLC=D-Glucose; ICIT=Isocitrate; LAC=D-Lactate; MAL=D-Malate; MK=Menaquinone; MKH2=Menaquinol (reduced Menaquinone);

NAD=Nicotinamide adenine dinucleotide; NADH=Nicotinamide adenine dinucleotide – reduced; NADP=Nicotinamide adenine dinucleotide phosphate; NADPH=Nicotinamide adenine dinucleotide phosphate – reduced; OAA=Oxaloacetate; PEP=Phosphoenolpyruvate; PYR=Pyruvate; R5P=D-Ribose; RU5P=Ribulose 5-phosphate; S7P=Sedoheptulose 7-phosphate; SUCC=Succinate; SUCC-COA=Succinyl-CoA; XU=D-Xylulose; XU5P=D-Xylulose 5-phosphate; XYL=D-Xylose.

Fig. 2. *B. succiniciproducens* performance in pure glucose fermentations at varying initial concentrations (60, 80, and 100 g/L). (A) Bacterial growth, (B) glucose consumption as a function of time and glucose utilization at the end of the fermentation, and (C) SA production.

Fig. 3. *B. succiniciproducens* performance in mock media fermentations at varying concentrations of sugars. (A) Bacterial growth, (B) SA production, (C,D,E,F) and sugar consumption as a function of time and sugar utilization at the end of the fermentation in the different media. “Mock DDAPH” contains four sugars (xylose, glucose, galactose, and arabinose), acetate, furfural, and HMF, mimicking the concentrations of actual DDAPH at starting sugar content of 60 and 80 g/L of. “Mock sugars” media contains the same four sugars than “mock DDAPH”, at the corresponding concentrations, but without inhibitors.

Fig. 4. *B. succiniciproducens* performance in biomass hydrolysate, DDAPH, at varying initial concentrations of sugars (40 and 60 g/L), in pure xylose (60 g/L), and “mock DDAPH” (60 g/L). (A) Bacterial growth, (B) SA production, and (C,D,E,F) sugar

consumption as a function of time in the different media. “Mock DDAPH” contains four sugars (xylose, glucose, galactose, and arabinose), acetate, furfural, and HMF, mimicking the concentrations of actual DDAPH at starting sugar content of 60 g/L. Concentrations are shown in Table 1.

Fig. 5. Furfural detoxification in different fermentation media and byproducts generated by *B. succiniciproducens* as a function of time. (A) Furfural detoxification by the bacterium after 73 h of fermentation, (B) LA, (C) FA, and (D) AA production.

Fig. 6. Evaluation of metabolic and fermentation parameters in *B. succiniciproducens* fermentations. (A) Analysis of the computed net NAD+ generation in the whole set of fermentations conducted in the current study. (B) SA and acid metabolic yields obtained in the different fermentations performed in the current study from diverse carbon sources; (C) SA productivity in DDAPH at an initial sugar content of 40 and 60 g/L as well as in “mock DDAPH” at an initial sugar content of 60 g/L. The productivity of “Without lag DDAPH-60g/L” is calculated from the fermentations in DDAPH-60 g/L without lag phase (from 24 h onwards).

Tables

Table 1. Composition of DDAPH at the different dilutions utilized in the current study.

Compounds (g/L)	100% DDAPH	58% DDAPH[a]	43% DDAPH[a,b]	29% DDAPH[b]
Total sugars	**138.2**	**~80**	**~60**	**~40**
Glucose	15.0	8.7	6.6	4.4
Xylose	99.0	57.3	43.0	28.7
Galactose	8.0	4.6	3.5	2.3
Arabinose	15.6	9.0	6.8	4.5
Potential inhibitors				
Acetic acid	3.8	2.2	1.6	1.1
Furfural	1.4	0.8	0.6	0.4
HMF	0.3	0.2	0.1	0.1

[a] These dilutions were utilized for "mock sugars" and "mock DDAPH" fermentations.

"Mock sugars" did not contain AA, furfural, and HMF.

[b] These dilutions were utilized for DDAPH fermentations.

Table 2. Byproduct generation by *B. succiniciproducens* in batch fermentations. The values presented correspond to the concentrations obtained at the end of the fermentation. The split ratios of the major fermentation products are also presented (the sum of lactic acid (LA), formic acid (FA), acetic acid (AA), and succinic acid (SA) is defined to be 1).

Media	**Acid**					**Molar**			
	SA (g/L)	**LA (g/L)**	**FA (g/L)**	**AA (g/L)**	**Total* (g/L)**	**LA**	**FA**	**AA**	**SA**
Glucose 60 g/L	29.5 ± 1.1	10.3 ± 1.5	2.9 ± 0.2	5.2 ± 0.0	47.9 ± 1.87	0.22	0.12	0.17	0.49
Glucose 80 g/L	30.9 ± 0.3	13.1 ± 0.9	2.9 ± 0.3	4.5 ± 0.1	51.4 ± 1.0	0.27	0.12	0.14	0.48
Glucose 100 g/L	24.6 ± 0.8	12.7 ± 0.0	3.0 ± 0.0	4.0 ± 0.5	44.3 ± 0.94	0.29	0.14	0.14	0.43
Mock DDAPH 60 g/L	25.9 ± 0.1	7.8 ± 0.2	2.3 ± 0.0	5.3 ± 0.0	41.3 ± 0.22	0.19	0.11	0.20	0.49
Mock DDAPH 80 g/L	22.1 ± 0.45	8.8 ± 0.1	2.7 ± 0.1	5.8 ± 0.1	39.4 ± 0.48	0.22	0.13	0.22	0.43
Mock sugars 60 g/L	26.0	7.4	2.0	5.2	40.6	0.19	0.10	0.20	0.51
Mock sugars 80 g/L	23.2	8.3	2.4	5.4	39.3	0.21	0.12	0.21	0.46
Xylose 60 g/L	28.1 ± 0.5	7.0 ± 0.3	3.3 ± 0.1	6.5 ± 0.2	44.9 ± 0.62	0.16	0.14	0.22	0.48
DDAPH 60 g/L	30.6 ± 0.32	2.6 ± 0.1	3.9 ± 0.1	8.7 ± 0.2	45.8 ± 0.40	0.06	0.16	0.28	0.50
DDAPH 40 g/L	24.1 ± 0.05	3.6 ± 0.5	2.3 ± 0.3	6.1 ± 0.1	36.1 ± 0.59	0.10	0.13	0.26	0.52

[a]Total: includes the sum of the acids SA, LA, FA, and AA.

Table 3. Lumped reactions for the production of acids in *B. succiniciproducens*.

		Glucose
	Succinate	1.0 ADP + 2.0 CO_2 + GLC + 2.0 NADH --> 1.0 ATP + 2.0 NAD + 2.0 SUCC
	Lactate	1.0 ADP + GLC --> 1.0 ATP + 2.0 LAC
	Acetate/Formate	3.0 ADP + GLC + 2.0 NAD --> 2.0 AC + 3.0 ATP + 2.0 FOR + 2.0 NADH
	Acetate/CO_2	3.0 ADP + GLC + 4.0 NAD --> 2.0 AC + 3.0 ATP + 2.0 CO_2 + 4.0 NADH
Redox Balanced*	Maximum ATP	2.0 ADP + GLC --> AC + 2.0 ATP + FOR + SUCC
	Maximum Succinate	1.29 ADP + 0.86 CO_2 + GLC --> 1.29 ATP + 1.71 SUCC
		Xylose
	Succinate	0.8 ADP + 2.0 CO_2 + 2.0 NADH + 1.2 XYL --> 0.8 ATP + 2.0 NAD + 2.0 SUCC
	Lactate	0.8 ADP + 1.2 XYL --> 0.8 ATP + 2.0 LAC
	Acetate/Formate	2.8 ADP + 2.0 NAD + 1.2 XYL --> 2.0 AC + 2.8 ATP + 2.0 FOR + 2.0 NADH
	Acetate/CO_2	2.8 ADP + 4.0 NAD + 1.2 XYL --> 2.0 AC + 2.8 ATP + 2.0 CO_2 + 4.0 NADH
Redox Balanced*	Maximum ATP	1.8 ADP + 1.0 CO_2 + 1.2 XYL --> 1.0 AC + 1.8 ATP + 1.0 FOR + 1.0 SUCC
	Maximum Succinate	1.09 ADP + 0.86 CO_2 + 1.2 XYL --> 1.09 ATP + 1.71 SUCC

*Abbreviations in the table: GLU: glucose; XYL: xylose; SUCC: succinate; FOR: formate; AC: acetate; LAC: lactate.

Figures

Fig. 1

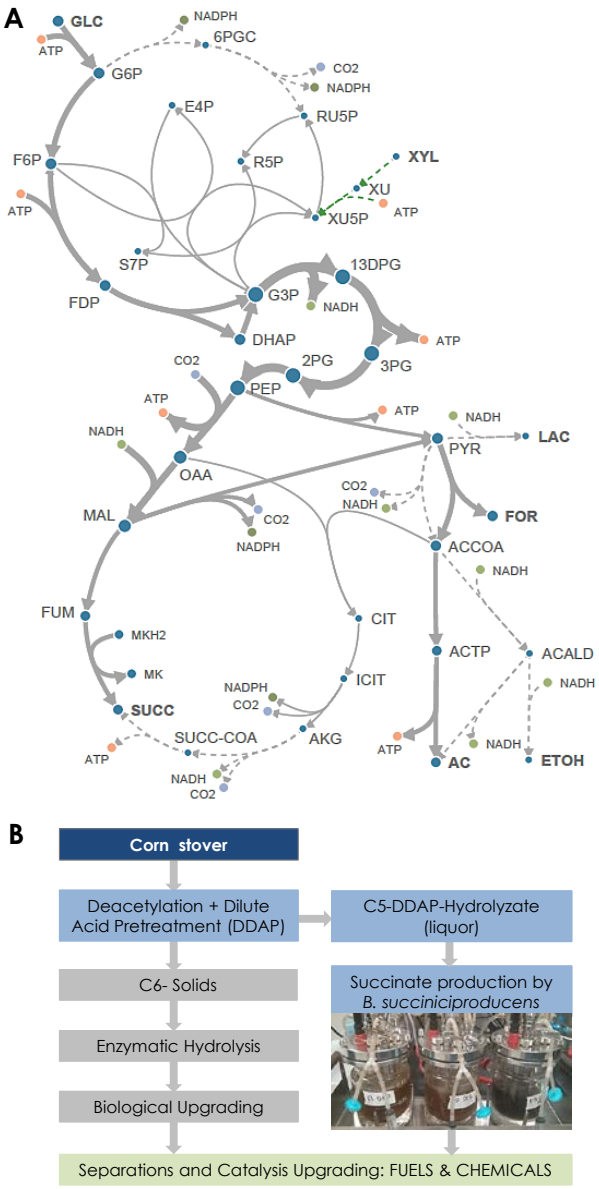

Fig. 2

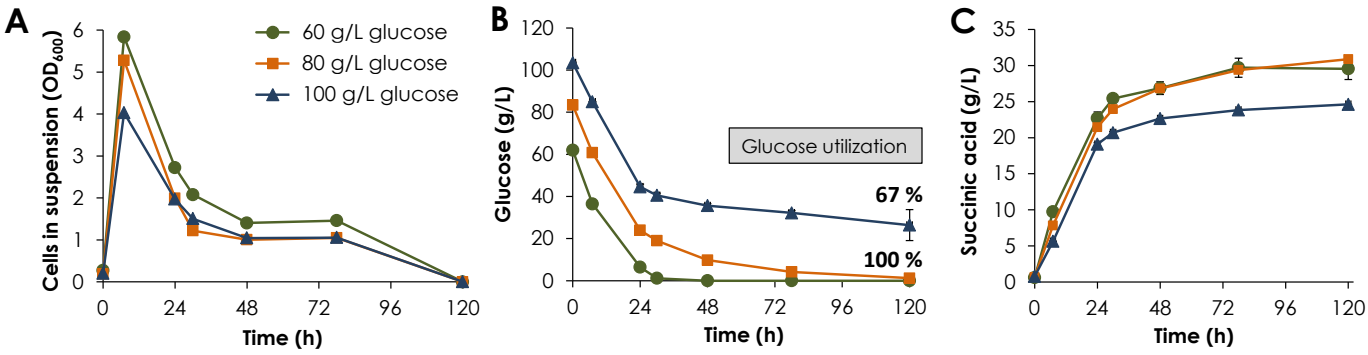

Fig. 3

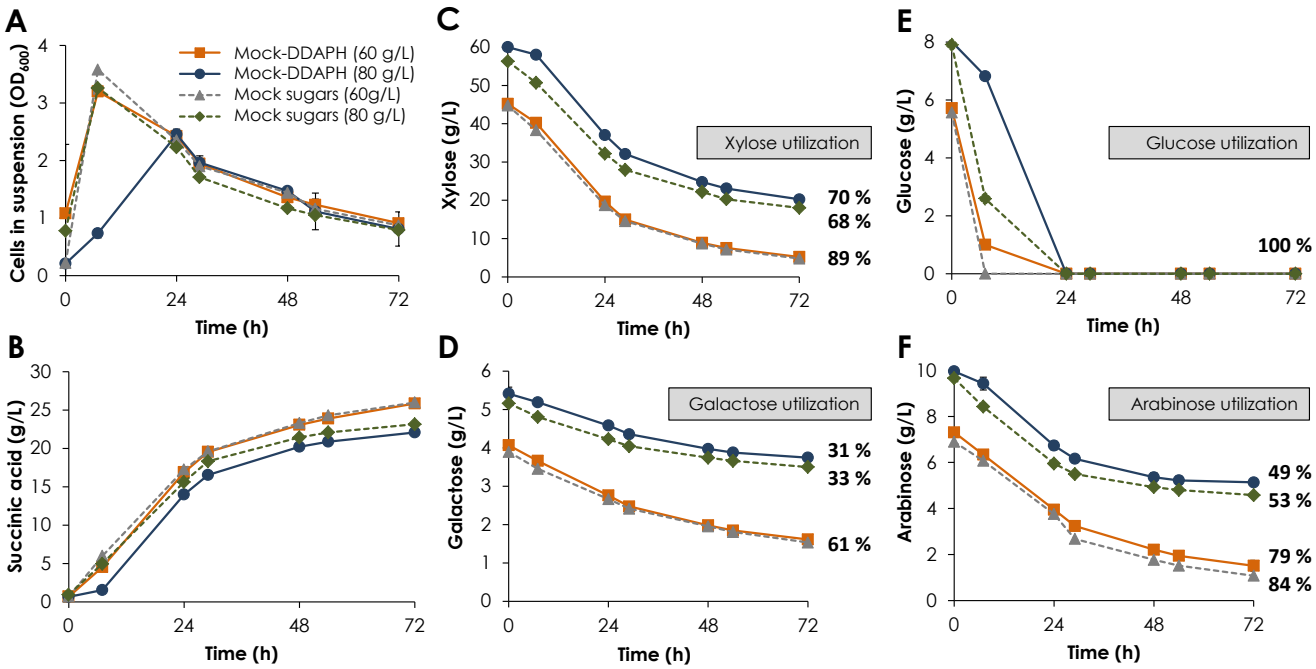

Fig. 4

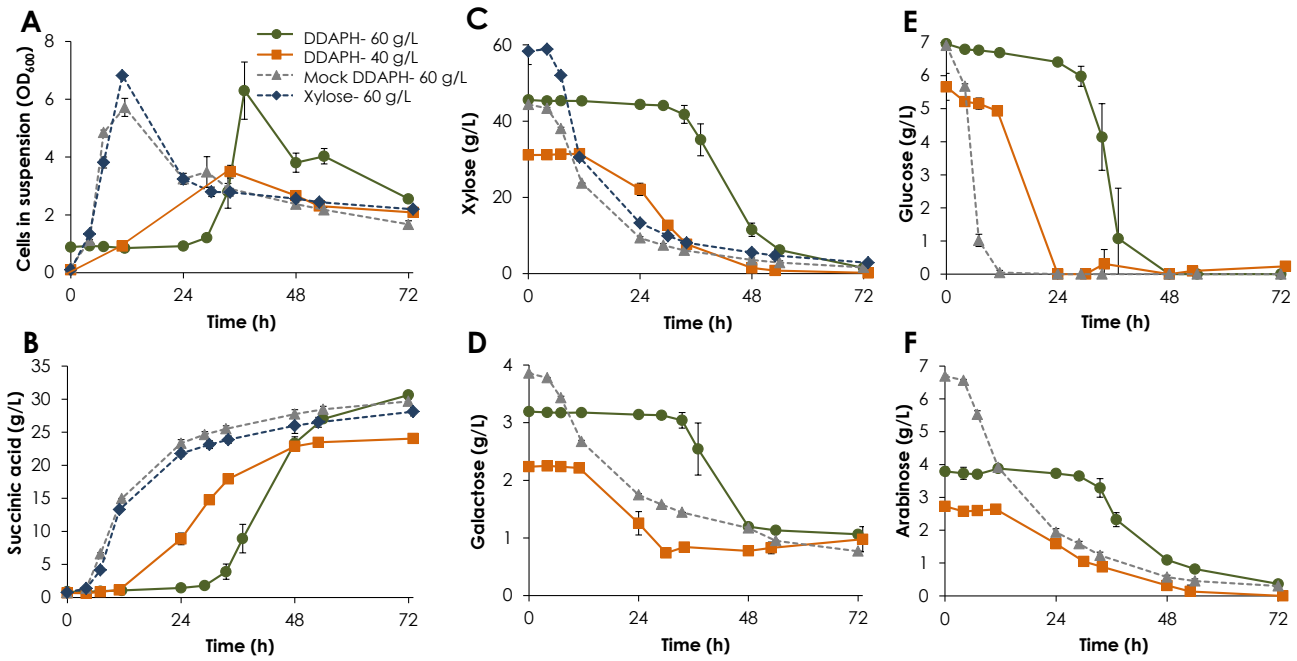

Fig. 5

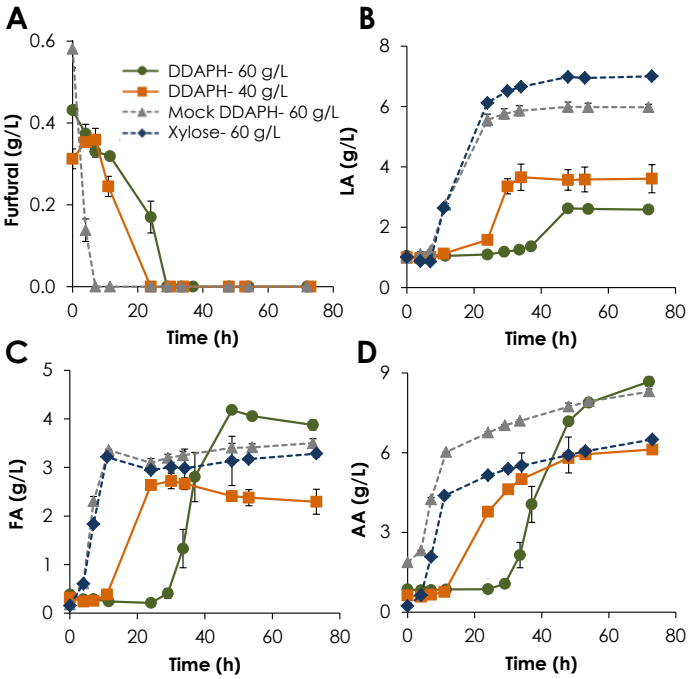

Fig. 6