Effects of *Clostridium beijerinckii* and medium modifications on acetone butanol ethanol (ABE) production from switchgrass

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ABSTRACT

The presence of lignocellulose derived microbial inhibitory compounds (LDMICs) in lignocellulosic biomass (LB) hydrolysates is a barrier to efficient conversion of LB hydrolysates to fuels and chemicals by fermenting microorganisms. Results from this study provide convincing evidence regarding the effectiveness of metabolically engineered *C. beijerinckii* NCIMB 8052 for the fermentation of LB-derived hydrolysates to acetone butanol ethanol (ABE). The engineered microbial strain (*C. beijerinckii*_SDR) was produced by integration of an additional copy of a short-chain dehydrogenase/reductase (SDR) gene (*Cbei*_3904) into the chromosome of *C. beijerinckii* NCIMB 8052 wildtype where there was control by a constitutive *thiolase* promoter. The *C. beijerinckii*_SDR and *C. beijerinckii* NCIMB 8052 wildtype were used for comparative fermentation of non-detoxified and detoxified hydrothermolysis pretreated switchgrass hydrolysates (SH) with and without $(NH_4)_2CO_3$ supplementation. In the absence of $(NH_4)_2CO_3$, fermentation of non-detoxified SH with *C. beijerinckii*_SDR resulted in the production of 3.13- and 2.25-fold greater quantities of butanol (11.21 g/L) and total ABE (20.24 g/L), respectively, than 3.58 g/L butanol and 8.98 g/L ABE produced by *C. beijerinckii*_wildtype. When the medium for fermentation of the non-detoxified SH was supplemented with $(NH_4)_2CO_3$, concentrations were similar for butanol (9.5 compared with 9.2 g/L) and ABE (14.2 compared with 13.5 g/L) produced by *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype, respectively. Furthermore, when *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype were cultured in detoxified SH medium, *C. beijerinckii*_SDR produced 1.11- and 1.18-fold greater quantities of butanol and ABE, respectively, than when there was culturing with *C. beijerinckii*_wildtype. When the combined results of the present study are considered, conclusions are that the microbial strain and medium

modifications of the fermentation milieu resulted in greater production of fuels and chemicals from non-detoxified LB hydrolysates.

Keywords: ABE fermentation; butanol; acetone; ammonium carbonate; switchgrass hydrolysate; LDMIC; short-chain dehydrogenase/reductase; *Clostridium beijerinckii*

Running title: Butanol production from lignocellulosic biomass

CONTRIBUTION TO THE FIELD STATEMENT

The results presented in this manuscript provide convincing evidence regarding the effectiveness of metabolically engineered *C. beijerinckii* NCIMB 8052 for the fermentation of LB-derived hydrolysates, SH, to acetone butanol ethanol (ABE). Given the surging research into next-generation fuels, especially butanol that is produced by solventogenic *Clostridium* species, there is a dire need to develop industrial strain of solventogenic *Clostridium* species capable of metabolizing cheap and readily available substrates such as lignocellulosic biomass (LB). We sought to address this need in this work. We demonstrate for the first time that integration of an additional copy of a short-chain dehydrogenase/reductase (SDR) gene (*Cbei*_3904) into the chromosome of *C. beijerinckii* NCIMB 8052 will result in improved strain with capacity to produce more butanol from crude substrate (LB) than from pure substrate (glucose). Furthermore, our findings provide evidence that a simple fermentation medium modification can result in significant improvement in the fermentation of non-detoxified LB by *C. beijerinckii* NCIMB 8052 wildtype. These findings are likely to stimulate broad interest given their value to researchers focusing on improving the economics of biofuel production.

1. INTRODUCTION

Clostridium species can ferment a wide variety of substrates such as starch, disaccharides, hexoses, pentoses, glycerol, cellulose, and syngas into industrially important chemicals and environmentally compatible fuels (butanol, ethanol, isopropanol, and hexanol; Ezeji et al. 2007a; Philips et al., 2015; Liu et al., 2015; Sandoval-Espinola et al., 2017; Sun et al., 2019). Solventogenic *Clostridium* species exhibit a biphasic fermentation pattern characterized by acid (acetic acid and butyric acid) production in the exponential phase (acidogenesis) and solvent production (acetone, butanol, and ethanol; solventogenesis) in the second (stationary phase). During acidogenesis, there is production of H_2, CO_2, acetate, and butyrate resulting in a decrease in culture pH. During solventogenesis, there is marked change in metabolism resulting in uptake of the acids produced during the acidogenic phase along with sugar substrates, which are metabolized into for acetone, butanol, and ethanol (ABE; Jones and Woods, 1986; Veza, et al., 2021). A typical batch fermentation for producing ABE using *Clostridium* species results in the molar ratio of 3:6:1 for acetone, butanol, and ethanol, respectively.

Butanol or ABE is currently produced at laboratory scale by fermenting food crops such as corn and sugarcane, which raises concerns over human food security. Consequently, exploring non-food substrates—lignocellulosic biomass (LB) such as energy crops, agricultural residues, and farm wastes—for biofuel production is being considered a "panacea" for preventing or reducing prospective competition between food sources and industrial raw materials for biofuels production (Palmqvist and Hahn-Hagerdal, 2000; Ezeji et al., 2007; Isar and Rangaswamy, 2011; Zhang and Ezeji, 2013; Okonkwo et al., 2016; Sodre, et al., 2021). LB is composed of polymeric sugar forms (cellulose and hemicellulose) and lignin. The compact nature of LB, however, makes it recalcitrant to enzymatic hydrolysis to release fermentable monomeric sugars (glucose, xylose, and arabinose).

89 Hence, there is need for a pretreatment process prior to enzyme-mediated hydrolysis, and this
90 typically involves application of heat and acid/alkaline to facilitate breakdown of the lignin matrix
91 of LB. Nonetheless, in addition to releasing fermentable sugars, the pretreatment process releases
92 acetate from the hemicellulose component of LB alongside generation of a plethora of toxic
93 phenolic and furanic aldehydes generally referred to as lignocellulose-derived microbial inhibitory
94 compounds (LDMICs; Ezeji et al., 2007b; Veza, et al., 2021, Sodre, et al., 2021). LDMICs such
95 as furanic aldehydes (e.g., furfural and hydroxymethylfurfural-HMF) and phenolics
96 (hydroxybenzaldehyde, ferulic acid, syringic acid etc.) impair the growth and capacity of
97 fermenting microorganisms to utilize sugars thereby resulting in low yield of biofuels (Ezeji et al.,
98 2007b; Ezeji and Blaschek 2008; Baral and Shah, 2014; Ujor et al., 2016). The presence of
99 LDMICs in hydrolysates, therefore, is a major impediment to the use of LB for biofuel production.
100 Various researchers have explored LB hydrolysates (LBH) detoxification processes such as
101 overliming (Qureshi et al., 2010, Zhang et al., 2018), use of activated carbon (Dong et al., 2018,
102 Liu et al., 2015a), and media optimization strategies such as glycerol (Ujor et al., 2014) and
103 allopurinol (Ujor, et al., 2015) supplementations, to mitigate the inhibitory effects of LDMICs on
104 fermenting microorganisms. These strategies, however, have associated costs, therefore, leading
105 to relatively greater biofuel production costs (Liu et al., 2015). Intermittent addition of substrates
106 during fermentation has been explored as means of circumventing LDMICs-mediated toxicity.
107 With this strategy, there was enhanced bioconversion of LBH to butanol, thereby raising the
108 prospect of eliminating the need for detoxification of hydrothermolysis pretreated LBH prior to
109 fermentation (Adesanya et al., 2022).

110 To further reduce or eliminate cost associated with detoxification of LBH prior to
111 fermentation, metabolic engineering of solventogenic *Clostridium* species for increased tolerance

112 to LDMICs has been viewed as a possible strategy to markedly improve efficacy and efficiency,
113 and consequently economics of fermenting undetoxified LBH into butanol (Agu et al., 2019).
114 Towards this goal, a LDMIC-tolerant strain of *C. beijerinckii* was engineered to overexpress a
115 short-chain dehydrogenase/reductase (SDR) (Okonkwo et al., 2019). This approach was based on
116 results from a previous genome-wide transcriptional study that showed significant upregulation of
117 the open reading frame *Cbei*_3904 in *C. beijerinckii*, which encodes an NAD(P)H-dependent SDR
118 when furfural was supplemented in the growth medium (Zhang and Ezeji, 2013). The SDR
119 encoded by *Cbei*_3904 has been shown to be involved in the transformation of furfural and HMF
120 to their respective less inhibitory alcohols (furfuryl alcohol and 2,5-bis-hydroxymethylfuran-HMF
121 alcohol) in *C. beijerinckii* (Zhang et al., 2015). The resulting metabolically engineered strain of *C.*
122 *beijerinckii* (*C. beijerinckii*_SDR) however, has not been evaluated for tolerance to LDMICs in
123 undetoxified LBH. The present study, therefore, was conducted to evaluate the capacity of *C.*
124 *beijerinckii*_SDR to ferment undetoxified switchgrass hydrolysate (SH) to butanol.

125 **2. MATERIALS AND METHODS**

126 **2.1. Production of switchgrass hydrolysates**

127 Dried *Panicum Virgatum L.* (Alamo Switchgrass) was collected from the Gasification
128 Laboratory at Oklahoma State University and processed using a hammer mill equipped with a 2
129 mm sieve. The comminuted switchgrass was pretreated in a 1 L Parr reactor (Parr series 4520, Parr
130 instrument company, Moline IL, USA) at a loading rate of 10% solids at 200 °C for 10 min (Pessani
131 et al., 2011; Liu et al., 2015a). After cooling, the pretreated switchgrass was vacuum filtered with
132 Whatman No 4 filter paper to separate solids from the liquid component containing mainly
133 degraded hemicellulose (xylose) according to a previously described method (Adesanya et al.,
134 2022). The solids were washed four times with deionized water and enzymatically hydrolyzed

using Accellerase 1500 (gifted by DuPont, Rochester, NY, USA) in a shaker water bath, at 50 °C and 250 rpm as described previously (Liu et al., 2015a; Adesanya et al., 2022).

2.2. Detoxification of hydrolyzed switchgrass hydrolysates

A portion of the SH was detoxified using activated carbon to reduce the concentrations of LDMICs produced during the pretreatment. Detoxification was conducted with Calgon rod shaped activated carbon (AP4-60, Calgon Carbon Corporation, Pittsburgh, PA) using the treatment regimen described by Adesanya et al. (2022).

2.3. Bacterial strains and culture conditions

Clostridium beijerinckii NCIMB 8052 was purchased from the American Type Culture Collection (Manassas, VA, USA) as *C. beijerinckii* ATCC 51743. In an earlier study, *C. beijerinckii*_SDR was constructed by integrating the open reading frame *Cbei*_3904 (which encodes an SDR) into the chromosome of *C. beijerinckii* NCIMB 8052 to obtain the LDMIC-tolerant *C. beijerinckii*_SDR (Okonkwo et al., 2019). The chromosomally integrated *SDR* gene was constitutively expressed by placing it under the control of thiolase promoter from *C. acetobutylicum* ATC 824. *Clostridium* strains (*C. beijerinckii*_SDR and *C. beijerinckii* NCIMB 8052) were stored as spore suspensions in ddH_2O at 4 ℃ (Han et al., 2013). Spores for each strain (400 µL) were reactivated using heat shock treatments at 75 ℃ for 3 min prior to inoculation of 10 mL anoxic Tryptone-Glucose-Yeast extract (TGY) medium (Ezeji et al., 2013; Liu et al., 2015). The culture medium was incubated at 35 ℃ overnight (12 – 13 h) during which $OD_{600\ nm}$ of 0.9 to 1.1 was attained. Approximately 2 mL of overnight culture of each strain (10 %, v/v) was used to inoculate 18 mL of fresh anoxic TGY medium to increase the quantity of the preculture. These new cultures were incubated at 35 ℃ until OD ~ 0.9 to 1.1 was attained (3 – 4 h), after which they

were used to inoculate the fermentation medium. All inoculations, handling, and incubation processes were performed in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) with a modified atmosphere of 82% N_2, 15% CO_2, 3% H_2. The TGY medium in loosely capped bottles and test tubes was maintained overnight in the anaerobic chamber to allow for removal of residual oxygen before fermentation was initiated (Ezeji et al., 2013; Liu et al., 2015b; Sun et al., 2020).

2.4. ABE fermentation of non-detoxified switchgrass hydrolysates

Fermentation was conducted in 150-mL Pyrex screw-cap bottles with a 50 mL working volume. To initiate fermentation, non–detoxified SH (44.5 mL) was transferred into pre-sterilized 150-mL screw-cap bottles which was then supplemented with 1% (v/v) of each of the P2 buffer, mineral, and vitamin stock solutions (**Table 1**; Ezeji et al., 2003). Sterile yeast extract (1 mL of 50 g/L stock) was aseptically added to the non-detoxified SH fermentation medium followed by the addition of 3 mL (6%, v/v) of *C. beijerinckii*_SDR or *C. beijerinckii*_wildtype preculture.

For fermentations using the modified medium [with $(NH_4)_2CO_3$ supplementation], 44.0 mL of non-detoxified SH was transferred into another set of 150-mL Pyrex screw-cap bottles. Modified P2 buffer, mineral, and vitamin stock solutions (1%, v/v each) was added (**Table 1**). Yeast extract (1 mL of 50 g/L stock) was added to the medium containing the non-detoxified SH resulting in a 1 g/L final concentration. Also, $(NH_4)_2CO_3$ (500 µL of 200 g/L stock) was added resulting in a final concentration of 2 g/L in the fermentation medium.

2.5. Fermentation of detoxified switchgrass hydrolysates to ABE

Fermentation was conducted in 150-mL Pyrex screw-cap bottles containing a 50 mL working volume of fermentation medium. *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype (6%,

v/v) were separately inoculated into the detoxified SH fermentation medium as described above for the non-detoxified SH fermentation medium. As with the undetoxified SH medium, another set of fermentation cultures was established with the addition of $(NH_4)_2CO_3$ to a final concentration of 2 g/L. P2 medium (60 g/L glucose; 1 g/L yeast extract) was used as positive control. For the positive control, 45.5 mL of P2 medium was transferred into another set of 150-mL Pyrex screw-cap bottles. Subsequently, the P2 buffer, mineral, and vitamin stock solutions (1%, v/v each) were added using the methods described above. The compositions of the P2 buffer, minerals, and stock solutions are presented in **Table 1**. All fermentation experiments were conducted in triplicate. Samples (1 mL) were collected from each bottle at the time of initiation of the fermentation (0 h) and subsequently at 12 h intervals for 72 to 120 h to determine pH, OD_{600nm}, sugar, butanol, acetone, ethanol, acetic and butyric acid concentrations.

2.6. Analytical methods

Optical density was determined at 600 nm using a DU® 800 spectrophotometer (Beckman Coulter Inc., Brea, CA) to estimate the changes in the growth of the *C. beijerinckii* strains during fermentation. Gas chromatography was conducted to quantify the concentrations of acetone, butanol, ethanol, acetic, and butyric acids using a 7890A system (Agilent Technologies 7890, Agilent Technologies Inc., Wilmington, DE), according to a previously described method (Ujor et al., 2021). Sugar concentrations (glucose, xylose, and arabinose) were analyzed using HPLC (Waters, Milford, MA, USA) equipped with evaporative light scattering detector (Waters, Milford, MA, USA) according to a previous method (Ujor et al., 2021). The concentrations of the LDMICs were quantified using HPLC according to the method of Agu et al., 2016. The ABE yield was determined by dividing the total grams of ABE produced by the total grams of glucose or sugars utilized during fermentation, while ABE productivity was calculated by dividing the maximum

amount of ABE (g/L) produced by the corresponding fermentation time (h). Glucose utilization rate was calculated by dividing the total concentration of glucose used to produce the maximum ABE by the corresponding fermentation time.

2.7. Statistical analyses

A one-way ANOVA and student t-test analyses were performed using the general linear model (GLM) procedure of SAS OnDemand for Academics 3.1.0 (SAS Institute Inc., Cary, NC). The Least Square Difference (LSD) test procedure was used for evaluation of treatment means. There was determination of values for maximum optical density readings, sugar utilization during the fermentation periods, as well as maximum concentrations, yield, and production of butanol and ABE. Mean differences in these values because of using different *C. beijerinckii* strains and/or different fermentation medium compositions were tested for significance when there was a $P \geq 0.05$.

3.0. RESULTS

3.1. Production and detoxification of switchgrass hydrolysate (SH)

The hydrothermolysis pretreatment method was efficacious for deconstructing switchgrass biomass. The composition of the pretreated solids has been reported by Adesanya et al. (2022). Two batches of SH were utilized. The SHs were subjected to enzymatic hydrolysis to produce fermentable monomeric sugars. After hydrolysis, Batch 1 of the SH was detoxified using activated carbon to remove/reduce LDMICs while the SH in Batch 2 was not detoxified. Glucose concentrations of Batches 1 (detoxified) and 2 (non-detoxified) of the SH were 58.7 and 61.2 g/L, respectively (**Table 2**). The concentrations of LDMICs in the medium containing the detoxified and non-detoxified SH are presented in **Table 2**. In the medium based on the detoxified SH, the

concentrations of furfural, HMF, vanillic acid, syringic acid, p-coumaric acid, hydroxybenzaldehyde were 2-, 1.06-, 3.7-, 6.9-, 1.8- and 1.6-fold lower, respectively, when compared to the concentrations in the non-detoxified SH medium.

3.2. Fermentation profiles of the *C. beijerinckii* strains during growth in non-detoxified and detoxified SH

3.2.1. The growth profiles of *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR

The increase in growth by *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR was rapid, attaining maximum $OD_{600\ nm}$ of 5.7 and 5.9, respectively, in the glucose P2 medium (positive control; **Fig. 1**). Similarly, there was marked increase in growth for both *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR, reaching maximum $OD_{600\ nm}$ of 6.3 and 6.1, respectively, in the medium containing the detoxified SH (**Fig. 1**). Similarly, when the detoxified SH medium was supplemented with $(NH_4)_2CO_3$, there was discernible rapid increase in the growth of *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR, attaining maximum $OD_{600\ nm}$ of 6.8 and 7.7, respectively (**Fig. 1**). Conversely, when *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR were cultured in non-detoxified SH medium, there was reduced growth for both strains, attaining maximum $OD_{600\ nm}$ of 1.9 and 4.4, respectively, (**Fig. 1**). As expected, there was an increase in population size for *C. beijerinckii*_wildtype and *C. beijerinckii* resulting when there was fermentation in non-detoxified SH medium supplemented with $(NH_4)_2CO_3$ reaching $OD_{600\ nm}$ of 5.4 and 6.2, respectively (**Fig. 1**).

3.2.2. pH, acetic and butyric acid concentrations in the fermentation cultures of *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR

The pH, acetic acid, and butyric acid concentration values are depicted in **Figures 2, 3 and 4** for cultures of both strains of *C. beijerinckii* NCIMB 8052 and *C. beijerinckii*_SDR grown in non-detoxified and detoxified SH. As expected, during fermentation of non-detoxified SH without $(NH_4)_2CO_3$ supplementation, the pH of the fermentation medium decreased to less than 5.4 (**Fig. 2A**) at 12 h with a concomitant increase in acetic (**Fig. 3A**) and butyric acid concentrations (**Fig. 4A**). While the pH increased after 12 h with *C. beijerinckii*_SDR, the pH did not increase significantly in cultures inoculated with *C. beijerinckii*_wildtype (**Fig. 2A**). Meanwhile, when non-detoxified SH medium was supplemented with $(NH_4)_2CO_3$, the pH for both *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR increased after 12 h of fermentation (**Fig. 2B**) with a concomitant decrease in acetic acid concentration (**Fig. 3A**), a trend that is similar to the pH of the P2 medium control (**Fig. 2E, 3E, and 4E**). Notably, the butyric acid concentration remained relatively small in the fermentation media (**Fig. 4A and B**). To determine the extent to which the resulting pH and acid profiles of *C. beijerinckii*_wildtype during fermentation of non-detoxified SH negatively affected the cells (in addition to the effects of the LDMICs), the SH was detoxified by utilizing activated carbon to reduce the concentrations of LDMICs (**Table 2**). The pH and acid profiles of *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR improved markedly during fermentation of detoxified SH and showed the typical pH profile of ABE fermentation – pH decreases before and increases after 12 h of fermentation (**Fig. 2C**). Furthermore, prior to 12 and after 12 h of fermentation, the observed decrease and increase in culture pH coincided with an increase and decrease in butyric acid concentration, respectively (**Fig. 4C**). The observed fluctuations in culture pH and acid concentration are due to acid production and re-assimilation of acidic constituents by *C. beijerinckii*_wildtype- and *C. beijerinckii*_SDR, typical of what occurs with the exponential growth/acidogenic and solventogenic phases of ABE fermentation, which

indicate relatively good physiological state of the culture during growth in detoxified SH or non-detoxified SH with $(NH_4)_2CO_3$ supplementation. Similarly, when *C. beijerinckii*_wildtype- and *C. beijerinckii*_SDR were grown in detoxified SH medium supplemented with $(NH_4)_2CO_3$, the pH of the cultures decreased and later increased as fermentation progressed (**Fig. 2D**). Conversely, at the same fermentation timepoints, the concentration of acids increased which later decreased as fermentation progressed (**Fig. 3D, and 4D**). Notably, the acetic acid content of the fermentation medium at 0 h (**Fig. 3**) was from ammonium acetate, which is contained in the P2 buffer.

3.2.3. Sugar utilization during fermentation in SH

Most of the pentose content of the SH after pretreatment by hydrothermolysis was recovered during the washing process leaving mostly cellulose which was later hydrolyzed to glucose using a commercial cellulase. **Table 3** shows total glucose uptake by the LDMICs-tolerant *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype during the fermentation of non-detoxified SH, non-detoxified SH with $(NH_4)_2CO_3$ supplementation, detoxified SH, and detoxified SH with $(NH_4)_2CO_3$ supplementation. While the LDMIC-tolerant *C. beijerinckii*_SDR readily utilized the glucose in non-detoxified SH as a carbon source for energy metabolism and ABE production, such that greater than 57 g/L glucose was utilized in 84 h, the *C. beijerinckii*_wildtype was not efficient in utilizing the glucose in non-detoxified SH (**Table 3**). Consequently, approximately 25 g/L glucose was utilized by *C. beijerinckii*_wildtype during the same period. This translates into a 2.3-fold greater glucose utilization by *C. beijerinckii*_SDR when compared to *C. beijerinckii*_wildtype. For the SH detoxified with activated carbon, detoxification did not translate to improved sugar utilization by *C. beijerinckii*_SDR. However, there was improved glucose utilization by *C. beijerinckii*_wildtype leading to an increase in consumed glucose from 25 (from non-detoxified) to 46 g/L (detoxified; **Table 3**). Interestingly, during fermentation of non-

detoxified SH with $(NH_4)_2CO_3$ supplementation, both microbial strains utilized similar quantities of glucose at a similar rate (**Table 3**), even though the growth of *C. beijerinckii*_SDR was greater than that of *C. beijerinckii*_wildtype (**Fig. 1B**). Specifically, *C. beijerinckii*_SDR utilized 42.4 g/L glucose in non-detoxified SH with $(NH_4)_2CO_3$ supplementation, resulting in 1.3-fold less glucose utilized in non-detoxified SH without $(NH_4)_2CO_3$ supplementation. The rate of glucose utilization by *C. beijerinckii*_SDR in non-detoxified SH medium with $(NH_4)_2CO_3$ supplementation was similar (0.59 g/L/h) to that of *C. beijerinckii*_wildtype (**Table 3**). Furthermore, when SH was detoxified with activated carbon and the medium was supplemented with $(NH_4)_2CO_3$, the overall glucose utilization by *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype was also similar. *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype utilized approximately 48 g/L and 47 g/L glucose, respectively, at the rates of ~0.68 and ~0.66 g/L/h, respectively (**Table 3**).

3.2.4. ABE production in SH

*C. beijerinckii*_SDR produced more total ABE than *C. beijerinckii*_wild type in all the media (non-detoxified SH, non-detoxified SH with $(NH_4)_2CO_3$ supplementation, detoxified SH, and detoxified SH with $(NH_4)_2CO_3$ supplementation). Notably, the quantities of acetone produced by *C. beijerinckii*_SDR were greater than that produced by *C. beijerinckii*_wildtype. Acetone concentration in the medium peaked at 8.0 g/L during fermentation of non-detoxified SH without $(NH_4)_2CO_3$ supplementation by *C. beijerinckii*_SDR, which was 2.5-fold greater than that (3.24 g/L) produced by *C. beijerinckii*_wildtype (**Fig. 5A**). When the non-detoxified SH medium was supplemented with $(NH_4)_2CO_3$, the concentration of acetone in the medium was similar for both fermentations with *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype (**Fig. 5B**). Likewise, during fermentation of detoxified SH or detoxified SH with $(NH_4)_2CO_3$ supplementation, there were greater acetone concentrations in the medium inoculated with *C. beijerinckii*_SDR than in *C.*

314 *beijerinckii*_wildtype cultures. With *C. beijerinckii*_SDR, acetone concentrations were 4.4 g/L
315 (**Fig. 5D**) and 4.7 g/L (**Fig. 5C**) when the SH was detoxified with and without $(NH_4)_2CO_3$
316 supplementation, respectively. With *C. beijerinckii*_wildtype, acetone concentrations in the
317 detoxified with and without $(NH_4)_2CO_3$ supplementation media were 3.4 g/L (**Fig. 5D**) and 3.8 g/L
318 (**Fig. 5C**), respectively. There was a marked difference in ethanol production during fermentation
319 in non-detoxified SH without $(NH_4)_2CO_3$ supplementation and in P2 medium (**Fig. 6A and 6E**).
320 During fermentation of non-detoxified SH, without $(NH_4)_2CO_3$ supplementation, both strains of *C.*
321 *beijerinckii* exhibited similar ethanol profile until 48 h. At the 60-h time point in cultures of *C.*
322 *beijerinckii*_SDR, there was a decrease in ethanol concentration and subsequently, an increase at
323 72 h, while in cultures of *C. beijerinckii*_wildtype there was a sustained increase in ethanol
324 concentration up to 96 h of fermentation (**Fig. 6A**). The highest ethanol concentration observed
325 with *C. beijerinckii*_SDR was 1.48 g/L at 72 h, while *C. beijerinckii*_wildtype produced 2.5 g/L
326 at 96 h. Notably, fermentation with *C. beijerinckii*_SDR in the P2 control medium led to greater
327 quantities of ethanol production than was observed during fermentation with *C.*
328 *beijerinckii*_wildtype in the same conditions (**Fig. 6E**). The ethanol production profiles of *C.*
329 *beijerinckii*_wildtype and *C. beijerinckii*_SDR during fermentation of non-detoxified SH with
330 $(NH_4)_2CO_3$ supplementation and detoxified SH with $(NH_4)_2CO_3$ supplementation were similar
331 (**Fig. 6B and 6D**).

332 Fermentation with *C. beijerinckii*_SDR led to 11.2 g/L and 20.2 g/L butanol and ABE respectively
333 (at 84 h of fermentation), when the substrate was non-detoxified SH with no $(NH_4)_2CO_3$
334 supplementation, which is 3.1- and 2.2-fold greater than the maximum butanol and ABE
335 concentrations, observed with *C. beijerinckii*_wildtype (**Fig. 7A and 8A and Table 3**). Although
336 the 20.2 g/L ABE produced by *C. beijerinckii*_SDR in non-detoxified SH medium is greater than

that it produced in P2 medium control (16.1 g/L ABE), the associated ABE productivity of 0.24 g/L/h (**Table 3**) was 1.5-fold less than that of the P2 medium control where the ABE productivity was 0.36 g/L/h. The relatively poor ABE productivity performance was because while it took *C. beijerinckii*_SDR 84 h to produce maximum ABE of 20.23 g/L in non-detoxified SH medium without $(NH_4)_2CO_3$ supplementation, it took *C. beijerinckii*_SDR only 48 h to achieve maximum ABE concentration of 16.1 g/L in P2 medium control. When the non-detoxified SH medium was supplemented with $(NH_4)_2CO_3$, however, *C. beijerinckii*_SDR produced 9.5 g/L butanol and 14.2 g/L ABE, which are 1.03- and 1.06- fold greater than the butanol (9.2 g/L) and ABE (13.35 g/L) produced by *C. beijerinckii*_wildtype (**Fig. 7B and 8B** and **Table 3,** respectively). Interestingly, without $(NH_4)_2CO_3$ supplementation in non-detoxified SH medium, *C. beijerinckii*_SDR produced 1.2- and 1.42-fold greater butanol and ABE (**Fig. 7A and 8A** and **Table 3**) respectively, than it produced with $(NH_4)_2CO_3$ supplementation.

To evaluate the effect of SH detoxification by activated carbon on butanol and ABE production, butanol and ABE concentrations were assessed during fermentation in detoxified SH with and without $(NH_4)_2CO_3$ supplementation. While *C. beijerinckii*_wildtype produced 11.05 and 15.09 g/L butanol and ABE, respectively, during fermentation of detoxified SH with no $(NH_4)_2CO_3$ supplementation, *C. beijerinckii*_SDR produced 12.32 and 17.86 g/L butanol and ABE, respectively, which were 1.11- and 1.18-fold, respectively, greater than the concentrations produced with *C. beijerinckii*_wildtype (**Table 3**). When the detoxified SH medium was supplemented with $(NH_4)_2CO_3$, *C. beijerinckii*_SDR produced 17.13 and 10.73 g/L ABE and butanol, respectively, which were 1.1- and 1.05-fold greater than the respective concentrations produced with *C. beijerinckii*_wildtype (15.57 g/L ABE and 10.25 g/L butanol). Further, *C. beijerinckii*_SDR produced 9.99 and 16.1 g/L butanol and ABE, respectively, while *C.*

*beijerinckii*_wildtype produced 9.66 and 14.08 g/L butanol and ABE, respectively, during the fermentation of control P2 medium (**Fig. 7E and 8E**), which appear to validate the greater efficacy of *C. beijerinckii*_SDR as compared with *C. beijerinckii*_wildtype to produce ABE.

4. 0. DISCUSSION

Overcoming the challenges associated with the production of butanol and ABE, particularly overcoming the hurdles posed by LDMICs has dominated research efforts during the past decade (Sarangi, and Sonil 2018; Sodre, et al., 2021). Consequently, the goal of the present study was to evaluate the capacity of *C. beijerinckii* strain constitutively overexpressing a short-chain dehydrogenase/reductase (SDR; *Cbei*_3904) gene to convert non-detoxified SH to butanol and ABE. Additionally, we assessed whether modification of the fermentation medium by $(NH_4)_2CO_3$ supplementation results in a greater metabolism of the SH-derived glucose to butanol. The SDR superfamily of proteins consists of NAD(P)H-dependent oxidoreductases involved in a single step reduction of aldehydes such as furans to lesser toxic alcohols (Zhang et al., 2015). Overexpression of SDR genes in non-*Clostridium* species has led to improved detoxification, hence greater tolerance to furans by these microorganisms (Almeida et al., 2008, Chung et al., 2015, Kim et al., 2017, Wang et al., 2013). Although the results from various studies indicate that concentrations as high as 2.0 g/L furfural enhances growth and solvent production in *C. beijerinckii*-wildtype (Ezeji et al., 2007b), larger concentrations of furans and other LDMICs, especially phenolic compounds (such as vanillin, p-coumaric acid, syringic acid, hydroxybenzaldehyde etc. even at small concentrations), are inhibitory to microorganisms involved in the production of butanol and ABE. The presence of phenolic LDMICs alongside furans in hydrolysates produced after pretreatment and hydrolysis of LB, therefore, is a barrier to effective bioconversion LB hydrolysates to fuels and chemicals.

There were 2.3-, 2.2- and 3.1-fold greater cell population, total ABE, and butanol concentrations, respectively, by the *C. beijerinckii*_SDR compared to the *C. beijerinckii*_wildtype during the fermentation of non-detoxified SH pretreated with hydrothermolysis procedures and without $(NH_4)_2CO_3$ supplementation. The lower cell density in cultures of *C. beijerinckii*_wildtype is an indication that there was marked inhibition of growth by the LDMICs present in the undetoxified SH. This contrasted with the profiles of *C. beijerinckii*_SDR grown in undetoxified SH (**Table 3**). The extent of inhibition observed for *C. beijerinckii*_wildtype during the fermentation of non-detoxified SH is not in agreement with the levels of LDMICs present in the SH (**Table 2**). This is because *C. beijerinckii*_wildtype can effectively tolerate such levels of LDMICs (**Table 2**) during growth and fermentation, and can detoxify up to 20-, 16-, 4- and 2-mM furfural, HMF, 4-hydroxybenzaldehyde, and p-coumaric acid, respectively (Ezeji et al., 2007; Zhang and Ezeji, 2014). It is possible that the hydrothermolysis pretreatment method used in the pretreatment of switchgrass for this study may have generated additional LDMICs that could not be detected by the HPLC based analytical procedure described by Agu et al. (2016). Nonetheless, the results from the present study provide further evidence that *C. beijerinckii*_SDR can detoxify or tolerate LDMICs at the concentrations present in the SH, as evidenced by the increased cell population during the fermentation period (**Fig. 1A**). This is further evidenced by the effective conversion of SH-derived glucose to butanol and ABE during fermentation of undetoxified SH by *C. beijerinckii*_SDR (**Fig.7A and 8A**). Although the robustness of *C. beijerinckii*_SDR in terms of fermentation efficacy was highlighted during the fermentation of non-detoxified SH, the fermentation profile of this strain (butanol and ABE production) during fermentation of non-detoxified SH with $(NH_4)_2CO_3$ supplementation further underscored its potential.

Comparing the growth patterns of *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype in non-detoxified SH, with and without $(NH_4)_2CO_3$ supplementation alongside the acid production profiles, the inhibitory actions of LDMICs (**Table 2**) appeared to be compounded by the acetic acid content of the SH (**Fig. 3**). The acetic acid concentrations at 0 h in fermentations with *C. beijerinckii*_SDR (9.74 g/L) or *C. beijerinckii*_wildtype (7.92 g/L) were greater than the acetic acid concentrations (~3-4 g/L) typically present at the initiation time point of ABE fermentation. These relatively lesser acetic acid concentrations at 0 h are desired to maintain a pH range that supports microbial population growth and ABE production. In the present study, the acetic acid produced during pretreatment of switchgrass and that from the buffer component of the P2 medium resulted in a greater acetic acid concentration of the SH fermentation medium at 0 h (**Fig. 3**). Because ABE fermentation with *C. beijerinckii* is bi-phasic, acetic acid produced during the acidogenic/microbial population growth phase exacerbates the considerably greater acetic acid concentration of the fermentation medium. Consequently, there were two types of adaptive challenges during the fermentation period for *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype in the present study. The greater than typical acid concentration in the fermentation medium appears to be the major inhibitory factor to the growth and survival of *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR during fermentation. The lower concentrations of protonated acetic and butyric acid and greater buffering capacity provided because of supplementation with $(NH_4)_2CO_3$ may have contributed to the enhanced growth observed for *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype during fermentation of non-detoxified SH (**Fig. 1B**).

While these larger cell populations led to 2.6- and 1.5-fold increase in butanol and ABE production, respectively, for *C. beijerinckii*_wildtype, supplementation of the non-detoxified SH medium with $(NH_4)_2CO_3$ supplementation had a negative effect on ABE production in cultures

inoculated with *C. beijerinckii*_SDR. Consequently, there was 1.2- and 1.4-fold less butanol and ABE production (**Table 3**), respectively, during the growth of *C. beijerinckii*_SDR in non-detoxified SH with $(NH_4)_2CO_3$ supplementation than in non-detoxified SH medium without $(NH_4)_2CO_3$ supplementation (**Table 3**). These findings are consistent with the previous findings of Han et al. 2013 where supplementation of fermentation medium with carbonates resulted in greater buffering of the medium and larger cell populations of the microorganisms under investigation. While supplementation of the fermentation medium with $CaCO_3$ at ≥4 g/L resulted in a marked increase in ABE production, there was only a slight increase in total ABE production with $(NH_4)_2CO_3$ supplementation (Han et al., 2013). For ABE fermentation using *Clostridium* species, pH is a major indicator of the acidogenic growth phase and effects on ABE production (Gottwald & Gottschalk, 1985). It should be noted that the association between the magnitude of the population growth and ABE production is not always linear (Han et al., 2013). During the solventogenic phase of the fermentation in which is characterized by ABE production, maintenance of the fermentation medium pH in the range of 5.0 to 5.5 is optimal for ABE production while a higher pH typically leads to acid accumulation (Bahadur and Saraj 1960; Bahl et al. 1982; Monot et al., 1984; Wu et al., 2017). The pH range of the medium during the solventogenic phase was 5.2 to 5.6 in cultures of *C. beijerinckii*_SDR grown in non-detoxified SH without $(NH_4)_2CO_3$ supplementation. These conditions resulted in the maximum ABE production of 20.24 g/L (**Table 3**). Conversely, *C. beijerinckii*_SDR maintained pH above 5.5 in the non-detoxified SH medium supplemented with $(NH_4)_2CO_3$ during solventogenic phase of the fermentation (**Fig. 2B**). It is possible, therefore, that the buffering effect of $(NH_4)_2CO_3$ resulted in a sustained pH greater than 5.5, which led to enhanced growth of *C. beijerinckii*_SDR that was in

450 turn sub-optimal for ABE production. Hence, there was a lesser ABE production with *C.*
451 *beijerinckii*_SDR in non-detoxified SH medium with $(NH_4)_2CO_3$ supplementation.

452 As expected, both *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype produced higher butanol and
453 ABE concentrations in detoxified SH without $(NH_4)_2CO_3$ supplementation than in the P2 medium
454 (**Fig. 8, Table 3**). The improvement was because the LDMICs present in the SH that was pretreated
455 using hydrothermolysis procedures had been markedly reduced by the activated carbon
456 detoxification treatment. Consequently, the growth of *C. beijerinckii*_SDR in the detoxified SH
457 was rapid, leading to high glucose consumption thereby, achieving a maximum ABE of 17.86 g/L
458 in 48 h compared to a maximum ABE of 15.09 g/L in 72 h for the *C. beijerinckii*_wildtype.
459 Furthermore, the ABE productivity of 0.37 g/L/h achieved with *C. beijerinckii*_SDR was greater
460 than the ABE productivity of 0.27 g/L/h observed for *C. beijerinckii*_wildtype (**Table 3**). The ABE
461 yield with both *C. beijerinckii*_wildtype and *C. beijerinckii*_SDR varied between 0.32 and 0.37 g
462 ABE/g glucose during fermentation of SH or P2 medium control. There was no trend or pattern of
463 ABE production that was indicative of factors or conditions that affected the ABE yield. It is
464 possible that some carbons were diverted to the production of compounds such as formic and lactic
465 acid as these compounds are typically produced relatively in marked amounts by solventogenic
466 *Clostridium* species under unfavorable growth conditions.

467 The capacity of *C. beijerinckii*_SDR to increase in population size and ferment non-
468 detoxified SH relative to that of *C. beijerinckii*_wildtype in substrates replete with LDMICs and
469 high levels of acetic acid indicates that overexpression of the SDR (*Cbei*_3904) in *C. beijerinckii*
470 conferred some resiliency to the strain in conditions that were inhibitory to *C.*
471 *beijerinckii*_wildtype. Although the protein product of *Cbei*_3904 has been re-annotated as a
472 tri/tetra-hydroxynaphthalene reductase-like enzyme, it is still an oxidoreductase and SDR

473 superfamily domains (https://www.ncbi.nlm.nih.gov/protein/WP_012060066.1;
474 https://www.kegg.jp/dbget-bin/www_bget?cbe:Cbei_1071+cbe:Cbei_2398+ cbe:Cbei_3904).
475 The tri/tetra-hydroxynaphthalene reductases are specifically involved in fatty acid biosynthesis,
476 co-factor (biotin) metabolism, and reduction of alternate phenolic compounds and cyclic ketones
477 in bacteria (Schätzle et al., 2012, Okonkwo et al., 2019). This background information leads to the
478 suggestion that the protein product resulting from expression of *Cbei*_3904 is possibly involved in
479 modulating lipid composition of the cell membrane of *C. beijerinckii*_SDR and consequently,
480 fortifying the membrane integrity, thus, improving tolerance to LDMICs. Interestingly,
481 modulation of lipid biosynthesis and composition occurs in *Saccharomyces cerevisiae* in response
482 to the presence of organic acids and phenolic compounds during fermentation of LB hydrolysates
483 (Guo et al., 2018). Overexpression of the *OLE1* gene that encodes for the protein responsible for
484 the synthesis of monounsaturated fatty acids in *S. cerevisiae* leads to an increased monounsaturated
485 fatty acid content of the plasma membrane and conferment of enhanced tolerance to the deleterious
486 effects of acetic acid and phenolic compounds (Guo et al., 2018). Considering the tolerance of *C.*
487 *beijerinckii*_SDR to the LDMICs in non-detoxified SH, the capacity to grow and convert glucose
488 contained in SH to ABE, it is likely that a similar mechanism (as in *S. cerevisiae*; Guo et al., 2018)
489 might account for the robust capacity of *C. beijerinckii*_SDR to grow in and ferment non-
490 detoxified SH to ABE.

491 The large quantities of acetone produced by *C. beijerinckii*_SDR in comparison to *C.*
492 *beijerinckii*_wildtype during fermentation in all the media evaluated in the present study including
493 in the P2 medium control (**Fig. 5**) is noteworthy. This may be due to greater utilization of
494 NAD(P)H because of insertion of additional copy of SDR gene in *C. beijerinckii*, hence, reduced
495 availability of NAD(P)H for butanol production. Integration of the *Cbei*_3904 into the genome of

C. beijerinckii under the control of a constitutive promoter (thiolase) ensures continued production of the associated enzyme during both the acidogenic and solventogenic phases of growth (Okonkwo et al. 2019). As an NAD(P)H-dependent oxidoreductase, continued expression of *Cbei*_3904, which likely promotes fatty acid biosynthesis, co-factor (biotin) metabolism, and NAD(P)H-consuming reduction of phenolic compounds (Schätzle et al., 2012; Okonkwo et al., 2019), may be in direct competition with butanol production, which is also NAD(P)H-dependent. A limiting quantity of NAD(P)H in the cytoplasm of *C. beijerinckii*, even for a short period, can have ramifications that includes accumulation and decarboxylation of acetoacetic acid to acetone and CO_2 (Han et al., 2011) and increased growth of the population of *C. beijerinckii* (**Fig. 1**) due to the abundance of NAD^+ for glycolysis (Ujor et al., 2014, Zhang et al., 2012). In the presence of LDMICs (**Fig. 5A and C**) and to sustain cell population, active detoxification of LDMICs by *C. beijerinckii* requires repartitioning of NAD(P)H utilization for different processes, with potential beneficial effect on non-NAD(P)H-dependent acetone production, which facilitates the ability of the cell to reabsorb and convert acetic acid to a neutral product (acetone).

5. 0. CONCLUSION

In the present study, the capacity of a genetically engineered strain of *C. beijerinckii* NCIMB 8052 (*C. beijerinckii*_SDR) to tolerate the deleterious effects of LDMICs and produce ABE with hydrothermolysis pretreated SH was evaluated. Additionally, the effect of fermentation medium modification by $(NH_4)_2CO_3$ supplementation on the fermentation profile of *C. beijerinckii*_SDR was investigated. Supplementation of SH with $(NH_4)_2CO_3$ led to improved growth of *C. beijerinckii* strains and ABE production. Use of non-detoxified SH along with metabolically engineered *C. beijerinckii*_SDR to produce ABE is a promising combination as it produced 1.26-fold (20.24 g/L ABE) more ABE than the positive (16.1 g/L ABE). This result underscores the

519 robustness of *C. beijerinckii*_SDR for ABE production using undetoxified hydrothermolysis
520 pretreated SH. Moreover, our results make a case for metabolic engineering as a tool for rewiring
521 metabolic networks in fermenting microorganisms towards enhanced production of fuels and
522 chemicals using cheap biomass substrates.

523 **AUTHOR CONTRIBUTIONS**

524 TCE, HKA, and VCU conceived, designed, and supervised the study. TO, YA, and CCO
525 performed the experiments. TO and YA analyzed the data. TO and TCE wrote the first draft of the
526 manuscript. All authors contributed to the writing of the final draft and approved the submitted
527 version.

528

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720 **Tables and Figures**

721 **Table 1:** Compositions of fermentation and preculture media and stock solutions used in the
722 fermentation of SH by *Clostridium beijerinckii*

Medium Component	Formula	Amount g/L
Switchgrass Hydrolysates		
Non-detoxified SH glucose		61.2
Detoxified SH hydrolysate glucose		58.7
Yeast extract		1
Glucose P2 medium		
Glucose	$C_6H_{12}O_6$	60
Yeast Extract	-	1
TGY medium		
Tryptone	-	30
Glucose	$C_6H_{12}O_6$	20
Yeast Extract	-	10
L-Cysteine	$C_3H_7NO_2S$	1
P2 buffer stock solution		
Potassium phosphate monobasic	KH_2PO_4	50
Potassium phosphate dibasic	K_2HPO_4	50
Ammonium acetate	$NH_4CH_3CO_2$	220
Adjusted P2 buffer stock solution		
Potassium phosphate monobasic	KH_2PO_4	50
Potassium phosphate dibasic	K_2HPO_4	50
Ammonium acetate	$NH_4CH_3CO_2$	50
Vitamins		
p-(4)-Aminobenzoic acid	$C_7H_7NO_2$	0.1
Thiamine	$C_{12}H_{17}N_4OS^+$	0.1
Biotin	$C_{10}H_{16}N_2O_3S$	0.01
Minerals stock solution		
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	20

Manganese sulfate heptahydrate	$MnSO_4 \cdot 7H_2O$	1
Ferrous sulfate heptahydrate	$FeSO_4 \cdot 7H_2O$	1
Sodium chloride	NaCl	1
Supplement		
Yeast extract stock		50
Ammonium carbonate stock	$(NH_4)_2CO_3$	200

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724 **Table 2:** Concentrations of glucose and LDMICs in detoxified and non-detoxified SH. Standard
725 deviation for glucose reading represents duplicates (n = 2)

Compound	**Non-detoxified SH (g/L)**	**Detoxified SH (g/L)**
Sugar		
Glucose	61.2 ± 0.29	58.7 ± 0.74
LDMICs	**mg/L**	**mg/L**
HMF	9.52	8.95
Furfural	6.03	2.93
Vanillic Acid	2.20	0.59
Syringic Acid	6.15	0.89
Coumaric Acid	1.06	0.59
Hydroxybenzaldehyde	2.92	1.88

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731 **Table 3**: Summary of data from the fermentation of SH by *C. beijerinckii*_SDR and *C.*
732 *beijerinckii*_wildtype. Standard deviation for readings represents triplicates (n = 3)*

Medium		*C. beijerinckii*_SDR	*C. beijerinckii*_wildtype
Non-detoxified SH without medium modification	Glucose consumed (g/L)	57.71± 0.90[a]	24. 89 ± 2.30[d]
	Maximum butanol (g/L)	11.21 ± 0.60[b]	3.58 ± 0.30[f]
	Glucose utilization rate (g/L/h)	0.60 ± 0.01[d]	0.26 ± 0.10[e]
	Maximum ABE (g/L)	20.24 ± 0.8 [a]	8.98 ± 0.47[g]
	ABE yield (g/g)	0.35 ± 0.00[b]	0.36 ± 0.01[a]
	ABE productivity (g/L/h)	0.24 ± 0.00[e]	0.15 ± 0.00[g]
Non-detoxified SH with medium modification	Glucose consumed (g/L)	42.44 ± 1.20[c]	41.48 ± 0.70[c]
	Maximum butanol (g/L)	9.50 ± 0.59 [e]	9.19 ± 0.75 [e]
	Glucose utilization rate (g/L/h)	0.59 ± 0.01[d]	0.58 ± 0.02[d]
	Maximum ABE (g/L)	14.2 ± 0.6[e]	13.5 ± 0.80[f]
	ABE yield (g/g)	0.34 ± 0.00[b]	0.36 ± 0.01[a]
	ABE productivity (g/L/h)	0.30 ± 0.00[d]	0.37 ± 0.01[a]
Detoxified SH without medium modification	Glucose consumed (g/L)	57.86 ± 0.60[a]	45.9 ± 1.96[b]
	Maximum butanol (g/L)	12.32 ± 0.17[a]	11.05 ± 1.23[b]
	Glucose utilization rate (g/L/h)	0.80 ± 0.01[a]	0.64 ± 0.02 [c]
	Maximum ABE (g/L)	17.86 ± 0.25[b]	15.09 ± 1.5 [d]
	ABE yield (g/g)	0.32 ± 0.00 [c]	0.33 ± 0.02[bc]
	ABE productivity (g/L/h)	0.37 ± 0.00[a]	0.21 ± 0.00 [f]
Detoxified SH with medium modification	Glucose consumed (g/L)	48.35 ± 0.80[b]	47.2 ± 1.20[b]
	Maximum butanol (g/L)	10.72 ± 0.07 [c]	10.25 ± 0.28 [d]
	Glucose utilization rate (g/L/h)	0.68 ± 0.00 [b]	0.66 ± 0.00[bc]
	Maximum ABE (g/L)	17.13 ± 0.34[c]	15.57 ± 0.60 [d]
	ABE yield (g/g)	0.37 ± 0.00[a]	0.36 ± 0.00[a]
	ABE productivity (g/L/h)	0.36 ± 0.00 [b]	0.32 ± 0.00 [c]

*Statistical analysis was carried out to assess significant difference between *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype and, the different medium compositions for each parameter. Same letter superscripts represent no significant differences between *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype, and across the different medium compositions.

Figure Legends

Figure 1: Estimated population profiles for *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype during fermentation of SH and P2 medium control.

Figure 2: pH profiles of the culture medium during fermentation of SH and P2 medium control by *C. beijerinckii*_SDR or *C. beijerinckii*_wildtype

Figure 3: Acetic acid concentration profiles in the fermentation media using *C. beijerinckii*_SDR or *C. beijerinckii*_wildtype

Figure 4: Butyric acid concentration profiles in the fermentation media using *C. beijerinckii*_SDR or *C. beijerinckii*_wildtype

Figure 5: Acetone concentration profiles in cultures of *C. beijerinckii*_SDR or *C. beijerinckii*_wildtype grown in SH and P2 medium control

Figure 6: Ethanol concentration profiles in cultures of *C. beijerinckii*_SDR or *C. beijerinckii*_wildtype grown in SH and P2 medium control

Figure 7: Butanol concentration profiles in cultures of *C. beijerinckii*_SDR or *C. beijerinckii*_wildtype grown in SH and P2 medium control

Figure 8. ABE concentrations in cultures of *C. beijerinckii*_SDR and *C. beijerinckii*_wildtype grown in SH and P2 medium control