Engineering *Clostridium saccharoperbutylacetonicum* for enhanced isopropanol-butanol-ethanol (IBE) production from lignocellulosic biomass through acetic acid pretreatment

by

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Abstract

The objective of this study was to achieve efficient biofuel production from lignocellulosic biomass through the development of a novel biomass pretreatment method in combination with the metabolic engineering of microbial strains for efficient conversion of biomass feedstock. First, an innovative biomass pretreatment method was developed using acetic acid (AA) as the treatment reagent considering its various advantages compared to the conventional dilute acid pretreatment method and the benefit of AA for biobutanol production; then, the hyper-butanol producing strain *Clostridium saccharoperbutylacetonicum* N1-4 was engineered for enhanced acid re-assimilation and acetone-butanol-ethanol (ABE) production from the acetic-acid-pretreated biomass; further, *C. saccharoperbutylacetonicum* was engineered for enhanced production of isopropanol-butanol-ethanol (IBE; which can be used directly as a fuel source rather than ABE) from the acetic-acid-pretreated biomass.

For the biofuel production from lignocellulosic biomass, most biomass pretreatment processes need to use some chemical reagent as the catalyst to overcome the biomass recalcitrance barrier. Such reagents are usually severe inhibitors for the subsequence fermentation process. Therefore, in many cases, the liquid prehydrolysates fraction (LPF) after the pretreatment is discarded, which is a tremendous wasting of materials and leads to additional pollution. Biobutanol produced from ABE fermentation process has been of great interests recently due to its high value as a biofuel or biochemical. During the ABE fermentation, AA is produced and then re-assimilated as a carbon source. Thus, AA is a substrate rather than an inhibitor for biobutanol production. In this study,

we employed AA as the chemical catalyst for the pretreatment of switchgrass which then be used for ABE production through simultaneous saccharification and fermentation (SSF) with hyper-butanol producing *C. saccharoperbutylacetonicum* N1-4. Through systematic investigation of pretreatment conditions and fermentation, we concluded that the pretreatment with 3 g/L AA at 170 ^{o}C for 20 min is the optimal conditions for switchgrass pretreatment leading to efficient biobutanol production. Both LPF and solid cellulosic fraction (SCF) of the pretreatment biomass are highly fermentable. In the fermentation with the LPF/SCF mixture, 8.6 g/L butanol (corresponding to a yield of 0.16 g/g) was obtained. Overall, here we demonstrated an innovative biomass pretreatment strategy for efficient carbon source utilization and biobutanol production.

ABE fermentation generally has two phases: in the acidogenesis phase, fatty acids (acetic acid and butyric acid) are accumulated, while in the solventogenic phase, fatty acids are re-assimilated and converted into solvents. Therefore, the improvement of acid re-assimilation capability in the *Clostridium* host can possibly enhance the solvent production. In addition, acetic acid is often a significant component in the biomass prehydrolysates after pretreatment (especially when acid-based biomass pretreatment approach is employed). Thus, the enhancement of acid re-assimilation in *Clostridium* has practical significance for biofuel production from lignocellulosic biomass. Here, we overexpressed key genes of the ABE fermentation pathways in *C. saccharoperbutylacetonicum* to enhance the acid re-assimilation and solvent production. First, the native *sol* operon (*ald-ctfA-ctfB-bcd*) was overexpressed under the strong constitutive thiolase promoter (P_{thl}), generating PW2 strain. Fermentation results demonstrated that the acid re-assimilation was improved in the host strain and ABE production has been increased to 31.4 g/L (vs. 26.4 g/L in JZ100 strain as the control). Although the ethanol production has been increased by six times, the butanol production has not been significantly increased in the engineered strain. In order to further drive the carbon

flux from C2 metabolites to C4 metabolites and ultimate butanol production, the key genes including *hbd, thl, crt* and *bcd* (expression cassette, or EC) in the butanol production pathway was further overexpressed under P_{thl} besides the *sol* operon overexpression as in PW2, generating PW3 strain. Compared to the control, the butanol and acetone production in PW3 was increased by 8% and 18% respectively. The final total solvent production increased by 12.4% than the control, but was 10% lower than PW2 (mainly because of the dramatic increase of ethanol production in PW2). In PW3, both *sol* operon and EC were overexpressed with P_{thl}, which could lead to competition for the same RNA polymerase for the expression of multiple genes. To avoid this issue and further improve ABE production, a new strain PW4 was constructed to express *sol* operon with P_{thl} but EC with ferredoxin gene promoter (P_{fdx}). The fermentation results demonstrated that, however, the production of all the solvents in PW4 was actually slightly lower than those in PW3. Moreover, we evaluated the effect of acetic acid concentrations on the solvent production in the engineered strains, and the maximum level of solvent production was achieved when 4.6 g/L acetate was supplemented. Therefore, SSF was carried out with PW2 and PW3 using switchgrass biomass pretreated with 3 g/L acetic acid (which ends up with approximately 4.6 g/L in the fermentation medium). 15.4 g/L total ABE (with a yield of 0.31 g/g) was produced in both PW2 and PW3, which was significantly higher than that in JZ100. This study demonstrated that the overexpression of key genes for acid re-assimilation and solvent production can significantly enhance ABE production in solventogenic clostridia.

Acetone is highly corrosive to engine parts, and thus cannot be used as a fuel source. For this reason, the acetone produced during ABE fermentation is often considered as an undesirable byproduct. Biologically, acetone can be converted into isopropanol by the secondary alcohol dehydrogenase. Isopropanol, and thus the isopropanol-butanol-ethanol (IBE) mixture, can be used

a valuable biofuel. In this study, we attempt to metabolically engineer the hyper-ABE producing *C. saccharoperbutylacetonicum* N1-4 strain for IBE production. First, we overexpressed the secondary alcohol dehydrogenase (*sadh*) gene from *C. beijerinckii* B593 in *C. saccharoperbutylacetonicum* on a plasmid, generating PW5 strain. A *hydG* gene (encoding a putative electron transfer protein) is right downstream of *sadh* within the same operon in the *C. beijerinckii* B593 genome. Therefore, additionally, we overexpressed *sadh-hydG* gene cluster together in *C. saccharoperbutylacetonicum* to evaluate the effect of *hydG* for isopropanol production, generating PW6 strain. Fermentation results indicated that in both PW5 and PW6, high levels of isopropanol were produced with no acetone production was detected. Comparatively, PW6 produced slightly higher isopropanol (10.2 g/L vs. 9.4 g/L in PW5) and total IBE. However, overall the performance of PW6 for solvent production is very similar to that of PW5. To eliminate the issue with plasmid-based overexpression such as instability and the requirement of antibiotics for cell cultivation and fermentation, we further integrated *sadh* or *sadh-hydG* into the chromosome of *C. saccharoperbutylacetonicum*, and generated strains PW8 and PW9. In PW8, there was 4.8 g/L acetone and 4.0 g/L isopropanol produced, while in PW9, up to 9.5 g/L isopropanol was produced with only 0.4 g/L acetone was detected. This indicated that the co-overexpression of *hydG* with *sadh* through chromosomal integration had significant positive effects on the conversion of acetone to isopropanol. In order to further enhance the solvent production, we additionally overexpressed in PW9 the *sol* operon (*ald-ctfA-ctfB-adc*), the expression cassette EC (*thl-hbd-crt-bcd*), or *sol* in combination with EC, generating strains PW10, PW11, and PW12, respectively. The fermentation characterization indicated that PW10 had significantly elevated ethanol production, as well as 25% higher isopropanol with slightly decreased butanol production, leading to a significant increase in total solvent titer (34.2 g/L vs.

27.6 g/L in PW9) and yield (0.48 g/g vs. 0.40 g/g in PW9). In PW11, the butanol production increased to 17.9 g/L while ethanol production decreased to 0.4 g/L; however, the isopropanol and final total solvent production was very similar to that in PW9. In PW12, with the co-overexpression of *sol* operon and EC, the production of isopropanol, butanol, and ethanol increased to 11.7 g/L, 17.3 g/L, and 1.1 g/L respectively comparing to PW9, resulting in a slight increase in total solvent yield. Finally, SSF was carried out with PW9 and PW10 using the acetic-acid-pretreated switchgrass as the feedstock, and the final solvent titer reached 13.7 g/L and 16.2 g/L, corresponding to the solvent yield of 0.27 g/L and 0.32 g/g in PW9 and PW10, respectively. The engineered strains in this study (PW9, PW10, PW11) produced the highest total IBE that has ever been reported in the batch fermentation with solventogenic clostridia. Our results indicated that the acetic-acid-pretreated biomass can be efficiently converted into biofuel using the metabolically engineered *Clostridium* hosts. Overall, this study demonstrated an innovative approach for biofuel production by combining a tailored biomass pretreatment method and metabolic engineering of microbial workhorse for enhanced conversion of lignocellulosic carbon source for biofuel production.

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List of Abbreviations

AA	acetic acid
ABE	acetone, butanol, and ethanol
AFEX	ammonia fiber explosion
ATP	adenosine triphosphate
BA	butyric acid
Cassette EC	including thiolase (*thl* CSPA_RS03020), β-hydroxybutyryl-CoA dehydrogenase (*hbd* CSPA_RS02150), crotonase (*crt* CSPA_RS2130), and butyryl-CoA dehydrogenase (*bcd* CSPA_RS2150)
CBP	consolidated bioprocessing
crRNA	CRISPR RNA
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats and associated protein 9
DNA	deoxyribonucleic acid
DSB	double strand breakage
DP	degree of polymerization
gRNA	guide RNA
HDR	homology directed repair
HMF	5-hydroxyl furfural
HPLC	high performance liquid chromatography
hydG	the gene encoding a putative electron transfer protein
IBE	isopropanol-butanol-ethanol
IEP	intron-encoded protein
LPF	liquid prehydrolysates fraction
NADH	reduced nicotinamide adenine dinucleotide

NHEJ	non-homologous end-joining
ORF	Open Reading Frame
PAM	protospacer-adjacent motif
RAM	retrotransposition-activated selection marker
RM	restriction-modification
RNA	ribonucleic acid
sadh	the gene encoding the secondary alcohol dehydrogenase
SCF	solid cellulosic fraction
SHF	separate hydrolysis and fermentation
SOE-PCR	overlapping extension PCR
sol operon	consisting genes encoding NAD-dependent aldehyde dehydrogenase (*ald* CSPA_RS27680), butyrate-acetoacetate CoA transferase subunits A/B *(ctfA* CSPA_RS27685; *ctfB* CSPA_RS27690), and acetoacetate decarboxylase (*adc* CSPA_RS27695)
spyCas9	*Streptococcus pyogenes* bacterium Cas9
SSF	simultaneous saccharification and fermentation
TGY	tryptone-glucose-yeast extract
TPC	total phenolic compounds
tracrRNA	a trans-activating crRNA

I. Introduction

The finite nature of fossil fuel and the associated environmental implications provide an impetus for alternative bio-based fuels and chemicals from renewable resources. Solvents such as acetone, butanol, and ethanol produced through solventogenic clostridial fermentation (and thus this process is also termed as ABE fermentation) represent important potential renewable fuels and/or chemicals. Biobutanol has been of particular interest because of its various advantages as a biofuel and considerable value as an industrial chemical (Jones & Woods, 1986). As a fuel source, butanol has many superior properties over ethanol, such as higher energy content, less evaporation, less corrosive effect, not hygroscopic nature, similar feature as gasoline (thus can be blended at any percentage blend gasoline), and compatibility with existing infrastructure (Peter Dürre, 2007). As an industrial chemical, butanol has been widely used in the production of polymeric plastics, surface coatings/paintings, elastomers, lacquers and surface cleanser, and it has been used as the diluent for brake fluid formulations and the solvent for manufacturing of antibiotics, vitamins and hormones (Green, 2011).

In the ABE fermentation, conventional feedstock was exclusively food-based sugar or starch, occupying 60-80% of overall production cost, which made this fermentation process failed to compete with the petrochemical industry (Jones & Woods, 1986; Taconi, Venkataramanan, & Johnson, 2009). Recently, the skyrocketing price of oil, the increasing concern about environmental problems, the high cost of conversional starch (maize, wheat, millet, rye, etc.) or sucrose-based (molasses) substrates and the advancement of biotechnology have led to a renewed

interest in biobutanol production with lignocellulosic-based substrate (S. Y. Lee et al., 2008). Lignocellulosic feedstock, which represents a cheap, abundant and renewable carbon source, is primarily composed of cellulose, hemicellulose and lignin. Lignin, cross-linking with hemicellulose through covalent and hydrogen bond, forms a three-dimensional matrix, which embraces the crystallized cellulose fibers and protects the carbohydrate from external degradation (Badiei, Asim, Jahim, & Sopian, 2014). Thus, a pretreatment process is required to break this matrix and improve cellulose bioconversion efficiency, and most of the known pretreatment processes utilize chemical reagents (either diluted acid, alkaline or organosolv based) (Paulova, Patakova, Branska, Rychtera, & Melzoch, 2015). These reagents, however, even at low levels, are usually severe inhibitors (besides the phenolic inhibitors generated from the degradation of biomass) for the subsequent microbial fermentation processes (T. C. Ezeji, N. Qureshi, & H. P. Blaschek, 2007; Paulova et al., 2015). In addition, significant level of acetic acid is usually produced during the biomass pretreatment process, which is also a strong inhibitor, for example, for the microbial ethanol fermentation process (Wei, Oh, Million, Cate, & Jin, 2015).

ABE fermentation is a unique bi-phasic process, in which at the first phase (acidogenesis), carbohydrate carbon sources are degraded into acids (mostly acetic and butyric acids) while at the second phase (solventogenesis), the acids generated from the first phase are re-assimilated and converted into solvents along with additional consumption of carbohydrate (Jones & Woods, 1986). In this sense, acetic acid (and butyric acid) is a substrate rather than an inhibitor for ABE production. Indeed, it has been reported by different groups that the supplementation of exogenous

acetate can efficiently improve butanol production and stabilize the ABE fermentation process (C.-K. Chen & H. P. Blaschek, 1999; Chih-Kuang Chen & Hans P Blaschek, 1999; Gu et al., 2009). Therefore, if acetic acid is employed as the reagent in biomass pretreatment, this reagent along with the acetic acid generated during the pretreatment can both be utilized for ABE production. In such a method, no exogenous chemical reagent is introduced, and thus can avoid its inhibition on the butanol fermentation. In addition, the acetic acid (as a weak organic acid) pretreatment can potentially generate lower level phenolic inhibitors when compared to the regular pretreatment process (with strong chemical reagents involved) under similar conditions.

With acetic acid as the reagent for pretreatment, it could lead to even higher concentration of total acetic acid. Thus, it would be beneficial if the acetic acid re-assimilation capability of the butanol producing *Clostridium* strains can be further improved through metabolic engineering. In *Clostridium saccharoperbutylacetonicum* N1-4 (a well-recognized hyper-butanol producer; was selected as the strain that I primarily work with in this study), solventogenic genes are organized in a polycistronic solvent producing *sol* operon consisting genes encoding NAD-dependent aldehyde dehydrogenase (*ald* CSPA_RS27680), butyrate-acetoacetate CoA transferase subunits A/B (*ctfA* CSPA_RS27685; *ctfB* CSPA_RS27690), and acetoacetate decarboxylase (*adc* CSPA_RS27695), among which *ctfA/ctfB* are the major genes responsible for acids re-assimilation (Kosaka, Nakayama, Nakaya, Yoshino, & Furukawa, 2007).

With the re-assimilation of acids, acetoacetate is produced followed by being transformed to acetone through the catalysis by acetoacetate decarboxylase (*adc*) (Jones & Woods, 1986). Along

with improved butanol production (with acids re-assimilation), acetone production will also be increased. Acetone is a valuable solvent; however, it (and thus ABE mixture) cannot be used as a fuel source because of its corrosive nature. Co-production of acetone therefore causes lower yield of fuel alcohols. There were recently considerable efforts to convert acetone into isopropanol by introducing the secondary alcohol dehydrogenase (*sadh*) gene into ABE-producing hosts (Dusséaux, Croux, Soucaille, & Meynial-Salles, 2013; Y. S. Jang et al., 2013; J. Lee et al., 2012). Isopropanol, as a secondary alcohol, itself is a valuable fuel source (as well as a useful chemical), and thus the Isopropanol-Butanol-Ethanol (IBE) mixture can be directly used as a fuel source. This would simplify the downstream end product recovery process and can save a lot of energy.

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated 9 (CRISPR-Cas9) system is an RNA-guided immune system in bacteria and archaea that is able to recognize and cleave foreign invasive DNAs, such as phage or plasmids (Sorek, Lawrence, & Wiedenheft, 2013; Y. Wang et al., 2015). The type II-A CRISPR-Cas system of *Streptococcus pyogenes*, which requires a mature CRISPR RNA (crRNA), a trans-activating crRNA (tracrRNA), and DNA endonuclease Cas9, has been engineered as a cutting-edge genome engineering tool for both eukaryotic and prokaryotic cells (Chylinski, Le Rhun, & Charpentier, 2013). For genome editing in bacteria, this system is primarily functional as a tool for selecting edited cells against non-edited background cells thereby leading to high efficiency of genome engineering (Y. Wang et al., 2016). So far, CRISPR-Cas9-based tools have shown their versatility for gene deletion or insertion and have been reported as successfully utilized in various bacteria

(Chung et al., 2017; W. Jiang, Bikard, Cox, Zhang, & Marraffini, 2013; S. Wang, Dong, Wang, Tao, & Wang, 2017; Y. Wang et al., 2015), with attractive features such as ease of use, high efficiency, strong adaptability, and multiplex targeting ability.

Research objectives

Based on the background information discussed above, the first objective was, to develop a biomass pretreatment method using acetic acid as the treatment reagent. Alamo switchgrass was used as feedstock. It can be pretreated under relatively mild conditions. Pretreatment will be carried out in Parr reactor with different acetic acid concentrations and pretreatment temperatures. The pretreated biomass will be characterized by quantifying the sugars concentration and levels of representative inhibitors, and the fermentability of the pretreated biomass will be evaluated by batch fermentation with *C. saccharoperbutylacetonicum* N1-4 in order to systematically evaluate the acetic-acid pretreatment.

The second objective of this study was to develop *C. saccharoperbutylacetonicum* (from N1-4 (HMT) type strain) strains for enhanced acetic acid re-assimilation and improved biosolvent production through metabolic engineering. The whole *sol* (*ald*, *cftA/ctfB* and *adc*) operon was overexpressed based on plasmid. Moreover, the cassette EC including thiolase (*thl* CSPA_RS03020), β-hydroxybutyryl-CoA dehydrogenase (*hbd* CSPA_RS02150), crotonase (*crt* CSPA_RS2130), and butyryl-CoA dehydrogenase (*bcd* CSPA_RS2150), were also overexpressed on a plasmid to enhance the conversion of acetyl-CoA to butyryl-CoA and improve bio-solvent production.

The third objective of this study was to further engineer *C. saccharoperbutylacetonicum* to efficiently convert acetone produced from native ABE pathways into isopropanol. The secondary alcohol dehydrogenase (*sadh*) genes from *C. beijerinckii* NRRL B593 was chromosomally integrated into the host strain to convert acetone into isopropanol using CRISPR-Cas9 system. In addition to the *sadh* gene, the *hydG* gene encoding a putative electron transfer protein was also integrated together because the *hydG* gene consists of operon with the *sadh* gene in the chromosome of *C. beijerinckii* NRRL B593. Thus, the effects of *hydG* (in combination with *sadh*) for the conversion of acetone to isopropanol was evaluated. After obtaining the mutants, fermentation optimization will be performed using the pretreated biomass from the first objective. Overall, this study was to achieve efficient biofuel production from lignocellulosic biomass through the development of a tailored biomass pretreatment method in combination with the metabolic engineering of microbial strains for efficient conversion of biomass feedstock.

II Literature Review

II.1 *Clostridium* solvent fermentation

II.1.1 Background

Because the world supply of fossil fuels is limited and will eventually fail to meet the global demand for energy, tremendous research around the world has focused on the production of biofuels such as ethanol and butanol obtaining from renewable resources (Qureshi, Saha, & Cotta, 2007). *n*-Butanol (butanol hereafter unless otherwise indicated) is a linear four-carbon alcohol with a formula of C_4H_9OH, which can be used as a valuable fuel source. Because of the longer hydrocarbon chain and being fairly non-polar, butanol is more similar to gasoline than ethanol. It has been demonstrated that butanol can be applied directly in regular vehicle engines without any engine modification (Jones & Woods, 1986). Butanol has a relative lower Reid vapor pressure, seven times less than ethanol and 27 times less than gasoline, making it safer to handle and especially when used in hot area and hot weather (Andersen, Anderson, Wallington, Mueller, & Nielsen, 2010). Because of the less corrosive feature, butanol can be delivered through existing gasoline supply infrastructure while ethanol must be transported via rail, barge or truck. Moreover, butanol contains more energy per unit volume than ethanol and almost as much as gasoline (29.2 MJ/L for butanol versus 21.2 MJ/L for ethanol; and 32.0 MJ/L for gasoline) (**Table II-1**) (Peter Dürre, 2007). In this sense, butanol is considered to be the next generation biofuel after ethanol, and a fuel source much superior over ethanol. Besides its use as a promising biofuel, butanol can also be used as a valuable chemical feedstock for many industries. For example, it

was recognized as an excellent building block for synthetic rubber, and it can also be used as a paint thinner, solvent in coating applications as with lacquers, ambient-cured enamels, and as a component of hydraulic and brake fluids (Y.-S. Jang, Malaviya, Cho, Lee, & Lee, 2012; Tashiro, Yoshida, Noguchi, & Sonomoto, 2013).

Table II-1. Comparison of properties of butanol, ethanol and gasoline

	Gasoline	Butanol	Ethanol
Molecular formula	C4-C12	C_4H_9OH	C_2H_5OH
Octane number	80-99	96	108
Density (g/mL)	0.72-0.78	0.81	0.79
Air fuel ratio	14.6	11.2	3.0
Boiling point (°C)	25-215	118	78.4
Energy content (MJ/L)	32.0	29.2	21.2
Water solubility (%) at 25℃	<0.01	9.1	100.0
Reid vapor pressure (kPa)	60-62	2.2	16.0

Butanol can be produced from either chemical synthesis approach or biological fermentation route (S. Y. Lee et al., 2008). For the chemical synthesis of butanol, Oxo synthesis, Reppe synthesis, and crotonaldehyde hydrogenation are the three most important steps (**Fig. II-1**). For the biobutanol production through fermentation process, solventogenic *Clostridium* spp., is the most commonly used workhorse. The fermentation is termed as acetone-butanol-ethanol (ABE) fermentation because of its main products including acetone, butanol and ethanol. When comparing with chemical approaches, there are several advantages for fermentation, including broad substrate utilization (such as agricultural wastes and lignocellulose biomass), simpler and

milder processes, less energy extensive and environmentally benign (Balan, 2014; Ibrahim, Ramli, Kamal Bahrin, & Abd-Aziz, 2017; Y.-S. Jang, Malaviya, et al., 2012).

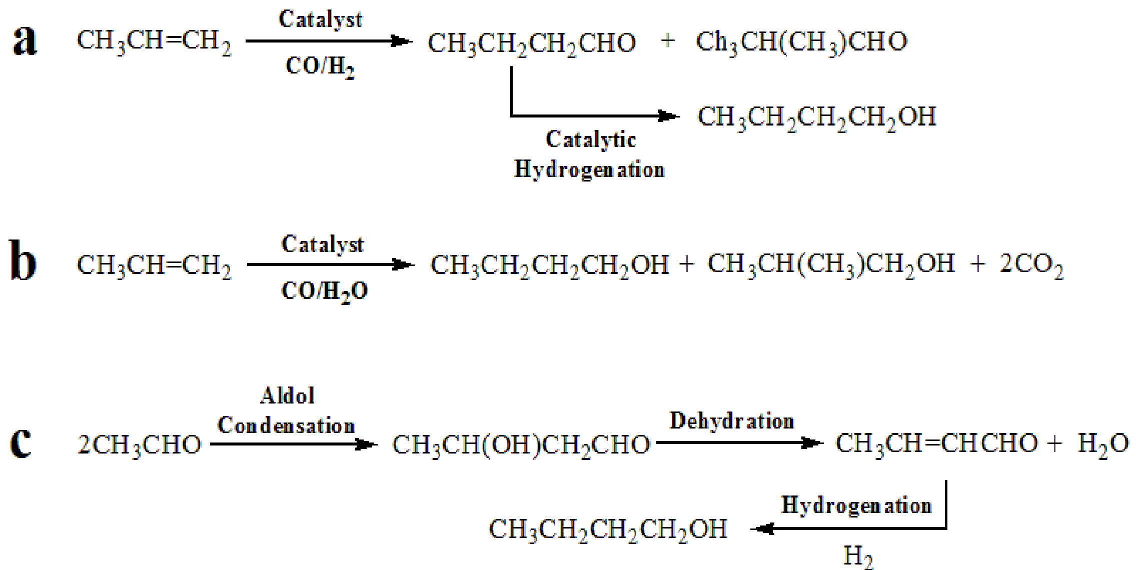


Fig. II-1. Chemical synthesis of butanol. (a) Oxo synthesis, (b) Reppe process, (c) crotonaldehyde hydrogenation (S. Y. Lee et al., 2008).

The microbial fermentation production of butanol was first reported by Pasteur in 1861, while it was only in 1905 that Schardinger (Schardinger, 1905) reported the microbial fermentation production of acetone. Started from as early as 1910s, industrial production of butanol was mainly based on the clostridial ABE fermentation (Jones & Woods, 1986). Between 1912 and 1914, Weizmann isolated and studied several strains; one of them was called BY and later named *Clostridium acetobutylicum*. This organism had many unique properties, including the ability to use a variety of substances and to produce high yields of butanol and acetone (Gabriel, 1928). At

that time, acetone was the primary interest (while butanol was considered as a useless byproduct) due to its application in the production of cordite in the first World War. Over the history, maize, wheat, and rye were all used for ABE fermentation at that time (T. C. Ezeji et al., 2007). Till the first part of 20th century, ABE fermentation was performed at a large industrial scale and ranked second in importance only to ethanol fermentation. In 1945, it was reported that two-third of the industrially used biobutanol was produced through fermentation in the United States (Jones & Woods, 1986). But later, this process lost competitiveness due to the increase of feedstock costs and advancement of the petrochemical industry. In recent years, however, the fluctuating price of crude oil price and increasing concerns about environmental deterioration have renewed the interest in biological production of butanol (S. Y. Lee et al., 2008; Zverlov, Berezina, Velikodvorskaya, & Schwarz, 2006).

II.1.2 Microorganism and metabolism

Non-pathogenic solventogenic clostridia are used as the primary workhorse for ABE fermentation; they are rod-shaped, spore-forming gram-positive bacteria and typically strict anaerobes. Solventogenic clostridia can utilize a large variety of substrates from monosaccharides including many pentoses and hexoses to polysaccharides (Jones & Woods, 1986). The well-known *Clostridium* species for solvent production include *C. acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum,* and *C. saccharobutylicum* (Keis, Shaheen, & Jones, 2001). Among them, *C. acetobutylicum* is often called the 'Weizmann Organism' as it was applied in the industrial Weizmann process for ABE production; it is the most extensively studies with most

genetic information available. It has also been known for high level solvent production and good resistance to lignocellulosic inhibitors (Y.-S. Jang, Malaviya, et al., 2012). *C. beijerinckii* is another widely species with good performance and genetic stability in solvents production. Recently, extensive transcriptional analyses and genetic studies have been performed, especially for these two species (Brüggemann & Gottschalk, 2009). *C. saccharoperbutylacetonicum* N1-4 (HMT) can naturally produce very high levels of solvent and possesses various advantageous features. This strain has been broadly studied on fermentation characteristics including the desirable fermentation media and fermentation conditions (Tiam mun, Ishizaki, Yoshino, & Furukawa, 1995; S. Wang, Dong, Wang, et al., 2017).

In all these solventogenic clostridial strains, the metabolic pathways are very similar (Peter Dürre, 2005). Typically, ABE fermentation by solventogenic clostridia is a unique bi-phasic process. First, in the acidogenesis phase, carbohydrate carbon sources are degraded into acids (mostly acetic and butyric acids), hydrogen and carbon dioxide. Then in the solventogenic phase, the acids generated from the first phase are re-assimilated and converted into solvents along with the additional consumption of carbohydrate, as shown in **Fig. II-2**. During the acidogenesis, the accumulation of acids in the fermentation broth imposes growth inhibition for the cells. Especially at a low pH, the butyrate and acetate produced will present as the undissociated form and can pass through the cytoplasmic membrane via diffusion. Inside the cells, the acids will dissociate to form salts and protons due to the internal higher pH. Thus, the proton gradient across the membrane will be destroyed and cessation of cell growth will occur. By decreasing the concentration of

acids, this effect is mitigated during the solventogenesis phase (Maddox et al., 2000; Martin, Petitdemange, Ballongue, & Gay, 1983). During these processes, the pH first decreases to lower levels due to the accumulation of acids and then increases after solventogenesis is initiated (Jones & Woods, 1986).

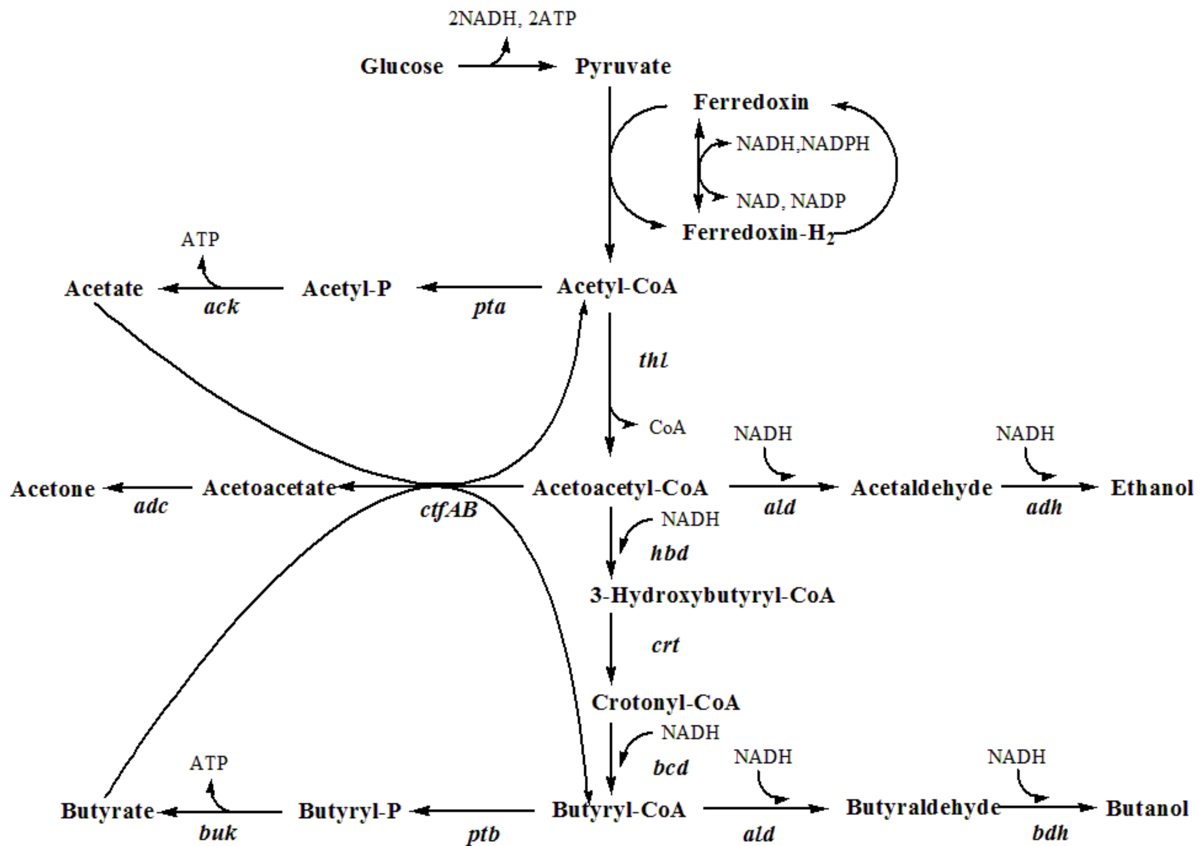


Fig. II-2. Metabolic pathways in solventogenic clostridia. Reactions which predominate during acidogenesis and solventogenesis are indicated by dotted and solid arrows, respectively. Thick arrows indicate reactions which activate the whole fermentative metabolism. Gray letters indicate genes and enzymes for the reactions. CAC and CAP numbers are the ORF numbers in genome and megaplasmid, respectively.

At the solventogenic phase, however, the end-product butanol (as well as other products) is also highly toxic to the microorganism. When butanol concentration reached a certain level, it will affect the integrity of cell membranes and thus inhibit the cell in a manner dramatically disrupting the membrane-associated functions, such as sugar uptake and solvents synthesis (C.-K. Chen & H. P. Blaschek, 1999; Y.-S. Jang, Lee, et al., 2012; Jones & Woods, 1986). It has been reported that *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 can tolerate up to 13 g/L butanol (Tashiro et al., 2013; Tomas, Beamish, & Papoutsakis, 2004). When the *C. acetobutylicum* ATCC 824 cell was exposed to the butanol solution at the threshold level of 16 g/L, it was completely degenerated by the autolysin excreted by itself (Van Der Westhuizen, Jones, & Woods, 1982). Many groups had studied the genetic modification of the wild type strain to enhance butanol tolerance. For example, in the pGROE1 mutant of *C. acetobutylicum* ATCC 824, the butanol tolerance was enhanced and the subsequent butanol concentration was improved from the practical maximum of 13 g/L to 18.5 g/L (Tomas et al., 2004). The mutant BA101, generated through chemical mutagenesis of *C. beijerinckii* NCIMB 8052, was able to produce 19 g/L of butanol with a butanol tolerance up to 21 g/L in a batch fermentation (C.-K. Chen & H. P. Blaschek, 1999). However, overall, the relatively low concentration of solvents produced during the fermentation is still a major limitation for the economically viable industrial production of this bioprocess.

The detailed metabolic pathways in the fermentation with the model microorganism *C. acetobutylicum* are shown in **Fig. II-2.** The glucose is first metabolized via glycolysis process

with a conversion of 1 mole to 2 moles of pyruvate, with a net production of 2 moles of adenosine triphosphate (ATP) and 2 moles of reduced nicotinamide adenine dinucleotide (NADH). Then the pyruvate is catalyzed by pyruvate ferredoxin oxidoreductase (*pfor*) to produce acetyl-CoA and reduced ferredoxin. During the acidogenic phase, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA by means of two analogous steps which firstly result in the production of the corresponding acyl-phosphate and ATP by phosphotransacetylase (*pta*) and phosphate butyryltransferase (*ptb*), respectively. Later, the acetate kinase (*ack*) and butyrate kinase (*buk*), which are analogous but distinct enzymes, mediate the formation of acetate and butyrate, respectively. Butyryl-CoA is formed from acetyl-CoA following a metabolic pathway with four enzymes involved: thiolase (*thl*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), crotonase (*crt*) and butyryl-CoA dehydrogenase (*bcd*) (Brüggemann & Gottschalk, 2009; Jones & Woods, 1986; S. Y. Lee et al., 2008). The onset of solventogenic phase involves a switch of the carbon flow from the acid-producing pathways to the solvent-producing pathways. Acetate and butyrate is metabolized by acetoactyl-CoA:acetate/butyrate-CoA transferase (*ctfA/ctfB*) into acetyl-CoA, butyryl-CoA and acetoacetate. Then, the acetoacetate is split into acetone and carbon dioxide by acetoacetate decarboxylase (*adc*). The acetyl-CoA and butyryl-CoA goes through two different pathways. In one pathway, acetyl-CoA is converted to ethanol by acetaldehyde dehydrogenase (*adhE*) and ethanol dehydrogenase (*adhA, adhB*); in another, butyryl-CoA is converted to butanol by butyraldehyde dehydrogenase (*adhE*) and butanol dehydrogenase (*bdhA, bdhB*). Among these enzymes, *ctfA/ctfB* are genes responsible for acids re-assimilation (Kosaka

et al., 2007), *adc* is responsible for acetone production, and the cassette EC, including *thl, hbd, crt* and *bcd* are responsible for the conversion of acetyl-CoA to butyryl-CoA (Hou et al., 2013). The final end product ethanol and butanol are mainly produced under the action of the bifunctional protein AdhE (aldehyde/alcohol dehydrogenase).

II.1.3 Clostridial isopropanol-butanol-ethanol fermentation

Even though the ABE fermentation has been performed worldwide at industrial scale in the past, it is currently considered as a less economically viable process than ethanol for biofuel production with respect to carbon recovery; the acetone produced during this process cannot be used as a fuel due to its corrosiveness to engine parts that are composed of rubber or plastic (P. Dürre, 1998). Reducing acetone production has been an important objective of clostridial metabolic engineering (J. Lee et al., 2012). Hence, it is desirable to suppress the formation of acetone or to convert it into another product that can be directly used as a fuel source.

The attempt to suppress the acetone production by metabolic engineering have been demonstrated to negatively decrease butanol production and accumulate the acetic and butyric acids (Y. Jiang et al., 2009; Tummala, Junne, & Papoutsakis, 2003). As a defensive mechanism against low culture pH, acetate and butyrate are re-assimilated by *ctfA/ctfB* to acetyl-CoA and butyryl-CoA, respectively, with concomitant production of acetone by *adc*. Thus, acetone formation is essential in the cell culture for cytosolic detoxification to increase the culture pH in response to acetic acid and butyric acid to produce butanol and ethanol (Zhao , Tomas , Rudolph, Papoutsakis, & Bennett, 2005). Hence, the conversion of acetone into another fuel source is more

desirable instead of suppressing the acetone production in the metabolic pathway. Isopropanol, as a simple secondary alcohol, shows a higher energy density than acetone (23.9 MJ/L vs 22.6 MJ/L) and can also be used as a fuel additive for the preparation of high-octane gasoline (Peralta-Yahya & Keasling, 2010). Some *C. beijerinckii* strains such as NRRL B593 can naturally produce isopropanol, instead of acetone, together with butanol and ethanol (Hiu, Zhu, Yan, & Chen, 1987). The IBE mixture can be directly used as a fuel source, which could save tremendous energy for the downstream recovery process. However, the IBE production titer (a total concentration of 5.87 g/l) and rate (a productivity of 0.12 g/L/h) with the natural strain are generally very low (**Table II-2**). Also no efficient genetic modification tools has been developed for these strains so far, which can be used to further improve their performance (Dusséaux et al., 2013).

Hence, there is a strong interest to transform the native ABE producers into efficient IBE producers by introducing the 'acetone-to-isopropanol' pathway through metabolic engineering. This has been demonstrated in several laboratories (Dai et al., 2012; Dusséaux et al., 2013; Y. S. Jang et al., 2013) by overexpressing the secondary alcohol dehydrogenase (SADH, encoded by *sadh*) from *Clostridium beijerinckii* NRRL B-593 (GenBank ID: AF157307.2) in the ABE-producing hosts. A summary of IBE production with various engineered solventogenic clostridia strains from recent literatures is shown in **Table II-2**. From these results, however, many limitations still exist for these genetically engineered strains, such as low efficiency in converting acetone into isopropanol, limited total solvent production, and genetic instability due to the plasmid-based overexpression.

Table II-2. Comparison of IBE production in various native and engineered clostridial strains

Strains	Ethanol (g/L)	Isopropanol (g/L)	Butanol (g/L)	IBE (g/L)	IBE yield (g/g)	Reference
Clostridium beijerinckii B593 (DSM 6423)	-	2.2	3.7	5.9	0.30	(Survase et al., 2011)
C. acetobutylicum ATCC 824-ADH integration	2.1	2.5	10.8	18	0.37	(Bankar et al., 2015)
C. acetobutylicum ATCC 824 (*sadh*)	1.5	8.8	13.7	24.4	0.35	(Collas et al., 2012)
C. acetobutylicum Rh8 (psADH)	1.3	7.6	15	23.9	0.31	(Dai et al., 2012)
C. acetobutylicum ATCC 824 (pIPA3)	0.8	6.1	10.2	17.1	0.28	(J. Lee et al., 2012)
C. acetobutylicum PJC4BK (pIPA3-Cm2)	1.9	4.4	14.1	20.4	0.3	(J. Lee et al., 2012)
C. acetobutylicum ATCC 824 *Δbuk* (pCLF952)	1	4.8	14.6	20.4	0.33	(Dusséaux et al., 2013)

II.2 Metabolic engineering in *Clostridium*

Metabolic engineering has been found its application in solventogenic clostridia for investigating the complex metabolism, improving the solvent production, as well as obtaining a new product through introducing heterologous pathways. Various metabolic engineering tools have been developed for various solventogenic clostridia species (Al-Hinai, Fast, & Papoutsakis, 2012; S. Y. Lee et al., 2008; Y. Wang et al., 2013). However, compared to the model organisms *Escherichia coli, Saccharomyces cerevisiae*, and *Bacillus subtilis*, the development of genetic engineering tools and metabolic engineering successes have lagged far behind (Joseph, Kim, & Sandoval, 2018).

II.2.1 Plasmid transformation

For the genetic manipulation of clostridial strains, often the most difficult step is to establish an efficient method for introducing foreign DNA into host cells (Michael E. Pyne, Bruder, Moo-Young, Chung, & Chou, 2014). Thus, establishing a methodology for efficient transfer of plasmid DNA into the host is the premise for *Clostridium* genetic modification. There are two primary DNA transfer methods within *Clostridium*: conjugation and transformation (Michael E. Pyne et al., 2014). Bacterial conjugation involves direct cell-to-cell transfer of plasmid DNA from one donor species to the target, or recipient, species, while transformation involves the uptake of DNA by competent cells. Transformation is more commonly employed in *Clostridium* due to its technical simplicity, better reliability, independence from donor species, and mostly high efficiency (Purdy et al., 2002). There are two primary approaches for transforming bacterial cells:

heat shock and electroporation. For Gram-positive *Clostridium*, transformation via electroporation is generally employed because of its high efficiency than heat shock approach (Oultram et al., 1988). Due to the different features of the membrane among different strains, there is almost no commonality in transformation protocols that can be applied among different species or strains without rigorous optimization.

The presence of highly active restriction-modification (RM) systems is the most probable factor responsible for hindering the electro-transformation. If improperly methylated plasmid is used to transform even highly competent cells, few or no transformants will be obtained (L. D. Mermelstein, Welker, Bennett, & Papoutsakis, 1992). Before attempting to transform an uncharacterized strain of *Clostridium*, it is necessary to assay crude cell lysates for the presence of Type II restriction endonucleases by incubation of the active lysate in the presence of unmethylated plasmid DNA. Once the RM systems is identified, efforts can be made to protect the recognition sequences via methylation on transforming plasmids (L. Mermelstein & Papoutsakis, 1993). The development of an efficient DNA methylation system was an important step, which can be performed *in vivo* by expressing the methyltransferase in *E. coli* cloning strains or, ideally, *in vitro* if the methyltransferase is commercially available. For example, it has been demonstrated that many *Clostridium* species produce isoschizomers of the *E. coli* Dam (DNA adenine methylase) RM systems (Roberts, Vincze, Posfai, & Macelis, 2014), while most common laboratory *E. coli* strains are Dam^+. Thus, the Dam^- *E. coli* hosts can be selected to prepare the restrict plasmid DNA and such barriers can thus be overcome. Mermelstein and Papoutsakis (L.

Mermelstein & Papoutsakis, 1993) reported that methylation of the shuttle vectors with φ3TI methyltransferase (encoded by *B. subtilis* phage φ3TI) prior to transformation greatly reduces or prevents the degradation of the transforming plasmid DNA by the attack of a strong restriction system (*Cac824I*) present in *C. acetobutylicum*.

The physical barrier imposed by the Gram-positive cell wall is another factor responsible for hindering the transformation of plasmid DNA to a recipient cell (Aune & Aachmann, 2010). Since the cell wall is continuously remodeled throughout the course of bacterial growth, the growth phase of cells at the time of harvest is often critical and it is usually optimal to use mid-logarithmic-phase cells for electro-transformation. The structure and density of the cell wall can also be altered by the formulation of the growth medium and washing with appropriate electroporation buffer, including the pH and buffer type and strength, in addition to the presence of cell-wall-disrupting agents and associated osmotic stabilizers (Aune & Aachmann, 2010). For example, 10% glycerol solutions can be efficiently used to electrotransform the plasmids for *C. beijerinckii,* while the HEPES, MOPS and SMP buffering systems have been shown to be good for the permeabilization of other clostridial strains (Klapatch, Guerinot, & Lynd, 1996; Nakayama et al., 2007; S. Wang, Dong, Wang, et al., 2017). Despite the plasmid-based overexpression strategies had been widely used in *Clostridium* genus, many limitations still exist, such as the instability of plasmid vector and the requirement of antibiotics for the cultivation to maintain the plasmid.

II.2.2 Genome editing technologies

For modifying genes on the genome, success with homologous recombination has been demonstrated in solventogenic clostridia strains. Homologous recombination achieves genetic modification through either the 'single-crossover' mechanism in which an integrated plasmid serves as an insertional mutagen, or the 'double crossover' mechanism in which an introduced alternative allele is exchanged with the wild-type allele (Brüggemann & Gottschalk, 2009). The former type of mutant is inherently unstable, while the latter type has proven difficult to isolate, partly because the use of negative selection markers.

In solventogenic clostridia strains, the retrohoming of Mobile Group II introns has been widely used for genome modification in clostridia (Heap, Pennington, Cartman, Carter, & Minton, 2007; Y. Wang et al., 2013). This technology works by inserting an intron into chromosomal DNA through the plasmid-based monocistronic expression of a ribonucleoprotein complex comprising RNA in a lariat configuration (acting as a ribozyme) and an intron-encoded protein (IEP). The Mobile Group II introns are minimally dependent on host factors, as the IEP (LtrA in the model system based on the *Lactococcus lactis* Ll.LtrB intron) performs multiple activities: maturase for facilitating RNA splicing, endonuclease for cleavage of the DNA strand opposite the RNA splice, and reverse transcriptase which uses intron RNA as template to insert DNA into the host chromosome. The host DNA repair machinery replaces intron RNA with DNA, completing the insertion (Joseph et al., 2018). The term Targetron, which is based on Group II introns, was first used to refer to targeted Group II introns when the L1.LtrB intron was further modified to include

a retrotransposition-activated selection marker (RAM, often an antibiotic resistance gene), providing a means to select for successful targeting events (Zhong, Karberg, & Lambowitz, 2003). The RAM is inserted into the domain IV of the intron inactivated by the insertion of a Group I intron which is self catalytically spliced out of mRNA in an orientation dependent manner. The marker gene and group I intron are oriented in the opposite directions such that it is only spliced out of the L1.LtrB mRNA, and a functional marker gene can only be expressed after successful chromosomal insertion occurs. ClosTron was developed as an adaptation of Targetron technology for efficient gene targeting specifically in *Clostridium* species. The original ClosTron plasmid, pMTL007, tailored the commercially available *E. coli* Targetron vector, pACD4k-C, to include standardized genetic parts such as promoters, origins of replications and RAMs suitable for efficient gene editing in *Clostridium* (Heap et al., 2007). It has been employed in targeted gene disruption across the *Clostridium* genus including *C. acetobutylicum*, *C. beijerinckii, C. botulinum*, and *C. difficile* (Joseph et al., 2018). Even though these systems have been widely used, they have several drawbacks. First, its efficiency heavily relies on the precise prediction of insertion sites using a specific algorithm; Second, the perturbation achieved with TargeTron is virtually merely an insertion-based disruption, but not a true deletion, and its capability for gene integration is very limited; moreover, the intron insertion is not stable and may be spliced back out by the intron-encoding protein (Heap et al., 2007; Y. Wang et al., 2013). Thus, a facile and efficient method capable of performing precise, scarless, and stable genome manipulations is desirable to carry out efficient metabolic engineering on solventogenic clostridia.

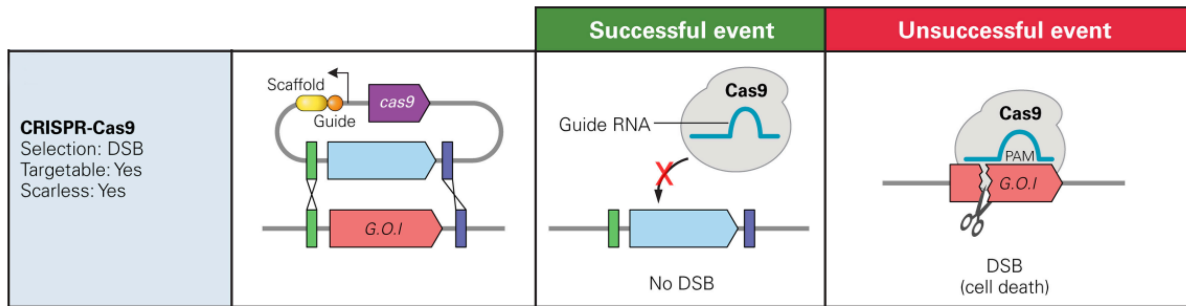


Fig. II-3. CRISPR-Cas9 used for genome editing in *Clostridium* spp. and its mechanism of selection. G.O.I: the native gene of interest (Joseph et al., 2018).

II.2.3 CRISPR based editing

The clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein (Cas or Cas9) system is an RNA-guided immune system in bacteria and archaea that can efficiently confers resistance to foreign genetic elements such as plasmids and phages (Sorek et al., 2013). Recently, the CRISPR-Cas9 system has been engineered as a cutting-edge genome engineering tool for both eukaryotic and prokaryotic cells (Jinek et al., 2012; S. Wang, Dong, Wang, et al., 2017). In this system, a mature CRISPR RNA (crRNA) and the trans-activating tracrRNA complex are co-processed together to make a dual tracrRNA-crRNA, directing the Cas9 nuclease to the site-specific DNA sequence with the protospacer-adjacent motif (PAM). Recently, the CRISPR-Cas9 system from *Streptococcus pyogenes* (*spy*Cas9) has been exploited for genome engineering in various organisms, including for example, both the Gram-positive *S. pneumonia* and *Lactobacillus reuteri* and the Gram-negative *E. coli* (W. Jiang et al., 2013; Oh & van Pijkeren, 2014). In the case of *spy*Cas9, the PAM consensus sequence is NGG, providing many possible target sites (Chylinski et al., 2013). By delivering the Cas9

nuclease complexed with a synthetic gRNA into a cell, the cell's genome can be cut at a desired target site, allowing existing genes to be removed and/or new ones added. For the gene editing in bacteria, this system is primarily functional as a tool for selecting edited cells against non-edited background cells thereby leading to high efficiency of genome engineering. Genetic mutations can be introduced through non-homologous end-joining (NHEJ) or homology directed repair (HDR) by providing a DNA editing template (Hsu, Lander, & Zhang, 2014). The endonuclease activity of Cas9 can then lead to a double strand breakage (DSB). So far, CRISPR-Cas9 based tools have shown their versatility for gene deletion or insertion and have been reported as successfully utilized in various bacteria strains (Chung et al., 2017; W. Jiang et al., 2013; S. Wang, Dong, Wang, et al., 2017; Y. Wang et al., 2015), with attractive features such as ease of use, high efficiency, strong adaptability, and multiplex targeting capability. The use of CRISPR-Cas9 represents a major advancement in *Clostridium* gene editing as scarless and markerless edits are enabled.

Table II-3. CRISPR-Cas9 based genetic editing and gene repression in *Clostridium* spp. (Joseph et al., 2018).

Species	Homology arm length (bp)	Transformation Eff. (CFU/μg)	Editing efficiency (%)	Cas9 promoter	Gene targeted	Desired edit	Reference
C. acetobutylicum	664	NR	100	*tet* (inducible)	upp	DNM	(Wasels et al., 2017)
	500	NR	100		upp	66 bp del	
	1000	NR	100		upp	306 bp rep	
C. beijerinckii	1000	NR	67	*spollE*	pta	50 bp del	(Y. Wang et al., 2016)
	1000	$1.05*10^2$	80	*bgaL* (inducible)	pta	50 bp del	
	1000	$3.94*10^2$	0		pta	1500 bp del	
	1000	$2.92*10^2$	87		pta	1614 bp del	
	1000	NR	>99		pta	SNM	
	1000	NR	NR	*spollE*	spo0A	262 bp del	(Y. Wang et al., 2015)
C. autoethanogenum	NR	NR	>50	*tet* (inducible)	caethg_0385	del	(Nagaraju et al., 2016)
	NR	NR	>50		caethg_05552	del	
C. acetobutylicum	500	0.2	100	*thl*	cac1502	rep w/trunc.	(Bruder et al., 2016)
	1000	0.38	100		cac1502	rep w/trunc	
	1000	0.4	NR		cac1502	rep w/Pthl::afp	
C. ljungdahlii	NR	NR	100	*ptb*	pta	1000 bp del	(H. Huang et al., 2016)
	NR	NR	>75		adhE1	2600 bp del	
	NR	NR	100		ctf	1200 bp del	
	NR	NR	>50		pyrE	570 bp del	
C.saccharoperbutylacetonicum N1-4	1000	$1.5*10^4$	NR	*bgaL* (inducible)	buk	del	(S. Wang, et al., 2017)
	1000	$1.6*10^4$	75		pta	del	
C. pasteurianium	1000	2.6	100	*thl*	cpaAIR	567 bp del	(Michael E Pyne et al., 2016)

In several *Clostridium* species, the CRISPR-Cas9 system has been used as a counter-selection tool to select for homologous recombination mutants (Bruder et al., 2016; H. Huang et al., 2016; Nagaraju et al., 2016; S. Wang, Dong, Wang, et al., 2017; Y. Wang et al., 2015) **(Table II-3)**. Because *Clostridium* species lack of efficient NHEJ systems, a Cas9-mediated chromosomal DSB results in cell death (T. Xu, Li, He, Van Nostrand, & Zhou, 2017). Thus, to select for successful homologous recombination events, one can selectively eliminate non-edited members of the population by targeting the wild type sequence **(Fig. II-3)**. However, there are still some limitation for genome engineering in *Clostridium* using CRISPR-Cas9. For example, simultaneous constitutive expression of the sgRNA and Cas9 protein often resulted in few or no transformed colonies in the presence of a homologous repair donor vector, as DSBs result in cell death before recombination can occur (Bruder et al., 2016; Qi Li et al., 2016; Nagaraju et al., 2016; S. Wang, Dong, Wang, et al., 2017). This can be addressed by placing Cas9 expression under the control of an inducible promoter, such as lactose promoter (S. Wang, Dong, Wang, et al., 2017; Y. Wang et al., 2016). Another strategy is to use a two-plasmid system, where the donor DNA and sgRNA are introduced separately from the Cas9 gene. This method requires two separate transformation events, which avoids the transformation of very large plasmids (Wasels et al., 2017). Wasels et al. reported successful recombinants were isolated at a rate up to 100% with commonly observed efficiencies of greater than 50% by using these methods. *Clostridium* genome engineering has made much progress recently in the development of synthetic biology tools, although it still lags behind other model organisms (e.g., *E. coli*).

II.3 Chemical composition of lignocellulosic biomass

Feedstock is typically the greatest fraction of cost in ABE fermentation. Thus, employing the cheapest feedstock and reaching the theoretical maximum conversion to solvent will result in the best ABE production economics. In the early twentieth century, the feedstocks of the commercial ABE fermentation were primarily food-based starches from maize, potatoes and wheat, or sugars from molasses (Jones & Woods, 1986). However, these food-based starches have a number of problems and the most contentious issue is 'fuel vs food'. As the solvent are produced directly from food crops, the rise in demand for feedstock has led to an increase in the volume of crops being diverted away from the global food market. This has been blamed for the global increase in food prices over the last couple of years (Y.-S. Jang, Malaviya, et al., 2012). It had been estimated that the cost of food-based feedstock accounted for 60-80% of the total production cost in the commercial process (Taconi et al., 2009). Therefore, the resurgence of this bioprocess for ABE production depends largely on the availability of low-cost and abundant feedstocks.

Lignocellulosic biomass is an attractive renewable energy feedstock supplies, due to its abundant availability domestically and globally. It is superior to other feedstock due to two major reasons: (1) it has no competition with food supplies; and (2) it shows the desired physical and chemical properties suitable for production, harvest, handling, storage, and transportation (Balan, 2014). The estimated annual potential availability of biomass in the U.S. is more than 1 billion tons by 2030 and the annual worldwide production is 10-50 billion tons (DOE, 2016).

The precise chemical composition of lignocellulosic materials varies on the types of species, sources, and even parts of a given plant. In general, it is mainly composed of cellulose (40-50%), hemicellulose (15-30%), lignin (16-35%) and a small amount of extractives (1-5%) **(Table II-4)** (Balan, 2014; Biswas, Uellendahl, & Ahring, 2015). Cellulose and hemicellulose are polymers of carbohydrates and their building blocks are monosaccharides such as glucose, xylose, and mannose. Lignin is a polymer of complex aromatic alcohols. All these components are bundled in a recalcitrant form. Depending on the species, the chemical compositions could exhibit significant variations, especially on the hemicellulose and lignin content. Generally, the forestry biomass contains higher lignin content while the agricultural residues and herbaceous biomass are richer in the hemicellulose content. Moreover, even within the same type of lignocellulose, the chemical composition could also be different as determined by the age of plant, growth conditions, climate, etc. (Bensah & Mensah, 2013).

Table II-4. Chemical composition of selected lignocellulosic biomass (dry weight basis) (Biswas et al., 2015)

Lignocellulosic biomass	Cellulose, %	Hemicellulose, %	Lignin, %
Corn stover	37–42	20–28	18–22
Sugarcane bagasse	26–50	24–34	10–26
Wheat straw	31–44	22–24	16–24
Hardwood stems	40–45	18–40	18–28
Softwood stems	34–50	21–35	28–35
Rice straw	32–41	15–24	10–18
Barley straw	33–40	20–35	8–17
Switch grass	33–46	22–32	12–23
Energy crops	43–45	24–31	19–12
Manure solid fibers	8–27	12–22	2–13
Municipal organic waste	21–64	5–22	3–28

Cellulose, as the primary constituent in biomass (30-45 wt% of dry biomass), is a polysaccharide polymerizing with glucose as its monomer, which condenses through β (1-4)-glycosidic bonds. The molecular of cellulose has a linear, unbranched structure, which consists 10,000 to 15,000 glucose units, and the degree of polymerization (DP) could stretch up to 17,000 (Harmsen, Huijgen, Bermudez, & Bakker, 2010). The individual cellulose molecules are linked together to form elementary microfibrils, in which aggregated by intermolecular hydrogen bonding into larger subunits called fibrils. It is reported that the inter-and-intra molecular hydrogen bonds remained robustly stable until subjecting to severe conditions at 320 ℃ and 25 MPa, at which the fibers started to de-crystallized into amorphous form (Deguchi, Tsujii, & Horikoshi, 2006).

Hemicellulose, as the second major component in biomass (10-30 wt% of dry biomass), is a non-homogeneous and branched polysaccharides made up of hexose (D-glucose, D-galactose and D-mannose), pentose (D-xylose and L-arabinose), acetyl group and uronic acids (D-glucuronic acid and D-galacturonic acid) (G ŕio et al., 2010). Unlike cellulose, hemicelluloses generally occur in the form of hetero-polysaccharides with branches and result in a lower DP of approximately 100-200. Hemicellulose found in hardwood trees and herbaceous biomass is predominantly xylan with some glucomannan, while in softwoods it is mainly rich in galactoglucomannan and contains only a small amount of xylan (G ŕio et al., 2010). Due to the randomly amorphous branched structure and low DP, hemicellulose is highly susceptible to be hydrolyzed by acid or cellulase enzyme.

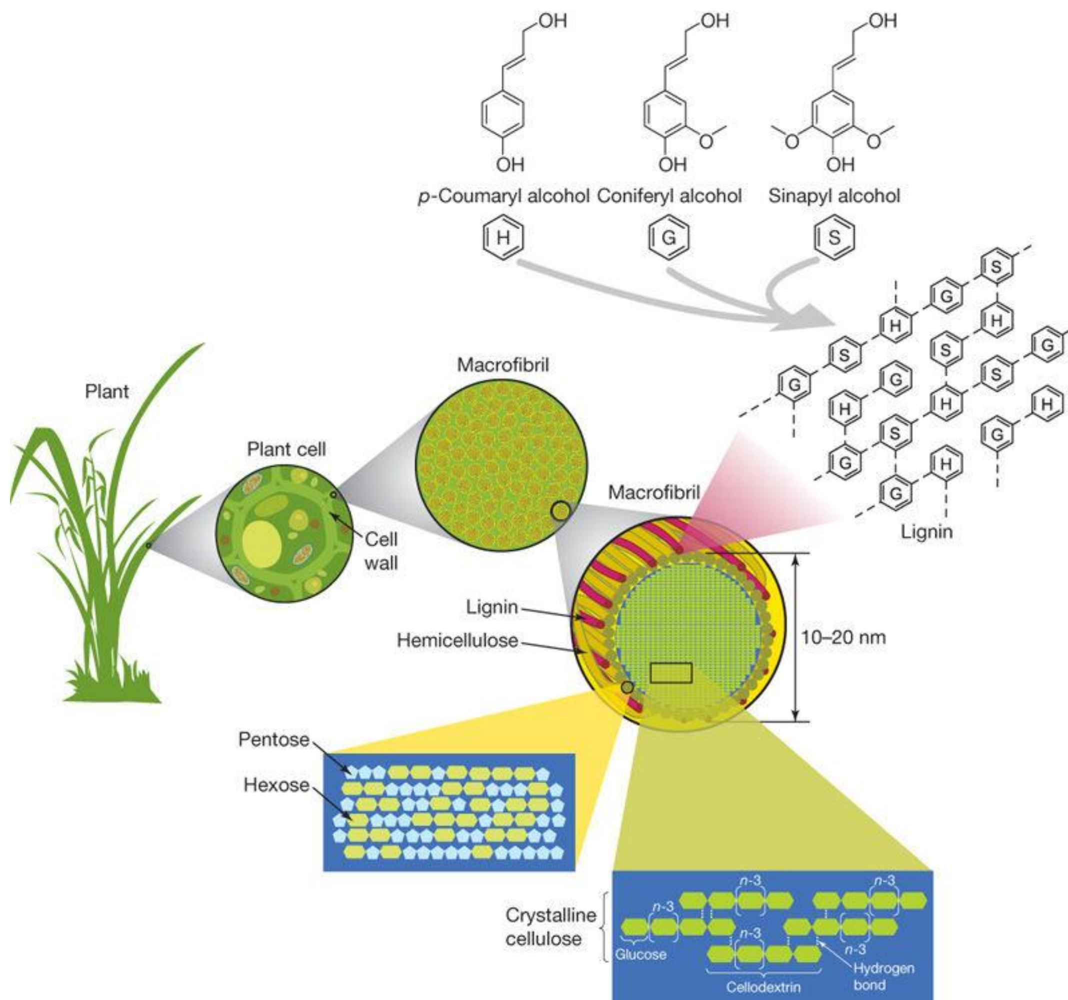


Fig. II-4. Structure of lignocellulose (Rubin et al., 2008)

Lignin, as the major non-carbohydrate component in biomass (10-30 wt% of dry biomass), is a complex copolymer of three phenyl propionic alcohol monomers, including ρ-coumaryl, coniferyl and sinapyl alcohol. In contrast to cellulose, lignin forms a three-dimensional network randomly cross-linking with hemicellulose through the covalent and hydrogenic bonds. This cross-linked network forms a matrix and embraces the crystallized cellulose fibers, protecting the fibers from being enzymatically or mechanically damaged (Harmsen et al., 2010). As shown in

Fig II-4, the microfibrils contain alternating phases of highly ordered (crystalline) and randomly oriented (amorphous) cellulose embedded in a matrix of hemicellulose. The cellulose and hemicellulose fractions are covered in an amorphous layer of lignin (Mosier et al., 2005). Thus, the presence of lignin and hemicellulose makes the access of cellulase enzymes to cellulose becomes difficult, reducing the efficiency of the later hydrolysis and fermentation process. Therefore, a pretreatment process is necessary before hydrolysis and fermentation based on the lignocellulosic materials. Because lignin is more abundant in the woody biomass than the agricultural residues or herbaceous biomass, woody biomass is more recalcitrant than the grass or agricultural residues and thus requires more harsh pretreatment conditions (Kumar & Wyman, 2009).

II.4 Bioconversion of lignocellulosic biomass to ABE

A multi-stage bioconversion process is needed to convert lignocellulosic biomass to the advanced ABE product. These processes include (1) pretreatment to disrupt the recalcitrant structure of lignocellulosic biomass and make cellulose accessible to hydrolytic enzymes, (2) hydrolysis to hydrolyze the carbohydrate polymers to fermentable monosaccharides, (3) ABE fermentation, and (4) product recovery (**Fig. II-5**). As compared to other fermentation feedstocks like commercial glucose and starch biomass, lignocellulosic biomass required extra pretreatment, hydrolysis and detoxification processes for sugar production before fermentation to biobutanol production (Mosier et al., 2005).

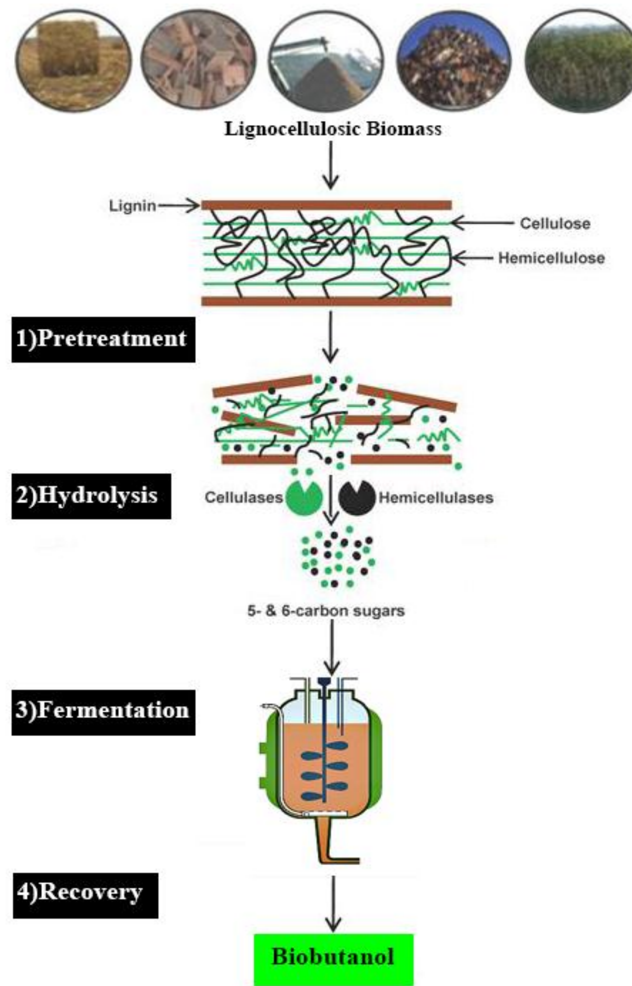


Fig. II-5. Schematic picture for the conversion of lignocellulosic biomass to biobutanol

II.4.1 Pretreatment

The overall purpose of pretreatment is to break down the shield matrix formed by lignin and hemicellulose, to disrupt the crystalline structure and reduce the degree of polymerization of cellulose. This process is a crucial step for overall process in cellulosic biobutanol production because it is one of the most costly processing steps and the challenges has been widely reported (Balan, 2014). An effective pretreatment process should (a) maximize the digestibility of

carbohydrates in the subsequent enzymatic or acid hydrolysis; (b) minimize loss of cellulose and hemicellulose composition; (c) maximize the production of other valuable co-products, e.g. lignin; (d) minimize the toxicity to subsequent enzymatic hydrolysis and/or fermentation; (e) minimize the operational cost for capital and operational; (f) be scalable to industrial size; and (g) minimize solid-waste residues (Humbird et al., 2011; Ibrahim et al., 2017; Mosier et al., 2005; Peralta-Yahya & Keasling, 2010). Lignin starts decomposed at temperature higher than 220 ℃, while hemicellulose at 180 ℃ and cellulose at 280 ℃ (Bahrin et al., 2012). A high denaturation temperature needs high energy input, makes the pretreatment become a tedious process, while a low denaturation temperature cannot disrupt the shield matrix efficiently. So far, however, none of the pretreatment technologies have completely satisfied all these listed criteria. Hence, more research efforts are required to improve the pretreatment for lignocellulosic biomass.

Over the decades, various pretreatment technologies have been developed, which normally can be categorized into four categories: physical, physico-chemical, chemical and biological methods. Physical pretreatment methods include comminution (including dry, wet, and vibratory ball milling), grinding, chipping, irradiation or combination of them. The effects of physical pretreatment involve the reduction in the particle size, the degree of crystallinity, polymerization and the increase of accessible surface area for the cellulase enzyme (Kumar & Wyman, 2009; Mosier et al., 2005). However, this type of pretreatment is lightly used as a major industrial option due to high equipment and energy cost. Physico-chemical is the method in combination of both chemical and physical process. The widely investigated processes include steam explosion

(autohydrolysis or with addition of SO_2), ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP), liquid hot-water pretreatment (LHW), supercritical Fluid (SCF) Pretreatment, CO_2 explosion and microwave-chemical pretreatment (Taherzadeh & Karimi, 2008). Depending on the pretreatment reagents, chemical pretreatment can be categorized as acid-, alkali-, organic solvent-based. Different types of chemical pretreatments have different effects on the change of the structure of lignocellulose (Kumar & Wyman, 2009; Mosier et al., 2005). Under acid-catalyzed pretreatment (using mineral acids such as H_2SO_4, HCl, H_3PO_4, and HNO_3 or organic acids like fumaric, maleic, and acetic acid), hemicellulose is solubilized to monomeric xylose leaving the cellulose and lignin behind. The pretreatment under alkaline conditions are typically characterized as lignin degradation and removal (Balan, 2014; Kumar & Wyman, 2009). Organosolv pretreatments employ organic solvents like methanol, ethanol, acetone, ethylene glycols, and tetrahydrofurfuryl alcohol at either neutral or acidic conditions to extract hemicellulose and lignin portion from biomass (Lai, Tu, Li, & Yu, 2014). Ionic liquid pretreatment has been advocated as a green method to fractionate carbohydrates from lignin through dissolution cellulose portion of biomass. However, the cost of ionic liquid and catalyst required for pretreatment are major bottlenecks preventing commercialization of this technology (Cruz et al., 2013; Qiang Li et al., 2009). Biological pretreatment is to effectively degrade lignin but very little cellulose in biomass by using the microorganisms like brown, white, and soft-rot fungi (Sanchez & Cardona, 2008). These processes are operated at mild conditions and require low capital costs when compared to expensive reactor systems required for physical/chemical

pretreatment processes. However, the biological process is a relatively slow process requiring several days to pretreat the biomass. Furthermore, the sugar conversion after the microbial pretreatment process is lower when compared to chemical pretreatment (Sun & Cheng, 2002).

Among these pretreatment categories, chemical methods are the most widely used methods in industry because of the reasonable high yield with low cost and short reaction time. And among chemical pretreatment methods, acid catalyzed pretreatment is one of the most widely performed for lignocellulosic biomass and used for ABE fermentation (T. Ezeji, N. Qureshi, & H. P. Blaschek, 2007; Ibrahim et al., 2017; Nanda, Dalai, & Kozinski, 2014). The detailed features of acid catalyzed pretreatments methods are discussed below.

Treatment of lignocelluloses with acid to break the matrix of biomass has received considerable research attention over the years and nearly commercialized in a wide variety of biomass types (Digman et al., 2010; Wyman et al., 2011; J. Xu, Thomsen, & Thomsen, 2009b). Based on the acid concentrations, acid-catalyzed pretreatment can be divided into concentrated acid pretreatment (30-70%) and dilute acid pretreatment (0.5-2%). The advantages of concentrated acid pretreatment at low temperature (40 ℃) includes low temperature treatment, low production of degradation products, and the capacity to directly saccharify the cellulose and hemicellulose portion from various types of lignocellulose into sugars without subsequent enzymatic hydrolysis (Balan, 2014). However, the corrosion of equipment, acid recovery, and neutralization waste when acid is not recovered appear to be the major limitation in this method. (Harmsen et al., 2010; Kumar & Wyman, 2009).

In contrast to the concentrated acids, dilute acid pretreatment at high temperature (100-200 ℃) has become a state of the art technology for pretreating any lignocellulosic biomass especially for herbaceous and agricultural lignocellulose, such as switchgrass, corn stover, and rice/wheat straws (Harmsen et al., 2010; Mosier et al., 2005). This type of pretreatment significantly improves the hemicellulos hydrolysis to fermentable sugars and dissolving lignin with less degradation. It results in high recovery of the hemicellulosic sugars in the pretreatment liquid, and in a solid cellulose fraction with enhanced enzymatic convertibility (Carrasco et al., 1994). Unfortunately, dilute acid pretreatment also has some drawbacks, such as the reactor needs to be resistant to the corrosive acid especially at elevated temperature, gypsum will be formed during neutralization after treatment with acid, and acid reagent left in the prehydrolysates are usually severe inhibitors for the subsequent microbial fermentation processes (Jönsson, Alriksson, & Nilvebrant, 2013).

II.4.2 Detoxification

During the pretreatment, additional inhibitors can be generated from the degradation of the biomass in prehydrolysates. Furan aldehydes and aliphatic acids are carbohydrate degradation products, while lignin is the main source of phenolic compounds, as indicated by guaiacyl (4-hydroxy-3-methoxyphenyl) and syringyl (4-hydroxy-3,5-dimethoxyphenyl) moieties found in many phenolics (Jönsson et al., 2013). The phenolic compounds are known to increase biological membrane fluidity and can cause loss of cellular integrity, thereby affecting its role in selective barriers and enzyme matrices (Heipieper, Weber, Sikkema, Keweloh, & de Bont, 1994). They

were found to significantly affect both cell growth and ABE production of *Clostridium* (T. Ezeji et al., 2007). In addition, significant level of acetic acid is usually produced during the biomass pretreatment process, which is also a strong inhibitor, for example, for the microbial ethanol fermentation process (Wei et al., 2015).

Several methods can be taken to avoid problems caused by inhibitors. One way is to genetically modify the microorganisms adaptive to the toxic environment or to improve the strain tolerance to lignocellulosic hydrolysates. It has been achieved by overexpressing homologous or heterologous genes encoding enzymes that confer resistance towards specific inhibitors (Almeida et al., 2007; Cerisy et al., 2017; Gorsich et al., 2006; Guo et al., 2012; Petersson et al., 2006). An NADPH-dependent alcohol dehydrogenase (ADH6p) was identified and reported as one of the key enzymes responsible for HMF and furfural reduction in *S. cerevisiae*. The further overexpression of the corresponding ADH6 gene generated a yeast strain with at least 4-fold increased HMF uptake in defined medium under both aerobic or anaerobic conditions (Petersson et al., 2006). It was reported that a *C. beijerinckii* NCIMB 8052 mutant strain *C. beijerinckii* IB4 that can tolerate to high level of inhibitors was screened by low-energy ion implantation and used for butanol fermentation. Evaluation of toxicity showed that this mutant had a higher level of tolerance than parent strain for five out of six phenolic compounds tested (the exception was vanillin) (Guo et al., 2012).

The other way to solve this problem caused by inhibitors is to detoxify the pretreatment prehydrolysates. This includes techniques of physical, chemical and biological treatment to

remove or modify inhibitors (Jönsson & Martín, 2016; Mosier et al., 2005; Nanda et al., 2014). Physical treatments, which include evaporation, steam stripping, solvent extraction, aqueous two-phase extraction, absorption (activated carbon) and supercritical liquid, normally tend to remove the inhibitors rather than modify their chemical structures. Chemical treatments, which include alkaline detoxification, detoxification with reducing agents, ion exchange and chemical extraction, are mainly conducted to convert inhibitors into less toxic compounds. Biologically, many fermenting microorganisms are able to detoxify weak inhibitors such as aldehydes and carboxylic acids. This type of detoxification method is mainly dependent on microorganism types, the inoculation size and the chemical structure of the inhibitors (Almeida et al., 2007).

Activated carbon adsorption. Among those methods, activated carbon adsorption is inexpensive, easy to integrate into the process, and able to remove inhibitors selectively (K. Liu et al., 2015; Miyafuji et al., 2003; Solange In Mussatto, es, Roberto, & es, 2001; Solange Inês Mussatto & Roberto, 2004). This method has been studied in detoxification of prehydrolysates due to their excellent absorptivity. With the high degrees of microporosity, it can improve the fermentability of prehydrolysates by removing furans and phenolic compounds but not reducing the fermentable sugar concentrations. It was reported that the removal of phenolic compounds was dependent on hydrolysate/charcoal ratio, treatment time and treatment temperature (Solange In Mussatto et al., 2001). By increasing the treatment temperature for activated carbon, it was found to increase the adsorption of phenolic compounds and furans due to the increase in the hydrophobicity (Miyafuji et al., 2003). Moreover, it is also attractive since activated carbon can

be derived from biomass char, a low value byproduct of the thermochemical conversion of biomass through pyrolysis.

II.4.3 Enzymatic hydrolysis

Pretreatment process disrupts the recalcitrant structure, fractionates the main component, and enhances biomass digestibility for downstream processes. However, the key issue of the biorefinery has not been addressed until the carbohydrates of lignocellulose are decomposed into fermentable sugars (Jørgensen, Kristensen, & Felby, 2007). Thus, the next step of degradation of lignocellulose into fermentable sugars is hydrolysis which is normally done by enzymes.

In comparison to other hydrolysis methods (e.g. concentrated acid hydrolysis), enzymatic hydrolysis has several key advantages, such as lower corrosion, milder operation conditions, higher sugar yields, lower energy input and lower level of inhibitory products (Taherzadeh & Karimi, 2007). Cellulases and hemicellulases are usually used to hydrolyze cellulose and hemicellulose into fermentable monomeric sugars. The commonly used hydrolases include exo-1,4-β-D-glucanases (CBH), endo-1,4-β-D-glucanases (EG), 1,4-β-D-glucosidases (BG), endo-1,4-β-D-xylanases, 1,4-β-D-xylosidases, endo-1,4-β-D-mannanases, and 1,4-β-D-mannosidases (Jørgensen et al., 2007). These enzymes work synergistically to hydrolyze polysaccharides into monosaccharides by creating new accessible sites for each other. Optimal conditions for cellulases have been reported as temperature of 40-50 ℃ and pH 4-5, while optimal assay conditions for hemicellulases are often similar (Maitan-Alfenas, Visser, & Guimarães, 2015). Typically, three steps, namely, transportation of enzymes from bulk solution to the substrate

surface, adsorption of the enzymes to the substrates, hydrolysis of the polysaccharides into subunits, and desorption of enzymes and products back to the bulk solution, are involved in the process of enzymatic hydrolysis (Walker & Wilson, 1991). As a result, biomass sugars are released by hydrolyzing the cellulose and hemicellulose. Enzymatic digestibility is often correlated with the characteristics of lignocellulose (such as chemical composition, porosity, degree of crystallinity and polymerization, accessible surface area, surface charge, etc.), the enzyme-related factors (such as specific activity, nonspecific binding, end-products inhibition), hydrolysis reaction conditions (pH, temperature and agitation speed), and etc. (Cara et al., 2007; Jørgensen et al., 2007; Saha, Iten, Cotta, & Wu, 2005; Taherzadeh & Karimi, 2007). However, the intrinsic problems associated with enzymatic hydrolysis are long hydrolysis time, expensive catalyst, and end-product inhibition (Balan, 2014; Jørgensen et al., 2007; Taherzadeh & Karimi, 2007).

II.4.4 Microbial fermentation

In terms of the methods for fermentation of lignocellulosic feedstocks into butanol by *Clostridium* strains, mainly three process concepts have been developed: separate hydrolysis and fermentation (SHF), consolidated bioprocessing (CBP), and simultaneous saccharification and fermentation (SSF) (Balan, 2014; Taherzadeh & Karimi, 2007; Tengborg, Galbe, & Zacchi, 2001; Walker & Wilson, 1991).

The SHF, which is the most traditional method for biofuels production, is a two-stage process in which the hydrolysis of biopolymers to sugars and sugar fermentation are conducted separately.

The optimal conditions for most of commercial enzyme (SPEZYME® CP or Cellic® CTEC-2) are at the temperature of 50±5 ℃ and pH of 4.0-5.0, whereas most *Clostridium* strains have better solvent production performance at temperature below 35 ℃ and broth pH of 6.5±1 (Baral et al., 2016; T. C. Ezeji et al., 2007). The main advantage of SHF is that both hydrolysis and fermentation are performed at their optimal conditions. The major drawback of SHF, however, is the accumulation of sugars in hydrolysis step may cause end-product inhibition to enzymes which will decrease the sugar yield and the slow rate of enzymatic hydrolysis and fermentation process, which largely compromises the overall bioconversion yields (Olofsson, Bertilsson, & Lidén, 2008).

CBP is a relatively new bioconversion strategy that allows the enzyme production, hydrolysis and fermentation to biofuels occurring in one single process without enzyme addition (Balan, 2014). Notably, the production of enzymes used in hydrolysis is produced in situ in the fermentation vessel leading to significantly reduce the capital cost for operation and purchasing enzymes. Since this process takes place in a single step, the choice of microorganism is of great importance. The microorganism chosen must have enzymatic machinery to produce both a variety of hemicellulases and cellulases as well as produce high solvent titers (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010). However, few wild-type microorganisms are able to fulfill all these functions and thus most of the cultures are recombinant strains. Additionally, this bioprocess suffers from low bioconversion yield and excessive byproducts (Olson, McBride, Joe Shaw, & Lynd, 2012).

SSF, which was first introduced by Gauss et al. in 1976 (Gauss, Suzuki, & Takagi, 1976), combined the two separate unit processes of enzymatic hydrolysis and fermentation into one single step. The sugars produced by enzymes will be immediately consumed by microorganisms which minimizes the risk of contamination by other saccharolytic organisms and keeps substrate levels low thus avoiding inhibition of both the fermentative organisms and the end-product inhibition problem (Alvira et al., 2010; Balan, 2014). Additionally, SSF procedure would also save substantial equipment and operation cost by the combination of two separate processes into one step (Olofsson et al., 2008). The challenge of SSF is the compromise on optimal conditions for the hydrolysis and the fermentation, resulting in lower efficiency and lower solvent yields. Moreover, due to the water-insoluble property of lignocellulose, however, the SSF is limited to work under relatively low solid loadings, resulting in a dilute product concentration, which may potentially increase the difficulty for products recovery (T. C. Ezeji et al., 2007).

III. Development of an acetic acid pretreatment method for biomass pretreatment and efficient ABE production from acetic acid pretreated switchgrass with *Clostridium saccharoperbutylacetonicum*

Abstract

For the biofuel production from lignocellulosic biomass, most biomass pretreatment processes need to use some chemical reagent as the catalyst to overcome the biomass recalcitrance barrier. Such reagents are usually severe inhibitors for the subsequence microbial fermentation process. Therefore, in many cases, the liquid prehydrolysates fraction (LPF) after the pretreatment is discarded, which is a tremendous wasting of materials and leads to additional pollution. Biobutanol produced from the acetone, butanol and ethanol (ABE) fermentation process has been of great interests recently due to its high value as a biofuel or biochemical. During the ABE fermentation, acetic acid (AA) is produced and then re-assimilated as a carbon source. Thus, AA is a substrate rather than an inhibitor for biobutanol production. In this study, we employed AA as the chemical catalyst for the pretreatment of switchgrass which then be used for ABE production through simultaneous saccharification and fermentation (SSF) with hyper-butanol producing *Clostridium saccharoperbutylacetonicum* N1-4. Through the systematic investigation of the pretreatment conditions and fermentation, we concluded that the pretreatment with 3 g/L AA at 170 ^{o}C for 20 min is the optimized conditions for switchgrass pretreatment leading to efficient biobutanol production. Both LPF and solid cellulosic fraction (SCF) of the pretreatment biomass are highly

fermentable. In the fermentation with the LPF/SCF mixture, 8.6 g/L butanol (corresponding to a yield of 0.16 g/g) was obtained. Overall, here we demonstrated an innovative biomass pretreatment strategy for efficient carbon source utilization and biobutanol production.

Keywords: Pretreatment; Acetic acid; *Clostridium saccharoperbutylacetonicum* N1-4; switchgrass; acetone, butanol and ethanol (ABE) fermentation; butanol

III.1 Introduction

It is a globally recognized that the energy crisis due to the exhaustion of fossil fuels is a big program that human beings are facing in the near future. The production of bioenergy from renewable resources are considered as a promising solution to the energy issue as well as the associated environmental problems. For the bioenergy production, lignocellulosic biomass is widely considered as a sustainable feedstock because it is inexpensive, highly abundant and broadly distributed (T. Ezeji et al., 2007; S. Liu, 2015). Prior to converting the lignocellulosic biomass into bioenergy through microbial fermentation, a pretreatment process is generally required to overcome the biomass recalcitrance barrier. For most of the known pretreatment processes, chemical reagents (such as diluted acid, alkaline or organosolv) are usually used as the catalysts for breaking down the recalcitrant structure of the biomass (Paulova et al., 2015). These reagents, even at low levels, are severe inhibitors (besides the phenolic inhibitors generated from the degradation of biomass during the pretreatment) for the subsequent microbial fermentation processes (T. Ezeji et al., 2007; Paulova et al., 2015). In addition, significant level of acetic acid

(AA) is usually produced by cleaving off the acetyl group in the hemicellulose backbone during the biomass pretreatment process (Helmerius, von Walter, Rova, Berglund, & Hodge, 2010), which is also a strong inhibitor, for example, for the microbial ethanol fermentation process (Wei et al., 2015). With such problems, the liquid prehydrolysates fraction (LPF) after the biomass pretreatment is usually discarded, which is a tremendous wasting of materials and leads to additional pollution.

Recently, biobutanol produced from renewable biomass carbon sources through the clostridial acetone, butanol and ethanol (ABE) fermentation has been of great interest, because it not only can be used as a valuable fuel source with various advantages over ethanol, but also has vast applications as a chemical feedstock in many industries (Peter Dürre, 2008). As a fuel, butanol has comparable energy content as gasoline, which is much higher than ethanol. Compared to ethanol (the well accepted biofuel as an additive to gasoline), butanol is also less soluble in water, less evaporative, and less hygroscopic, making it easier to handle and more compatible with the existing pipeline infrastructure and regular vehicle engines. As a biochemical, butanol can be used in food, cosmetic, phamaceutical, plastic industries (Peter Dürre, 2007). ABE fermentation is a unique bi-phasic process. In the first phase (acidogenesis), carbohydrate is degraded into acids (mostly AA and butyric acid, or BA), while in the second (solventogenesis), the acids generated from the first phase are re-assimilated and converted into solvents along with the uptake of additional carbohydrate (Jones & Woods, 1986). In this sense, AA (as well as BA) is a substrate rather than an inhibitor for biobutanol production. Indeed, it has been reported by various

researchers that the supplementation of exogenous acetate can efficiently improve butanol production and stabilize the ABE fermentation process (C.-K. Chen & H. P. Blaschek, 1999; Chih-Kuang Chen & Hans P Blaschek, 1999; Gu et al., 2009).

Therefore, if AA is employed as the biocatalyst in biomass pretreatment, this reagent along with the AA generated during the pretreatment can both be utilized for biobutanol production. In such an approach, no exogenous chemical reagent is introduced, and thus can save cost and meanwhile avoid its inhibition on the butanol fermentation. In addition, the AA (as a weak organic acid) pretreatment can potentially generate lower level phenolic inhibitors when compared to the regular pretreatment process (with strong chemical reagents involved) under similar conditions. Thus, the LPF from the biomass pretreatment with AA could be possibly utilized as the carbon source (rather than discarded) for ABE fermentation, thus ending up with more comprehensive and efficient utilization of the biomass carbon source and minimizing pollution.

Hildebrand solubility parameter is considered a numerical estimate of the interaction between different materials, with similar values indicating good solubility (Burke, 1984). For example, solvents which display good lignin solubility have Hildebrand solubility parameter close to 11 (Quesada-Medina, López-Cremades, & Olivares-Carrillo, 2010). AA has a value of 10.1, and therefore is conferred to be a very effective reagent for lignin solution and biomass pretreatment (Pan & Sano, 1999). Previously, Xu et al. (J. Xu et al., 2009b) reported that the pretreatment with 10 g AA/kg on raw corn stover at 195 ℃ for 15 min resulted in xylose recovery up to 81.82% in the prehydrolysates. In another report, the same research group (J. Xu, Thomsen, & Thomsen,

2009a) reported that pretreatment of corn stover with combined AA and lactic acid yielded a higher glucan recovery and the simultaneous saccharification and fermentation (SSF) led to a high ethanol yield (88.7% of the theoretical yield based on pure glucose). However, to our best knowledge, there was no research so far in which biomass was pretreated with AA and then used for the downstream butanol production purpose by taking advantage of unique acid re-assimilation feature of ABE fermentation.

Therefore, in this study, the objective was to explore AA as an innovative and efficient reagent for biomass pretreatment, and meanwhile utilize the pretreated biomass for biobutanol production. We systematically optimized the conditions for the biomass pretreatment. Both the solid cellulosic fraction (SCF) and LPF were successfully fermented for efficient butanol production. In addition, with the mixture of SCF/LPF, high titer and yield for the solvent production was achieved after the fermentation. This study provides valuable references for developing an efficient, economical and sustainable bioprocess for biofuel production from low-value lignocellulosic biomass.

III.2 Material and methods

III.2.1 Feedstock, enzymes, microorganism and reagents

The Alamo-I switchgrass (*Panicum virgatumis*) was provided by Ceres, Inc. (Thousand Oaks, CA). Before the pretreatment, the biomass was milled to pass through a 0.25-inch screen and then stored at room temperature. The content of glucan, xylan, and lignin in the untreated switchgrass (in % based on dry weight) was 35.6±0.66, 19.2±0.32, and 20.0±0.15, respectively (**Table III-1**).

Comparing with the wooden biomass, the herbaceous switchgrass biomass is less recalcitrant and thus can be processed more easily under relatively mild pretreatment conditions (McLaughlin & Adams Kszos, 2005). The commercial enzyme cocktail Cellic CTec2 was obtained from Novozymes (Franklinton, NC) for the hydrolysis purpose. The enzyme activity was determined as 119 FPU/ml using Whatman #1 filter paper as the substrate, and the β-glucosidase activity as 343 IU/mL using p-nitrophenyl-β-D-glucoside (PNPG) as the substrate. *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923, = ATCC 27021) was obtained from DSMZ (Braunschweig, Germany) and used for ABE fermentation in this study. All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

III.2.2 Pretreatment

For the pretreatment, 200 ml stainless steel batch reactors (1.375"ID x 6"L) were used to hold the biomass slurry, which were heated up in the oven of a gas chromatography (GC; an old GC has been modified and repurposed for precisely heating up the reactor) (Pallapolu, 2016). Switchgrass (10 g) was loaded into the tubular reactor at a liquid/solid weight ratio of 10:1. Various amounts of AA (0 g, 30 g, 70 g, or 110 g per kg of dry biomass, thus equivalent to 0, 3, 7 and 11 g/L) were applied for the biomass pretreatment at three different temperatures (150, 170 or 190 ℃) for 20 min. After the pretreatment, the slurry was immediately fractionated into a SCF and an LPF through vacuum filtration using a filter paper (Whatman®, Grade 802 Fluted, size 32.0 cm). The SCF was washed with tap water (300 mL for each run) for five times and dried at room

temperature. Then it is stored in a climate cabinet at 25 ℃ and 65% relative humidity. The LPF was collected and stored at 4 ℃ until later use. All the experiments were performed in duplicate.

III.2.3 Detoxification

Detoxification was performed with activated carbon to the LPF to improve its ability to be hydrolyzed and fermented in the following steps. Granular activated carbon with particle size of 20-40 mesh was used for this purpose. Before use, the activated carbon was rinsed with DI water on a filter paper to remove the impurities and then dried at 45 ℃ in an oven for an overnight. The adsorbent was then loaded into the LPF at a ratio of 5% (w/v). The mixture was then incubated in a shaker at 150 rpm of agitation and 60 ℃ for 6 h to reach the adsorption equilibrium. The detoxified LPF was recovered through centrifugation. Then, the pH was adjusted to around 6.5 with 5 N of sodium hydroxide. Microfiltration (with 0.45-μm filter) was then applied to remove the suspended particles inside. The chemical composition of the detoxified LPF was then analyzed.

III.2.4 Enzymatic hydrolysis.

Enzymatic hydrolysis of the LPF was carried out by mixing 100×50 mM sodium citrate buffer (pH 4.8) to make a total volume of 100 mL. The cellulase at a loading of 15 FPU/g glucan was added, and the reaction mixture was incubated in a shaker at 150 rpm of agitation and 50 ℃. Samples were taken at various time intervals (0, 3, 6, 12, 24, 36 and 72 h) and centrifuged to remove the insoluble materials (solid phase). The glucose or xylose yield (%) at the specific time was calculated based on the amount of glucose or xylose in the liquid phase, as a percentage of the theoretical total sugars available in the original feedstock. Each enzymatic hydrolysis was carried

out in duplicate.

III.2.5 Batch fermentation

The *C. saccharoperbutylacetonicum* culture was maintained in the glycerol stock at -80 ℃. To prepare the seed culture, 1 mL of the glycerol stock was anaerobically inoculated into 100 mL tryptone-glucose-yeast extract (TGY) medium containing 30 g/L of tryptone, 20 g/L of glucose, 10 g/L of yeast extract, and 1 g/L of L-cysteine. The TGY culture was incubated in an anaerobic chamber under an N_2-CO_2-H_2 (volume ratio of 85:10:5) atmosphere at 35 ℃ for 12 to 14 h till the OD_{600} reaching ~0.8 (S. Wang, Dong, Wang, et al., 2017), which would then be used as the inoculum for the fermentation.

When SCF and/or LPF was used as the substrate, SSF were performed. Batch fermentations were carried out in 500 mL bioreactors (GS-MFC, Shanghai Gu Xin biological technology Co., Shanghai, China) with a 250 mL working volume. The modified P2 (MP2: by eliminating the ammonium acetate within the P2 medium) medium contains the following (in g/L): KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; NaCl, 0.01; p-aminobenzoic acid, 0.001; thiamine-HCl, 0.001; biotin, 0.00001. For the fermentation with SCF, pretreated SCF (based on the total glucose and xylose concentration of 20 g/L) along with yeast extract (2 g/L), tryptone (6 g/L), and MP2 medium were mixed together. For the fermentation with LPF, the MP2 medium along with yeast extract (2 g/L) and tryptone (6 g/L) was directly supplemented into the LPF, making a final volume of 100 ml. The mixture was then filter-sterilized by passing through a VWR bottle top filtration (0.2 μm PES) (VWR) and then decanted into a

sterile serum bottle. For the fermentation with the mixture of SCF and LPF, similar as the fermentation with LPF, the MP2 medium along with yeast extract (2 g/L) and tryptone (6 g/L) was firstly supplemented into the LPF, making a final volume of 100 ml. Then, the SCF (based on the ratio of SCF and LPF after pretreatment from certain amount of biomass) was added.

For all the fermentation, the initial pH was adjusted to 6.5 with 2 N NaOH. To generate an anaerobic condition, oxygen-free nitrogen was sparged through the fermentation broth starting several hours before the inoculation until the cell culture initiated its own gas production. The Cellic CTec2 enzyme of 15 FPU/g glucan and active growing preculture (5% v/v) were added at the same time to initiate the fermentation. The fermentation was performed at 30 ℃ with 150 rpm agitation for 96 h with the pH controlled > 5.0. All fermentations were performed in triplicates.

III.2.6 Analytical procedure

The lignin and carbohydrate composition of SCF was analyzed following the National Renewable Energy Laboratory protocol (NREL/TP-510-42618) (Sluiter, Ruiz, Scarlata, Sluiter, & Templeton, 2005). The chemical composition of LPF was analyzed via secondary hydrolysis as described in the protocol (NREL/TP-510-42623) (Sluiter et al., 2006). The LPF was characterized for the carbohydrate content (oligosaccharide and monomeric sugars) and degradation products (AA, furfural, HMF and total phenolic compounds (TPC)). The amount of oligosaccharides in LPF was calculated by subtracting the monomeric sugar content in the LPF from the total monomeric sugar content after secondary hydrolysis. The TPC was determined using the Folin-Ciocalteu (F-C) assay (Ainsworth & Gillespie, 2007). In brief, 100 μL of LPF, standard (gallic acid) or 95%

(vol/vol) methanol blank was added into 2 ml microtubes and mixed with 200 μL of F-C reagent by vortex. The total volume was made to 1.1 mL by adding 800 μl sodium carbonate into each tube and incubate at room temperature for 2 h. Transfer 200 μl sample, standard or blank from the assay tube to a clear 96-wells microplate and read the absorbance of each well at 765 nm using a spectrophotometer Tecan infinite M100 pro (Tecan Trading AG, Switzerland). The calibration curve was obtained in a similar manner to that described for samples using standards solutions of gallic acid. The results were expressed as mg per gram of dry material (mg g^{-1} DW).

The sugar analysis was performed with an Agilent 1260 Infinity HPLC system (Agilent Technologies, CA) equipped with a refractive index detector (RID) and a 300 mm × 7.8 mm (i.d.), 9 μm Aminex HPX-87P column and a 30 mm × 4.6 mm (i.d.) guard column (Bio-Rad, Hercules, CA). Nano-pure water was used as the mobile phase at an isocratic flow rate of 0.6 mL/min and the temperature was maintained at 85 ℃ during the 35 minutes elution. The fermentation products were quantified with this HPLC system and a Varian MetaCarb 87H Column 300 × 7.8 mm along with a 50 × 4.6 mm MetaCarb 87H guard column (Agilent Technologies, CA). 0.005 N H_2SO_4 was used as the mobile phase at an isocratic flow rate of 0.6 mL/min, and the temperature of the column was maintained at 25 ℃ during the elution.

All of the analyses were carried out using SAS software (SAS Institute Inc, Cary, NC). *p*-values below the conventional 5% threshold were regarded as significant.

III.2.7 Mass balance calculation

The recovery rate and solvent yield from the pretreatment were calculated according to the literature (J. Xu et al., 2009b). In details, the recovery rate (of either glucan or xylan) was obtained by dividing the mass of glucan (or xylan) in both the SCF and LPF after pretreatment with the mass of glucan (or xylan) in the original biomass used for the pretreatment.

$$\text{Recovery}_{Glucan} = \frac{Mass_{Glucan\ in\ SCF} + Mass_{Glucan\ in\ LPF}}{Mass_{Glucan\ in\ raw\ biomass}} \times 100\%$$

$$\text{Recovery}_{Xylan} = \frac{Mass_{Xylan\ in\ SCF} + Mass_{Xylan\ in\ LPF}}{Mass_{Xylan\ in\ raw\ biomass}} \times 100\%$$

The solvent yield was calculated by dividing the solvent of the generated in the fermentation medium with the total carbohydrate added before fermentation:

$$\text{Solvent yield}\ \% = \frac{Solvent_{g/L}}{Total\ carbohydrate_{g/L}} \times 100\%$$

III.3 Results and discussion

III.3.1 Biomass pretreatment and composition analysis

Alamo switchgrass was selected as the lignocellulosic feedstock in this study. In comparison with the wooden biomass, it is less recalcitrant and thus can be processed easily with relatively mild pretreatment conditions (X. Wang, Zhang, Wang, & Wang, 2016). Generally, the biomass pretreatment through the hydrothermal approach is carried out at temperatures between 160 and 230 ℃ for a residence time of 10-60 minutes. One of the primary objectives for hydrothermal pretreatment is to remove as much hemicellulose as possible from the biomass (F. Huang & Ragauskas, 2013). Using AA as the chemical catalyst in the biomass pretreatment, the recalcitrance of the biomass can be overcome at relative milder conditions, and thus lead to

improved yield of sugars and biofuel production.

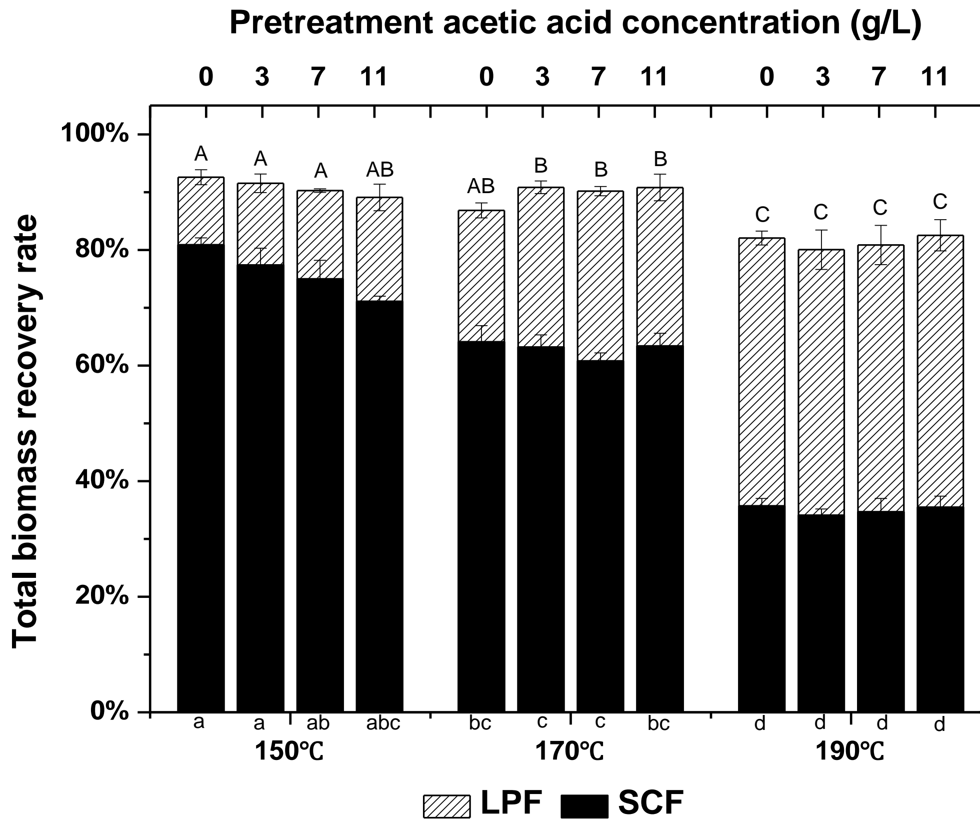


Fig. III-1. Total biomass recovery rate in SCF and LPF with different pretreatment conditions. The values represent the means of duplicated samples, and the error bars represent standard deviations. Within each measurement, bars containing the same letter (uppercase for LPF and lowercase for SCF) are not significantly different at the 0.05 level based on Turkey's HSD test.

Table III-1. The chemical composition of the raw switchgrass and solid cellulosic fraction (SCF) after pretreatment.

Pretreatment condition (g acetic acid/L)		Composition of SCF (%)			
		Glucan	Xylan	Carbohydrate	Lignin
Raw switchgrass		35.6±0.6	19.2±0.8	65.5±1.2	22.6±0.3
150 ℃	0	34.9±2.3	20.9±0.2	64.5±2.9	28.0±0.6
	3	35.3±3.1	19.6±1.0	61.9±3.2	28.3±0.9
	7	37.1±0.7	19.3±0.7	63.8±0.9	29.2±0.7
	11	35.7±2.4	17.7±0.6	60.1±2.8	29.5±0.3
170 ℃	0	44.5±0.8	11.8±1.1	62.6±3	31.1±1.1
	3	45.1±1.9	10.7±1.1	61.9±1.4	32.4±1.3
	7	48.2±2.6	7.5±0.7	61.7±2.2	35.0±1.7
	11	48.8±0.3	6.7±0.9	61.3±1.3	35.8±0.6
190 ℃	0	45.3±1.6	15.5±0.3	62.2±1.1	29.1±0.5
	3	44.6±2.0	15.1±0.1	61.4±2.3	30.5±0.4
	7	45.7±2.1	17.2±0.3	64.8±1.9	29.6±0.4
	11	44.5±1.7	15.6±1.3	61.7±1.4	30.3±0.3

All results shown are average value ±standard deviations from duplicated experiments.

In this study, for the pretreatment, 10 g of dried switchgrass (for each reaction) was mixed with 100 ml water, and then pretreated under four different AA concentrations (0, 3, 7, 11 g/L) at three different temperatures (150 ℃, 170 ℃, 190 ℃; and thus totally 12 different pretreatment conditions) for 20 min. The composition of the SCF of the pretreated biomass (as compared to the untreated raw biomass) was illustrated in **Table III-1**. In the raw switchgrass, the total carbohydrate (cellulose and hemicellulose) represented approximately 65.5% (wt/wt; the same below unless otherwise indicated) and the total lignin accounted for 22.6%. After pretreatment, especially when the pretreatment temperature was above 170 ℃, the glucan content in the SCF increased while the xylan content decreased significantly. The total biomass recovery rate decreased significantly from above 70 % to lower than 40% when the pretreatment temperature was increased from 150 ℃ to 190 ℃ (**Fig. III-1**). At 170 ℃, when the AA concentration increased from 0 to 11 g/L, the xylan content in the SCF degraded remarkably from 11.8% to 6.7%, while the other components (glucan and lignin) increased slightly (**Table III-1**). At 150 ℃ (the mildest condition employed in this study), the residual cellulose, hemicellulose, lignin and total sugars were almost unchanged compared to the raw biomass. On the other hand, the SCF composition was almost not influenced by the increase of AA concentration under this pretreatment condition. This indicated that 150 ºC was not effective for the biomass pretreatment. While at 190 ℃, most of the components were carbonized due to this harsh pretreatment condition, as illustrated by the low biomass recovery rate in **Fig. III-1**, demonstrating that this high temperature was unfavorable for the biomass pretreatment. Therefore, based on the SCF composition under various pretreatment

temperature conditions, 170 ℃ was likely the optimal temperature for the switchgrass pretreatment in this study.

The composition of the LPF was presented in **Table III-2**. At 170 ℃ when no AA was added, a total of 15.5 g/L sugars with xylose (7.7 g/L) as the dominant component was detected in the LPF. With the increase of AA concentration for the pretreatment, more sugars were released with 21.0 g/L (11.1 g/L xylose) was detected when 7 g/L AA was used. When 11 g/L AA was employed, the total sugars decreased slightly possibly due to the degradation. It should be noticed that, with 7 g/L AA was used for the pretreatment, the majority of xylose was as the oligomer (9.1 g/L) and with only a small portion in the form of the monomer (2.1 g/L). It has been reported that the hydrothermal pretreatment (also called self-hydrolysis) works by cleaving off the acetyl group in the hemicellulose backbone, and simultaneously releasing the polysaccharides and AA into the LPF (Helmerius et al., 2010). This could be confirmed by the increase of acetate concentration in the LPF and decrease of hemicellulose (xylan) content in SCF after the pretreatment as illustrated in **Table III-1** & **Table III-2**. At 150 ℃, with the increase of added AA concentration from 0 to 11 g/L, the total sugar in LPF increased from 7.5 to 12.7 g/L. For xylose, however, even when 11 g/L AA was used, only 3.6 g/L was detected most of which was oligomer. Therefore, again, these results suggested that 150 ^{o}C was not adequate for the biomass pretreatment. On the other hand, at 190 ℃, the concentration of all the sugars in LPF (generated under conditions with various AA concentrations) was very low, because most of released sugars were further degraded into other products such as furfural and HMF (**Fig. III-1** and **Table III-2**). The concentration of these side

products (HMF, furfural and phenolic compounds) increased with the pretreatment temperature and reached the highest at 190 ℃. Taken together, 170 ℃ was concluded as the optimal temperature for the switchgrass pretreatment, based on the analysis of both the SCF and LPF compositions. Therefore, for the following steps, the biomass pretreated at 170 ºC (with various concentrations of AA employed for the pretreatment) was subjected to further processing and fermentation.

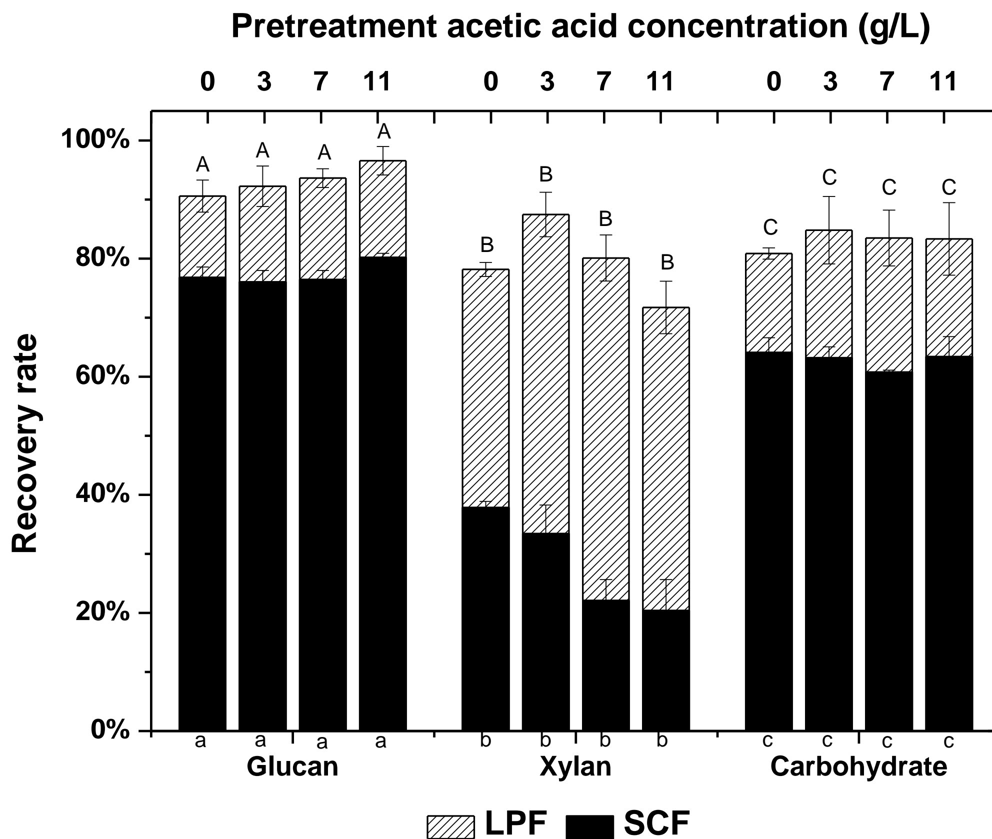


Fig. III-2. Carbohydrate recovery rate in SCF and LPF under 170 ℃ pretreatment with different acetic acid concentration. The values represent the means of duplicated samples, and the error bars represent standard deviations. Within each measurement, bars containing the same letter (uppercase for LPF and lowercase for SCF) are not significantly different at the 0.05 level based on Turkey's HSD test.

Table III-2. Chemical composition of LPF.

Pretreatment conditions		Composition of carbohydrate (g/L)							By-products (g/L)			
		Glucose			Xylose							
Temperature	Acetic acid (g/L)	Monomer	Oligomer	Total	Monomer	Oligomer	Total	Total Carbohydrate	Acetate	HMF	Furfural	Phenolic compound
150 ℃	**0**	2.1±0.1	1.8±0.2	3.8±0.4	0.1±0.1	0.7±0.1	0.8±0.1	7.5±0.1	2.2±0.3	0.2±0.3	0.2±0.1	3.8±0.1
	3	2.2±0.2	2.1±0.3	4.3±0.2	0.2±0.3	1.7±0.2	1.9±0.3	9.8±0.3	3.9±0.2	0.2±0.3	0.2±0.1	4.0±0.1
	7	2.0±0.2	2.3±0.6	4.4±0.4	0.2±0.2	2.4±0.5	2.6±0.2	10.6±0.4	6.2±0.4	0.2±0.1	0.3±0.1	4.2±0.2
	11	2.4±0.1	2.7±0.4	5.1±0.4	0.3±0.2	3.4±0.1	3.6±0.3	12.7±0.1	9.7±0.8	0.3±0.2	0.6±0.1	4.4±0.2
170 ℃	**0**	1.0±0.1	3.9±0.2	4.9±0.4	0.6±0.1	7.1±0.4	7.7±0.4	15.5±0.1	2.4±0.4	0.4±0.4	0.7±0.2	6.2±0.2
	3	1.6±0.1	4.2±0.3	5.8±0.5	0.8±0.1	9.5±0.5	10.4±0.5	20±0.1	4.9±0.6	0.5±0.5	0.8±0.1	6.3±0.2
	7	1.6±0.3	4.5±0.5	6.1±0.3	2.1±0.1	9.1±0.4	11.1±0.5	21±0.5	7.8±0.6	0.7±0.3	1.3±0.2	6.4±0.2
	11	1.8±0.3	4.0±0.6	5.8±0.5	2.7±0.2	7.2±0.8	9.8±0.7	18.4±0.5	11.7±0.8	0.8±0.6	1.7±0.3	6.5±0.1
190 ℃	**0**	1.3±0.2	1.0±0.5	2.3±0.6	0.1±0.1	0.1±0.1	0.2±0.1	3.1±0.5	3.5±0.4	1.3±0.1	3.2±0.2	11.8±0.2
	3	1.1±0.1	1.0±0.3	2.1±0.5	0.1±0.0	0.1±0.1	0.2±0.1	2.8±0.2	5.3±0.3	1.4±0.6	2.9±0.3	11.9±0.2
	7	0.6±0.0	1.2±0.3	1.8±0.2	0.13±0.0	0.1±0.0	0.2±0.0	3.0±0.1	6.6±0.0	1.3±0.5	2.6±0.2	12.3±0.2
	11	0.3±0.1	2.1±0.1	2.4±0.1	0.12±0.1	0.2±0.1	0.3±0.2	2.2±0.1	10.8±0.1	1.8±0.5	3.7±0.3	12.3±0.3

All results shown are average value ± standard deviations from duplicated experiments.

The recovery rate of glucan, xylan and the total carbohydrate from the pretreatment at 170 ℃ was calculated and illustrated in **Fig. III-2**. For the glucan, only a small fraction was released into the LPF, varying from 13.8% without AA added to 17.2% with 7 g/L added. However, more than 75% of the glucan was conserved in the SCF. The total glucan recovery rate was more than 90% for under all the pretreatment conditions. In the opposite, most of the xylan was released into the LPF, ranging from 40.3% (when no AA was added) to 58% (when 7 g/L AA was used). The recovery rate of xylan in the SCF decreased with the increase of the AA concentration, from 37.8% (0 g/L AA) to 20.4% (11 g/L AA). These results indicate that the xylan recovery in LPF was much more subjected to the influence of AA concentration. However, when the recovery rate of the total carbohydrate was considered, it was not significantly influenced by the AA concentration employed for the pretreatment, with a recovery rate of 60.8%-61.1% was observed within the SCF. The recovery rate in LPF was only 16.8% when no AA was added, and slightly increased when various concentrations of AA was employed for the pretreatment (**Fig. III-2**). Overall, these results indicated that the concentration of AA employed for the pretreatment at 170 ℃ did not significantly influence the total carbohydrate recovery rate.

III.3.2 Detoxification of LPF

During the pretreatment, hexose and xylose can be degraded to 5-hydroxyl furfural (HMF) and furfural (Jönsson et al., 2013). Additionally, lignin-degraded compounds partially precipitate as high-molecular-weight insoluble particles with the other part dissolved as soluble phenolic compounds in the LPF. As shown in **Table III-2**, generally, the concentration of HMF, furfural

and TPC increased with the temperature and AA concentration employed for the pretreatment. Comparatively, the temperature played a more significant role than AA concentration for the generation of these degradation products. Under the same pretreatment condition, the TPC concentration was much higher than that of furfural and HMF.

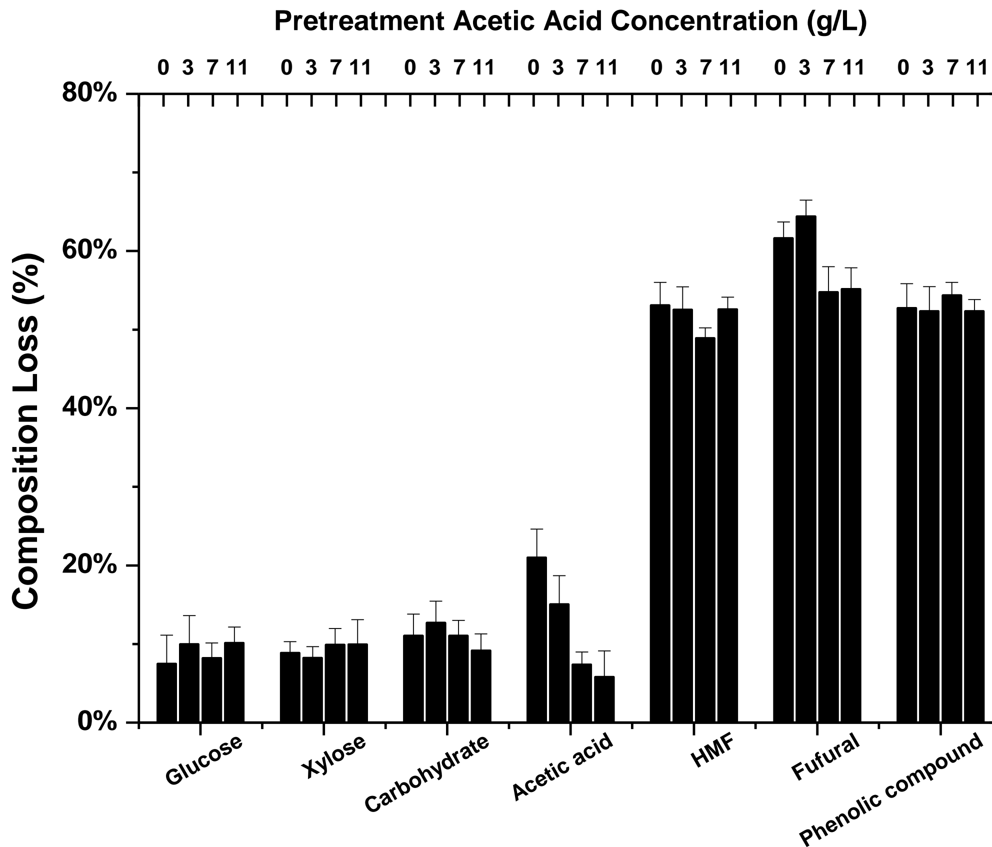


Fig. III-3. Effects of activated carbon detoxification on composition decrease percentage of 170 ℃ pretreated LPF. The values represent the means of duplicated samples, and the error bars represent standard deviations.

These degradation production (furfural, HMF, and TPC) are all common inhibitors for the downstream fermentation process (Jönsson et al., 2013). As a preliminary test, we tried to carry

out SSF with *C. saccharoperbutylacetonicum* N1-4 for ABE production using the LPF as the substrate. The results showed no cell growth and solvent production (data not shown). Based on our previous experiment (Yao et al., 2017), furfural or HMF at the level of < 3 g/L in the fermentation medium does not have significant inhibition on *C. saccharoperbutylacetonicum* for ABE fermentation, while the soluble phenolic compounds (p-coumaric, ferulic acid, vanillic acid, 4-hydroxybenzoic acid, etc.) derived from lignin, are much more toxic. These phenolic compounds inhibit the cell growth even at a very low concentration (< 1 g/L). The inhibitory mechanism of these phenolic compounds has been proposed as disrupting the function of cell membrane via hydrophobic interaction (K. Liu et al., 2015).

To decrease the toxicity of LPF, a detoxification procedure is necessary prior to the hydrolysis and fermentation. Various methods for the detoxification of biomass hydrolysates have been previously reported including neutralization, over-liming, evaporation, ion exchange resin adsorption and activated carbon adsorption (Jönsson et al., 2013). Activated carbon has been used as an adsorbent for hundreds of years in wastewater, drinking water, refinery waste, and chemical clarification applications. Comparing to other detoxification methods, activated carbon adsorption is less costly, easy to operate, and the activated carbon is easy to be regenerated (Jönsson et al., 2013). Numerous studies have reported to use activated carbon adsorption for the detoxification on the prehydrolysates prior to the fermentation (Berson, Young, Kamer, & Hanley, 2005; Foo & Hameed, 2010; K. Liu et al., 2015), and this method has been proven to remove phenolic compounds effectively (Larsson, Reimann, Nilvebrant, & Jönsson, 1999). Thus, activated carbon

adsorption was applied in this study for the detoxification of LPF generated at 170 ℃ with various levels of AA for the pretreatment.

As shown in **Fig. III-3**, after the activated carbon adsorption, HMF and furfural in the LPF were decreased by 50%-60% for the LPF from all pretreatment conditions. Similarly, the TPC concentration decreased by > 50%. This effectiveness of activated carbon adsorption on the removal of these inhibitors has also been reported previously (Berson et al., 2005; Larsson et al., 1999; K. Liu et al., 2015). It is known that activated carbon detoxifies the pre-hydrolysate by physical adsorption and such efficient removal of the inhibitors is probably due to the high affinity of these compounds to the activated carbon (Berson et al., 2005). A common yet unfavorable feature is that detoxification will also cause the adsorption of carbohydrates. Results in **Fig. III-3** showed that the decrease of glucose, xylose and total carbohydrate was only around 8%, 9% and 11%, which was much lower than that for the removal of inhibitors. Additionally, the results also showed that the adsorption of inhibitors and carbohydrates did not change remarkably for the LPF obtained from the pretreatment with different AA concentrations applied. The adsorption has also removed 6%-21% unbound AA (with higher percentage of AA been removed when lower AA was employed for the pretreatment). However, most of the AA is still left in the solution, which could be used as the carbon source for the following ABE fermentation. All these results indicated that activated carbon adsorption is an ideal detoxification approach for selectively removing the inhibitors while keeping most sugars and acetate in LPF.

III.3.3 The effect of detoxification on enzymatic hydrolysis

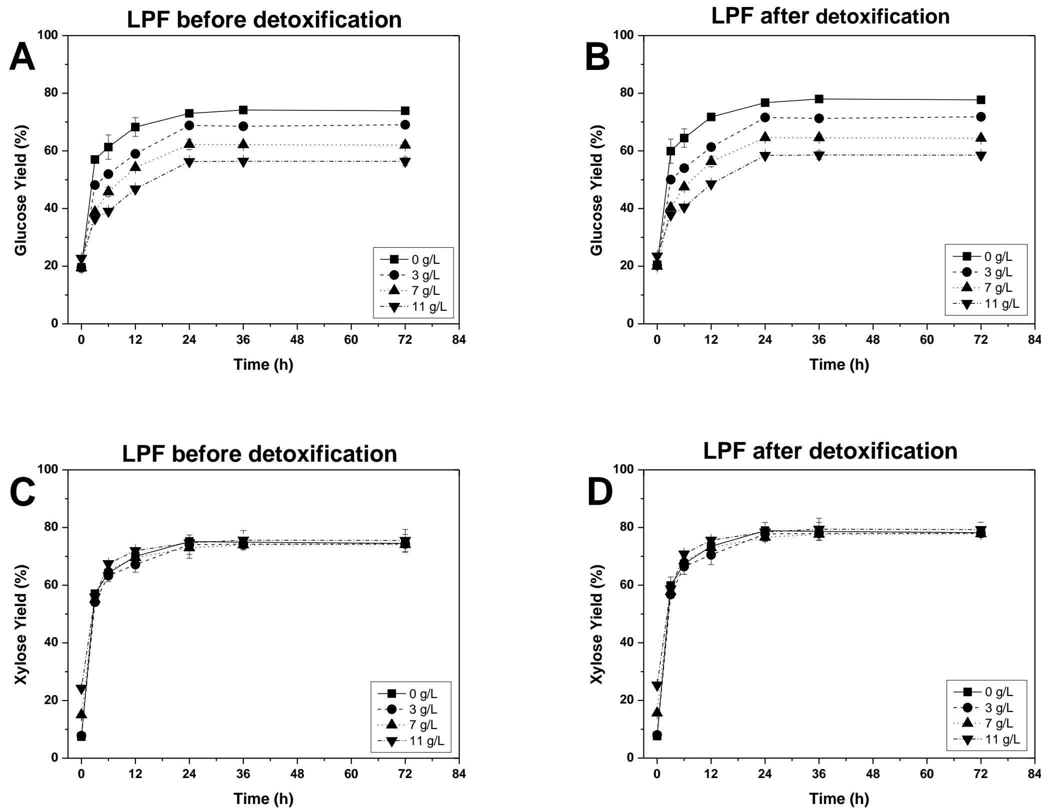


Fig. III-4. Effect of detoxification on the enzymatic hydrolysis of glucan (A and B) and xylan (C and D) in 170 ℃ pretreated LPF substrates. The values represent the means of duplicated samples, and the error bars represent standard deviations.

Enzymatic hydrolysis was carried out with an enzyme loading of 15 FPU/g glucan for both the detoxified LPF and undetoxified LPF, to evaluate the effects of detoxification on the enzymatic hydrolysis. As shown in **Fig. III-4**, without detoxification, the glucan-to-glucose yield reached a maximum of 73.9% in the LPF without AA added for the pretreatment, and a minimum of 56.4% in the LPF when 11 g/L AA was used for the pretreatment. With detoxification, the maximum of glucose yield increased slightly to 77.7% in the LPF without AA added for the pretreatment, while

a minimum of 58.5% (also slightly increased from the case without detoxification) was achieved in the LPF when 11 g/L AA was used for the pretreatment. For the xylan-to-xylose hydrolysis yield, neither the detoxification nor the amount of AA used for the pretreatment had a significant difference. For LPF from all pretreatment conditions, the final hydrolysis yield of xylose was around 78%. On the other hand, the hydrolysis kinetics were similar to each other as well for the detoxified and undetoxified LPF from the same pretreatment conditions. These results indicated that detoxification did not significantly influence the enzymatic hydrolysis in term of the hydrolysis kinetics or final sugar yield.

III.3.4 Simultaneous Saccharide and Fermentation (SSF) of SCF and LPF

For the ABE production from lignocellulosic feedstocks, SSF has been proven to be a preferable approach with various advantages when compared to the separate hydrolysis and fermentation (SHF). In SSF, the feedback inhibition of sugars on cellulases is mitigated because the sugars are consumed by the fermenting organism as soon as it is formed. On the other hand, the saccharification (enzymatic hydrolysis) and fermentation are carried out in the same reactor, which simplifies the operation and decreases the cost (Guan, Shi, Tu, & Lee, 2016). Generally, for the regular biomass pretreatment, the LPF which contains high levels of inhibitors (including the chemical reagents for pretreatment and the degradation products from the biomass) is discarded and not used for the fermentation. However, in this study, AA was used as the chemical reagent which can be used as a carbon source for ABE fermentation, and also the biomass is pretreated in a relatively mild condition. Besides, the acid-based pretreatment can help release large fraction of

the hemicellulose into the LPF. Therefore, with the detoxification process, we expect that the LPF (and thus the mixture of LPF-SCF mixture) from this study could be used for efficient fermentation for ABE production. Thus, in this study, we carried out SSF using LPF, SCF and LPF-SCF mixture respectively for ABE production. An enzyme loading of 15 FPU/g glucan was employed for each fermentation. A control fermentation was meanwhile performed using Avicel (20 g/L) as the feedstock. The fermentation was run for 96 h with the results illustrated in **Fig. III-5** and **Table III-3**.

For all the fermentations, the reducing sugars (glucose and xylose) were completely consumed by the end of the fermentation (data not shown). This demonstrated that the activated carbon detoxification was very effective for removing the inhibitory compounds in the LPF and enabling the successful fermentation (especially in the LPF and the mixture). In the SCF, 1.5 to 2.4 g/L AA was detected at the end of the fermentation (**Table III-3**). The production of BA was generally low, with 0.7 g/L (with no AA added for the biomass pretreatment) and 0.3 g/L (with 3 g/L AA added for the biomass pretreatment) produced. While there was no detectable BA in the fermentation with SCF generated through the pretreatment with 7 or 11 g/L AA employed. The butanol production increased from 3.0 g/l to 4.3 g/L with the increase of pretreatment AA concentration from 0-11 g/l, along with the increase of butanol yield from 0.15 to 0.21 g/g-sugar correspondingly. The total ABE, like butanol, also increased with the increase of pretreatment AA concentration (**Fig. III-5**).

Table III-3. Simultaneous saccharification and fermentation of 170 ℃ pretreated biomasses

Pretreatment methods		Starting sugars (g/L)	Acid (g/L)		Solvent Yield (g/g)	
Fractions	**Acetic acid (g/L)**		**Acetic**	**Butyric**	**Butanol**	**ABE**
SCF	0	20	2.4±0.2	0.7±0.2	0.15[a]	0.25[a]
	3	20	1.7±0.0	0.3±0.1	0.17[b]	0.29[b]
	7	20	1.6±0.3	0.0±0.0	0.20[c]	0.32[c]
	11	20	1.5±0.1	0.0±0.0	0.21[c]	0.33[c]
LPF	0	12.8	3.1±0.2	1.6±0.1	0.25[d]	0.33[d]
	3	16.4	4.5±0.1	1.8±0.3	0.25[d]	0.35[d]
	7	16.4	6.9±0.2	1.6±0.3	0.12[e]	0.22[e]
	11	14.9	11.1±0.9	1.2±0.4	0.07[f]	0.17[f]
Mix	0	50.0	1.7±0.4	1.6±0.2	0.14[gh]	0.23[g]
	3	53.6	3.8±0.4	1.7±0.1	0.16[g]	0.26[h]
	7	53.5	5.8±0.3	1.6±0.2	0.13[h]	0.24[gh]
	11	52.1	10.5±0.0	1.1±0.2	0.08[i]	0.16[i]
Avicel	1.6	20	1.3±0.1	0.8±0.1	0.24	0.39

The data presented are averages of three independent analyses, and error bars represent standard deviations.
Values followed by the same letter within the same section are not significantly different at the 0.05 level based on Tukey's HSD test.

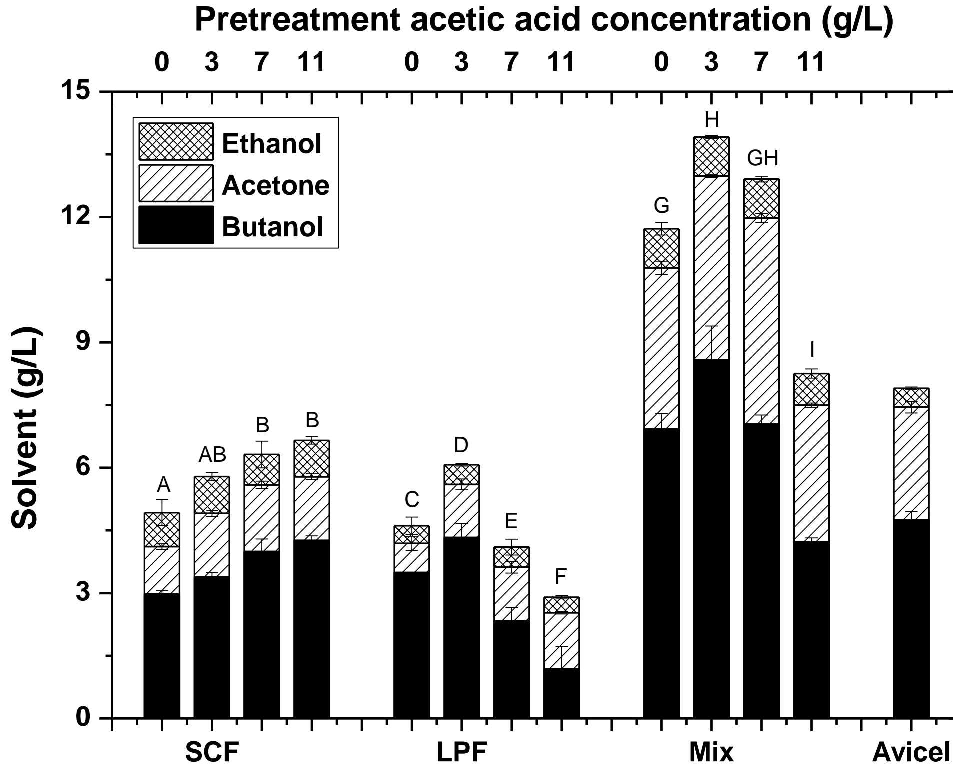


Fig. III-5. Solvent production by *C. saccharoperbutylacetonicum* N1-4 strain with pretreated biomass as substrate. The samples were examined following 96 h of incubation at 30 ℃. The values represent the means of triplicated samples, and the error bars represent standard deviations. Within each measurement in the same section, bars with the same letter are not significantly different at the 0.05 level based on Turkey's HSD test.

For the fermentation with Avicel (used as a control for the fermentation with SCF), 1.3 g/L AA and 0.8 g/L BA was produced. While this BA level was comparable, the AA level was only about half of that from the fermentation with SCF (with 0 g/L AA employed for the pretreatment). In the clostridial metabolic pathways, other than glycolysis, the main route for energy (ATP) generation is through the AA and BA production pathway. In the fermentation with pretreated biomass as

substrate, the recalcitrant structure and some toxic substance after pretreatment will inhibit the strain growth to some extent. To compensate such inhibition and sustain the cell growth, the cell will direct more carbon flow for the AA and BA formation to generate energy, leading to the increased production of acid (especially AA, because the AA production pathway is more efficient for energy generation than the BA production pathway). There was 4.8 g/L butanol and 8.0 g/L total ABE produced in the fermentation with Avicel. The final titer and yield for the solvent production were all higher than those from the fermentation with SCF (**Fig. III-5** and **Table III-3**).

For the fermentation with LPF, the final AA concentration was kept approximately at the same level as in the original LPF. This indicated that the produced AA during the fermentation has been mostly re-assimilated; however, not all the AA in the medium could be re-assimilated. The BA production was at similar levels (from 1.2-1.8 g/L) in all the fermentations with the LPF generated in pretreatment with different AA concentrations. However, these values were much higher than that from the fermentation with SCF. There was high level AA in the LPF (but not in the SCF); the re-assimilation of AA led to increased BA production. For the butanol production, the final concentration ranged from 1.2 to 4.3 g/L depending on different levels of pretreatment AA concentration, with corresponding yields varied from 0.07 to 0.25 g/g-sugar. The lowest butanol production (1.2 g/L) was observed at 11 g/L pretreatment AA concentration, while the highest butanol production (4.3 g/L; corresponding to the highest yield of 0.25 g/g) was obtained with when 3 g/L AA was employed for the pretreatment. Similar as butanol production, the total ABE

reached the highest concentration of 6.1 g/L (corresponding to a yield of 0.35 g/g) at 3 g/L pretreatment AA concentration, while decreased to 2.9 g/L (a yield of 0.17 g/g) when 11 g/L AA was used for the pretreatment. Based on the results from the fermentation with LPF, 3 g/L AA is an appropriate concentration for the switchgrass pretreatment, which could lead to the highest solvent production in the following fermentation. This is different from the results for the SCF fermentation (in which the solvent production increased with the increase of AA level used for the pretreatment). This is because, the AA used for the pretreatment (along with those generated in the pretreatment) all ended up in the LPF (but not SCF). For the SCF, the higher AA used, the harsher condition for the pretreatment (and thus the best accessibility for the enzymatic hydrolysis and microbial fermentation); while in LPF, when AA was too high (> 7 g/L), it will inhibit the cell growth and fermentation (although it benefits at a lower concentration).

Finally, the SCF and LPF was mixed together and used as the carbon source for the SSF. At the end of the fermentation, there was still tremendous AA left depending on the different conditions (that is the AA concentration used for the pretreatment). However, compared to the fermentation with LPF, clearly under each condition, more AA has been re-assimilated. This is because in the mixture more carbon source (sugars) was available, and thus led to more efficient AA re-assimilation, as well as high level of solvent production. However, the BA production was at the similar levels as compared to the fermentation with LPF. The butanol and total ABE production ranged from 4.2-8.6 g/L and 8.3-13.9 g/L, respectively, corresponding to the yield of 0.08-0.16 g/g and 0.16-0.26 g/g, respectively. The lowest solvent production was observed when 11 g/L AA

was employed for the pretreatment, while the highest solvent production was obtained at 3 g/L AA for the pretreatment. When the AA concentration used for the pretreatment at 0-3 g/L, the solvent production with the SCF/LPF mixture was lower than that with LPF but higher than that with SCF. However, when the AA concentration for the pretreatment was high (7-11 g/L), on the contrary, the solvent production with the mixture was higher than that with LPF but lower than that with SCF. Taken together, these results demonstrate that when no AA (0 g/L AA) was employed for the pretreatment, the biomass recalcitrance barrier could not be effectively overcome, thus leading to lower enzyme digestibility and fermentability of the SCF. While when high concentration (7-11 g/L) of AA was used, the degradation products as well as the high concentration AA in the LPF will inhibit the fermentation. Therefore, 3 g/L AA was determined as the optimal concentration for the switchgrass pretreatment for the ABE production purpose with *C. saccharoperbutylacetonicum* N1-4.

On the other hand, a large fraction of the AA within the LPF has not been re-assimilated for the biosolvent production. Therefore, metabolic engineering efforts are desired to enable the strain for enhanced acid re-assimilation for the fermentation of the AA-pretreated lignocellulosic biomass.

III.4 Conclusion

In the present study, AA was explored as a catalyst for efficient hydrothermal pretreatment of switchgrass. The pretreatment biomass was further utilized as the carbon source for biobutanol production through ABE fermentation taking advantage of the acid re-assimilation capability of the *Clostridium* strain. Our results demonstrated that the pretreatment with 3 g/L AA at 170 ^{o}C for

20 min is the optimized conditions for switchgrass pretreatment under which most of the xylan was released into the LPF while most of the glucan was reserved in the SCF. After detoxification with activated carbon, both the LPF and SCF could be fermented for butanol production through SSF. In the fermentation with the SCF/LPF mixture, 8.6 g/L butanol and 13.9 g/L ABE was obtained, corresponding to high yields of 0.16 g/g and 0.26 g/g, respectively. The results from this study demonstrated an innovative and efficient strategy for comprehensive conversion of lignocellulosic biomass into high value biofuel.

IV. Enhancement of acid re-assimilation and biosolvent production in *Clostridium saccharoperbutylacetonicum* by overexpressing key genes in the ABE fermentation pathway

Abstract

Biobutanol produced through the well-known clostridial acetone-butanol-ethanol (ABE) fermentation process has attracted a lot of attention recently due to its value to be used as a biofuel source or biochemical with various industrial applications. ABE fermentation generally has two phases: in the acidogenesis phase, fatty acids (acetic acid and butyric acid) are accumulated, while in the solventogenic phase, fatty acids are re-assimilated and converted into solvents. Therefore, the improvement of acid re-assimilation capability in the *Clostridium* host strain can possibly enhance the solvent production. In addition, acetic acid is often a significant component in the biomass prehydrolysates after pretreatment (especially when acid-based biomass pretreatment approach is employed). Thus, the enhancement of acid re-assimilation in *Clostridium* has practical significance for biofuel production from lignocellulosic biomass. In this study, we overexpressed key genes of the ABE fermentation pathways in *Clostridium saccharoperbutylacetonicum* N1-4 to enhance the acid re-assimilation and solvent production in the host. First, the native *sol* operon (*ald-ctfA-ctfB-bcd*) was overexpressed under the strong constitutive thiolase promoter (P_{thl}), generating PW2 strain. Fermentation results demonstrated that the acid re-assimilation was improved in the host strain and the ABE production has been increased to 31.4 g/L (vs. 26.4 g/L

in JZ100 strain as the control). Although the ethanol production has been increased by six times (4.9 g/L vs. 0.7 g/L in the control), the butanol production has not been significantly increased. In order to further drive the carbon flux from C2 metabolites to C4 metabolites and ultimate butanol production, the key genes including *hbd, thl, crt* and *bcd* (expression cassette, or EC) in the butanol production pathway was further overexpressed under P_{thl} besides the *sol* operon overexpression as in PW2, generating PW3 strain. Compared to the control strain JZ100, the butanol and acetone production in PW3 was increased by 8% and 18% respectively. The final total solvent production in PW3 increased by 12.4% than the control, but was 10% lower than PW2 (mainly because of the dramatic increase of ethanol production in PW2). In PW3, both *sol* operon and EC were overexpressed with P_{thl}, which could lead to competition for the same RNA polymerase for the expression of multiple genes. To avoid this issue and further improve ABE production, a new strain PW4 was constructed to express *sol* operon with P_{thl} but EC with ferredoxin gene promoter (P_{fdx}). The fermentation results demonstrated that, however, the production of all the solvents in PW4 was actually slightly lower than those in PW3. Moreover, we evaluated the effect of acetic acid concentrations on the solvent production in the engineered strains, and the maximum level of solvent production was achieved when 4.6 g/L acetate was supplemented. Therefore, simultaneous saccharification and fermentation (SSF) was carried out with PW2 and PW3 using switchgrass biomass pretreated with 3 g/L acetic acid (which ends up with approximately 4.6 g/L in the fermentation medium). Results showed that 15.4 g/L total ABE (with a yield of 0.31 g/g) was produced in both PW2 and PW3, which was significantly higher than that in JZ100. This study

demonstrated that the overexpression of key genes for acid re-assimilation and solvent production can significantly enhance ABE production in solventogenic clostridia.

Keywords: acetate; *Clostridium saccharoperbutylacetonicum* N1-4; *sol* operon; expression cassette EC; acid re-assimilation; metabolic engineering

IV.1 Introduction

Lignocellulosic biomass is considered as the most promising long-term feedstock for the production of biofuels (T. Ezeji et al., 2007). The biomass typically needs to be pretreated prior to subsequent enzymatic hydrolysis to monomeric sugars and fermentation to biofuels. Pretreatment, however, generates a wide range of toxic compounds from the degradation of carbohydrates, lignin and extractives, which may significantly inhibit microbial fermentation. The most common fermentation inhibitors in hydrolysate are furan derivatives (furfural and 5-hydroxymethylfurfural), phenolic compounds (such as coumaric acid, ferulic acid, syringaldehyde, and vanillin), and weak acids (mainly acetic acid and formic acid) (Jönsson & Martín, 2016). Among these degradation compounds, acetic acid, resulted from the hydrolysis of acetyl groups of hemicellulose, has been known to be the most prevalent organic acid accumulated in the hydrolysate of hardwoods and annual plants and is a potential severe inhibitor for various microbial fermentation process (such as ethanol fermentation with yeast).

Bio-butanol produced from renewable carbon sources through the clostridial acetone-butanol-ethanol (ABE) fermentation is of great interest, because it not only can be used as an important renewable fuel that has various advantages over ethanol, but also has vast applications as a chemical feedstock in many industries (Y.-S. Jang, Malaviya, et al., 2012). The ABE fermentation was successfully operated in the industrial scale for biosolvent production in the early half of 20th century, but it gave way to chemical solvent synthesis from petroleum for economic reasons (Jones & Woods, 1986). Recently, the ABE fermentation received revived attention because of the fluctuating price and limited availability of petroleum oil and the surplus of waste lignocellulosic biomass materials that can be utilized as inexpensive fermentation substrates (Balan, 2014). Among the well-known solventogenic clostridial strains (from species of *C. acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum* and *C. saccharobutylicum*) that can perform efficient ABE production, *C. saccharoperbutylacetonicum* N1-4 (ATCC 27021) can naturally produce very high levels of solvent and possesses various advantageous features (Tiam mun et al., 1995; S. Wang, Dong, Wang, et al., 2017).

Generally, ABE fermentation is a unique bi-phasic process, in which at the first phase (acidogenesis), carbohydrate carbon sources are degraded into acids (mostly acetic and butyric acids), while at the second phase (solventogenesis), the acids generated from the first phase are re-assimilated and converted into solvents along with the consumption of additional carbohydrates (Jones & Woods, 1986). In this sense, acetic acid (and butyric acid) is a substrate rather than an inhibitor for biobutanol production. Actually, the supplementation of additional acetate in

chemically defined fermentation medium was found to increase and stabilize solvent production by *C. beijerinckii* NCIMB 8052 and *C. beijerinckii* BA101 (C.-K. Chen & H. P. Blaschek, 1999; Chih-Kuang Chen & Hans P Blaschek, 1999). However, such effects have not been previously investigated or demonstrated in *C. saccharoperbutylacetonicum* N1-4. Furthermore, little work has been done to develop robust *C. saccharoperbutylacetonicum* strains for enhanced acid re-assimilation and elevated solvent production.

In *C. saccharoperbutylacetonicum* N1-4, phosphotransacetylase (*pta*) and acetate kinase (*ack*) are responsible for the acetic acid production from acetyl-CoA and phosphotransbutyrylase (*ptb*) and butyrate kinase (*buk*) are for the butyric acid production from butyl-CoA. Solventogenic genes are organized in a polycistronic solvent-producing *sol* operon consisting genes encoding NAD-dependent aldehyde dehydrogenase (*ald*; CSPA_RS27680), butyrate-acetoacetate CoA transferase subunits A/B (*ctfA/ctfB*; CSPA_RS27685/CSPA_RS27690), and acetoacetate decarboxylase (*adc*; CSPA_RS27695), among which *ctfA/ctfB* are genes primarily responsible for acid re-assimilation (Kosaka et al., 2007). Along with the re-assimilation of acids, acetoacetate is produced followed by being transformed to acetone through the catalysis by *adc*. The cassette EC, including thiolase (*thl* CSPA_RS03020), β-hydroxybutyryl-CoA dehydrogenase (*hbd* CSPA_RS02150), crotonase (*crt* CSPA_RS2130), and butyryl-CoA dehydrogenase (*bcd* CSPA_RS2150), are responsible for the conversion of acetyl-CoA to butyryl-CoA (Hou et al., 2013). The final end products ethanol and butanol are mainly produced under the action of the bifunctional aldehyde/alcohol dehydrogenase (encoded by *adhE*).

To improve alcohol titers and butanol-selective production, metabolic flux in the solventogenic biosynthesis and flow of carbon pathways have been enhanced through metabolic engineering in solventogenic clostridial strains. In *C. acetobutylicum*, the butanol reached an extremely high productivity (2.64 g $L^{-1}h^{-1}$) in a long-term fermentation through overexpressing the *thl, ctfA/B* and *adhE1* genes as well as knocking out the *pta* and *buk* genes (S.-H. Lee, Kim, Kim, Cheong, & Kim, 2016). By expressing the *sol* operon, optimizing the promoter of *aad* and co-expressing *thl*, total alcohol titers and butanol selectivity have been significantly increased (Sillers, Al‐Hinai, & Papoutsakis, 2009; Tummala et al., 2003). Hou *et al*. overexpressed the cassette EC (*thl, hbd*, *crt* and *bcd*) as well as the *adhE* and *ctfAB* genes from *sol* operon in *C. acetobutylicum*, resulting in 18.9 g/L of final butanol titer and 0.71 mol of butanol yield per mol of glucose consumed in batch fermentation. Recently, in *C. saccharoperbutylacetonicum* strain N1-4, the overexpression of *sol* operon increased ethanol production by 400% with enhanced acid re-assimilation, and the overexpression of EC significantly increased the butanol production (by 13.7%) and selectivity (73.7%) (S. Wang, Dong, & Wang, 2017).

Previously, we have developed a biomass pretreatment method using acetic acid as the treatment reagent and used the biomass hydrolysates for ABE production. However, in that process, since elevated level of acetate is generated in the biomass hydrolysates, it can result in incomplete acetate re-assimilation and potential inhibition for cell growth. Therefore, in this study, our objective was to develop a robust strain with enhanced acid re-assimilation through metabolic engineering, to boost biosolvent production from acetic-acid-pretreated biomass. Various key

genes related to acid re-assimilation and alcohol biosynthesis pathways including the *sol* operon (*ald-ctfA-ctfB-adc*) and cassette EC (*thl-hyd-crt-bcd*) were overexpressed in *C. saccharoperbutylacetonicum* N1-4. Fermentation results demonstrated that the engineered strain have reinforced capability for acid re-assimilation and solvent production, and can efficiently convert the acetic-acid-pretreated biomass into ABE.

IV.2 Material and methods

IV.2.1 Strains and growth conditions

Table IV-1. Strains and plasmids used in this study

Strains	Description and Relevant Characteristics	Source
E. coli ER2523 (NEB express)	*fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 Δ(mcrCmrr) 114::IS10*	New England Biolabs
C. saccharoperbutylacetonicum		
N1-4	DSM 14923 (= ATCC 27021), wild type strain	DSM
JZ100	N1-4 harboring pJZ100	This work
PW2	N1-4 harboring pPW2	This work
PW3	N1-4 harboring pPW3	This work
PW4	N1-4 harboring pPW4	This work
Plasmids		
pTJ1	CAK1 ori Amp^r Erm^r	(Y. Wang et al., 2013)
pJZ100	pTJ1 derivate; Expression vector under the control of the thiolase promoter (P_{thl})	This work
pSH7	pTJ1 derivative; CAK1 ori Amp^r Erm^r::cassette EC (*thl hbd crt bcd*)	(S. Wang, Dong, & Wang, 2017)
pPW1	pSH7 derivative; containing additional P_{thl}-XhoI-T_{thl}	This work
pPW2	pJZ100 derivative; containing additional *sol* operon *(ald ctfA ctfB adc)*	This work
pPW3	pPW1 derivative; containing additional *sol* operon *(ald ctfA ctfB adc)*	This work
pPW4	pSH7 derivative; containing additional P_{fd}-*sol* operon	This work

All bacterial strains used in this study are listed in **Table IV-1**. NEB® Express Competent *E. coli* ER2523 (New England Biolabs Inc., Ipswich, MA) was used for cloning and vector

maintenance. It was grown aerobically at 37 ℃ in the Luria-Bertani (LB) medium supplemented with 100 μg/ml of ampicillin (Amp) as needed. *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923 = ATCC 27021) was obtained from DSMZ (Braunschweig, Germany) and grown anaerobically at 35 ℃ in the tryptone-glucose-yeast extract (TGY) medium containing 30 g/l of tryptone, 20 g/l of glucose, 10 g/l of yeast extract, and 1 g/l of L-cysteine (Yao et al., 2017). 30 μg/ml of clarithromycin (Cla) was supplemented as needed for *C. saccharoperbutylacetonicum* mutant selection and cultivation.

IV.2.2 Plasmids construction

Table IV-2. Primers sequences

Primers	Sequence (5’→3’)
YW32	GTTTTCCCAGTCACGACGTT
YW33	TTGCTGCTCATGCAGATGAT
YW1075	TCTATAAAATTTTAGGAGGTCAAACATGATTAAAGACACGCTAGTTTCTATAA
YW1076	TATCATAGTAACCTTTTTAAATCTTAATTTATATTATTTAAGGGAAAGATAATCATGTACAACC
YW1177	GTAATACTAAAACTGAATTGATTGG
YW1178	GTTATATCCCGCCGTCAACCACCATCAAACAGGATTTTCG
YW1179	CTGTTTGATGGTGGTTGACGGCGGGATATAACATGAGC
YW1180	GTCGAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCG
YW2459	AAAGTTACTGTAGTTAGTATGGGACTTC
YW2460	AACAACTGGTATTAGTAATACTAAAACTGA
YW2491	AACCATCACACTGGCGGCCGTTTAAATATTATTATATGTGAGAAAAAATAAATTTG
YW2492	CTTTAATCATCTAGAACACCTCCTAATAAATTG
YW2493	GGTGTTCTAGATGATTAAAGACACGCTAG
YW2494	TTGGGCCCTCTAGATGCATGTTATTTAAGGGAAAGATAATCATG

The plasmids and primers used in this study are presented in **Table IV-1** and **Table IV-2**, respectively. All DNA primers were synthesized by Integrated DNA Technologies (Coralville, Iowa). The plasmids pTJ1 (Y. Wang et al., 2016) and pSH7 (S. Wang, Dong, & Wang, 2017) were used as mother vectors for the recombinant plasmid construction. The plasmid pSH7 has been

previously derived from pTJ1 containing the expression cassette EC (*thl-hyd-crt-bcd*) from *C. saccharoperbutylacetonicum* N1-4 (S. Wang, Dong, & Wang, 2017). All cloning PCR was performed using the high-fidelity DNA polymerases, Phusion (New England BioLabs Inc., Ipswich, MA), PrimeSTAR (Takara Bio USA, Inc., Mountain View, CA), or Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd., Nanjing, China). The *thiolase* promoter (P_{thl}) and terminator (T_{thl}) were amplified from *C. saccharoperbutylacetonicum* N1-4 using primer pairs of YW1177/YW1178 and YW1179/YW1180, respectively. After being fused together through overlapping extension PCR (SOE-PCR) with primers YW1177 and YW1180, this P_{thl}-T_{thl} fragment (containing two *BseR*I restriction enzyme sites in the middle between P_{thl} and T_{thl}) was inserted between the *Apa*I and *Bam*HI restriction enzyme sites of pTJ1 through Gibson Assembly (NEBuilder® HiFi DNA Assembly Master Mix, New England Biolabs Inc., Ipswich, MA) (Gibson et al., 2009), generating pJZ100. On the other hand, the same P_{thl}-T_{thl} fragment was inserted into the *Xho*I site of pSH7 through Gibson Assembly, generating pPW1. The *sol* operon including *ald, ctfA, ctfB* and *adc* (Kosaka et al., 2007) was amplified from *C. saccharoperbutylacetonicum* N1-4 using primers YW1075 and YW1076, followed by being inserted into the *Bse*RI sites of pJZ100 and pPW1, generating pPW2 and pPW3, respectively. To obtain *sol* operon with the ferredoxin promoter (P_{fd}), two fragments of P_{fd} and *sol* operon were amplified first from *C. saccharoperbutylacetonicum* N1-4 with primer pairs of YW2491 and YW2492 and of YW2493 and YW2494, respectively. Then the desirable P_{fd} - *sol* was generated through SOE-PCR with primers YW2491 and YW2494, followed by being inserted into the XhoI site of pSH7, generating

pPW4. All the plasmid constructs were verified through Sanger sequencing performed by ACGT, Inc. (Wheeling, IL).

IV.2.3 DNA transformation and mutant verification

The transformation of *C. saccharoperbutylacetonicum* N1-4 was carried out with electroporation following the protocol as previously described (S. Wang, Dong, Wang, et al., 2017). Briefly, *C. saccharoperbutylacetonicum* N1-4 was cultivated anaerobically at 35 ℃ in TGY medium until the optical density at 600 nm (OD_{600}) reached 0.8-1.0. The cells were harvested immediately through centrifugation at 4,200 g at 25 ℃ for 10 min. The cell pellets were washed once with SMP buffer (270 mM sucrose, 1 mM $MgCl_2$, and 5 mM sodium phosphate, pH 6.5) with the same volume as the original volume of the bacterial culture at room temperature and then re-suspended in 1/20 volume of SMP butter, obtaining the competent cells. Immediately, 1.0 μg of plasmid DNA was mixed with 400 μl of competent cells and transferred into a pre-cooled 0.2 cm electroporation cuvette. The whole mixture within the cuvette was then incubated in ice for 20 minutes. The whole process was carried out by transferring the cell culture in and out the anaerobic chamber to avoid exposing the cell to oxygen (the centrifugation needed to be performed outside of the chamber). A Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA) connected to the anaerobic chamber was used to deliver the electronic pulse with the following conditions: 1,000V of voltage, 25 μF of capacitance and 300 Ω of resistance. Afterwards, the cells were transferred into 1.6 ml of TGY and incubated at 35 ℃ for 2-4 h for recovery. The recovered cells were plated onto pre-warmed TGY plates containing clarithromycin and incubated

anaerobically at 35 ℃. After 1-2 days, Cla-resistant colonies would grow and were picked for colony PCR (cPCR) to confirm the presence of plasmid using primers YW32 and YW33 (for pPW2) or YW2459 and YW2460 (for pPW3 or pPW4). The generated recombinant strains were named based on the harbored plasmid as *C. saccharoperbutylacetonicum* PW2, *C. saccharoperbutylacetonicum* PW3, *C. saccharoperbutylacetonicum* PW4 and *C. saccharoperbutylacetonicum* JZ100 (as a control strain).

IV.2.4 Fermentation

Batch fermentation was carried out with a model solution containing 80 g/L glucose, 2 g/L yeast extract, 6 g/L tryptone and filter-sterilized P2 or modified P2 (MP2) medium. The P2 medium contains (in g/L): KH_2PO_4, 0.5; K_2HPO_4, 0.5; CH_3COONH_4, 2.2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; NaCl, 0.01; p-aminobenzoic acid, 0.001; thiamine-HCl, 0.001; and biotin, 0.00001. The MP2 medium is the same as P2 medium except that 2.2 g/L of CH_3COONH_4 in P2 was replaced with 2 g/L $(NH_4)_2SO_4$.

To investigate the effect of acetate on the solvent production in various strains, the fermentation was performed in 250 mL serum bottles with a working volume of 100 mL. Before the fermentation, the stock solution of glucose, and that of mixed yeast extract and tryptone were both set to pH 6.8, sparged with N_2 for 10 min and autoclaved at 121 ºC for 20 min. After being cooled down, they were put into the anaerobic chamber. Along with filter-sterilized MP2 or P2 stock solutions with different concentration of sodium acetate, all the necessary components were mixed together to reach the designated composition for each fermentation. This included 80 g/L glucose,

2 g/L yeast extract, 6 g/L tryptone, and various concentrations (0-12.6 g/L; there is around 1.6 g/L acetate in the P2 medium) of acetate. Then, active growing preculture (at OD_{600} of 0.8-1.0 grown in TGY) was inoculated into the fermentation with an inoculum ratio of 5% (v/v). Then all the bottles were put into a shaker incubator and the fermentation was carried out at 30 ℃ with 150 rpm agitation without pH control. All the serum bottles were kept sealed during the fermentation to maintain the anaerobic condition. All fermentations were performed in triplicates.

Large-scale batch fermentation was performed in BioFlo 115 benchtop bioreactors (New Brunswick Scientific Co., Enfield, CT) with a working volume of 1.5 liters. Model solution of the same composition as described above was added into the reactor and then autoclaved. Oxygen-free nitrogen was flushed through the broth starting overnight or at least several hours before the inoculation (until the fermentation culture initiated its own gas production).

The cell culture was propagated anaerobically in the TGY medium until the OD_{600} reached ~0.8. Then the culture was inoculated into the reactor at 5% (vol/vol) inoculum ratio to start the fermentation. The temperature was controlled at 30±1 ℃ and the agitation was maintained at 55 rpm. The pH was controlled > 5.0 throughout the fermentation by adding 6 M NaOH. Samples were taken throughout the fermentation to monitor the cell density, sugar consumption, and endproduct production. Each fermentation was conducted in duplicate. The pH profile was automatically recorded by the NBS BioCommand software (New Brunswick Scientific Co, Inc., Edison, NJ) in real time throughout the fermentation.

IV.2.5 Analytical procedures

Cell growth was monitored by measuring OD_{600} with the Ultrospec 10 cell density meter (Amersham Biosciences Corp., Piscataway, NJ). An Agilent 1260 Infinity HPLC system (Agilent Technologies, CA) was used for the analyses of sugars and fermentation endproducts. The various compounds were separated with a Varian MetaCarb 87H Column 300 $\times$ 7.8 mm along with a 50 $\times$ 4.6 mm MetaCarb 87H guard column (Agilent Technologies, CA) and then detected with the refractive index detector (RID). 0.005 N H_2SO_4 was used as the mobile phase at an isocratic flow rate of 0.6 mL/min, and the temperature of the column was maintained at 25 ℃ during the elution.

All the statistical analyses were performed using SAS® University Edition software (SAS Institute Inc, Cary, NC). *p*-values of less than 0.05 were regarded as significant.

IV.3 Results and discussion

IV.3.1 Effects of *sol* operon overexpression on solvent production

As shown in **Fig. IV-1**, the kinetics for cell growth and sugar consumption of *C. saccharoperbutylacetonicum* PW2 are similar to *C. saccharoperbutylacetonicum* JZ100. However, interestingly, the pH profile of PW2 firstly decreased to around 5.5 and then increased back, and further decreased again after 20 h. However, the pH never reached a point < 5.0 in the whole process. The acetate level decreased from the very beginning of the fermentation, and leveled off at 0.3 g/L from 36 h till the end of the fermentation. The peak level of butyrate production in PW2 was only about 1/3 of that in JZ100, and there was no detectable butyrate production at the end of the fermentation. These results about acetate and butyrate production indicated that PW2 has

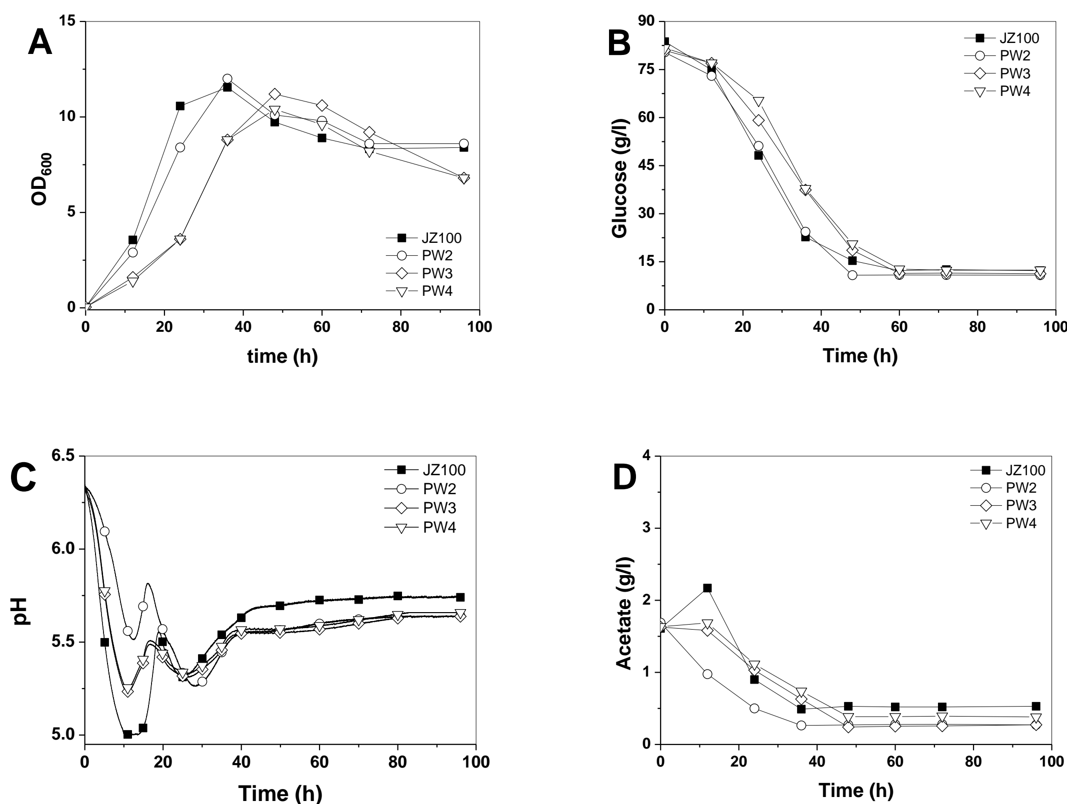
significantly enhanced acid re-assimilation capability because of the overexpression of *sol* operon. Significantly higher ethanol has been produced (4.9 g/L vs. 0.7 g/L in JZ100) in PW2. Also, the acetone and butanol production in PW2 has been improved by 19.2% and 3.5% respectively compared to the control. Overall, the total solvent production was improved to 31.4 g/L (compared to 25.1 g/L in JZ100), which was the highest among all the strains constructed in this study (**Fig. IV-1I**). It is not surprising that the overexpression of *ctfA/B* and *adc* has led to significantly increased acetone production. The *ald* gene encodes an alcohol dehydrogenase; it has the activity for catalyzing both ethanol and butanol production. The overexpression of *ald* led to six times more ethanol production in PW2 than in JZ100, however, the butanol production in PW2 was only slightly increased. This might be because *ald* within the *sol* operon has higher specificity for ethanol production rather than for butanol production. On the other hand, it was a more 'cost-efficient' pathway (less reducing power is needed) for the cell to convert acetyl-CoA to ethanol rather than to butyryl-CoA and further butanol.

Previously, *sol* operon has been overexpressed in *C. saccharoperbutylacetonicum* driven by the P_{thl} from *C. beijerinckii* NCIMB 8052, which led to slightly decreased butanol production and marginally increased ABE production compared to the control strain (S. Wang, Dong, & Wang, 2017). In this study, we employed the native P_{thl} for the overexpression of *sol* operon and resulted in enhanced butanol and much more elevated ABE production. This suggested that the native promoter enabled superior activity of the overexpressed genes, and thus a preferable option.

Table IV-3. Summary of the fermentation results for *C. saccharoperbutylacetonicum* strains

	Strains			
Characteristics	JZ100	PW2	PW3	PW4
Glucose consumed (g/L)	71.4±1.0[a]	69.6±1.5[a]	69.7±1.2[a]	69.3±1.1[a]
Acetone (g/L)	7.3±0.6[a]	8.7±0.5[a]	8.6±0.4[a]	8.2±0.4[a]
Ethanol (g/L)	0.7±0.1[a]	4.9±0.2[b]	1.2±0.1[a]	1.0±0.1[a]
Butanol (g/L)	17.1±0.6[a]	17.7±0.2[a]	18.4±0.3[a]	17.0±0.4[a]
Final solvents (g/L)	25.1±0.8[a]	31.4±0.7[b]	28.2±1.1[ab]	26.3±1.1[ab]
Final solvent yield (g/g)	0.35±0.01[a]	0.45±0.01[b]	0.41±0.01[ab]	0.38±0.02[ab]
Acetic acid (g/L)	0.5±0.4[a]	0.3±0.3[a]	0.3±0.1[a]	0.4±0.2[a]
Butyric acid (g/L)	0.1±0.1[a]	0±0[a]	0±0[a]	0±0[a]

All results shown are average value ± standard deviations from duplicated experiments. Values followed by the same letter with in row are not significantly different at the 0.05 level based on Tukey's HSD test.



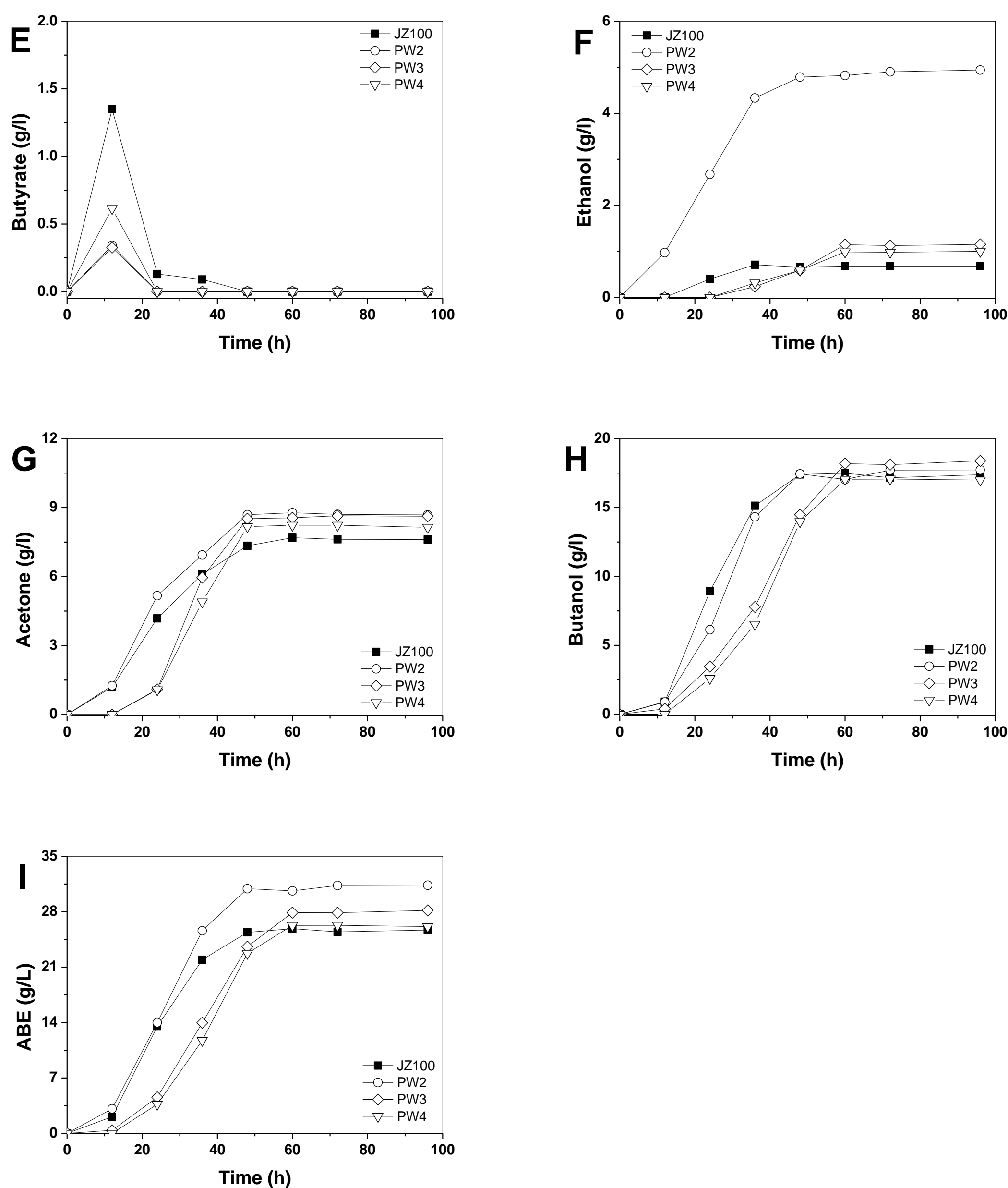


Fig. IV-1. Batch fermentation profiles of *C. saccharoperbutylacetonicum* mutant strains in the P2 medium with 1.6 g/L acetate. (A) Cell optical density; (B) Glucose consumption; (C) pH; (D) Acetate production; (E) Butyrate production; (F) Ethanol production; (G) Acetone production; (H) Butanol production; (I) Total acetone butanol and ethanol. Fermentation was carried out in replicates, with one batch reported here as the representative.

IV.3.2 Effects of combined overexpression of *sol* operon and EC on solvent production

Further, we overexpressed both *sol* operon and cassette EC in *C. saccharoperbutylacetonicum* to see whether such a strategy can have complementary and synergistic effects on the solvent production. As shown in **Fig. IV-1**, the constructed strain PW3 (with one P_{thl} to drive the *sol* operon expression, and another P_{thl} to drive EC expression; **Table IV-3**) demonstrated the delayed growth at the beginning of the fermentation when compared to JZ100. The sugar consumption was a little delayed when compared to JZ100. The pH initially decreased to ~5.25 (and never reached a point < 5.0 as JZ100 did) and then increased back. For the acetate production, there was no noticeable increase from the beginning of the fermentation, and then increased to lower levels after 12 h of the fermentation, and only 0.3 g/L acetate was detected at the end. The peak value of the butyrate in PW3 was lower than in JZ100, and by the end of the fermentation, there was no butyrate left. These results indicated that the co-overexpression of *sol* operon in PW3 brought an efficient acid re-assimilation. After the fermentation, there was 1.2 g/L ethanol produced, which as slightly higher than JZ100, but much lower than that in PW2. The acetone production was similar to that in PW2 and 17.8% higher than that in JZ100. The butanol production reached 18.4 g/L in PW3, which was the highest among all these strains. In addition, 28.2 g/L total ABE was produced in PW3, which was 12.4% higher than that in JZ100, but 10.2% lower than that in PW2. Therefore, briefly, by co-expression of *sol* operon and EC in PW3, we did see the combined benefits for enhanced acid re-assimilation and elevated butanol production.

Since we used P_{thl} for the overexpression of both the *sol* operon and EC in PW3, we speculated

that the competition for the same RNA polymerase could have impeded the expression of the target genes. On the other hand, this could also possibly negatively influence the native thiolase gene expression due to the promoter titration effects (Sillers et al., 2009). Therefore, further improved performance of the recombinant strain (particularly PW3) could be possibly achieved by overexpressing these two gene clusters using two different strong constitutive promoters. The promoter of the ferredoxin gene (P_{fdx}) has been known as another constitutive strong promoter (Woods, 1995). Therefore, we constructed *C. saccharoperbutylacetonicum* PW4 by overexpressing EC under P_{thl} and *sol* operon under P_{fdx}. As shown in **Fig. IV-1** and **Table IV-3**, the phenotype for the cell growth, sugar consumption, and endproduct production in PW4 were all very similar to PW3. Actually, there was slightly decreased production for acetone, ethanol and butanol. The final total solvent in PW4 was 6.7% lower than that in PW3. Such results were unanticipated but not surprising. P_{fdx} is a very strong promoter; however, it might not be as strong as P_{thl}, and thus the solvent production in PW4 was not further increased (but decreased) as compared to PW3.

IV.3.3 Effects of supplemented acetate on the fermentation with various recombinant strains

With the metabolic engineering efforts in this study, we attempted to enhance the re-assimilation capability in the host strain and thus elevated solvent production. In the lignocellulosic biomass hydrolysates (especially when acetate is used as the chemical reagent for the pretreatment), higher levels of acetate (than that exists in the P2 medium) are expected. Therefore, we further systematically evaluated the performance of various recombinant strains constructed in this study

when various concentrations of acetate were supplemented. The fermentation was carried out in the serum bottle with a 100ml working volume.

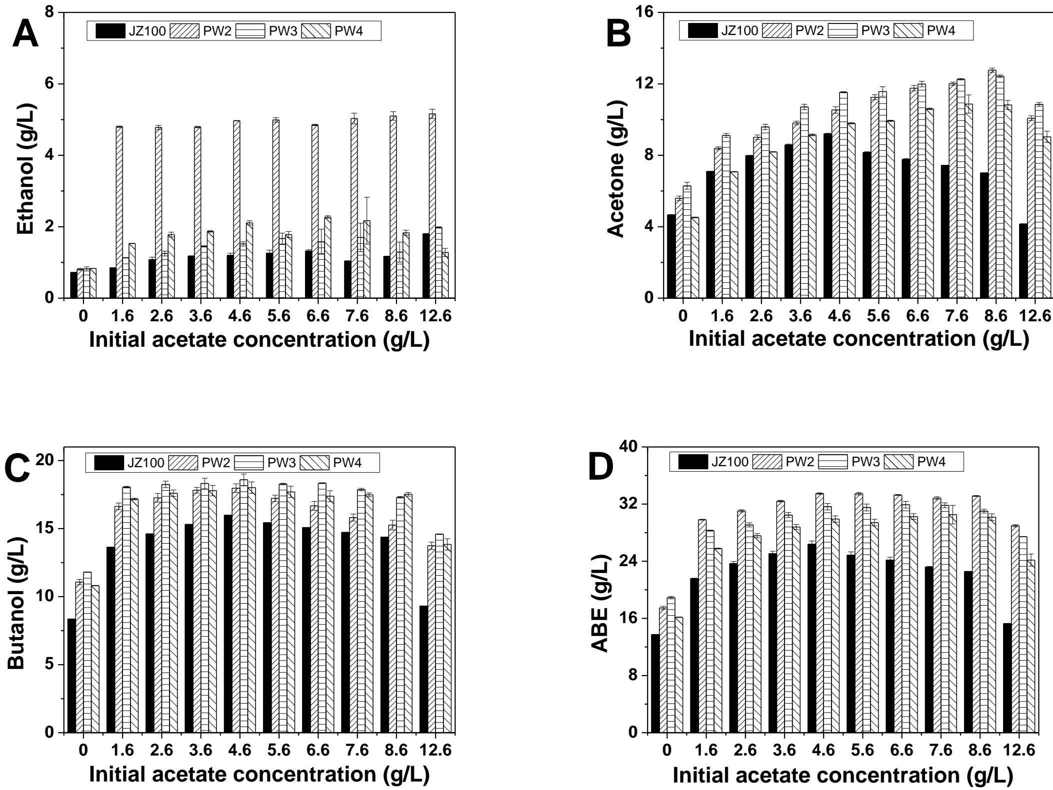


Fig. IV-2. The effect of added acetate on solvent production by *C. saccharoperbutylacetonicum* mutant strains. Cultures were grown in 100 ml MP2 medium plus 80g/L glucose and 0-17.5 g/L mM sodium acetate. The samples were examined following a 96-h fermentation. Values are the means of triplicate sample; error bars standard deviations.

As shown in **Table IV-4** and **Fig. IV-2**, when there is no acetate supplemented, the solvent production was low for all strains, indicating that appropriate amount of acetate is required for the buffering purpose and enhancement of solvent production during ABE fermentation (Chih-Kuang Chen & Hans P Blaschek, 1999). However, comparatively, all the engineered strain produced more

solvent than the control strain, in the order of PW3 > PW2 > PW4 > JZ100. This indicated that the overexpression of *sol* operon is critical for the solvent production especially when there is no acetate is supplemented. Previously it has been reported that when acetate was supplemented in the ABE fermentation with *C. beijerinckii* NCIMB 8052, the CoA transferase activity was elevated and thus led to enhanced solvent production. While when there is no acetate supplemented as the case here, the overexpression of *sol* operon could offset the inefficiency of sol operon activity in the WT strain and thus significantly increased the solvent production. With the increase of supplemented acetate concentration, the production of acetone, ethanol, butanol as well as the total ABE in each strain generally increased and reached the maximum level when 4.6 g/L acetate was supplemented. With the further increase of the supplemented acetate concentration (from 5.6-8.6 g/L), the solvent production in JZ100 gradually decreased; however, the solvent production in the engineered strains kept at the similar level with some marginal decrease (as that when 4.6 g/L acetate was supplemented). This indicated that, with the overexpression of key genes in the solvent production pathway (especially with the overexpression of *sol* operon), the acid re-assimilation and solvent production was enhanced in the recombinant strains. From another perspective, we could say that the tolerance to the high level of fatty acids has been reinforced in the engineered strain compared to the control. However, when the supplemented acetated was increased to 12.6 g/L, the solvent production in all the strains has been remarkably decreased. It suggested that the high level of acetate under this condition is too toxic and thus the ABE production was inhibited even in the strains with the overexpression of the ABE pathway genes. Under all these conditions,

PW3 generally produced the highest levels of butanol, while PW2 produced the highest ethanol and thus highest total ABE (except for the condition when there is no acetate added) among all the strains (**Fig. IV-2D**).

Table IV-4. Summary of the fermentation results for *C. saccharoperbutylacetonicum* mutant strains with various initial acetate concentration.

	Strains [a]	Initial acetate concentration (g/L)									
		0	1.6	2.6	3.6	4.6	5.6	6.6	7.6	8.6	12.6
Glucose consumption (g/L)	ZJ100	44.9±1.1	70±0.3	74.6±0.3	75.7±1.1	76.7±0.9	76.6±1.0	76.1±0.9	72.6±0.2	70.1±0.1	42.7±0.1
	PW2	47.4±1.5	71.9±0.9	75.9±0.4	77.4±0.2	78.5±0.4	79.7±0.5	79.7±0.1	79.8±0	79.7±0.1	80±0
	PW3	45.5±0.1	69.6±0.3	70.4±0.3	76.6±1	79.5±1	79.1±0.1	78.8±0.8	79.7±0.5	80±0	80±0
	PW4	45.3±1.1	68.1±0.3	70.6±0.3	75.7±1	78.8±1	79.3±1	79.5±0.9	79.5±0.7	79.6±0.9	79±0
Acetate re-assimilation (g/L)	ZJ100	-0.4±0.2	1.1±0.1	1.7±0	2.6±0	3.2±0	3.6±0	4.1±0.1	3±0.4	3.4±0.4	2.4±0.1
	PW2	-0.1±0.1	1.3±0	2.4±0	3.2±0	3.9±0	4.5±0	4.8±0.1	5.4±0.2	5.7±0.1	7.2±0.2
	PW3	0±0	1.1±0	1.6±0	2.3±0	2.9±0	3.2±0	3.7±0	4.6±0	4.5±0	6.6±0.1
	PW4	0±0	1.3±0.2	1.6±0	2.2±0	2.7±0	2.8±0	2.8±0.1	2.9±0.1	3.6±0.1	6.1±0
Acetate (g/L)	ZJ100	0.4±0.2	0.6±0.1	0.9±0	1±0	1.4±0	2±0	2.5±0.1	4.6±0.4	5.2±0.4	10.2±0.1
	PW2	0.1±0.1	0.3±0	0.2±0	0.4±0	0.7±0	1.1±0	1.8±0.1	2.2±0.2	2.9±0.1	5.5±0.2
	PW3	0±0	0.5±0	1±0	1.3±0	1.7±0	2.4±0	2.9±0	3±0	4.1±0	6±0.1
	PW4	0±0	0.3±0.2	1.1±0	1.4±0	1.9±0	2.8±0	3.8±0.1	4.7±0.1	5±0.1	6.5±0
Butyrate (g/L)	ZJ100	0±0	0±0	0±0	0±0	0±0	0.4±0.1	1.3±0.1	1.5±0	1.2±0	1.9±0
	PW2	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0.2
	PW3	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	1.7±0.8
	PW4	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	1.7±0.5
ABE yield (g/g)	ZJ100	0.31±0.02	0.31±0.01	0.32±0.01	0.33±0.05	0.34±0.03	0.32±0.04	0.32±0.04	0.32±0	0.32±0	0.36±0.02
	PW2	0.37±0.13	0.41±0.09	0.41±0.13	0.42±0.11	0.43±0.18	0.42±0.14	0.42±0.15	0.41±0.09	0.42±0.12	0.36±0.13
	PW3	0.42±0.19	0.41±0.11	0.41±0.15	0.4±0.15	0.4±0.03	0.4±0.27	0.4±0.15	0.4±0.04	0.39±0.07	0.34±0.11
	PW4	0.36±0.02	0.38±0.01	0.39±0.01	0.38±0.05	0.38±0.03	0.37±0.04	0.38±0.04	0.38±0.51	0.38±0.26	0.31±0.32

[a] Cultures were grown in 100 ml MP2 medium plus 80g/L glucose and 0-17.5 g/L mM sodium acetate. The samples were examined following a 96-h fermentation. Values are the means of triplicate sample; error standard deviation.

IV.3.4 ABE production from acetic-acid-treated switchgrass through SSF

In section III, we have previously developed an innovative acetic-acid-based biomass pretreatment approach for enhanced biosolvent production. With acetic acid as the catalyst for biomass pretreatment, no additional chemical is introduced and thus saves cost and avoids the associated environmental pollution. In addition, generally with such a mild chemical reagent for biomass pretreatment, less amount of inhibitors will be generated compared to the conventional biomass pretreatment methods. Thus, the LPF from the acetic-acid-based pretreatment (which is usually discarded during the regular biomass pretreatment process because of its low fermentability) can be used for the fermentation along with the SCF to provide additional carbon source. The supplemented acetic acid for the pretreatment (along with the acetic acid generated during the process) will be re-assimilated as additional carbon source for ABE production in the clostridial fermentation, thus leading to improved solvent yield. However, with the high level of acetate in the hydrolysate, the WT strain for ABE fermentation cannot take up all the acetate for solvent production. We speculated that the constructed recombinant strains in this study could work better for the fermentation with this substrate. Based on the results with the model solutions as described above, the PW2 and PW3 strains demonstrated the best potential for solvent production when high levels of fatty acid existed in the medium. In addition, 4.6 g/L of supplemented acetate in the medium was likely the most appropriate for the solvent production **(Fig. IV-2).** Therefore, switchgrass (a total of 100 ml slurry containing a total amount of 10 g dry

biomass) was pretreated with 3 g/L acetic acid (which would generate around 4.6 g/L acetate in the hydrolysates) at 170 ^{o}C for 20 min. After processing, the LPF/SCF mixture was used for SSF with *C. saccharoperbutylacetonicum* PW2 and PW3. As shown in **Table IV-5**, compared to the control JZ100, PW2 and PW3 had more efficient acid (both acetic and butyric acids) re-assimilation, and produced significantly higher levels of acetone and ethanol and slightly higher butanol, with 15.4 g/L total ABE (20% higher than JZ100). The ABE yield was also ~20% higher in PW2 and PW3 than in JZ100.

Table IV-5. Summary of the pretreated biomass SSF results for *C. saccharoperbutylacetonicum* mutant strains

Characteristics	*C. saccharoperbutylacetonicum* N1-4		
	JZ100	PW2	PW3
Residual sugars (g/L)	3.9±0.1^{a}	3.5±0.1^{ab}	3.3±0.0^{b}
Acetone (g/L)	4.1±0.1^{a}	5.3±0.0^{b}	6.0±0.1^{c}
Ethanol (g/L)	0.7±0.0^{a}	1.8±0.1^{b}	1.2±0.1^{c}
Butanol (g/L)	8.0±0.1^{a}	8.3±0.1^{a}	8.3±0.1^{a}
Final solvents (g/L)	12.8±0.2^{a}	15.4±0.1^{b}	15.4±0.2^{b}
Final solvent yield (g/g)	0.26±0.01^{a}	0.31±0.00^{b}	0.31±0.01^{b}
Acetic acid (g/L)	4.4±0.1^{a}	3.2±0.1^{b}	3.9±0.0^{a}
Butyric acid (g/L)	1.5±0.1^{a}	0.6±0.0^{b}	0.6±0.0^{b}

All results shown are average value ± standard deviations from duplicated experiments. Values followed by the same letter with in row are not significantly different at the 0.05 level based on Tukey's HSD test.

IV.4 Conclusions

In this study, we overexpressed key genes in the ABE fermentation pathways in *C. saccharoperbutylacetonicum* N1-4 to enhance the acid re-assimilation and solvent production. First, the native *sol* operon (*ald-ctfA-ctfB-bcd*) was overexpressed under the strong constitutive

thiolase promoter and generated PW2 strain. Fermentation characterization indicated that the acid re-assimilation was improved in the host and the ABE production has been increased to 31.4 g/L. Although ethanol production has been increased by six times (4.9 g/L vs. 0.7 g/L in the control), butanol production has not been significantly increased. In order to further drive the carbon flux from C2 metabolites to C4 metabolites and butanol production, key genes in the expression cassette (EC) including *hbd, thl, crt* and *bcd* was further overexpressed under thiolase promote besides the *sol* operon overexpression as in PW2, generating PW3 strain. Compared to the control strain JZ100, the production of butanol and acetone in PW3 was increased by 8% and 18% respectively. The final total solvent production in PW3 increased by 12.4% than the control, but was 10% lower than PW2. To avoid the potential 'promoter titration' issue and further improve ABE production, a new strain PW4 was constructed to express *sol* operon with thiolase promoter but EC with ferredoxin gene promoter. Results demonstrated that, the production of all the solvents in PW4 was actually slightly lower than those in PW3, indicating that 'promoter titration' was not an issue in this case. Further, we evaluated the effect of acetic acid concentrations on the solvent production in the engineered strains, and the maximum level of solvent production was achieved when 4.6 g/L acetate was supplemented. Therefore, SSF was carried out with PW2 and PW3 using switchgrass biomass pretreated with 3 g/L acetic acid (which ends up with approximately 4.6 g/L in the fermentation medium). The fermentation ended up with 15.4 g/L total ABE (with a yield of 0.31 g/g) production in both PW2 and PW3, which was significantly higher than that in JZ100.

Overall, our results demonstrated that the overexpression of key genes for acid re-assimilation and solvent production can significantly enhance ABE production in solventogenic clostridia.

V. Engineering *Clostridium saccharoperbutylacetonicum* for high level Isopropanol-Butanol-Ethanol (IBE) production from acetic acid pretreated switchgrass using the CRISPR-Cas9 system

Abstract

Acetone is highly corrosive to engine parts that are composed of rubber or plastic, and thus it cannot be used as a fuel source. For this reason, the acetone produced during the acetone-butanol-ethanol (ABE) fermentation is often considered as an undesirable byproduct for biobutanol production. Biologically, acetone can be converted into isopropanol by the secondary alcohol dehydrogenase. Isopropanol, and thus the isopropanol-butanol-ethanol (IBE) mixture, can be used a valuable biofuel. In this study, we attempt to metabolically engineer the hyper-ABE producing *Clostridium saccharoperbutylacetonicum* N1-4 strain for IBE production. First, we overexpressed the secondary alcohol dehydrogenase (*sadh*) gene from *C. beijerinckii* B-593 in *Clostridium saccharoperbutylacetonicum* on a plasmid, generating PW5 strain. A *hydG* gene (encoding a putative electron transfer protein) is right downstream of *sadh* within the same operon in the *C. beijerinckii* B-593 genome. Therefore, additionally, we overexpressed *sadh-hydG* gene cluster together in *C. saccharoperbutylacetonicum* host in order to evaluate the effect of *hydG* for isopropanol production, generating PW6 strain. Fermentation results indicated that in both PW5 and PW6, high levels of isopropanol were produced with no acetone production was detected. Comparatively, PW6 produced slightly higher isopropanol (10.2 g/L vs. 9.4 g/L in PW5) and total

IBE. However, overall the performance of PW6 for solvent production is very similar to that of PW5. To eliminate the issue with plasmid-based overexpression such as instability and the requirement of antibiotics for cell cultivation and fermentation, we further integrated *sadh* or *sadh-hydG* into the chromosome of *C. saccharoperbutylacetonicum*, and generated strains PW8 and PW9. Fermentations results demonstrated that in PW8, there was 4.8 g/L acetone and 4.0 g/L isopropanol produced, while in PW9, up to 9.5 g/L isopropanol was produced with only 0.4 g/L acetone was detected. This indicated that the co-overexpression of *hydG* with *sadh* through chromosomal integration had significant positive effects on the conversion of acetone to isopropanol. In order to further enhance the solvent production, we additionally overexpressed in PW9 the *sol* operon (*ald-ctfA-ctfB-adc*), the expression cassette EC (*thl-hbd-crt-bcd*), or *sol* in combination with EC, generating strains PW10, PW11, and PW12, respectively. The fermentation characterization indicated that PW10 had significantly elevated ethanol production (5.2 g/L vs. 0.7 g/L in PW9), as well as 25% higher isopropanol (11.9 g/L vs. 9.5 g/L in PW9) with slightly decreased butanol production (16.5 g/L vs. 16.9 g/L in PW9), leading to a significant increase in total solvent titer (34.2 g/L vs. 27.6 g/L in PW9) and yield (0.48 g/g vs. 0.40 g/g in PW9). In PW11, the butanol production increased to 17.9 g/L while ethanol production decreased to 0.4 g/L; however, the isopropanol and final total solvent production was very similar to that in PW9. In PW12, with the co-overexpression of *sol* operon and EC, the production of isopropanol, butanol, and ethanol increased to 11.7 g/L, 17.3 g/L, and 1.1 g/L respectively comparing to PW9, resulting in a slight increase in total solvent yield. Finally, simultaneous saccharification and fermentation

(SSF) was carried out with PW9 and PW10 using the acetic-acid-pretreated switchgrass as the feedstock, and the final solvent titer reached 13.7 g/L and 16.2 g/L, corresponding to the solvent yield of 0.27 g/L and 0.32 g/g in PW9 and PW10, respectively. The engineered strains in this study (PW9, PW10, PW11) produced the highest total IBE that has ever been reported in the batch fermentation with solventogenic clostridia. In addition, our results indicated that the acetic-acid-pretreated biomass can be efficiently converted into biofuel using the metabolically engineered *Clostridium* host.

Keywords: *Clostridium saccharoperbutylacetonicum* N1-4; CRISPR-Cas9; chromosome integration; IBE production; secondary alcohol dehydrogenase; *sol* operon

V.1 Introduction

The limited supply and negative environmental problems as related to the use of petroleum-derived fuels and chemicals have led to the worldwide interest in renewable bio-based fuel and chemicals. Butanol, being a superior fuel over ethanol, has attracted an increased attention because of its advantageous properties including higher energy density and lower hygroscopicity (S. Wang, Dong, Wang, et al., 2017). It can be produced by the solventogenic clostridial strains through acetone-butanol-ethanol (ABE) fermentation. Even though the ABE fermentation has been performed worldwide at industrial scale previously, it is currently considered as less economical process than ethanol for biofuel production with respect to the lower carbon recovery. Particularly,

acetone produced during the ABE process cannot be used as a fuel due to its corrosiveness nature to engine parts that are composed of rubber or plastic (P. Dürre, 1998). Hence, it is desirable to suppress the formation of acetone or to convert it into another compound that can be used as a fuel source. For this reason, reducing acetone production has been an important objective for clostridial metabolic engineering (J. Lee et al., 2012).

The attempts to suppress the acetone production by metabolic engineering have been demonstrated to decrease butanol production and accumulate acetic and butyric acids (Y. Jiang et al., 2009; Tummala et al., 2003). A typical feature of ABE fermentation is a unique bi-phasic process, in which at the first phase (acidogenesis), carbohydrate carbon sources are degraded into acids (mostly acetic and butyric acids) while at the second phase (solventogenesis), the acids (causing low culture pH) generated from the first phase are re-assimilated and converted into solvents along with additional consumption of carbohydrates (Jones & Woods, 1986). As a defensive mechanism against low culture pH, acetate and butyrate are re-assimilated by CoA-transferase (*ctfA/B*) to acetyl-CoA and butyryl-CoA, respectively, with concomitant production of acetone by acetoacetate decarboxylase. Thus, acetone formation is essential in the cell growth for cytosolic detoxification to increase the culture pH for successful ABE production (Zhao et al., 2005). Hence, the conversion of acetone into another fuel source is more desirable than suppressing the acetone production in metabolic pathway.

Isopropanol, as a simple secondary alcohol, shows a higher energy density than acetone (23.9 MJ/L vs 22.6 MJ/L) and can also be used as a fuel additive for the preparation of high-octane

gasoline (Peralta-Yahya & Keasling, 2010). Some solventogenic clostridial strains such as *C. beijerinckii* NRRL B593 can produce isopropanol, instead of acetone, together with butanol and ethanol (Hiu et al., 1987). The IBE mixture can be directly used as a fuel source, which could save tremendous energy for the downstream recovery process. However, the IBE production titer (a total concentration of 5.87 g/l) and rate (a productivity of 0.12 g/L/h) with the natural strain are generally very low. Also no efficient genetic modification tools has been developed for these strains so far, which can be used to further improve their performance (Dusséaux et al., 2013).

Hence, there is a strong interest to transform the native ABE producers into efficient IBE producers by introducing the 'acetone-to-isopropanol' pathway through metabolic engineering. This has been demonstrated in several laboratories (Dai et al., 2012; Dusséaux et al., 2013; Y. S. Jang et al., 2013) by overexpressing the secondary alcohol dehydrogenase (SADH, encoded by the *sadh*) from *Clostridium beijerinckii* NRRL B-593 in the ABE-producing hosts. However, many limitations still exist for these genetically engineered strains for IBE production, such as low efficiency in converting acetone into isopropanol, limited total solvent production, and genetic instability due to the plasmid-based overexpression.

C. saccharoperbutylacetonicum N1-4 is known as a hyper-butanol producing strain. Besides, comparing to other solventogenic clostridial strains, it has various advantages including broader range of substrates (including both 5-carbon and 6-carbon monosaccharides, and disaccharides), more efficient re-assimilation of acids, and higher tolerance to lignocellulosic inhibitors (Hayashida & Ahn, 1990; Kosaka et al., 2007; Tashiro et al., 2004; Yao et al., 2017). Recently,

our lab has developed an efficient genome engineering system for this strain based on the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (CRISPR-Cas9) system (S. Wang, Dong, Wang, et al., 2017). This system is primarily functional as a tool for selecting edited cells against non-edited background cells thereby leading to high efficiency of genome engineering. With this system, versatile genome engineering purposes have been achieved with high efficiency in *C. saccharoperbutylacetonicum*, including both clean gene deletion and chromosomal gene integration.

In the present work, we aimed to develop a robust and genetically stable IBE producing strain by integrating *sadh* from *C. beijerinckii* NRRL B593 into the chromosome of *C. saccharoperbutylacetonicum* N1-4 using the CRISPR-Cas9 system. In addition to the *sadh* gene, the *hydG* gene encoding a putative electron transfer protein was also integrated together because the *hydG* gene is located right downstream of the *sadh* gene within the same operon in the chromosome of *C. beijerinckii* NRRL B593. By doing so, we attempted to evaluate the effects of *hydG* (in combination with *sadh*) on the conversion of acetone into isopropanol.

Previously, we reported that by overexpressing the *sol* operon in *C. saccharoperbutylacetonicum* N1-4, enhanced acid re-assimilation and elevated solvent (mostly ethanol and acetone) production were observed in the engineered strain. Also, the overexpression of the expression cassette (EC, consisted of *hbd, thl, crt* and *bcd*) which drives the carbon flux from C2 metabolites to C4 metabolites has led to increased butanol production and selectivity (S. Wang, Dong, & Wang, 2017). Therefore, here, the *sol* operon and EC were further introduced into

the engineered strain for IBE production (individually and simultaneously), attempting to further increase the final biosolvent production. First, the IBE production by the engineered strains was examined through batch fermentation using model solution (with 80 g/L glucose as the carbon source). Finally, the acetic-acid pretreated switchgrass biomass was used as the feedstock for the fermentation. Results demonstrated that lignocellulosic carbon source can be efficiently converted into IBE through fermentation using our engineered strains.

V.2 Material and methods

V.2.1 Strains, growth conditions, plasmids, and primers

All bacterial strains used in this study are listed in **Table V-1**. NEB® Express Competent *E. coli* ER2523 (New England Biolabs Inc., Ipswich, MA) was used for regular cloning and vector maintenance. It was grown aerobically at 37 ℃ in the Luria-Bertani (LB) medium supplemented with 100 µg/ml of ampicillin (Amp) as needed. *C. saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923 = ATCC 27021) was obtained from DSMZ (Braunschweig, Germany) and used as the mother strain in this study. The strain was grown anaerobically at 35 ℃ in the tryptone-glucose-yeast extract (TGY) medium containing 30 g/l of tryptone, 20 g/l of glucose, 10 g/l of yeast extract, and 1 g/l of L-cysteine (Yao et al., 2017). TGYL (TGY supplemented with 40 mM of lactose) was used to induce the Cas9 expression (see below). 30 µg/ml of clarithromycin (Cla) was added into either the TGY (named as TGYC) or TGYL (named as TGYLC) as needed for *C. saccharoperbutylacetonicum* mutant selection or cultivation. All the DNA primers used in this study were listed in **Table V-2**, and were synthesized by Integrated DNA Technologies (Coralville,

IA).

Table V-1. Strains and plasmids used in this study

Strains	Description and Relevant Characteristics	Source
E. coli ER2523 (NEB express)	*fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 Δ(mcrCmrr) 114::IS10*	New England Biolabs
C. saccharoperbutylacetonicum		
N1-4	DSM 14923 (= ATCC 27021), wild type strain	DSM
JZ100	N1-4 harboring pJZ100	This work
PW5	N1-4 harboring pPW5	
PW6	N1-4 harboring pPW6	
PW8	Derived from N1-4, with *sad*h$_{B\text{-}593}$ insertion	This work
PW9	Derived from N1-4, with *sad*h-*hydG*$_{B\text{-}593}$ insertion	This work
PW10	PW9 harboring pPW2	This work
PW11	PW9 harboring pSH7	
PW12	PW9 harboring pPW3	
Plasmids		
pTJ1	CAK1 ori Amp^r Erm^r	(Y. Wang et al., 2013)
pJZ100	pTJ1 derivate; Expression vector under the control of the thiolase promoter (P$_{thl}$)	This work
pSH7	pTJ1 derivative; CAK1 ori Amp^r Erm^r::cassette EC (*thl hbd crt bcd*)	(S. Wang, Dong, & Wang, 2017)
pPW2	pJZ100 derivative; contain additional *sol* operon *(ald ctfA ctfB adc)*	This work
pPW3	pSH7 derivative; contain additional *sol* operon *(ald ctfA ctfB adc)* with *thiolase* promoter	This work
pPW5	pJZ100 derivative; contain the *sad*h$_{B\text{-}593}$ gene	This work
pPW6	pJZ100 derivative; contain *sad*h-*hydG*$_{B\text{-}593}$ genes	This work
pPW7	pYW34 derivative; P$_{sRNA}$::20nt-spacer targeting on the gene of N1-4	This work
pPW8	pPW7 derivative; contain up homologous arm, P$_{thl}$-*sad*h$_{B\text{-}593}$-T$_{thl}$ and down homologous arm for gene insertion	This work
pPW9	pPW7 derivative; contain up homologous arm, P$_{thl}$- *sad*h-*hydG*$_{B\text{-}593}$-T$_{thl}$ and down homologous arm for gene insertion	This work

V.2.2 Plasmids construction

The plasmids pTJ1 were used as mother vector for the recombinant plasmid construction. The plasmid pSH7 is derived from pTJ1 with the *C. saccharoperbutylacetonicum* N1-4 cassette EC (*thl-hyd-crt-bcd*) and XhoI restriction enzyme site (S. Wang, Dong, & Wang, 2017). The plasmid pJZ100 was constructed to be used for the further construction of recombinant plasmids for gene

overexpression purpose. The promoter (P_{thl}) and terminator (T_{thl}) of the thiolase gene were amplified from *C. saccharoperbutylacetonicum* N1-4 using primer pairs of YW1177/YW1178 and YW1179/YW1180, respectively. After being fused together through overlapping extension PCR (SOE-PCR) with primers YW1177 and YW1180, this P_{thl}-T_{thl} fragment (containing two *Bse*RI restriction enzyme sites between P_{thl} and T_{thl}) was inserted between the *Apa*I and *Bam*HI digestion sites of pTJ1 through Gibson Assembly, generating pJZ100. This P_{thl}-T_{thl} fragment was also inserted into the XhoI digestion site of pSH7 through Gibson Assembly, generating pPW1.

Table V-2. Primers sequences

Primers	Sequence (5'→3')
YW32	GTTTTCCCAGTCACGACGTT
YW33	TTGCTGCTCATGCAGATGAT
YW484	AAAGTTAAAAGAAGAAAATAGAAATATAATCTTTAATTTGAAAAGATTTAAG
YW880	AGACGCATGGCTTTCAAAAA
YW881	CCGTTTACGAAATTGGAACAG
YW1075	TCTATAAAATTTTAGGAGGTCAAACATGATTAAAGACACGCTAGTTTCTATAA
YW1076	TATCATAGTAACCTTTTTAAATCTTAATTTATATTATTTAAGGGAAAGATAATCATGTACAACC
YW1177	GTAATACTAAAACTGAATTGATTGG
YW1178	GTTATATCCCGCCGTCAACCACCATCAAACAGGATTTTCG
YW1179	CTGTTTGATGGTGGTTGACGGCGGGATATAACATGAGC
YW1180	GTCGAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCG
YW1357	AGAATTTTAGGAGGTCAAACATGAAAGGTTTTGCAATGCTAGG
YW1358	TGCGAATGTGAACTTGTATACCTTCTACACATTTAGGATTCTTACAGTTTAAAC
YW1359	AGAATTTTAGGAGGTCAAACTAAGGAGGAACATATTTTATGAAAGGTTTTGC
YW1360	TGCGAATGTGAACTTGTATAGTTATAATATAACTACTGCTTTAATTAAGTCTTTTGGC
YW1432	TTGCTATTTCTAGCTCTAAAACGAAGATGCTGATACTTATACATGGTGGAATGATAAGGG
YW1433	TGTGATATGACTAATAATTAATTAATATTTTATACCAATAATCTAAATCAACAATTTCTTCATTATT TCTAAAAATTC
YW1434	AATTAATAATGTATCAGCATCTTCTGGATTATGAGATATATTAAGTTTTG
YW1435	GATGCTGATACATTATTAATTAATCAATCACTCACTAATTCAAATACTATTTTCC
YW1436	TTAGTATAAGGAATTAAGCTCAAATGAAGTAGATGAGTTTAAAAATATAAAATAG
YW1437	AGCTTAATTCCTTATACTAAATGTCAAGATAATGCAAAGGG
YW1438	CACTAGTAACCATCACACTGAGTTCAAATAATTTTAGATAAAGCAAACGAAATAGAAG
YW1978	TCTATACAATGGCAGGTCCAG
YW1979	GAGCCAGCATCAAATCCATAGGA

The open reading frame (ORF) of the *sadh* gene was amplified from *C. beijerinckii* NRRL B-

593 genomic DNA using primers YW1359 and YW1360, and then inserted into the *Bse*RI site of pJZ100, generating pPW5. The ORF of the *sadh-hydG* gene cluster (containing both *sadh* and *hydG* as an operon) was amplified from *C. beijerinckii* NRRL B-593 genomic DNA using primers YW1357 and YW1358, and then inserted into the *Bse*RI site of pJZ100, generating pPW6. The whole *sol* operon including *ald, ctfA, ctfB* and *adc* (Kosaka et al., 2007) was amplified from *C. saccharoperbutylacetonicum* N1-4 using primers YW1075 and YW1076, followed by being inserted into the *Bse*RI sites of pJZ100 and pPW1, generating pPW3, respectively.

The plasmid pYW34 carries the customized CRISPR-Cas9 system that can be used for versatile genome engineering purposes. It contains the Cas9 ORF from *S. pyogenes* under the control of a lactose inducible promoter and the chimeric gRNA sequence upstream of which the target sequence for the CRISPR system can be inserted and thus specific gene can be targeted for the gene editing purpose (Y. Wang et al., 2016). The plasmid pYW34 was used as the mother vector for the construction of the plasmid to insert *sadh* or *sadh-hydG* into the chromosome (**Fig. V-1**). First of all, the small RNA (sRNA, sCbei_5830) promoter (P_{sRNA}) was amplified from *C. beijerinckii* 8052 genomic DNA with primers YW484 and YW1432, with a 20-nt guiding sequences (5'-GTATAAGTATCAGCATCTTC-3'; targeting the intergenic region between 2376556 and 2376575) fused right downstream of P_{sRNA}, and then inserted into the *Btg*ZI site of pYW34 with Gibson Assembly, generating pPW7. To insert the desired gene into the chromosome through CRISPR-Cas9-assisted homologous recombination (Y. Wang et al., 2016), two homology arms (upstream arm and downstream arm; 1,000 bp for each) were amplified from *C.*

saccharoperbutylacetonicum N1-4 genomic DNA using the primer pairs of YW1433/YW1434 and YW1437/YW1438, respectively. The primer pairs of YW1435/YW1436 were used to amplify P_{thl}-*sadh*-T_{thl} or P_{thl}-*sadh-hydG*-T_{thl} with plasmids pPW5 or pPW6 as the template, respectively. The upstream arm, P_{thl}-*sadh*-T_{thl}, and the downstream arm were inserted into the *Not*I site of pPW7 in a single step through Gibson Assembly, generating pPW8. The upstream arm, P_{thl}-*sadh*-T_{thl}, and the downstream arm were inserted into the *Not*I site of pPW7 in a single step through Gibson Assembly, generating pPW9.

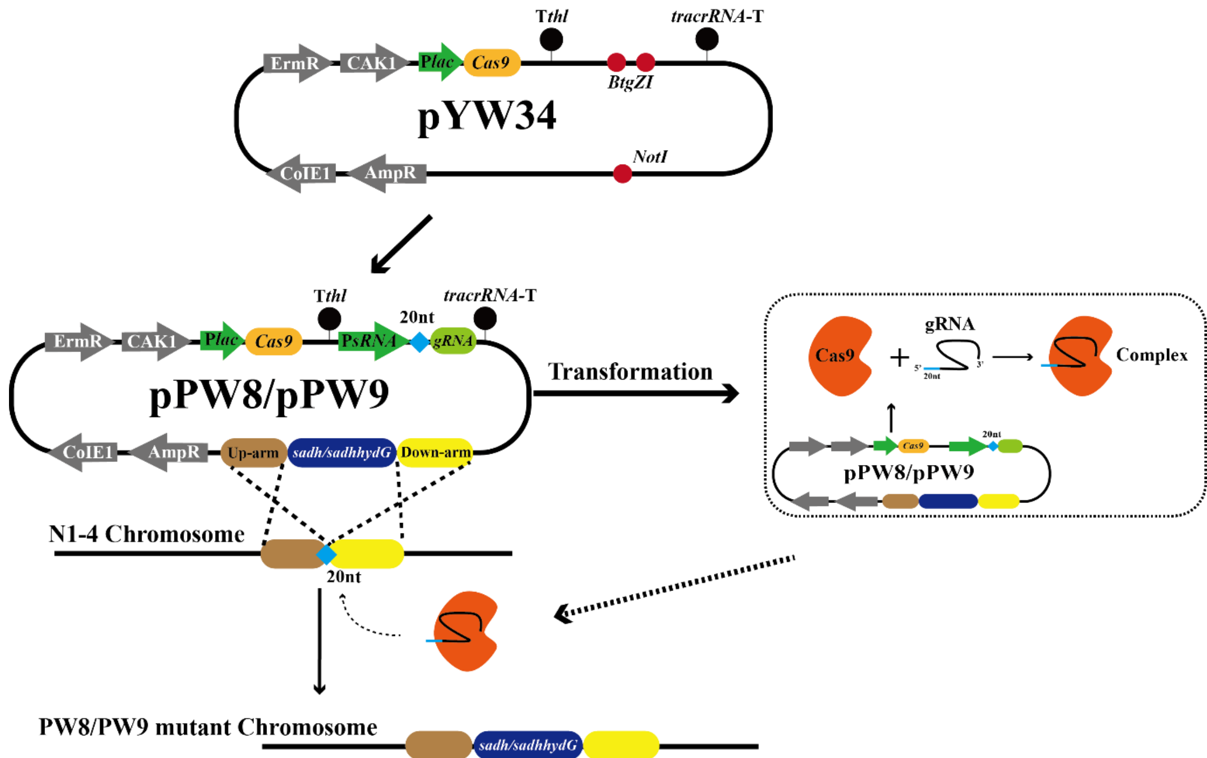


Fig. V-1. The CRISPR-Cas9 mediated genome editing in *C. saccharoperbutylacetonicum*

V.2.3 DNA transformation, mutant strains screening and plasmid curing

The transformation of *C. saccharoperbutylacetonicum* N1-4 was carried out through

electroporation following the protocol as described previously (S. Wang, Dong, Wang, et al., 2017). Briefly, *C. saccharoperbutylacetonicum* N1-4 was cultivated anaerobically at 35 ℃ in TGY medium until the optical density at 600 nm (OD_{600}) reached 0.8-1.0. The cells were harvested immediately through centrifugation at 4,200 g at 25 ℃ for 10 min. The cell pellets were washed once with 1x volume (as compared to the original volume of the cell culture) SMP buffer (270 mM sucrose, 1 mM $MgCl_2$, and 5 mM sodium phosphate, pH 6.5) at room temperature and then re-suspended in 1/20 volume of SMP buffer, obtaining the competent cells. Immediately, 1.0 µg of plasmid DNA was mixed with 400 µl of competent cells and transferred into a pre-cooled 0.2 cm electroporation cuvette; the whole mixture within the cuvette was then incubated on ice for 20 minutes. The whole process was carried out by transferring the cell culture in and out the anaerobic chamber to avoid exposing the cell to oxygen (the centrifugation needed to be performed outside of the chamber). A Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA) connected to the anaerobic chamber was used to deliver the electronic pulse with the following parameters: 1,000V of voltage, 25 µF of capacitance and 300 Ω of resistance. Afterwards, the cell culture was transferred into 1.6 ml of TGY and incubated at 35 ℃ for 2-4 h for recovery. The recovered cells were plated onto pre-warmed TGYC plates and incubated anaerobically at 35 ℃. After 1-2 days, Cla-resistant colonies would grow on the plate and were picked for colony PCR (cPCR) to confirm the presence of plasmid using primers YW32 and YW33 (for confirming pPW2, pPW3, pSH7, pPW5, pPW6, and pJZ100) or YW1435 and YW1438 (for confirming pPW8 and pPW9). The generated recombinant strain containing pPW5, pPW6 or pJZ100 was named as

C. saccharoperbutylacetonicum PW5, *C. saccharoperbutylacetonicum* PW6, or *C. saccharoperbutylacetonicum* JZ100 correspondingly. The positive colony containing pPW8 or pPW9 was then cultivated in TGYC liquid medium and subcultured successively to permit efficient homologous recombinant. After four generations of subculturing, the cell culture was transferred into the TGYLC liquid medium for the induction of Cas9 expression, and then further spread onto TGYLC plates. Colony PCR with primers YW1978 and YW1979 was used to detect the mutation. These two primers anneal to the chromosomal loci beyond the homologous recombination regions (and thus can only anneal to the chromosome but not the homology arms on the plasmid), ensuring the reliable detection of the positive mutants.

Once the desirable genome editing (gene insertion in this case) was achieved, the CRISPR-Cas9 plasmid in the mutant was then cured by subculturing the cells in the TGY liquid medium without Cla for 7-8 generations (Y. Li et al., 2012). Then the cells were spread onto TGY agar plates without Cla and incubated anaerobically at 35 ℃. Colony PCR was performed to confirm the curing of plasmids using primers YW880 and YW881 (targeting the antibiotics marker on the plasmid). Colonies lacking PCR products were picked and spread onto both TGY and TGYC plates to further confirm the loss of the plasmid. The clean mutant with plasmid cured should only be able to grow on the TGY plate. The generated mutant with the insertion of *sadh* or *sadh-hydG* in the chromosome was named as *C. saccharoperbutylacetonicum* PW8 and *C. saccharoperbutylacetonicum* PW9, respectively. Finally, the plasmid pPW2, pPW3, pSH7 was further transformed into PW9 strain, generating *C. saccharoperbutylacetonicum* PW10, *C.*

saccharoperbutylacetonicum PW11 and *C. saccharoperbutylacetonicum* PW12, respectively.

V.2.4 Batch fermentation

Batch fermentation was performed in BioFlo 115 benchtop bioreactors (New Brunswick Scientific Co., Enfield, CT) with a working volume of 1.5 liters. A model solution was prepared for the fermentation containing 80 g/L glucose, 2 g/L yeast extract, 6 g/L tryptone and filter-sterilized P2 medium. The P2 medium contains (in g/l): KH_2PO_4, 0.5; K_2HPO_4, 0.5; CH_3COONH_4, 2.2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; NaCl, 0.01; p-aminobenzoic acid, 0.001; thiamine-HCl, 0.001; and biotin, 0.00001. 30 μg/ml of Cla was supplemented for the fermentation when necessary.

The *Clostridium* strain was maintained in the glycerol stock in -80 ºC. To prepare the preculture, 1 ml of culture in glycerol stock was inoculated into TGY and propagated anaerobically until the OD_{600} reached ~0.8. Then preculture was inoculated into the reactor at 5% (vol/vol) inoculum ratio to start the fermentation. Oxygen-free nitrogen was flushed through the broth (starting overnight or at least several hours before the inoculation) until the culture initiated its own gas production. The temperature was controlled at 30 ±1 ℃ and the agitation was maintained at 55 rpm. The pH was controlled > 5.0 throughout the fermentation by adding 2 M NaOH. Samples were taken throughout the fermentation to monitor the cell density, sugar consumption, and endproduct concentration. The pH profile was automatically recorded by the NBS BioCommand software (New Brunswick Scientific Co, Inc., Edison, NJ) in real time throughout the fermentation. Each fermentation was conducted in duplicate.

V.2.5 Simultaneous saccharification and fermentation (SSF)

When the switchgrass hydrolysates were used as the carbon source, simultaneous saccharification and fermentation (SSF) was performed. As described in section III, the switchgrass was pretreated with 3 g/L acetic acid at 170 ºC for 20 min. Then the slurry was separated into the liquid prehydrolysates fraction (LPF) and solid cellulosic fraction (SCF). The LPF was detoxified through the activated carbon adsorption. Then the LPF and SCF were mixed together and used for the carbon source for SSF. The SSF was performed in 500 ml bioreactors (GS-MFC, Shanghai Gu Xin biological technology Co., Shanghai, China) with a working volume of 200 ml. The modified P2 medium (MP2; the sodium acetate was eliminated from P2) along with the LPF/SCF mixture as carbon source (supplemented with 2 g/L yeast extract and 6 g/L tryptone) was used for the fermentation. The anaerobic condition was generated by sparging oxygen-free nitrogen through the fermentation broth several hours before the inoculation until the cell culture initiates its own gas production. The Cellic CTec2 enzyme of 15 FPU/g glucan and active growing preculture (5% v/v) were added at the same time to initiate the fermentation. The fermentation was performed at 30 ℃ with 50 rpm agitation for 96 h with pH controlled > 5. All fermentations were performed in duplicate.

V. 2.6 Analytical Procedures

The cell growth was monitored by measuring OD_{600} using the Ultrospec 10 cell density meter (Amersham Biosciences Corp., Piscataway, NJ). An Agilent 1260 Infinity HPLC system (Agilent

Technologies, CA) was used for the analyses of sugars and fermentation endproducts. For the sugar analysis, the sample was separated with a 300 mm × 7.8 mm (i.d.), 9 µm Aminex HPX-87P column and a 30 mm × 4.6 mm (i.d.) guard column (Bio-Rad, Hercules, CA), and then detected with a refractive index detector (RID). Nano-pure water was used as the mobile phase at an isocratic flow rate of 0.6 mL/min and the temperature was maintained at 85 ℃ during the 35 minutes elution. The fermentation products were separated with a Varian MetaCarb 87H Column 300 × 7.8 mm along with a 50 × 4.6 mm MetaCarb 87H guard column (Agilent Technologies, CA) and then detected with the refractive index detector (RID). 0.005 N H_2SO_4 was used as the mobile phase at an isocratic flow rate of 0.6 mL/min, and the temperature of the column was maintained at 25 ℃ during the elution.

All the statistical analyses were performed using SAS® University Edition software (SAS Institute Inc, Cary, NC). *P* values of less than 0.05 were regarded as significant.

V.3 Results and discussion

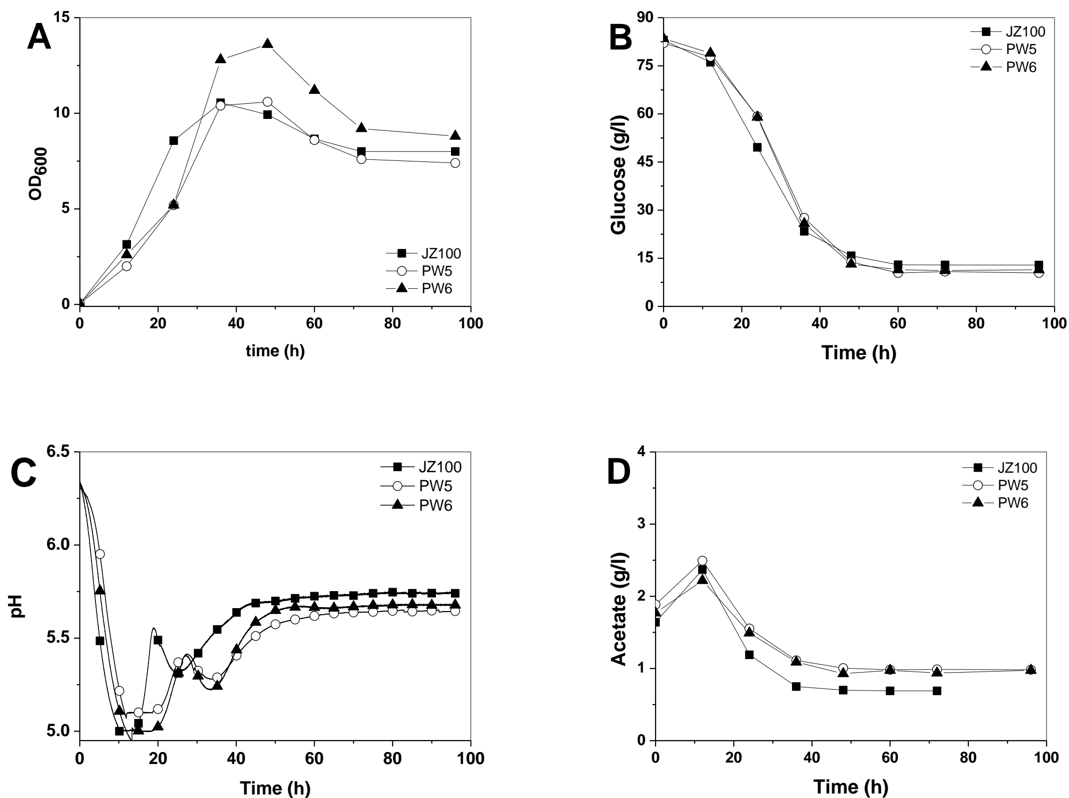
V.3.1 Engineer *C. saccharoperbutylacetonicum* for IBE production through plasmid-based overexpression

The secondary alcohol dehydrogenase gene (*sadh*) is the key gene that catalyzes the conversion of acetone into isopropanol in the *Clostridium* host. In the same operon as *sadh*, the gene (*hydG*) encoding a putative electron transfer protein locates right downstream of *sadh*. In order to investigate whether *hydG* has a positive effect for the conversion of acetone to isopropanol, here we constructed two strains: PW5 (harboring pPW5) with the overexpression of *sadh*, and pPW6

(harboring pPW6) with the overexpression of *sadh-hydG* (**Table V-1**). In both strains, the native promoter of the *thiolase* gene (P_{thl}) was selected to drive the expression of the heterologous gene, since P_{thl} has been reported as a strong and constitutive promoter (Tummala, Welker, & Papoutsakis, 1999). On the other hand, the strain JZ100 was constructed to serve as a control strain. Colony PCR results confirmed the existence of the plasmid in the corresponding host strain.

Batch fermentation was carried out and the results were illustrated in **Fig. V-2** and **Table V-2**. The cell growth in both PW5 and PW6 was slightly delayed at the early stage of the fermentation compared to JZ100 as the control. PW5 reached the similar maximum OD as JZ100, while PW6 grew to a much higher maximum OD (13.6 vs. 10.6 in JZ100; **Fig. V-2A**). Both PW5 and PW6 demonstrated similar sugar degradation profiles and consumed similar amount of glucose as JZ100 (**Fig. V-2B**). As shown in **Fig. V-2C**, the pH was around 6.3 at the beginning of all the fermentation, and then decreased quickly to 5.0 during the acidogenesis phase. For JZ100, the pH increased back to a high level from 5.0, and then decreased slightly to a lower value at around 30 h of the fermentation, followed by increase back and reached a plateau for the following time period. For PW5 and PW6, overall the pH profile followed the similar trend as JZ100 expect for a short 'suspension' for 6-8 hours after it decreased to the lowest value (from 12-20 hours). PW5 and PW6 produced slightly higher level of acetic acid and the same level of butyric acid as JZ100. However, interestingly, the peak level of butyric acid in PW5 and PW6 during the fermentation was remarkably lower than JZ100 (**Fig. V-2E**). This indicated that the overexpression of the isopropanol-producing pathway might have led to enhanced instantaneous carbon flux to

isopropanol production, and thus lower level for butyric acid production. The ethanol production was decreased by 56% in both PW5 and PW6 as compared to JZ100. As expected, in JZ100, 7.1 g/L acetone was produced and no isopropanol was detected. While in both PW5 and PW6, no acetone was detected while high levels of isopropanol were produced (9.4 g/L and 10.2 g/L respectively), indicating that the overexpression of either *sadh* alone or both *sadh* and *hydG* could efficiently convert all the acetone into isopropanol. Comparatively, PW2 produced slightly higher isopropanol, butanol and final total solvents (27.0 g/L in PW6, 25.9 g/L in PW5, and 24.9 g/L in JZ100), with a slightly higher solvent yield (0.39 g/g in PW6, 0.37 g/g in PW5, and 0.35 g/g in JZ100). However, such increase was not statistically significant (**Table V-3**).



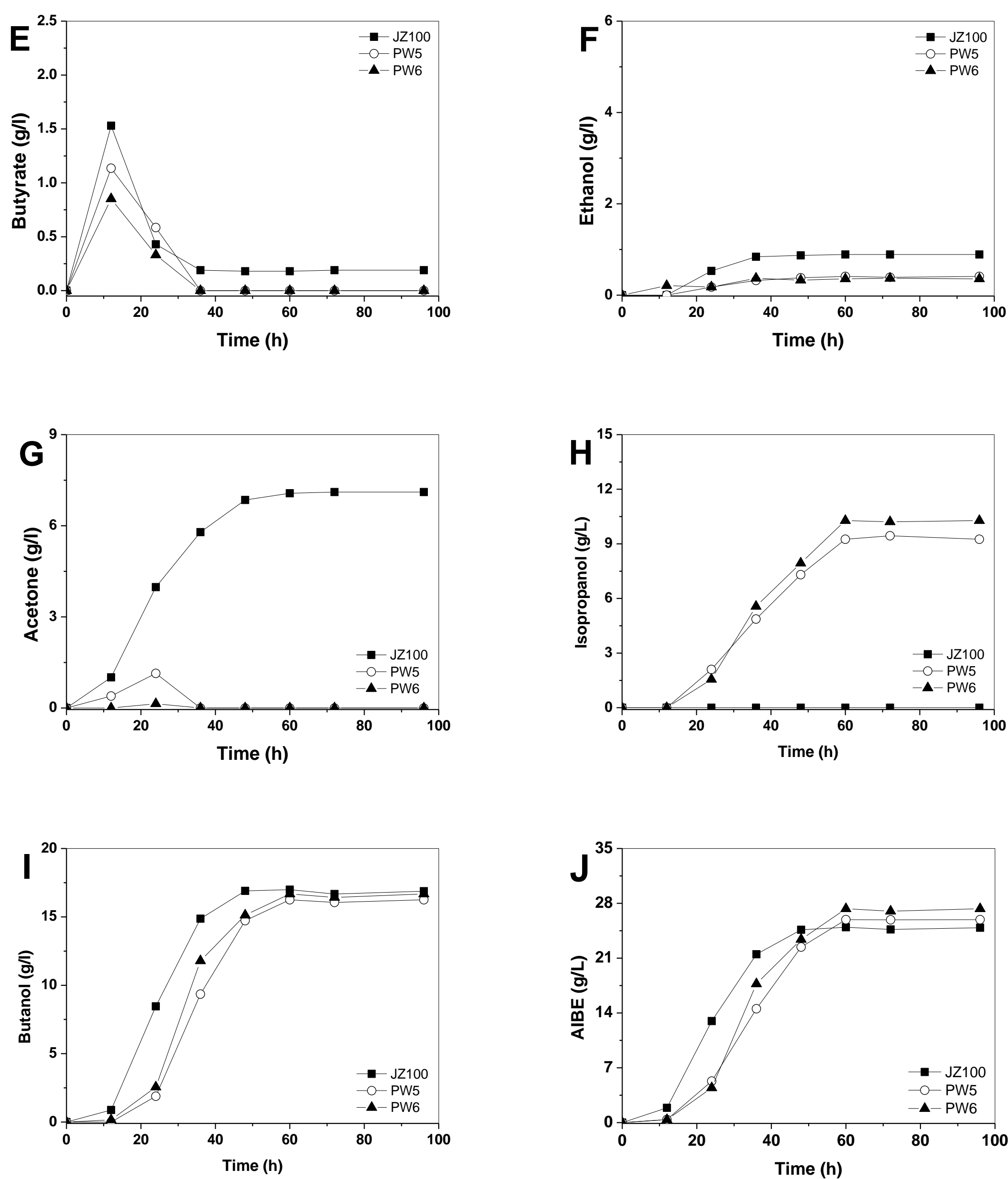


Fig. V-2. Batch fermentation profiles of *C. saccharoperbutylacetonicum* mutant strains. (A) Cell optical density; (B) Glucose consumption; (C) pH; (D) Acetate production; (E) Butyrate production; (F) Ethanol production; (G) Acetone production; (H) Isopropanol production; (I) Butanol production; (J) Total solvent production. Fermentation was carried out in replicates, with one batch reported here as the representative.

Table V-3. Summary of the fermentation results for *C. saccharoperbutylacetonicum* strains

Characteristics	*C. saccharoperbutylacetonicum* N1-4								
	WT	JZ100	PW5	PW6	PW8	PW9	PW10	PW11	PW12
Glucose consumed (g/L)	69.1±2.06^{a}	70.0±1.01^{a}	69.1±2.33^{a}	68.8±1.75^{a}	66.8±0.94^{a}	69.8±1.46^{a}	70.9±1.12^{a}	70.8±1.04^{a}	71.5±1.25^{a}
Acetone (g/L)	7.6±0.53^{a}	7.1±0.62^{a}	0.0±0.0^{b}	0.0±0.0^{b}	4.8±0.47^{c}	0.4±0.15^{b}	0.6±0.23^{b}	0.4±0.08^{b}	0.6±0.11^{b}
Isopropanol (g/L)	0.0±0.0^{a}	0.0±0.0^{a}	9.4±0.91^{b}	10.2±1.02^{b}	4.0±0.85^{c}	9.5±1.15^{b}	11.9±1.03^{b}	9.4±0.87^{b}	11.7±0.95^{b}
Ethanol (g/L)	0.7±0.08^{a}	0.9±0.02^{a}	0.4±0.13^{a}	0.4±0.11^{a}	0.7±0.27^{a}	0.7±0.15^{a}	5.2±0.18^{b}	0.4±0.16^{a}	1.1±0.31^{a}
Butanol (g/L)	17.4±0.85^{a}	16.9±0.62^{a}	16.1±0.63^{a}	16.4±0.76^{a}	16.8±0.48^{a}	16.9±0.96^{a}	16.5±0.86^{a}	17.9±0.81^{a}	17.3±1.05^{a}
Final solvents (g/L)	25.7±1.06^{a}	24.9±0.83^{a}	25.9±0.67^{a}	27.0±0.88^{a}	26.3±1.07^{a}	27.6±1.12^{a}	34.2±1.42^{b}	28.0±0.98^{a}	30.7±1.15^{ab}
Final solvent yield (g/g)	0.37±0.02^{a}	0.35±0.01^{a}	0.37±0.01^{a}	0.39±0.02^{ab}	0.39±0.01^{ab}	0.40±0.02^{ab}	0.48±0.02^{b}	0.40±0.02^{ab}	0.43±0.02^{ab}
Acetic acid (g/L)	0.5±0.3^{a}	0.7±0.41^{a}	1.0±0.19^{a}	0.9±0.25^{a}	0.6±0.33^{a}	0.6±0.18^{a}	0.2±0.10^{a}	0.0±0.0^{a}	0.0±0.0^{a}
Butyric acid (g/L)	0.1±0.11^{a}	0.2±0.13^{a}	0.2±0.01^{a}	0.2±0.05^{a}	0.3±0.03^{a}	0.2±0.02^{a}	0.0±0.0^{a}	0.2±0.03^{a}	0.0±0.0^{a}

All results shown are average value ±standard deviations from duplicated experiments. Values followed by the same letter with in row are not significantly different at the 0.05 level based on Tukey's HSD test.

Previously, the promoter of acetoacetate decarboxylase (*adc*) gene has been employed for the overexpression of *sadh* in *C. acetobutylicum* ATCC 824 for IBE production. The *adc* promoter is switched on at the onset of acetone production and is predominantly active during the solventogenic phase, and thus is expected to be sufficient for the conversion of the acetone into isopropanol in the host strain (J. Lee et al., 2012). However, the results demonstrated that the conversion of acetone into isopropanol was incomplete, with 0.1 g/L acetone left at the end of the fermentation (J. Lee et al., 2012). The P_{thl} is constitutively expressed at high levels throughout the fermentation process (Y. Wang, Li, Mao, & Blaschek, 2012). Therefore, the expression of *sadh* or *sadh-hydG* was induced much earlier than the initiation of acetone production. Thus, there would be enough and sustainable enzyme activity to ensure the completed conversion of acetone into isopropanol in PW5 and PW6. On the other hand, although PW6 produced slightly higher isopropanol and total solvents, the benefit of co-expressing *hydG* along with *sadh* in PW6 was not significant for the conversion of acetone to isopropanol when compared to PW5. In addition, with the conversion of acetone into isopropanol in PW5 and PW6, the butanol production was slightly decreased while the ethanol production was decreased by 56% (0.4 g/L in PW5 and PW6 vs. 0.9 g/L in JZ100) when compared to JZ100. Additional NAD(P)H needs to be consumed along with the conversion of acetone into isopropanol. Thus, such competition for reducing power has led to decreased butanol and ethanol production.

V.3.2 Engineer *C. saccharoperbutylacetonicum* for IBE production through chromosomal integration using CRISPR-Cas9

Although the plasmid-based overexpression of *sadh* or *sadh-hydG* was efficient for converting all the acetone into isopropanol in the host strain, the plasmid is not stable. Meanwhile, antibiotics need to be supplemented for the fermentation, which would complicate the operation and increase the cost. Moreover, the plasmid, as the non-essential genetic element, needs to replicate often enough to produce copies in amount allowing their distribution to the daughter cells after mother cell division. To bear such additional 'molecules', the cells need to consume more energy, and consequently produce more acid with more ATP generated and thus decreased final solvent production (Wegrzyn & Wegrzyn, 2002). Therefore, from the standpoint of industrial application, a stable chromosomal integration of the essential genes is highly desired. Previously, in our lab, we have developed the versatile CRISPR-Cas9 system for efficient genome engineering in various *Clostridium* strains (S. Wang, Dong, Wang, et al., 2017; Y. Wang et al., 2016). Here, we employed the CRISPR-Cas9 system to integrate *sadh* or *sadh-hydG* into the chromosome of *C. saccharoperbutylacetonicum* N1-4, and generated *C. saccharoperbutylacetonicum* PW8 and *C. saccharoperbutylacetonicum* PW9. As shown in **Fig. V-3**, the cPCR results confirmed the insertion of the desirable gene into the chromosome. Additionally, the Sanger sequencing results further verified the gene integration (data not shown).

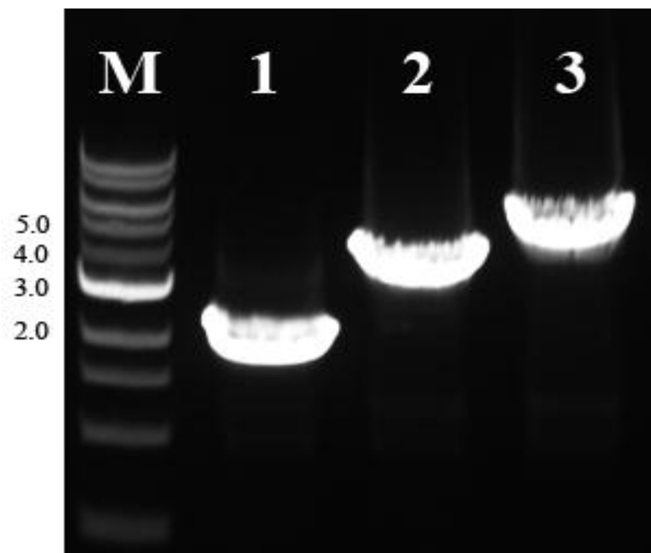
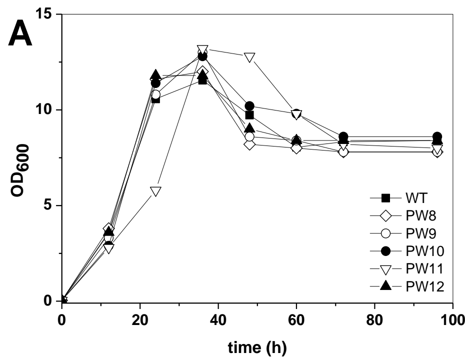
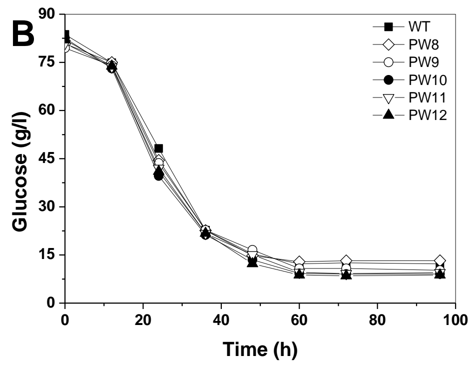


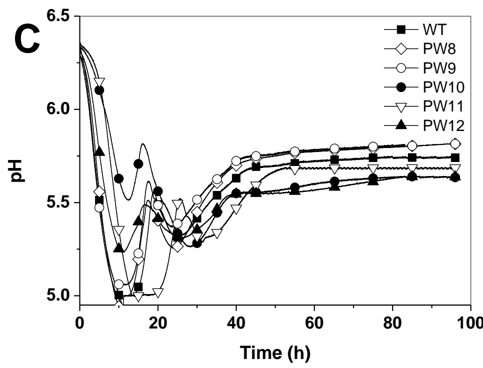
Fig. V-3. Colony PCR (cPCR) results: cPCR confirm the insertion of the s*adh*$_{B-593}$ or *sad*h-*hydG*$_{B-593}$ genes using primers YW1978 and YW1979 flanking the upstream and downstream of the homologous recombination region of *C. saccharoperbutylacetonicum* PW8 (Lane 2, 3,760 bp), *C. saccharoperbutylacetonicum* PW9 (Lane 3, 4,817 bp) and the wild type *C. saccharoperbutylacetonicum* N1-4 (Lane 1, 2,257 bp). The NEB 1-kb DNA ladder was used as the marker (lane M), with numbers on the right representing the band length.

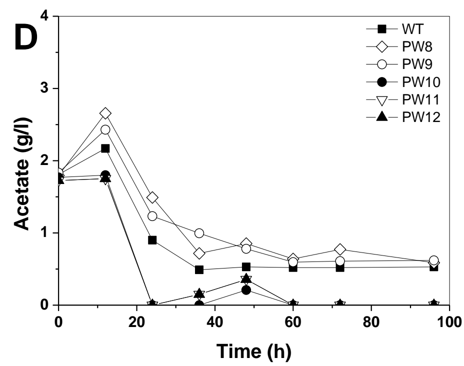
Fermentation was carried out with these strains (using wild type strain (WT) as the control). As shown in **Fig. V-4A**, the cell growth kinetics in PW8 was very similar to WT, while PW9 reached higher maximum OD (13.0 vs. 12.0 in PW8), indicating that the co-overexpression of *sadh-hydG* led to enhanced cell growth. The profiles for the sugar consumption in both PW8 and PW9 were very similar to WT, although PW8 consumed slightly less sugar while PW9 consumed slightly more sugar than WT (**Table V-3**). The pH profiles of PW8 and PW9 followed very similar trend as the WT, although generally they have slightly higher values (than that of WT) most of the time through the fermentation process. The peak value of the acetic acid in both PW8 and PW9 were higher than WT, while on the opposite, the peak value of butyric acid in both PW8 and PW9 were

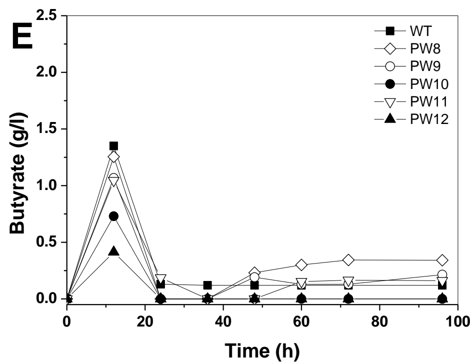
slightly lower than WT. Comparatively, PW8 has higher peak values for both acetic acid and butyric acid than PW9, although by the end of the fermentation, the production of both acids in these two strains were very similar. This indicated that, the co-expression of *hydG* along with *sadh* might have facilitated smoother acid re-assimilation during the fermentation. In PW8, there was 4 g/L isopropanol produced, and meanwhile, 4.8 g/L acetone was also detected (**Fig. V-4** and **Table V-3**). The production of other solvents (butanol and ethanol) was at the similar levels as WT, although the total solvent production and yield were slightly higher. These results suggested that the integration of one copy *sadh* can only convert part of the acetone into isopropanol. Comparatively, the multi-copy plasmid based overexpression of *sadh* in PW2 was more effective to completely convert the acetone into isopropanol in the host. Similar results have been reported previously by Bankar et al., that when only one copy of *sadh* was integrated into the chromosome, only about 50% of the acetone could be converted into isopropanol during the fermentation (Bankar et al., 2015). However, in their *C. acetobutylicum* mutant, the overall solvent production was much lower than the PW8 strain here (**Table V-5**). Interestingly, as shown in **Table V-3**, in the PW9 strain, almost all the acetone was converted into isopropanol (9.5 g/L isopropanol and 0.4 g/L acetone were detected at the end of the fermentation). The final total solvent was 5% and 7% higher than PW8 and WT, respectively (27.6 g/L in PW9, 26.3 g/L in PW8, and 25.7 g/L in WT) along with a slightly higher yield. Therefore, comparing PW9 to PW8, clearly, the expression of *hydG* along with *sadh* significantly enhanced the conversion of acetone to isopropanol.

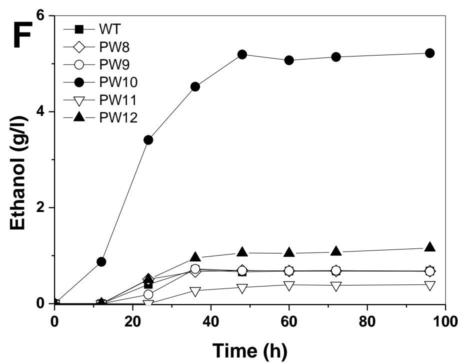

A
OD_{600}
time (h)
WT
PW8
PW9
PW10
PW11
PW12

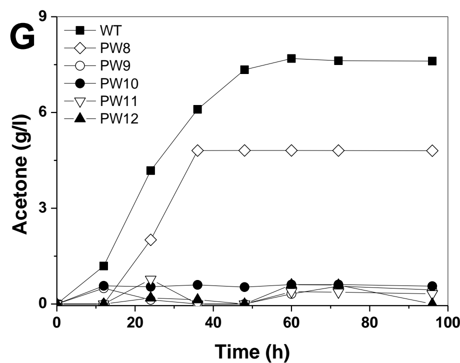

B
Glucose (g/l)
Time (h)
WT
PW8
PW9
PW10
PW11
PW12

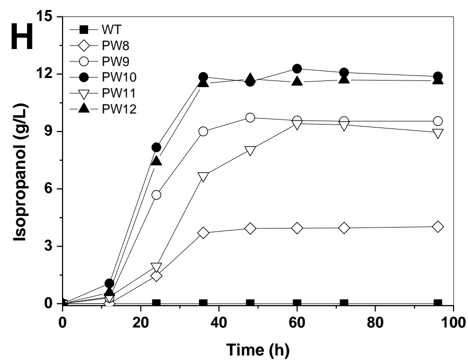

C
pH
Time (h)
WT
PW8
PW9
PW10
PW11
PW12


D
Acetate (g/l)
Time (h)
WT
PW8
PW9
PW10
PW11
PW12


E
Butyrate (g/l)
Time (h)
WT
PW8
PW9
PW10
PW11
PW12


F
Ethanol (g/l)
Time (h)
WT
PW8
PW9
PW10
PW11
PW12


G
Acetone (g/l)
Time (h)
WT
PW8
PW9
PW10
PW11
PW12


H
Isopropanol (g/L)
Time (h)
WT
PW8
PW9
PW10
PW11
PW12

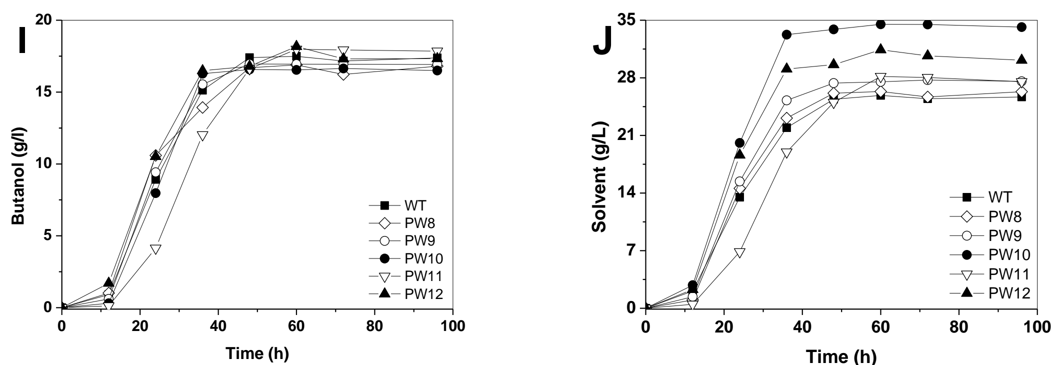


Fig. V-4. Batch fermentation profiles of *C. saccharoperbutylacetonicum* mutant strains. (A) Cell optical density; (B) Glucose consumption; (C) pH; (D) Acetate production; (E) Butyrate production; (F) Ethanol production; (G) Acetone production; (H) Isopropanol production; (I) Butanol production; (J) Total solvent production. Fermentation was carried out in replicates, with one batch reported here as the representative.

Although only one copy *sadh-hydG* was integrated into the chromosome, > 95% of the acetone could be converted into isopropanol. Previously, Jang et al. has co-overexpressed *sadh* and *hydG* in *C. acetobutylicum* for IBE production (Y. S. Jang et al., 2013). However, in their study, they co-expressed *hydG* simply because the *hydG* gene consists of the same operon with *sadh*; they did not include a strain with only *sadh* overexpressed, and thus the conclusion concerning the advantage for the co-expression of *hydG* could not be drawn. Although the function of the *hydG* gene has not been elucidated yet, we clearly demonstrate here this putative electron transfer protein has a positive effect for the reduction of acetone to isopropanol. We speculate that the expression of *hydG* might be beneficial for the redox balance in the host strain and lead to more reducing power available for the conversion of acetone to isopropanol.

V.3.3 Overexpress key genes in the solvent fermentation pathway to further enhance IBE production

Based on *C. saccharoperbutylacetonicum* PW9, we overexpressed the key genes in the solvent production pathway in attempting to further enhance the IBE production in the host. The *sol* operon consists of the key genes for the acid re-assimilation (*ctfA/B*) and the solvent production (*ald* for butanol/ethanol production and *adc* for acetone production). With the overexpression of the *sol* operon driven by P_{thl}, the cell growth kinetics in PW10 followed the similar trend as WT, and the peak OD value reached about the same level as PW9 (OD_{600}=13.0). PW10 consumed slightly more glucose than PW9. One interesting phenotype of PW10 was that the pH never decreased to 5.0 (~ 5.5 as the lowest value) before it increased back to the higher level (**Fig. V-4C**). This was because the overexpression of *sol* operon has significantly enhanced the acid re-assimilation capability in PW10, and thus the actual level of fatty acids in the fermentation was low and the pH would not decrease to < 5.0. Corresponding to the pH profile, the peak level of acetic acid and butyric acid in PW10 was much lower than PW9 or WT (**Figs. V-4D** and **V-4E**). In addition, by the end of the fermentation, there was only 0.2 g/L acetic acid left while the butyric acid was not even detectable (**Table V-3**). As shown in **Table V-3**, the ethanol production in PW10 has been significantly improved to 5.2 g/L compared to 0.7 g/L in PW9. The isopropanol production has also been increased to 11.9 g/L (compared to 9.5 g/L in PW9). However, the butanol production was kept at the similar level in PW10 as in PW9. The final total solvent production has been improved to 34.2 g/L (vs. 27.6 g/L in PW9), which was the highest among all the strains in this study (**Table V-3**).

Previously, we overexpressed *sol* operon in *C. saccharoperbutylacetonicum* N1-4 wild type strain for enhanced ABE production (S. Wang, Dong, & Wang, 2017). Compared to the control strain, the mutant *C. saccharoperbutylacetonicum* pSH1 had five times higher ethanol production, while the butanol and acetone production has been decreased by 17.6% and 15.1%, respectively. Totally, 24.3 g/L biosolvent was produced by the end of the fermentation, which was much lower than that in PW10 in this study. In PW10, compared to *C. saccharoperbutylacetonicum* pSH1, besides a new isopropanol pathway was integrated, the native *thl* promoter was used rather than the *thl* promoter from *C. beijerinckii* as used in pSH1. The results suggested that the native promoter could likely lead to enhanced gene expression and thus higher production of the endproducts. Previous research in another Gram-positive bacterium, *Bacillus*, has also shown that the best gene expression occurs when genes are driven by native promoters (F. D. Wilson, Flint, Deaton, & Buehler, 1994).

On the other hand, we overexpressed the expression cassette EC (*thl-hbd-crt-bcd*) in PW9 to evaluate its effects on IBE production. EC contains the key genes in the 'trunk' pathway responsible for the carbon flux from acetyl-CoA to butyryl-CoA. Thus, the overexpression of EC is expected to increase the selectivity of the C4 products over the C2 products in the mutant. As shown in **Fig. V-4A**, with EC overexpression in PW11, the cell growth was delayed than all the other strains. However, it reached the highest peak OD among all strains (OD_{600}=13.2) at around 40 h of the fermentation. The peak value of the acetic acid production was much lower than in PW9, but comparable to that in PW10. By the end of the fermentation, there was no detectable

acetic acid, and only 0.2 g/L butyric acid was produced. As illustrated in **Table V-3**, compared to PW9, the ethanol production in PW11 decreased to 0.4 g/L (vs. 0.7 g/L in PW9), while the isopropanol production was at the same level. The butanol production increased to 17.9 g/L (vs. 16.9 g/L in PW9). The total solvent production level was also kept at the similar level as in PW9, which is much lower than that in PW10.

Further, we expressed both *sol* operon and EC simultaneously in strain PW9 driven by the same P_{thl}, and generated the mutant PW12. The growth profile of PW12 is very similar to that of WT. The cell growth was not delayed as observed for PW11, and meanwhile the peak OD did not reach as high as PW11 (**Fig. V-4A**). The total sugar consumption was slightly increased compare to PW11; however, generally this is all similar as in other strains (**Fig. V-4B**). The pH initially decreased to ~5.25 (and did not reach a point <5.0 due to the enhanced acid re-assimilation). After 20h, the pH profile decreased slightly, and then further increased back to a plateau at ~5.5 which is comparable to that in pW10, but lower than all the other strains. Due to the efficient re-assimilation of fatty acids, the peak levels of the acetic acid and butyric acid was very low (the peak value of butyric acid was the lowest among all the strains). At the end of the fermentation, there is no detectable acetic or butyric acid (**Table V-3**). The isopropanol production reached 11.7 g/L in PW12, which was comparable to that in PW10 and 23.2% higher than that in PW9. Interestingly, the ethanol production in PW12 was much lower than PW10, indicating that tremendous flux in the 'trunk' pathway might have been successfully guided for the C4 products. However, the butanol production in PW12 was only slightly higher than in PW9 or PW10. The

total solvent production reached 30.7 g/L, which was ~10% higher than in PW9 or PW11, however, was ~10% lower than that in PW10.

V.3.4 Efficient IBE production from acetic-acid-treated switchgrass through SSF

In section III, we have developed an efficient acetic-acid-based biomass pretreatment approach for enhanced biosolvent production. With acetic acid as the catalyst for the pretreatment, no additional chemical reagent is introduced and thus saves cost and avoids the associated environmental pollution. Using acetic acid as the chemical catalyst to overcome the recalcitrance of the biomass at relative milder conditions, less amount of inhibitors will be generated compared to the conventional biomass pretreatment methods. Therefore, the LPF from the acetic-acid-based pretreatment (which is usually discarded after the regular biomass pretreatment process due to its low fermentability) can be used for the fermentation along with the SCF to provide additional carbon source. The supplemented acetic acid for biomass pretreatment (along with the acetic acid generated during the pretreatment) will be re-assimilated as additional carbon source for biosolvent production in the clostridial fermentation, thus leading to improved solvent yield. Indeed, the results in section III demonstrated that the acetic-acid-pretreated switchgrass can be used for efficient ABE production. However, with the re-assimilation of extra acetic acid, increased ratio of acetone will be produced during the ABE fermentation. As mentioned above, acetone is an unwanted product within the ABE mixture due to its undesirable features as a fuel source. In this sense, the IBE-producing strain generated in this study is highly appropriate to be employed for the fermentation of the acetic-acid-pretreated biomass.

SSF was carried out using the LPF/SCF mixture from the acetic-acid-pretreated switchgrass (from a total amount of around 50 g/L carbohydrate) with *C. saccharoperbutylacetonicum* PW9 and PW10. As shown in **Table V-4**, compared to WT, the PW9 strain converted almost all the acetone into isopropanol (with only 0.4 g/L acetone detected by the end of the fermentation). There was slightly higher level of acetic acid left at the end of the fermentation in PW9 than in WT, while the butyric acid production was very similar to that in WT. The production of ethanol, butanol, and final total solvent was all at similar levels as in WT. Compared to PW9, with the overexpression of *sol* operon in PW10, more acetic acid and butyric acid were re-assimilated, leading to enhanced solvent production. The total solvent titer and yield were 18.2% and 18.5% respectively higher than those in PW9. While the butanol production was still at the same level as in PW9 (and WT), the ethanol production was 1.4 times higher than that in PW9. In addition, enhanced fatty acid re-assimilation resulted in more acetone production. Although most of the acetone has been converted into isopropanol (with slightly higher isopropanol produced in PW10 than in PW9), more acetone still left (1.3 g/L in PW9 vs. 0.4 g/L in PW10). Altogether, these results demonstrated that the mutants generated in this study can be employed for efficient IBE production from lignocellulosic biomass.

The *sol* operon as well as EC were overexpressed on a multi-copy plasmid. It would be preferable to integrate them onto the chromosome of the host. We tried to integrate these genes onto the chromosome of PW9 using the CRISPR-Cas9 system. We constructed the vector for *sol* operon integration using CRISPR-Cas9 and transformed the plasmid into PW9; however, we failed

to isolate the positive mutant despite multiple attempts. We also tried to integrate additional copy of *sadh-hydG* into PW9 to further enhance the conversion of acetone into isopropanol (as there was still 0.4 g/L acetone production in the batch fermentation with PW9), but also failed. We speculate that this might be because, when an additional copy of exactly the same fragment is integrated into the chromosome, self-homologous recombination might have occurred among these identical fragments, causing genetic instability.

Table V-4. Summary of the pretreated biomass SSF results for *C. saccharoperbutylacetonicum* mutant strains

Characteristics	*C. saccharoperbutylacetonicum* N1-4		
	WT	PW9	PW10
Residual sugars (g/L)	1.4±0.58[a]	3.4±0.33[a]	2.4±0.27[a]
Acetone (g/L)	4.4±0.15[a]	0.4±0.09[b]	1.3±0.12[c]
Isopropanol (g/L)	0[a]	4.2±0.2[b]	4.6±0.36[b]
Ethanol (g/L)	0.9±0.02[a]	0.8±0.05[a]	1.9±0.12[b]
Butanol (g/L)	8.6±0.32[a]	8.4±0.26[a]	8.5±0.36[a]
Final solvents (g/L)	13.9±0.56[a]	13.7±0.65[a]	16.2±0.56[a]
Final solvent yield (g/g)	0.27±0.01[a]	0.27±0.02[a]	0.32±0.01[a]
Acetic acid (g/L)	3.8±0.31[a]	4.3±0.36[a]	3±0.23[a]
Butyric acid (g/L)	1.7±0.16[a]	1.6±0.12[a]	0.5±0.05[c]

All results shown are average value ± standard deviations from duplicated experiments. Values followed by the same letter with in row are not significantly different at the 0.05 level based on Tukey's HSD test.

On the other hand, with integration of one copy of *sadh-hydG*, there is still small amount of acetone left at the end of fermentation that has not been successfully converted into isopropanol. However, with the overexpression of *sadh* or *sadh-hydG* on a multi-copy plasmid in PW5 or PW6, acetone can be converted into isopropanol by 100%. Clearly, it is suggested that additional copy

of the gene could make a different for the overexpression. Therefore, we tried to integrate additional copy of *sadh-hydG* into a different locus of the PW9 chromosome. However, despite multiple attempts, no positive mutant was obtained. This might be because the integration of additional identical fragment into the chromosome could lead to undesired homologous recombination, thus causing genetic instability (Y. Li et al., 2012). One solution would be to do a codon optimization of the additional copy of the gene and thus the homologous recombination issue could be possibly avoided.

Interestingly, with all these genetic modifications, acetone, ethanol or isopropanol could be enhanced, but the butanol production has not been improved in any of these mutants (**Table V-3**). *C. saccharoperbutylacetonicum* N1-4 is naturally a hyper-butanol producer, with the butanol production already among the highest levels when compared to other ABE-producing strains. Therefore, the butanol production level might be close to the maximum that the strain can tolerate to. Therefore, the enhancement of the butanol tolerance in the host through metabolic engineering approaches might be able to further improve the butanol production in the host. Relevant research is currently underway in our lab.

Previously, various strains have been developed for IBE production by converting acetone into isopropanol in the ABE-producing host. We compared the results from this study to those from the previous literatures (**Table V-5**). It is indicated that our strain could produce the highest levels of IBE (as well as the solvent yield) that has ever been reported in a regular batch fermentation. Among all these strains from the previous report, *C. acetobutylicum* DSM 792-ADH is the only

one that has the isopropanol-production gene (*sadh*) integrated into the chromosome (Bankar et al., 2015). However, with the laborious allele-coupled exchange approach, the constructed mutant still contains a *ermB* marker inside of the chromosome. On the other hand, only around 50% of the acetone could be converted into isopropanol since only *sadh* was integrated into the chromosome. In this sense, the PW9 strain in this study is the first markerless clean mutant that has been engineered for IBE production. Our results demonstrated it as a promising workhorse for high level IBE production from low-value lignocellulosic carbon sources.

Table V-5. Comparison of the IBE production in this study with the previous reports

Strains	Ethanol (g/L)	Isopropanol (g/L)	Butanol (g/L)	IBE (g/L)	IBE yield (g/g)	Reference
C. saccharoperbutylacetonicum PW9	0.7	9.5	16.9	27.6	0.40	This work
C. saccharoperbutylacetonicum PW10	5.1	12.3	16.5	34.2	0.48	This work
Clostridium beijerinckii B593 (DSM 6423)	-	2.2	3.7	5.9	0.30	(Survase et al., 2011)
C. acetobutylicum ATCC 824-ADH integration	2.1	2.5	10.8	18	0.37	(Bankar et al., 2015)
C. acetobutylicum ATCC 824 (*sadh*)	1.5	8.8	13.7	24.4	0.35	(Collas et al., 2012)
C. acetobutylicum Rh8 (psADH)	1.3	7.6	15	23.9	0.31	(Dai et al., 2012)
C. acetobutylicum ATCC 824 (pIPA3)	0.8	6.1	10.2	17.1	0.28	(J. Lee et al., 2012)
C. acetobutylicum PJC4BK (pIPA3-Cm2)	1.9	4.4	14.1	20.4	0.3	(J. Lee et al., 2012)
C. acetobutylicum ATCC 824 *Δbuk* (pCLF952)	1	4.8	14.6	20.4	0.33	(Dusséaux et al., 2013)

V.4 Conclusions

In this study, we metabolically engineered the hyper-ABE producing *Clostridium saccharoperbutylacetonicum* N1-4 strain for IBE production. First, we overexpressed the secondary alcohol dehydrogenase (*sadh*) gene from *C. beijerinckii* B-593 on a plasmid, generating PW5 strain. We also overexpressed *sadh-hydG* gene cluster together in *C. saccharoperbutylacetonicum* host in order to evaluate the effect of *hydG* for isopropanol production, generating PW6 strain. Fermentation results indicated that in both PW5 and PW6, high levels of isopropanol were produced with no acetone production was detected. Overall the performance of PW6 is very similar to PW5 for solvent production, although comparatively the former produced slightly higher isopropanol and total IBE. In order to achieve genetically stable IBE production, we further integrated *sadh* or *sadh-hydG* into the chromosome of *C. saccharoperbutylacetonicum*, and generated strains PW8 and PW9. Fermentations results demonstrated that in PW8, only about 50% of the acetone could be converted into isopropanol, while in PW9, ~95% of the acetone can be converted into isopropanol. This indicated that the co-overexpression of *hydG* with *sadh* through chromosomal integration had significant positive effects on the conversion of acetone to isopropanol. To further enhance the solvent production, we additionally overexpressed in PW9 the *sol* operon (*ald-ctfA-ctfB-adc*), the expression cassette EC (*thl-hbd-crt-bcd*), or *sol* in combination with EC, generating strains PW10, PW11, and PW12, respectively. The fermentation characterization indicated that PW10 had significantly elevated ethanol production, as well as 25% higher isopropanol with slightly decreased butanol production,

leading to a significant increase in total solvent titer and yield when compared to PW9. In PW11, the butanol production increased to 17.9 g/L while ethanol production decreased to 0.4 g/L; however, the isopropanol and final total solvent production was very similar to that in PW9. In PW12, the production of isopropanol, butanol, and ethanol increased to 11.7 g/L, 17.3 g/L, and 1.1 g/L respectively comparing to PW9, resulting in a slight increase in total solvent yield. Finally, SSF was carried out with PW9 and PW10 using the acetic-acid-pretreated switchgrass, and the final solvent titer reached 13.7 g/L and 16.2 g/L, corresponding to the solvent yield of 0.27 g/L and 0.32 g/g in PW9 and PW10, respectively. Compared to the previous reports, the engineered strains in this study (PW9, PW10, PW11) produced the highest total IBE that has ever been reported in the batch fermentation with solventogenic clostridia. Our results meanwhile indicated that the acetic-acid-pretreated biomass can be efficiently converted into biofuel using the metabolically engineered *Clostridium* host.

VI. Future work

Although this work made good progress in the development of an innovative biomass pretreatment method using acetic acid as the treatment reagent and improvement of the biofuels production by metabolic engineering, more efforts are needed to further improve the technology developed in this study, towards ultimate technically and economically viable biofuel production.

Following primary tasks are proposed for the future to continue focusing on the bioprocess for IBE production from lignocellulosic feedstock.

The first task is to integrate *sol* operon into the chromosome of *Clostridium saccharoperbutylacetonicum* using the CRISPR-Cas9 system. With overexpression of *sol* operon using the multi-copy plasmid, the mutant strain demonstrated enhanced acid re-assimilation and solvent production. Compared to the plasmid-based gene overexpression, the chromosomal integration is more stable, and fermentation can be carried out without antibiotics, and thus would be a more preferable choice for an industry scale fermentation process. Therefore, the *sol* operon should be further integrated into the chromosome for stable expression.

Another suggestion for the future work is to integrate additional copies of *sadh-hydG* into the chromosome of *C. saccharoperbutylacetonicum* for further enhanced IBE production. As demonstrated above, PW5 and PW6 (with multi-copy plasmid-based overexpression of *sadh* and *sadh-hydG*, respectively) can produce high levels of isopropanol with no acetone produced. While in PW8 (with one copy of *sadh* integrated), only less than half of the acetone has been converted into isopropanol; and in PW9 (with one copy of *sadh-hydG* integrated), although most of the

acetone has been converted into isopropanol, there is still 0.4 g/L acetone left at the end of the fermentation. By comparing to the results from PW6 (and PW5), clearly, integration of additional copy of *sadh-hydG* can possibly help convert all acetone into isopropanol.

However, a potential pitfall for integration of multiple copies of genes into the chromosome is that homologous recombination might occur among these identical fragments, causing genetic instability. To avoid this, integration sites and/or the orientation should be carefully selected. Further, alternative gene codon usages should be employed to avoid this problem.

For biomass saccharification, enzymes accounts for a big cost. It has been estimated that hydrolysis enzymes could cost $2-$3 for production of one gallon of biofuel (Alvira et al., 2010). It is highly desirable to achieve low-cost enzyme production on site (in house) for efficient biomass saccharification and biofuel production. Therefore, another future work should be done is to produce crude enzymes in house for biomass hydrolysis using cellulolytic fungi, such as the well-known *Trichoderma reesei* RUT-C30 strain(D. B. Wilson, 2009). The crude enzyme can be first produced aerobically using lignocellulosic biomass as feedstock. After simple procedure for separation and removal of the cell biomass, the crude enzyme can be used directly for the simultaneous saccharification and fermentation (SSF) process.

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