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Direct Conversion of Xylan to Butanol by a Wild-Type *Clostridium* Species Strain G117

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ABSTRACT: Lignocellulosic biomass has great potential for use as a carbon source for the production of second-generation biofuels by solventogenic bacteria. Here we describe the production of butanol by a newly discovered wild-type *Clostridium* species strain G117 with xylan as the sole carbon source for fermentation. Strain G117 produced 0.86 ± 0.07 g/L butanol and 53.4 ± 0.05 mL hydrogen directly from 60 g/L xylan provided that had undergone no prior enzymatic hydrolysis. After process optimization, the amount of butanol produced from xylan was increased to 1.24 ± 0.37 g/L. In contrast to traditional acetone-butanol-ethanol (ABE) solventogenic fermentation, xylan supported fermentation in strain G117 and negligible amount of acetone was produced. The expression of genes normally associated with acetone production (*adc* and *ctfB2*) were down-regulated compared to xylose fed cultures. This lack of acetone production may greatly simplify downstream separation process. Moreover, higher amount of butanol (2.94 g/L) was produced from 16.99 g/L xylo-oligosaccharides, suggesting a major role for strain G117 in butanol production from xylan and its oligosaccharides. The unique ability of strain G117 to produce a considerable amount of butanol directly from xylan without producing undesirable fermentation byproducts opens the door to the possibility of cost-effective biofuels production in a single step.

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KEYWORDS: lignocellulose; xylan; *Clostridium*; butanol; biofuels

Introduction

The four carbon saturated alcohol butanol is considered to be one of the most promising alternative fuels due to its physicochemical similarities with gasoline (Cascone, 2008; Ranjan and Moholkar, 2012). Importantly, butanol can be generated as a product of anaerobic fermentation by a number of solventogenic *Clostridium* species (Dürre, 2008; Lütke-Eversloh, 2014). Butanol produced from a biological source is often termed "biobutanol." Sustainable biobutanol production is impeded by: (i) the high costs associated with acquisition and pre-processing of substrates for the fermentation process and (ii) the complexities of downstream purification of biobutanol (Gu et al., 2011).

Many traditional fermentation substrates, such as corn starch, are also human foods and so the price of these substrates fluctuates wildly and has been generally increasing in recent years (Gu et al., 2011). One strategy available for circumvention of high substrate costs is use of inexpensive and abundant feedstock materials as the fermentative substrate. Consequently, over the past decade considerable research efforts have been focused on cost-effective utilization of lignocellulosic biomass such as xylan, which constitutes the principal hemicellulosic component of plant wastes and represents one third of all renewable organic carbon available on earth (Bastawde, 1992; Prade, 1996). However, the β-1, 4-D-xylopyranose bonds that join xylose monomers in xylan must first be enzymatically hydrolyzed by a group of xylanases for effective fermentation to occur (Bajpai, 1997; Beg et al., 2001; Juturu and Wu, 2014). Current research efforts are focused on developing consolidated bioprocessing (CBP) strategies for biofuels production wherein microorganisms are used to hydrolyze and ferment inexpensive lignocellulosic materials directly into desired products without additional enzymes (Lynd et al., 2005). CBP is widely considered to be the best solution for cost-effective hydrolysis and fermentation of lignocellulosic biomass (Olson et al., 2012). Though several strains of *Clostridium cellulyticum*, including metabolically engineered and wild-type strains, have been reported to generate value-added products directly from cellulose (Higashide et al., 2011; Sizova et al., 2011; Yang et al., 2015), most of the reported studies using hemicellulose compounds of lignocellulose as a fermentation substrate have been focused on ethanol or hydrogen production rather than on butanol (la Grange et al., 2010; Lynd et al., 2005; Olson et al., 2012; Tolonen et al., 2011). However, no wild-type strains are known to produce butanol directly from cellulose or xylan, leaving a need for development of one-step strategies for biobutanol production from lignocellulosic materials.

Difficulties in downstream separation of butanol from the acetone and ethanol produced as byproducts through traditional ABE fermentation are another barrier to large scale production of butanol (Gu et al., 2011). To simplify the downstream separation process, approaches seeking to redirect carbon flow into fermentative

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pathways that generate only butanol through the use of metabolic engineering have received general interest. However, several reports suggest that elimination of acetone producing metabolic pathways is often associated with an undesirable decrease in overall butanol titer (Cornillot et al., 1997; Janatiidrissi et al., 1987; Nair and Papoutsakis, 1994; Tummala et al., 2003a,b). Despite this, Jiang et al. (2009) achieved significant success in engineering acetone production out of a solventogenic *Clostridium* by disrupting the acetoacetate decarboxylase gene (*adc*) in *C. acetobutylicum* EA 2018. This engineered strain produced a butanol concentration comparable to its wild-type strain while generating a dramatically decreased amount of acetone in the presence of the exogenous electron acceptor methyl viologen, which can alter the carbon flow towards butanol in ABE fermentation (Jiang et al., 2009). However, butanol production through such strategies has been conducted with expensive reducing sugars, like glucose, as the fermentative substrate. Hence, to obtain an economical yet simple process, it is desirable to use bacterial strains that can directly ferment lignocellulosic materials, such as xylan, to butanol as a principal product.

This study reports direct butanol production from xylan and xylo-oligosaccharides by a wild-type *Clostridium* sp. strain G117 isolated from grassland soil in Singapore. Compared to the traditional ABE fermentation process, this strain has the capability to produce acetone and butanol as the only products from fermenting glucose (Chua et al., 2013). Interestingly, acetone production was suppressed when strain G117 was fed solely with xylan, thereby laying the foundations for a butanol-only fermentation strategy using xylan as the sole carbon source.

Materials and Methods

All chemicals were purchased from Sigma–Aldrich unless specified otherwise.

Culture Medium and Cultivation

A wild-type *Clostridium* sp. strain G117 was cultivated under anaerobic conditions in 60 mL serum bottles containing 30 mL defined medium. The media composition used for the fermentation experiments was the same as described by He et al. (2003) except that 2-(*N*-morpholino) ethanesulfonic acid (MES) was used as the buffering agent to maintain a pH around 6.0. Xylan from beechwood (X4252), D-(+)-xylose (hereinafter called xylan and xylose, respectively) and xylo-oligosaccharides (XOS) were used as the carbon sources for fermentation. All experiments were carried out using a single carbon source, unless specified otherwise. For experiments involving xylan as the sole carbon source, xylan powder was weighed into serum bottles before addition of the fermentation medium under continuous nitrogen flushing, bottles were then sealed and autoclaved. For other cases, concentrated xylose/XOS stock solutions were sterilized separately and injected into the cultivation medium after autoclaving. Sterile yeast extract and vitamin stock solutions (Wolin et al., 1963) (used as additional nutrients) were injected separately into the sealed autoclaved culture bottles in selected experimental setups prior to inoculation. Fermentation studies conducted with xylose/XOS as the carbon source were inoculated with 6% [v/v] inoculum. Cultures grown in serum bottles were incubated in an orbital shaker at 150 rpm at 35°C with an initial pH of 6.5 and maintained at the desired pH using 9 M sodium bicarbonate solution until no further gas was produced from the serum bottles. Samples were taken at regular intervals and analyzed for the presence of acetone, butanol, ethanol, acetic acid, and butyric acid on a GC-FID as described below.

Gene Expression Studies Using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To perform gene expression studies related to acetone production, cultures of strain G117 were fed with either xylan or xylose. At 48 h of fermentation, 1 mL of cultures was harvested by centrifuging at 140,000 rpm and 4°C for 10 min (all in triplicates). Total RNA was extracted from the samples using a combined Trizol and RNeasy Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. Possible residual genomic DNA was removed using RNase-free DNase Kit (Qiagen, Hilden, Germany). Extracted RNA concentration was quantified using a NanoDrop such that equal quantities (242.75 ± 0.03 ng RNA from each sample) would be used to generate corresponding cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with RNase inhibitor and random hexa primer (both from Promega). cDNA samples were used as a DNA template for PCR amplification of the *adc* and Peptidase T (*pepT*) genes. The *pepT* gene (Wu et al., 2012) is a housekeeping gene for strain G117 and was used as an internal control to monitor efficiency of cDNA synthesis and PCR amplification. Details of the primers used for the experiments are shown in Table I. The obtained PCR products were analyzed by 1% agarose gel electrophoresis.

Quantitative Real-Time PCR (qPCR)

The functional gene acetaldehyde dehydrogenase (*adhE*, G117 has two *adhE* genes named *adhE1* and *adhE2*) is a butanol production

Table I. Specific primers for gene *adc, adhE1, adhE2,* and *pepT* in strain G117.

Gene	Primer-forward	Primer-reverse
adc	5′-CTT GCT GCT CCA GCG TTT C-3′	5′-GGC ATA GCC ATC ATC TCA AAT C-3′
ctfA	5′-ATC TGG ACT CGG AGG TGT-3′	5′-CAT CGG CTG ATA AAG GAA-3′
ctfB1	5′-TCT TTC GCA CTA ATA AGA GG-3′	5′-GTA TGT TGC ATT GCC ACT A-3′
ctfB2	5′-GAT AGG AGA ATC TGA CCC AG-3′	5′-TGC CCA CCT CTT ATT ATT G-3′
pepT	5′-TGA TGG AGG CGA GGA AGG TG-3′	5′-CAT TGT ATT CTT TGC AGA CCC TGG -3′
adhE1	5′-GAT AGA ATA GAT AAG TTC GGA GTA-3′	5′-CCC AAG ATC CAC AGC CAA G-3′
adhE2	5′-TGG AGT TGG AGC GGG AAA TAC-3′	5′-TAG CGA TTA TGC TTT GTT CTG ATGC-3′

related gene which is responsible for converting butyrl-CoA into butyraldehyde (Fig. 3). CoA transferase (*ctfAB*, G117 has three *ctfAB* genes: *ctfA*, *ctfB1*, and *ctfB2*) is another gene associated with acetone production. To compare the transcription ratio of gene acetoacetate decarboxylase (*adc*), *ctfAB* and *adhE12*, cDNA samples obtained from cultures at 48 h of fermentation were used as a DNA template for qPCR amplification using an ABI 7500 Fast real-time PCR system (ABI, Foster City, CA) with QuantiTect SYBR green as the PCR mixture reagents (Qiagen, GmBH, Germany). Corresponding data were analyzed with iQ5 software and the relative abundance was calculated based on cycle to threshold value (Ct value) of target genes (*adc*, *ctfA*, *ctfB1*, *ctfB2*, *adhE1*, *and adhE2*) and was normalized to the abundance of housekeeping gene *pepT* for comparison.

Optimization of Butanol Production From Xylan

To further improve butanol production through xylan fermentation, the impact of different parameters on target product output was evaluated. Diverse fermentation parameters like inoculum size, substrate concentration, along with levels of vitamin, trace elements, yeast extract, metal cofactors (Fe^{2+}, Cu^{2+}, Ca^{2+}), and redox regulators (nicotinic acid [NA], L-asparagine [LA]) were tested within the experiments using the method of "one factor at a time" and the butanol output was monitored. All experiments were carried out at least twice and the corresponding butanol concentrations achieved were determined after 120 h of incubation when gas production from the fermentation broth had ceased (fermentation completed).

Among the different parameters tested, four different factors: Fe^{2+}, Cu^{2+} vitamin, and yeast extract were found to impact achieved butanol concentrations. To further optimize butanol production, interactive effects of these four factors were determined using response surface methodology (RSM) (Merrill, 1994), wherein the fermentation parameters were used as the independent variable and the butanol concentration achieved within the experiments as the dependent variable.

Analytical Methods

Volatile fatty acids (i.e., acetic and butyric acids) and biosolvents (i.e., acetone, ethanol and butanol) were measured using a gas chromatograph (GC) (model 7890A, Agilent Technologies) equipped with a Duranbond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm; J&W) and a flame ionization detector (FID), while residual xylose or XOS concentrations were measured using a high-performance liquid chromatograph (HPLC) (model 1260 Infinity, Agilent Technologies) equipped with Agilent Zorbax Carbohydrate Analysis column (4.6 × 150 mm, 0.5 μm) and a refractive index detector (RID). The compositions of the gaseous products were measured using an Agilent GC7890 equipped with a thermal conductivity detector (TCD) as described previously (Bramono et al., 2011). The butanol mentioned in this study is 1-butanol unless specified otherwise. Samples of 1.3 mL were withdrawn from the desired cultures at pre-determined time points under sterile conditions. The samples were centrifuged at 4°C at 14000 rpm for 15 min and the supernatant of 475 μL mixed with 2 M HCL (25 μL) in 2 mL GC vials for analyzing the fatty acids and biosolvents, while another 400 μL of the above supernatant was pipetted into another vial for HPLC analysis of residual reducing sugars.

GC analysis of samples was carried out by injecting 1 μL samples (prepared as above) into the GC column maintained at 60°C initially for 2 min. The column temperature was increased at 15 °C/min to 230°C, and held for 1.7 min with Helium as the carrier gas, maintained at 1.5 mL/min flow rate. The concentrations of the target metabolites were determined from a five-point calibration curve prepared by running standard solutions containing known quantities of acetone, butanol, ethanol, acetic acid, and butyric acid. Data analysis was done using the Agilent ChemStation software.

HPLC analysis of the desired samples was carried out by injecting 3 μL samples into the column equilibrated with the mobile phase (acetonitrile and water (75:25, [v/v])) maintained at a flow rate of 1 mL/min and 40°C oven temperature. Commercially available pentose standards (i.e., xylose, xylobiose, and xylotriose) were used for preparing the corresponding calibration curves for identification and quantification of the residual sugars in the samples. Data analysis was done using the Agilent ChemStation software.

Xylanase activity in the culture supernatants (obtained by centrifuging at 14000 rpm, 4°C for 15 min) was measured according to the method described previously by Bailey et al. (1992) with slight modifications. Briefly, culture supernatants were amended with 1 mL 1 % [w/v] beechwood xylan and incubated at 55°C, pH 5.5 for 10 min. The liberated reducing sugars were measured according to the 3,5-dinitrosalicylic acid (DNS) assay method (Miller, 1959) using a Micro Plate Reader (Infinite 200 PRO, Tecan, Switzerland) with absorbance measured at 540 nm. The reducing sugar concentrations were then read-off by a calibration curve prepared by standard xylose (0–1 g/L). One international unit (U) was defined as the enzymatic activity required for the release of 1 μmol of xylose equivalents per unit volume and per minute of reaction.

Results and Discussion

Butanol Production From Pentose

To ascertain the ability of strain G117 to utilize pentose, it was first grown in presence of sugars like xylose and XOS. Strain G117 was found to efficiently utilize the pentose substrates under anaerobic conditions and butanol was observed in both xylose and XOS fed cultures within 24 h of inoculation (Fig. 1). Although the initial substrate concentration for both cases was kept constant at 60 g/L, the final substrate utilization was found to be higher in the case of xylose (66.67%) compared to that of XOS (28.3%) with a corresponding butanol concentration of 6.03 and 2.94 g/L, respectively. Interestingly, despite the disparity in the substrate utilization, the butanol yields for both substrates were found to be similar, that is, 0.16 ± 0.1 g butanol/g substrate.

As previously reported, solvent production by strain G117 is advantaged by its capability to produce only butanol and acetone compared to the conventional ABE fermentation pathway (Chua et al., 2013). In this study, the observed butanol to acetone ratio from xylose fermentation was found to be similar to that of

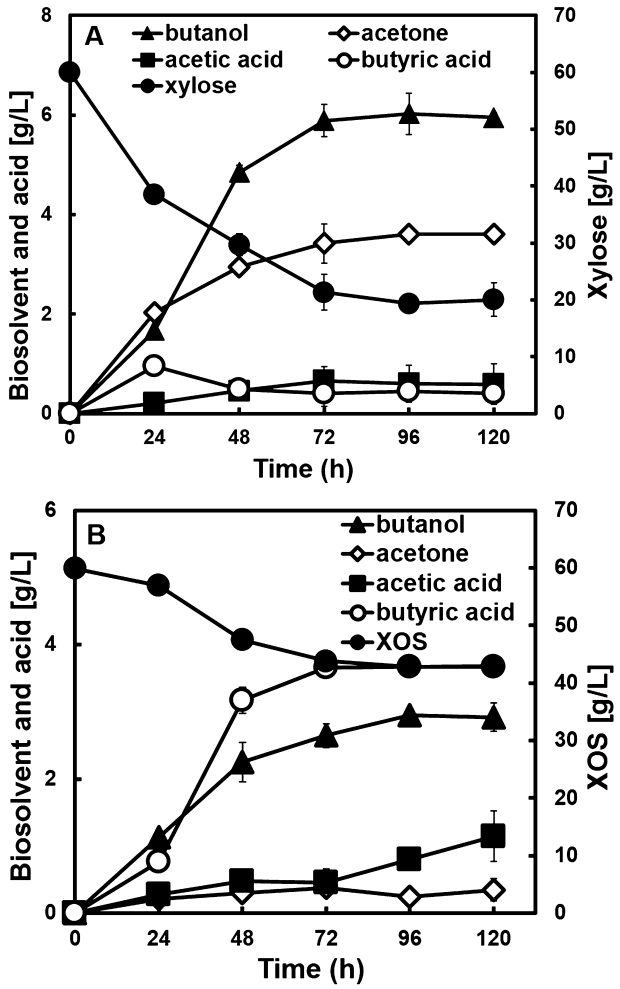

Figure 1. Biosolvent and acid production by *Clostridium* sp. strain G117 fed with (**A**) 60 g/L xylose, and (**B**) 60 g/L xylo-oligosaccharides. All experiments were conducted in biological replicates and error bars are from independent fermentation runs.

conventional ABE processes ($1.67 \pm 0.53 \approx 2{:}1$), however, the butanol to acetone ratio was significantly higher in the XOS fermentation (11.76 ± 3.58). Such a high butanol to acetone ratio from XOS fermentation is considered to be highly advantageous from a process commercialization perspective due to the complexities involved in the downstream separation of the solvent components from the fermentation broth. This ability to generate a higher ratio of butanol to acetone makes strain G117 a promising candidate for converting complex pentose polymers into butanol or similar value added products.

Direct Butanol and Hydrogen Production From Xylan by Strain G117

The breakdown of xylan polymers into monomers can be achieved through a group of enzymes collectively called xylanases. Draft genome sequence analysis of strain G117 revealed the presence of several xylanase related genes (Wu et al., 2012), indicating the genetic potential to breakdown xylan into more metabolically tractable monomers (Table II). Direct fermentation strategies were therefore attempted using strain G117 with 60 g/L xylan as the sole carbon source. The fermentation profile shown in Figure 2 indicates that strain G117 can produce butanol directly from xylan within 48 h with the concentration of butanol reached 0.85 ± 0.07 g/L after 72 h of incubation. Along with butanol, a cumulative yield of 53.4 ± 0.05 mL gaseous hydrogen was directly produced from xylan over the entire fermentation cycle. The proportion of hydrogen within the total gas produced was $42.4 \pm 1.4\%$ with the balance being CO_2.

As expected from anaerobic fermentation operations, butyric acid and acetic acid were also produced from xylan along with butanol. In contrast to xylose fermentation, XOS utilization by strain G117 was also accompanied with a considerable amount of butyric acid accumulation (Fig. 1), implying that complex sugars may have the potential to trigger systemic changes within the bacterial cell and prevent re-assimilation of the acids. Moreover, the butyric acid production from xylan fermentation was found to monotonically increase, reaching 4.4 g/L after 96 h as shown in Figure 2, though butanol production had stopped within 72 h. The accumulation of butyric acid from fermenting xylan, particularly in the later fermentation stage may further indicate the impact of complex sugars on triggering reduced assimilation of acids by the bacteria. Compared to simple sugars, xylan is a more complex carbon source that requires the action of xylanases and xylosidases to degrade it into xylose before fermentation. In typical ABE fermentation butyric acid is the precursor of butanol production (Lee et al., 2008), however the accumulation of butyric acid during fermentation of xylan may be due to: (i) the low level of enzymatic xylan hydrolysis leading to insufficient formation of the reducing

Table II. Xylanase related genes presented in *Clostridium* sp. strain G117.

Enzyme	Enzyme function	GI no.
endo-1,4-β-xylanase	Glycosidic bonds (backbone)	515776638, 150017882
β-D-xylosidase	xylobiose; attack the non-reducing ends of short xylooligosaccharides to liberate xylose	515776646, 515779697, 150017888
α-glucuronidase	Alpha-1,2 bonds between the glucuronic acid residues and backbond	
acetylxylan esterase	O-acetyl group	515777783
α-arabinofuranosidase	Alpha-arabinose	515776700, 515776853, 515779698, 150017222, 150018938, 150019780

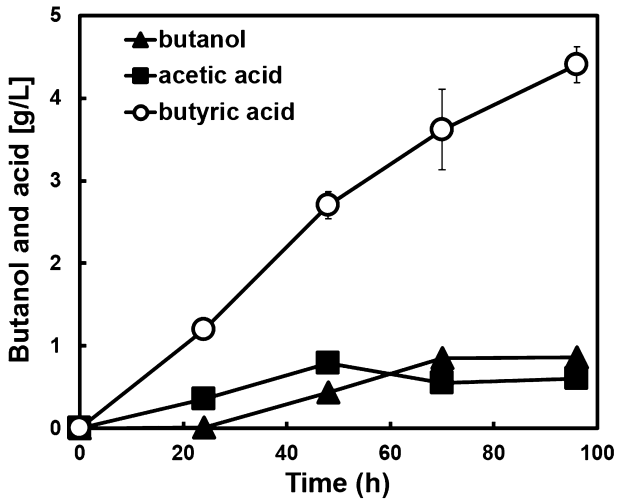

Figure 2. Biosolvent and acid production by *Clostridium* sp. strain G117 fed with 60 g/L xylan. All experiments were conducted in biological replicates and error bars are from independent fermentation runs.

sugar necessary for butanol production (Jones and Woods, 1986); (ii) two more NAD(P)H are needed for butanol formation by re-assimilation of butyric acid (Lütke-Eversloh and Bahl, 2011); (iii) the low expression level of *ctfAB* which is responsible for acid re-assimilation to biosolvents. Hence, an increase in enzymatic hydrolysis leading to higher xylose accumulation, overexpression of *ctfAB* in xylan fed cultures, or an increase in reducing potential may increase acid re-assimilation and subsequent butanol production.

Negligible Acetone Production From Xylan by Strain G117

Xylan fermentation by strain G117 was accompanied by an interesting phenomenon, wherein no acetone production was observed and butanol was the sole solventogenic product (Fig. 2). This observation implies that downstream butanol purification can be greatly simplified and thereby improve the overall process economics of butanol production (Ranjan and Moholkar, 2012). To the best of our knowledge, this is the first instance where butanol was the only solvent produced in the solventogenic stage (without acetone and ethanol production). To confirm whether acetone production was systemically repressed, expression levels of the *adc* gene were compared during xylan and xylose fermentation. As shown in Figure 3 (Lee et al., 2008), the acetoacetate decarboxylase encoding *adc* gene is responsible for converting acetoacetate to acetone (Petersen and Bennett, 1990) and disruption of *adc* in *Clostridium acetobutylicum* has been shown to result in reduced acetone production and a corresponding increase in the butanol to acetone ratio (Jiang et al., 2009). A comparison of the band intensity of *adc* PCR amplicons (Fig. 4A) indicates that *adc* expression in

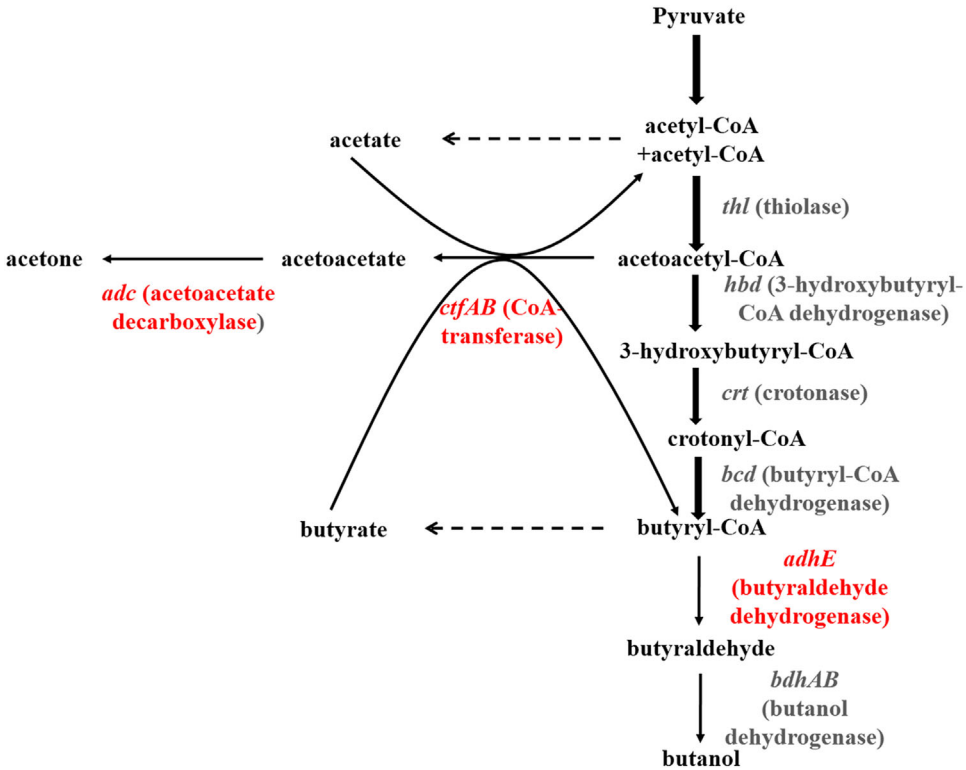

Figure 3. Typical metabolic pathway for acetone production by *Clostridium* species. Gray letters indicate genes and enzymes for the reaction. Dotted and solid arrows indicate reactions during acidogenic and solventogenic phase, respectively.

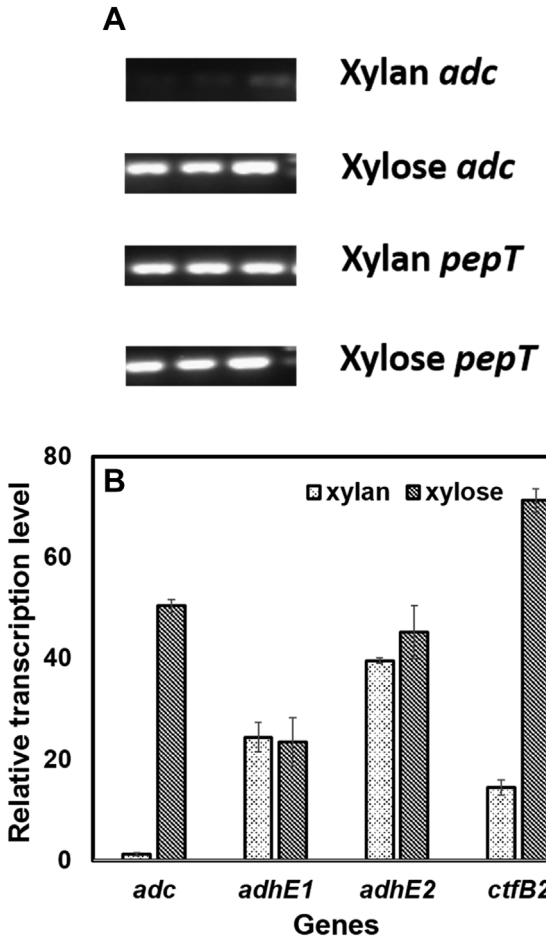

Figure 4. (**A**) Band intensities obtained from the PCR amplified products using specific primers of gene *adc* and *pepT*. Expression of housekeeping gene *pepT* remained constant in both xylan (60 g/L) and xylose (60 g/L) fed cultures while gene *adc* was expressed less with xylan than that with xylose. (**B**) Comparison of relative *adc, ctfB2,* and *adhE12* transcription levels between xylan (60 g/L) and xylose (60 g/L) fed cultures. Results were normalized to the expression level of housekeeping gene *pepT,* error bars are calculated from analytical errors.

xylan fed G117 cultures was significantly reduced. Since the same quantity of RNA was used for synthesis of the cDNA used as a template in the PCR cycle of each sample, the concentration of corresponding PCR amplified products is indicative of gene expression within the samples. In contrast, the expression of the house-keeping gene *pepT* was similar in the two growth conditions.

To confirm whether the lowered expression levels of the *adc* gene corresponds to the lowered acetone-production, the *adhE* (butanol generation associated gene), and *adc* (acetone generation associated gene) transcript levels were also quantified in both xylose and xylan fed cultures through quantitative real-time PCR (RT-qPCR). Figure 4B shows that the transcription levels of gene *adhE12* were comparable in xylose and xylan fed cultures while transcription of *adc* was lower in cultures fed with xylan. The disparity of the expression levels of *adc* accompanied with the comparable expression of *adhE* in xylan and xylose fed cultures suggests that the reduction of acetone production may result from inhibition of *adc*, possibly by electron flux balancing (Nakayama et al., 2011).

Additionally, it has been reported that acetone can be produced through non-enzymatic breakdown of acetoacetate, suggesting that *ctfAB*, converting acetoacetyl-CoA to acetoacetate, plays an important role in acetone generation (Han et al., 2011; Yu et al., 2015). Hence, transcription levels of *ctfA*, *ctfB1*, and *ctfB2* were compared in both xylose and xylan fed cultures through RT-qPCR. Results show that transcription levels of *ctfA* and *ctfB1* were comparable in both conditions, while *ctfB2* was transcribed at lower levels in xylan than xylose fed cultures (Fig. 4B). This suggests that

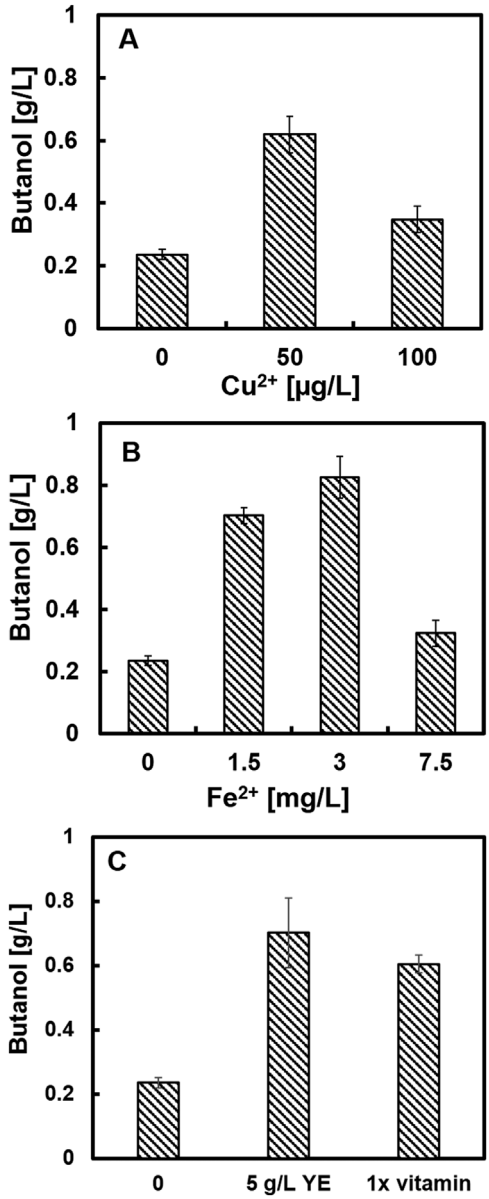

Figure 5. Effect of individual factors on butanol production from xylan. YE: yeast extract. All experiments were conducted in biological replicates and error bars are from independent fermentation runs.

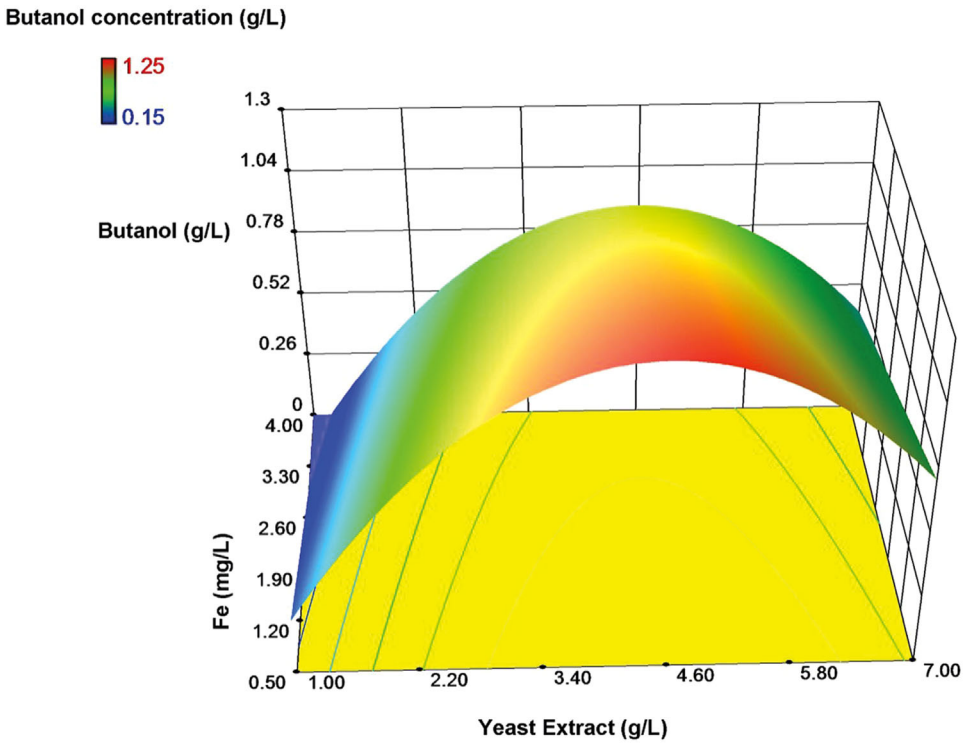

Figure 6. Response surface plot for butanol production from strain G117 using xylan as a substrate.

the negligible acetone production in strain G117 may result from the lower transcription levels of either or both *adc* and *ctfB2*. The lower expression level of *ctfB2* also provides a possible explanation for butyric acid accumulation in xylan fed cultures. Regarding the comparable transcription levels of *ctfA* and *ctfB1*, strain G117 may possess some unique metabolic properties accounting for its difference in *ctfA* and *ctfB1* expression from strains used in previous reports in *Clostridium tyrobutyricum* and *C. beijerinckii* NCIMB 8052. For example, unlike *C. tyrobutyricum* and *C. beijerinckii* NCIMB 8052, strain G117 produces negligible ethanol from reducing sugars such as glucose and xylose and there is no obvious acidogenic phase and solventogenic phase during the whole fermentation process (Chua et al., 2013). In addition, it also has been reported that disruption of *adc* resulted in a decrease of acetone production in *C. acetobutylicum* ATCC 824 as well as in other solventogenic *Clostridium* species (Jiang et al., 2009). This supports our finding that downregulation of *adc* is associated with a decrease of acetone production by solventogenic *Clostridium*.

Optimization of Butanol Production From Xylan

For efficient xylan utilization solventogenic bacteria need to degrade xylan in the surrounding environment before metabolizing the degraded substrates to butanol. The role of xylanase in this extracellular xylan hydrolysis suggests a correlation between enzyme activity and butanol production from xylan. However, our results demonstrate that higher xylanase activities did not correspond to increased butanol yields (data not shown). Interestingly, among the factors tested for increasing butanol production, four independent parameters (Cu^{2+}, Fe^{2+} vitamin, and yeast extract) seemed to improve butanol titer (Fig. 5). The interactive effects of these four variables were analyzed using RSM to obtain optimum conditions for butanol production from xylan by strain G117.

A total of 54 trials were conducted and the butanol concentration obtained from each trial was analyzed and fitted to a second-order polynomial equation which was used to draw a response surface plot as shown in Figure 6. This response surface plot indicates that maximum butanol production should be 1.25 g/L when Fe^{2+}, Cu^{2+}, and yeast extract concentrations are 1500 μg/L, 50 μg/L, and 5 g/L, respectively and the vitamin concentration is maintained as 1×. This analysis indicates that the impact of the yeast extract on butanol production significantly overshadow the effects of other individual factors. In the presence of yeast extract, factors like Fe^{2+}, Cu^{2+}, and vitamin concentrations did not seem to have a significant impact on the butanol production. Interestingly, even under such conditions the interactive effects of Fe^{2+} and vitamin solution showed significant contribution to butanol production. The less significant effects of individual factors may be due to the elemental composition of the yeast extract itself which is known to contain around 70 μg/g Cu^{2+}, 150 μg/g Fe^{2+}, and traces of vitamin B complex (Grant and Pramer, 1962). However, the Fe^{2+} concentration used in the RSM study was much higher than that present within yeast extract (ranging from 750 μg/L to 3750 μg/L) and the vitamin solution used consisted of various vitamins in addition to B-complex. Hence, although the effects of Fe^{2+} and vitamin were not as obvious as yeast extract

Table III. Comparison of biofuel production from lignocellulosic biomass in mono-cultures.

Organism	Relevant genotype	Substrate (g/L)	Product	Titer (g/L)	Butanol/Acetone ratio	Reference
C. japonicus Ueda 107	Metabolic engineered	Avicel (10)	Ethanol	<0.5		Gardner and Keating (2010)
C. phytofermentans ATCC700394	Wild type	Filter paper (10)	Ethanol	2.9		Tolonen et al. (2011)
C. thermocellum	Metabolic engineered	Avicel	Ethanol	1.7		Deng et al. (2013)
C. thermocellum	Metabolic engineered	Switchgrass	Ethanol	1.7		Yee et al. (2014)
C. thermocellum	Metabolic engineered	Avicel	Ethanol	5.61		Argyros et al. (2011)
C. cellulolyticum pWH320	Metabolic engineered	Crystalline cellulose (10)	Isobutanol	0.66		Higashide et al. (2011)
C. thermocellum	Metabolic engineered	Avicel	Isobutanol	5.4		Lin et al. (2015)
C. acetobutylicum 7	Wild type	Grass (30) and flour (30)	1-Butanol	4.1	1.78	Berezina et al. (2008)
C.cellulovorans	Metabolic engineered	Crystalline cellulose	Butanol	1.42		Yang et al. (2015)
C. phytofermentans ATCC700394	Wild type	Birchwood xylan (3)	Ethanol	0.46		Tolonen et al. (2011)
C. sp. strain G117	Wild type	Beechwood xylan	Butanol	1.25	N.A.	This study

individually, the interaction of these two factors significantly affected the butanol production from xylan by strain G117.

Table III presents a comparison of the results obtained through this study with those obtained from the limited avaiable literature reporting direct conversion of lignocellulosic biomass into butanol or other similar value-added products. Among these reports, *C. acetobutylicm* 7 was found to be capable of producing 1-butanol (4.1 g/L) from 30 g/L grass with a butanol/acetone ratio of 1.78 only when the medium was amended with 30 g/L flour (Berezina et al., 2008). Apart from wild type strains, numerous genetically modified strains have also been developed for the direct bioconversion of lignocellulosic biomass to biofuels. Among the previously reported metabolically engineered strains, *C. cellulolyticum* H10, engineered to extrachromosomally express *kivd yqhD alsS ilvCD* which generates 0.66 g/L iso-butanol from cellulose within 7–9 days can probably be considered as the best butanol producing strain (Higashide et al., 2011). Compared to theses previous studies, strain G117 shows significant advantages in several aspects including direct conversion of xylan to a higher amount of butanol as the only solventogenic product. This metabolic property of G117 can greatly improve the economic viability of biobutanol production both in terms of the associated substrate costs and the downstream separation complexities.

Conclusions

This study presents how a wild type *Clostridium* sp. strain G117 can be used as a potential candidate for direct conversion of xylan into butanol. Strain G117 was found to be particularly advantaged due to its capability to repress acetone production while fermenting xylan (fed with 60 g/L) thereby enabling it to produce butanol (1.25 g/L) as the sole solventogenic product within 5 days. Such observations can be considered highly promising for process commercialization where significant efforts need to be directed towards removal of unwanted solvents from the butanol following fermentation. The findings of this study thus offer fundamental knowledge for the future development of economically viable alternative fuel production strategies.

Authors' Contributions

Y.Y. designed and carried out the experiments. A.B. participated in the design of RSM study. T. L. involved in carry out the experiments. J. H. advised this study. All the authors contributed to the writing of this manuscript.

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