Simultaneous saccharification and fermentation of hemicellulose to butanol by a non-sporulating *Clostridium* species

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HIGHLIGHTS

- Simultaneous saccharification and fermentation of xylan to butanol by new strain MF28.
- Simultaneous fermentation of glucose and xylose to produce 11.9 g/L butanol.
- High yields of butanol on xylan and xylooligosaccharide were obtained.
- No acetone/ethanol byproducts were formed in converting lignocellulose to butanol.
- Non-sporulating strain MF28 is capable of continuous industrial-scale fermentation.

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ABSTRACT

Production of lignocellulosic butanol has drawn increasing attention. However, currently few microorganisms can produce biofuels, particularly butanol, from lignocellulosic biomass via simultaneous saccharification and fermentation. Here we report discovery of a wild-type, mesophilic *Clostridium* sp. strain MF28 that ferments xylan to produce butanol (up to 3.2 g/L) without the addition of saccharolytic enzymes and without any chemical pretreatments. Application of selective pressure from 2-deoxy-D-glucose facilitated isolation of strain MF28, which exhibits inactivation of genes (*gid* and *ccp* genes) responsible for carbon catabolite repression, thus allowing strain MF28 to simultaneously ferment a combination of glucose (30 g/L), xylose (15 g/L), and arabinose (15 g/L) to produce 11.9 g/L of butanol. Strain MF28 possesses several unique features: (i) non-sporulating, (ii) no acetone/ethanol, (iii) complete hemicellulose-binding enzymatic domain, and (iv) absence of carbon catabolite repression. These unique characteristics demonstrate the industrial potential of strain MF28 for cost-effective biofuel generation from lignocellulosic biomass.

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1. Introduction

Biobutanol produced from renewable biomass is a promising way to solve a growing demand for energy and the risks of climate change from increasing greenhouse gas emissions (Morone and Pandey, 2014). However, substrate cost remains a major factor influencing the economic viability of fermentative butanol production, accounting for up to 50% of the production cost (Green, 2011). Therefore, transition toward cheaper (non-edible) feedstocks offers the biggest opportunity for cost reduction and improved sustainability (Guan et al., 2016). Among the different types of biomass

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available, xylan, which is the second most abundant natural repository of xylose on Earth, is an attractive substrate for butanol production by microbial processes (Deutschmann and Dekker, 2012). Current industrial bioconversion of lignocellulosic biomass to biofuels, mainly ethanol, relies on separate hydrolysis process prior to fermentation, and requires extensive pretreatments, addition of costly enzymes, and detoxification to remove hydrolysate inhibitors generated from lignin and sugar degradation. Therefore, the development of consolidated bioprocessing (CBP) technology for converting cellulosic biomass into biofuels without adding enzymes is of high interest for economical biofuel production. However, the recalcitrance of hemicellulosic materials and the inability of microorganisms to efficiently ferment biomass hydrolysates still prevent commercial production of biofuels.

Development of engineered microbes or wild-type microbes for achieving saccharification and fermentation in a single bioreactor

has a great potential for value-added products production from cellulose (Sizova et al., 2011; Yang et al., 2015), while most of the reported studies using hemicellulose compounds of lignocellulosic biomass as a fermentation substrate have been focused on ethanol or hydrogen production rather than on butanol (Tolonen et al., 2011). However, few wild-type strains are known to produce butanol from cellulose or xylan via simultaneous saccharification and fermentation, leaving a need for development of one-step strategies for biobutanol production from lignocellulosic materials. Furthermore, inefficient co-fermentation of the three major reducing sugars (i.e., glucose, xylose and arabinose) derived from lignocellulosic biomass typically leads to incomplete sugar consumption due to carbon catabolite repression (CCR) (Yu et al., 2015). Poor butanol production from lignocellulosic biomass is thereby a common phenomenon observed in solventogenic *Clostridium* species. The aim of this study is to discover novel species exhibiting high productivity and yield when converting hemicellulosic xylan into butanol, while also being resistant to the effects of carbon catabolite repression and easy to cultivate for industrial applications. Finding a bacterium possessing a broad range of hydrolytic and solventogenic enzymes is, therefore, a potential pathway to fulfill the needs of economic conversion of biomass to butanol.

This study describes the characterization of a novel, mesophilic, wild-type *Clostridium* sp. strain MF28 capable of simultaneous saccharification and fermentation of hemicellulose and raw plant biomass to butanol in a consolidated process. Strain MF28 can also ferment both glucose and xylose simultaneously without exhibiting carbon catabolite repression. An unusual repressor gene in strain MF28 disables the expression of a crucial sporulation initiation gene cluster in the cell, making continuous or semi-continuous (fed-batch) industrial-scale fermentation possible.

2. Materials and methods

2.1. Media and cultivation

Cultures were grown and maintained in anaerobic, MES-buffered (20 mM) mineral salts medium BCM1 (biobutanol clostridial medium 1) containing $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.3 g/L), KH_2PO_4 (0.75 g/L), K_2HPO_4 (0.75 g/L), NH_4Cl (0.3 g/L), KCl (0.3 g/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.015 g/L), and NaCl (1 g/L), and reduced by addition of L-cysteine (0.2 mM), $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (0.2 mM) and DL-dithiothreitol (0.5 mM). 1 ml trace element mixture, 1 ml selenite-tungstate solution, and 0.1 ml resazurin solution were added aseptically per liter of medium (Li et al., 2014). Bottles (160 ml) containing 3% (wt/vol) of xylan were filled with 50 ml medium and sealed with butyl stoppers, autoclaved for 20 min, cooled to room temperature, and supplemented aseptically with 1 ml yeast extract solution (150 g/L) at a final concentration of 3 g/L. The pH was adjusted to 6.5. Agar plates (2%) were prepared with the same medium but containing 1% (wt/vol) xylan as the sole carbon and energy source. Agar plates were stored in an anaerobic chamber (Coy, USA) with a gas mixture of 85% N_2 , 10% H_2 and 5% CO_2 . All chemicals were reagent grade and were obtained from Sigma (St. Louis, MO), unless indicated otherwise. Gases (air, nitrogen, helium, hydrogen, and nitrogen-hydrogen- CO_2 mixture) were supplied by National Oxygen Pte Ltd (Singapore). For carbon assimilation and bioconversion kinetics studies, all cultures (triplicate for each experiment) were incubated in moderate temperature (37 °C) on a rotary shaker at 150 rpm, and un-inoculated controls were included to monitor potential non-biological activity. For substrate assimilation tests, actively growing MF28 cells (OD of ~2) of 3 ml were inoculated into the above mentioned medium (with a final total volume of 50 ml) supplemented with 6%

(wt/vol) of various carbon sources, as indicated, and maintained under identical conditions. Substrate assimilation tests were performed in biological triplicates and repeated at least twice to confirm results.

2.2. Enrichment of butanogenic microorganisms capable of assimilating xylan

The inocula for butanogenic microorganism screening were distinct aged spent mushroom substrate samples collected from a local farm. Enrichment was first carried out in 160 ml serum bottles filled with 50 ml anaerobic BCM1 medium containing xylan (3%, vol/wt) as the sole carbon source, and incubated for 5 days at 37 °C with a shaking speed of 100 rpm. After performing three subcultures of microorganisms tested, the resulting butanogenic culture (designated culture MF) was identified through detection of butanol in the medium.

2.3. Selection and isolation of xylan-assimilating butanogenic bacterium with resistance to carbon catabolite repression

Prior to isolation, enrichments in xylan-supplemented BCM1 medium were subjected to 2-deoxy-D-glucose (2-DG) (0.7 g/L) for 3 days in order to select a strain with resistance to 2-DG, thereby eliminating catabolite repression. Following three subcultures into fresh BCM1 medium, the culture demonstrating successive butanol production was spread directly on agar plates and incubated anaerobically at 37 °C for three days. Colonies arising on the plates were selected, re-streaked for further purification, and re-tested for butanol production and cell growth.

2.4. Conversions of xylan and plant biomass to butanol

Fermentation experiments with xylan as a substrate were carried out in a 3 L BIOSTAT® B plus bioreactor (Sartorius, Germany) (equipped with redox potential, temperature and pH probes) containing 1.5 L (working volume) of BCM1 medium at an agitation speed of 100 rpm and at 37 °C according to the cultivation method described previously (Li et al., 2014) with slight modifications. Briefly, 90 mL seed culture (OD = ~2) was inoculated into the bioreactor (6%, v/v). The initial pH of the medium was 6.2, and was allowed to drop to 5.3 as the culture progressed. Subsequently, the pH was automatically maintained at or above 5.3 by addition of 6 M NaOH or 3 M H_2SO_4 . Similar experiments with autoclaved plant biomass (switchgrass, corncob and hardwood) as a substrate in BCM1 were conducted as described above. Plant biomass was washed and ground to 80–120 mesh size powder prior to autoclaving. All experiments were performed in biological triplicates without any other pretreatment of biomass.

2.5. Adherence assay

Insoluble xylan was prepared as described previously (Irwin et al., 1994). Briefly, beechwood xylan (5 g) was suspended in 100 ml of deionized water. The mixture was then adjusted to pH 10.0 using 6 M NaOH, and stirred at 100 rpm for 1 h at room temperature. The pellets were harvested by centrifugation at 3000 × g for 10 min and resuspended in deionized water (adjusted to pH 7.0 using 1 M acetic acid) followed by washing twice with 10 volumes of deionized water. The resulting pellets were filtered with Whatman No. 1 paper and dried at 60 °C. An adherence assay of cells grown on insoluble xylan was performed as described previously (Bayer et al., 1983) with slight modification. *In vitro* assays were performed inside an anaerobic chamber. Cells used for adherence tests were prepared by centrifugation at 8000 g for 5 min at the appropriate time of growth and washed 3 times with PBS (pH

7.0). Cells were then resuspended in PBS to an optical density of 0.2 at 400 nm. The adhesion mixture consisted of 2 ml of 10% insoluble xylan and 1 ml of the cell suspension. Subsequently, the sample was mixed for 40 s and allowed to settle at room temperature for 30 min. The turbidity of the suspension was measured at 400 nm and compared with that of an identical cell suspension containing only PBS and no insoluble xylan suspension.

2.6. Enzymatic assays

For enzymatic activity assays, ten milliliter of cultures were harvested in the late exponential phase. The cell-free supernatant was measured for extracellular xylanase, while crude cell extracts of culture MF28 were assayed for solventogenic enzymes (butanol dehydrogenase (BDH), aldehyde dehydrogenase (ADH), and acetoacetate decarboxylase (AADC)) as well as for intracellular xylosidase. *In vitro* activity tests were performed inside an anaerobic chamber. Cell extraction, solventogenic enzyme activity assays, and determination of protein concentration in supernatant and cell extracts were conducted as described previously (Li et al., 2015). Xylanase activity was assayed by measuring the amount of reducing sugar released from beechwood xylan. One milliliter of reaction mixture containing 1% (w/v) of xylan and 100 μ l of enzyme sample (supernatant of culture MF28) in potassium phosphate buffer (50 mM, pH 6.0) was incubated at 37 °C for 15 min, after which the total amount of reducing sugar was measured. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 μ mol of reducing sugar (as xylose equivalent) per minute. The β -xylosidase activity was assayed by measuring the amount of *p*-nitrophenol released in the reaction mixture (1.1 ml) consisting of 0.9 mM *p*-nitrophenyl- β -D-xylopyranoside and 100 μ l of the enzyme sample (cell extract) in 100 mM MES buffer (pH 6.0) at 405 nm. The reaction mixture was incubated at 37 °C for 30 min. One unit was defined as the amount of enzyme that released 1 μ mol *p*-nitrophenol per minute. The amount of protein in the supernatants or cell extracts was determined using the DC protein assay Kit (BioRad, USA). All values of enzymatic assay were averaged from at least three independent extraction samples.

2.7. Molecular analyses

Extraction of genomic DNA or RNA from active cultures was performed as described previously (Li et al., 2015). Genomic DNA was used for enumeration of cells in the culture by quantitative PCR (qPCR) (ABI 7500 Fast Real-Time PCR system, Foster, CA, USA). qPCR was performed in 20 μ l reactions using Bioline SensiFAST SYBR Lo-ROX Kit. Prior to mRNA extraction, 2 μ l of Luciferase control mRNA (Promega) (diluted to 10^8 copies μ l⁻¹) was added into each sample in order to quantify mRNA loss during extraction and reverse transcription (RT). RT was performed immediately after RNA elution using the ABI High Capacity cDNA RT kit with random hexamer primers (Promega) in the presence of RNasin RNase inhibitor (Promega). Primers used in this study are listed in Table S1. The RT-PCR conditions were as follows: initial melt of 2 min at 95 °C, followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C, after the final cycle a melt curve analysis to determine primer specificity from 55 °C to 95 °C with a ramp speed of 1 °C per 10 s. Luciferase cDNA was quantified using the primers LucF and LucR. Transcripts of individual genes were quantified using primers specifically designed based on their gene sequences. The number of gene transcripts per cell was obtained by first normalizing for mRNA loss during extraction and cDNA synthesis using measured quantities of luciferase cDNA from each preparation, and then dividing by total cell number at each time point.

2.8. Analytical methods

Cell density was measured using a spectrophotometer (Biospec-1601; Shimadzu Co., Kyoto, Japan) at 600 nm after appropriate dilution. Sample preparation and quantification for butanol and other metabolites by gas chromatography were performed as described previously (Li et al., 2014). Concentration of residual xylan and components in switchgrass, corncob and hardwood samples were determined using quantitative saccharification from residual material after fermentation (Wolfrum et al., 2011; Izquierdo et al., 2014). Sugars including glucose, xylose, XOS (xylooligosaccharide) and other assimilation carbon sources were monitored using an Agilent 1100 HPLC with an ultraviolet (UV) light detector (210 nm) on a carbohydrate column (Phenomenex) at 30 °C, with 80/20 (v/v) of acetonitrile/water as a mobile phase at a flow rate of 1.0 ml/min (10 μ l injection volume). Detection limits are 0.1 g/L for carbohydrates, and 0.2 g/L for oligosaccharides.

2.9. Nucleotide sequence accession number

The 16S rRNA gene sequence and the whole genome sequence of *Clostridium* sp. strain MF28 have been deposited in GenBank under accession numbers KT368981 and CP014331. The functional genes involved in this study have been deposited in GenBank under accession numbers KT829486 to KT829521.

3. Results and discussion

3.1. Identification and characterization of a mesophilic strain MF28 capable of fermenting xylan to butanol

A sediment-free culture (designated culture MF) enriched from a two-year old spent mushroom sample was found to produce butanol in defined BCM1 medium supplemented with xylan as the sole carbon source at 37 °C. Culture MF was subjected to three consecutive subcultures in xylan-supplemented BCM1 medium added with 0.7 g/L of 2-deoxy-D-glucose (2-DG) for enrichment of a non-carbon catabolite repression (CCR) bacterium (Wick et al., 1957). The resulting culture MF was assayed for xylan hydrolyzing activity and resistance to 2-DG. Culture MF showed no carbon catabolite repression and high xylan hydrolytic activity (~ 2.7 U/mg-protein). Culture MF was inoculated onto agar plates and a total of 57 individual colonies were picked from the plates. The metabolites and xylanase activities of each isolate were determined. Among the 57 isolates, strain MF28 showed the highest butanol titer (1.8 g/L) with the highest xylanase activity (6.2 U/mg-protein).

The 16S rRNA gene sequence of strain MF28 shares 98% identity with that of *C. saccharobutylicum* strain NCP 262, 97% identity with both *C. beijerinckii* NCIMB 8052 and *C. diolis* strain SH1, and 84% identity with *C. xylanolyticum* strain DSM 6555T over 1502 bp. A phylogenetic tree based on the 16S rRNA gene sequence places strain MF28 within the lineage of the genus *Clostridium* (Fig. S-1). Strain MF28 grows optimally at pH 5.3 $\sim$ 5.9 and temperature of 35 $\sim$ 37 °C, however, it is also capable of growth over a broad range of pH (4.1 $\sim$ 7.9) and temperature (28 $\sim$ 47 °C) (Fig. S-2). This phenotype is different from several previously described xylan-fermenting thermophiles such as *C. thermolacticum* (Le Ruyet et al., 1985) and *C. clariflavum* (Sizova et al., 2011).

As a butanogenic microbe using xylan as a substrate, strain MF28 possesses an entire set of enzymes for the breakdown of xylan to produce butanol, including endo-1,4- β -xylanase (6.2 U/mg-protein), β -xylosidase/ α -L-arabinofuranosidase (9.6 U/mg-protein) and butanol dehydrogenase (0.2 U/mg-protein) (Table 1). Strain MF28 is unique because it is capable of simultaneous

Table 1

Specific enzymatic activities of key functional enzymes involved in the pathway of strain MF28.

Carbon source	Specific xylanase activity (U/ mg protein)	Specific xylosidase activity (U/ mg protein)	Specific butanol dehydrogenase (U/ mg protein)	Specific acetoacetate decarboxylase (U/mg protein)
Xylan	6.2	9.6	0.20	ND
Xylooligosaccharide	3.6	11.3	0.31	ND
Switchgrass	5.1	4.5	0.15	ND
Corn cob	5.4	5.2	0.16	ND
Hardwood	5.8	6.1	0.18	ND

ND, not detected (the detection limit is <0.01).

saccharification and fermentation of xylan to produce butanol, acetic acid, butyric acid, H₂ and CO₂. Another unique feature that distinguishes strain MF28 from other butanol-producing strains is that it produces only butanol without any by-products such as acetone and ethanol, from the fermentation of xylan. Compared with other known xylan-to-acids isolates (Le Ruyet et al., 1985; Sizova et al., 2011), strain MF28 shows great flexibility in utilizing various carbon substrates derived from lignocellulosic biomass (Table S2). In addition to xylan, xylooligosaccharides (XOS) and xylose, strain MF28 was also found to utilize mannose, galactose and arabinose (all derived from lignocelluloses) to produce butanol, and hence has high economical potential for biomass-based butanol production. Notably, strain MF28 co-fermented cellobiose and xylose synergistically to produce 9.9 g/L of butanol with a yield of 0.22 g/g (Fig. 1A). For the initial 48-h cultivation on 30 g/L xylose and 30 g/L cellobiose, strain MF28 unitized both xylose and

cellobiose simultaneously with an average consumption rate up to 0.22 g/L/h. This unique co-fermentation is an advance for ligno-cellulose to butanol technologies in both the saccharification and fermentation fronts. Co-fermentation of cellobiose/xylose by isolate MF28 makes it possible to use cellulase cocktails for saccharification (usually with limited β -glucosidase activities), thereby lowering the overall enzyme usage and cost associated with the cellulose saccharification process. Most traditional fungal cellulase cocktails require additional β -glucosidase to fully convert cellobiose into glucose. After spiking a combination of glucose (30 g/L), xylose (15 g/L), and arabinose (15 g/L) to mimic the composition of a biomass hydrolysate to the medium, strain MF28 rapidly consumed these three sugars simultaneously to produce 11.9 g/L of butanol with a yield of 0.21 g/g and a productivity of 0.10 g/L/h (Fig. 1B). Results were compared to that of a gene-modified *Clostridium* strain (producing 9.1 g/L butanol from

Fig. 1. Co-fermentation of mixtures of carbon sources and gene expression for carbon-catabolite-repression by strain MF28. (A) A mixture of 30 g/L of xylose and 30 g/L cellobiose (as the main components hydrolytically derived from biomass). (B) A mixture of 30 g/L of glucose, 15 g/L of xylose and 15 g/L of arabinose. Acetate and butyrate data were omitted for clarity. (C) Transcript levels of *gidA* and *gidB* genes (encoding glucose-inhibited division protein), and *ccp* gene (encoding catabolite control protein) fed with glucose (30 g/L) and xylose (30 g/L). (D) qPCR analysis to illustrate changes in the expression levels of selected genes involved in glucose and xylose catabolic pathways of strain MF28. The 12-h point represented the early-exponential phase as well as the early-solventogenesis phase, and the 36-h point represented late-exponential phase as well as the mid-solventogenesis phase. Error bars represent standard deviation of biological triplicate.

38.5 g/L glucose, 14.9 g/L xylose and 3.5 g/L arabinose) (Xiao et al., 2011). Amounts of acetic acid and butyric acid were observed to be low (1.6–2.1 g/L) in fermentation broths (data not shown), suggesting that the metabolic pattern was shifted toward more reduced metabolites as reflected by higher butanol-to-acid ratio (1.5–2.0). Unlike engineered butanogenic *Escherichia coli* and *C. sporogenes* BE01, strain MF28 produced butanol continuously and did not exhibit obvious acidogenic and solventogenic phase during the entire fermentation process (Chen et al., 2013a,b; Gottumukkala et al., 2013; Cheng et al., 2012). In addition, strain MF28 did not exhibit any carbon catabolite repression.

Monosaccharides and oligosaccharides such as glucose, mannose, galactose, arabinose, xylose, xylobiose and cellobiose that form as hydrolysis intermediates may inhibit polysaccharide utilization in microbial metabolisms. This inhibition is the result of carbon catabolite repression (CCR), and occurs in many of the microbial processes used to generate biofuels (Xiao et al., 2011; Yu et al., 2015). A key feature in the selection of strain MF28 was growth of enrichment cultures in the presence of 2-DG that posed a selection pressure. Accumulation of 2-deoxyglucose-6-phosphate rapidly leads to growth inhibition (Zhou et al., 2013) for strains showing CCR, thus a bacterial strain capable of growth in the presence of 2-DG could survive only if it is non-carbon catabolite repressing. Adding 2-DG facilitated isolation of strain MF28, which contains inactive genes (e.g., *gid* and *ccp* genes) for the carbon catabolite repressor cascade (Fig. 1C). Another feature in the selection of strain MF28 is the ability to efficiently co-ferment a mixture of glucose and xylose, a typical model mixture of hexose and pentose in lignocellulose. The high expression of a xylose transporter (*xyt*), xylose isomerases (*xymA* and *xymB*), and xylulokinase (*xylK*) (Fig. 1D) can apparently overcome two problems (i) the so-called “glucose repression effect” and (ii) the inability to efficiently ferment xylose resulting in an inherent rate-limiting step in the fermentation of sugar mixtures. This can be attributed to glucose transporter permease activity because strain MF28 carries out not only glycolysis but also the PPP (pentose phosphate pathway), both involving the transporter permease. Two glucose utilization channels are present in strain MF28, therefore no repression was observed for xylose utilization via the PPP pathway. This is a valuable characteristic of strain MF28 for future application in industrial biofuel production from lignocellulosic hydrolysates.

3.2. An unusual repressor controls the expression of a crucial sporulation initiation gene cluster in strain MF28

Surprisingly, a morphological analysis of the cells of strain MF28 indicated that no spores were formed in cultures during the 10 days of cultivation (Fig. S3A–C), suggesting that strain MF28 is asporogenic and solventogenic, unlike many other well-known Gram-positive *Clostridium* species, including *C. acetobutylicum* (Harris et al., 2002), *C. beijerinckii* (Ravagnani et al., 2000), and *Clostridium* sp. strain BOH3 (Li et al., 2014) that initiated sporulation at day 5 of fermentation (Fig. S3D). The sporulation gene cluster in the draft genome of strain MF28 consists of 39 genes, including 5 genes (*spo0*, *spoII*, *spoIII*, *spoIV* and *spoV*) for crucial stages of sporulation. Sporulation often leads to cessation of growth, causing major difficulties in continuous or semi-continuous (fed-batch) fermentation. An unusual repressor is present in the draft genome of strain MF28, *spoiH*, which encodes a sporulation initiation inhibitor and likely controls the expression of a crucial sporulation initiation gene cluster (Fig. 2A). Analysis of mRNA transcription by qPCR showed that expression of *spoII*, *spoIII* and *spoIV* genes were all below the detection limit, while *spo0A*, *spoV* and *spotr* genes exhibited negligible transcription levels in the exponential phase (Fig. 2A). This low expression could be due to the overexpression of the sporulation inhibition gene,

spoiH, resulting in no spore-formation for strain MF28. After supplementing 15 g/L of sugar at 48 and 72 h, culture MF28 maintained growth in the semi-continuous mode run (up to 10 days) (Fig. 2B). The success in semi-continuous (fed-batch) fermentation establishes the basis of industrial application potential of strain MF28. From a practical and fundamental point of view, a solvent-producing, non-sporulating strain is the most desirable microbe because most other solventogenic bacteria do not produce solvents following sporulation (Jones and Woods, 1986), making spore-forming bacteria not suitable for continuous or semi-continuous (fed-batch) industrial fermentations.

3.3. Simultaneous saccharification and fermentation of xylan, xylooligosaccharides and plant biomass to butanol

During xylan fermentation, cell growth of strain MF28 correlated tightly with the amount of substrate utilized (Fig. 3A). Strain MF28 produced a significant amount of butanol (3.2 g/L) from xylan via simultaneous saccharification and fermentation with a yield of 0.177 g/g. More importantly, when one xylose molecule is converted to the C4 compound butanol instead of the C3 compound acetone and C2 compound ethanol, the mass yield is expected to increase. Confirmation of this latter finding at the transcriptional level was achieved by using semi-quantitative reverse transcription PCR (Fig. 3B) and enzymes-related analysis involved in the pathway (Table 1 and Fig. S4). The semi-quantitative reverse transcription PCR confirmed transcriptional changes for the differentially expressed functional genes *xynA*, *xysB*, *bdhA*, *adhA*, *adc*, *ak*, and *bkA* (encoding endo-1,4-beta-xylanase, β -xylosidase/ α -L-arabinofuranosidase, NADPH-dependent butanol dehydrogenase, aldehyde dehydrogenase, acetoacetate decarboxylase, acetate kinase, and butyrate kinase, respectively) in strain MF28 during exponential and solventogenic growth phases when converting xylan to butanol. Strain MF28 consumed xylooligosaccharides (XOS) (xylobiose and xylotriose) faster than the precursor xylan, implying that the xylosidase activity is higher for XOS and that the breakdown of XOS by xylosidase is not rate-limiting in the xylan fermentation (Fig. 3 and Table 1). Strain MF28 produced 5.12 g/L butanol with a yield of 0.206 g/g from ~24.7 g/L of XOS mixture (16.49 g/L of xylobiose and 8.17 g/L of xylotriose) (Fig. 3C). The specific uptake rates, butanol yields, and butanol production rates of strain MF28 reached 125 mg/L/h, 177 mg/g, 22.2 mg/L/h on xylan; and 172 mg/L/h, 206 mg/g, 35.4 mg/L/h on XOS at day 6, respectively (Table 2). It should be noted that lower butanol concentration obtained after xylan fermentation (Fig. 3A and B) than that for model sugars (Fig. 1A and B) could be attributed to the fact that the concentration of the reducing sugars liberated from the enzymatic saccharification of xylan (xylose and xylooligosaccharides such as xylobiose and xylotriose) by enzymes produced (e.g., endo-1,4- β -xylanase, β -xylosidase, α -L-arabinofuranosidase) was lower than the concentration of the fermentable model sugars utilized (xylose, cellobiose and glucose). It could be due to the low efficiency of the enzymes produced by strain MF28 for the hydrolysis of the xylan to produce fermentable sugars such as xylose and xylooligosaccharides. In terms of carbon balance, the overall carbon recovery in the fermentation process was 96% (Table S3). These results build a foundation for industrial application of strain MF28 to produce butanol at significant titers and yields from xylan and XOS without addition of costly enzymatic (e.g. xylanase and xylosidase) and chemical pretreatments.

Interestingly, neither acetone nor ethanol was detected at the end of fermentation. Although the acetoacetate decarboxylase (AADC) encoded by *adc* gene is present in the genome of strain MF28, the absence of acetone as a byproduct is congruent with the observed absence of *adc* gene transcription (Fig. 3B). Annotation and deep analysis of the genome sequence revealed two genes,

Fig. 2. Non-sporulation initiation in strain MF28. (A) Transcriptional levels confirmed that an unusual repressor (*spoinh*) controls the expression of a crucial sporulation initiation gene cluster (*spo* family) in strain MF28. Error bars represent standard deviation of biological triplicate. (B) *Clostridium* sp. strain MF28 grows on semi-continuous mode run (fed-batch). Arrow indicates supplementing dosage interval point.

Fig. 3. Conversion of xylan and xylooligosaccharides to butanol and identification of key genes and proteins involved in the pathway. (A) One-pot butanol fermentation of strain MF28 grown on xylan (3%, wt/vol) in a 3 L bioreactor. (B) Reverse transcription-PCR analysis of the gene expression involved in the metabolic pathway in strain MF28 grown on xylan. (C) One-pot butanol fermentation of strain MF28 grown on 3% (wt/vol) of XOS (xylobiose and xylotriase) in a 3 L bioreactor. Error bars represent standard deviation of biological triplicate.

aarA and *aarB*, encoding acetoacetate metabolism regulatory proteins responsible for negative transcriptional regulation of *adc* present in strain MF28. The lack of acetone production is consistent

with the observed absence of *adc* transcripts (Fig. 3B). We suggest that overexpression of the *aarA* and *aarB* genes inhibited *adc* gene transcription, thereby exerting negative regulation on acetoacetate

Table 2

Biofuel (ethanol and butanol) production from lignocellulosic biomass by different mono-microbes.

Organism	Gene type	Carbon source	Product	Titer (g/L)	Yield (mg/g)	Productivity (mg/L/h)	Reference
<i>C. phytofermentans</i> ATCC700394	Wild type	Birchwood xylan	Ethanol	0.46	153	19.2	Tolonen et al. (2011)
<i>C. thermocellum</i>	Modified	Avicel	Ethanol	1.7	340	–	Deng et al. (2013)
<i>C. cellulovorans</i>	Modified	Crystalline cellulose	Butanol	1.42	200	5.9	Yang et al. (2015)
			Ethanol	1.60	190	6.7	
<i>S. cerevisiae</i> EG	Modified	Avicel	Ethanol	1.4	310	14.6	Fan et al. (2012)
<i>C. thermocellum</i>	Modified	Switchgrass	Ethanol	1.7	270	11.7	Yee et al. (2014)
<i>C. thermocellum</i>	Modified	Avicel	Isobutanol	5.4	170	72	Lin et al. (2015)
<i>C. clariflavum</i> strain 4-2a	Wild type	Xylan	Ethanol	0.041	13.7	0.7	Sizova et al. (2011)
		Steam-pretreated wood	Ethanol	0.042	14.0	0.7	
<i>C. sp.</i> strain MF28	Wild type	Beechwood xylan	Butanol	3.2	177	22.2	This study
		XOS	Butanol	5.1	206	35.4	
		Switchgrass	Butanol	0.91	72.2	1.9	
		Corn cob	Butanol	1.02	73.9	2.1	
		Hardwood	Butanol	1.53	98.1	3.2	

decarboxylase activity and acetoacetate metabolism and resulting in no formation of acetone in strain MF28. Additionally, measurements of enzyme activity indicated that the alcohol dehydrogenase (AdhE) in strain MF28 is not active on aldehyde, but is active on butyryl-CoA. This suggests that the AdhE in strain MF28 is distinct from the AdhE in other *Clostridium* strains, such as *Clostridium acetobutylicum* (Choi et al., 2012) and *Clostridium beijerinckii* (Ezeji et al., 2007) which is a bifunctional aldehyde/alcohol dehydrogenase responsible for production of both ethanol and butanol. In terms of butanol production from lignocellulosic biomass, difficulties in downstream separation of butanol from the acetone and ethanol produced as byproducts through traditional ABE fermentation are a key barrier to the industrial-scale production of biobutanol (Gu et al., 2011). This lack of acetone and ethanol production from xylan fermentation by strain MF28 may greatly simplify downstream separation processes, suggesting a potential role for strain MF28 in commercial butanol production.

To determine the feasibility of growing strain MF28 on raw plant biomass containing xylan (20–30% dry weight), particularly old woody materials, three model biomass substrates (switchgrass, corncob, and hardwood) were supplemented to culture medium. Strain MF28 yielded 0.9, 1.0, and 1.5 g/L of butanol from BCM1 medium containing 6% (wt/vol) untreated switchgrass, corncob, and hardwood, respectively (Fig. 4). No ethanol or acetone was detected in the culture media. These concentrations are comparable to the 0.028 g/L of butanol produced from ionic liquid pretreated switchgrass by engineered *E. coli* (Bokinsky et al., 2011); 4.1 g/L butanol and 2.3 g/L acetone from 30 g/L grass only when the medium was supplemented with 30 g/L flour by *C. acetobutylicum* 7 (Berezina et al., 2008). Similar levels of acetate (1.3–2.6 g/L) and butyrate (1.1–1.2 g/L) were produced from switchgrass and corncob, and slightly less (2.1 g/L and 0.8 g/L) from hardwood. Hydrogen was also produced at 10.7, 11.9 and 13.7 mmol/L from switchgrass, corncob, and hardwood, respectively (Fig. 4). Notably, strain MF28 shows a total conversion ($C_{\text{Total products/Biomass loaded}}$ of 0.093–0.12 g/g) and total yield ($Y_{\text{Total products/Sugar liberated}}$ of 0.39–0.57 g/g) in BCM1 medium supplemented with raw switchgrass, corncob, and hardwood, indicating the potential for conversion of this biomass to value added products (Fig. 4).

Table 2 summarizes a comparison of limited available reports on simultaneous saccharification and fermentation of lignocellulosic biomass into butanol or other similar value-added products (ethanol and isobutanol) by microbial isolates. For example, a wild type *C. phytofermentans* ATCC700394 was found to be capable of producing ethanol (0.46 g/L) from birchwood xylan (Tolonen et al., 2011). Apart from wild type strains, numerous genetically modified strains have also been developed for simultaneous saccharification and fermentation of lignocellulosic biomass to

Fig. 4. Conversion of lignocellulosic biomass to butanol by strain MF28. Cells were grown on BCM1 medium containing 6% (wt/vol) of raw switchgrass (SG), corncob (CC), and hardwood (HW), respectively. Total yield and total conversion denote total products/sugar liberated from biomass and total products/biomass loaded. Error bars represent standard deviation of biological triplicate.

biofuels. A recently reported engineered strain, *C. cellulovorans*, engineered to with an aldehyde/alcohol dehydrogenase, produces 1.42 g/L butanol and 1.60 g/L ethanol from cellulose, which can probably be considered as the best butanol producing strain (Yang et al., 2015). Compared to previous studies, strain MF28 exhibits significant advantages in several aspects including simultaneous saccharification and fermentation of xylan to a higher amount of butanol as the only solventogenic product and co-fermentation of major sugars derived from lignocellulosic biomass. This metabolic property of MF28 can notably facilitate the economic viability of biobutanol production in terms of both the associated substrate costs and the downstream separation complexities.

3.4. Contribution of xylan-binding domain to overall uptake of xylan

Adhesion to plant substrates is an important adaptation in some lignocellulolytic bacteria to enhance lignocellulolysis by increasing enzyme concentrations near the substrate and excluding

competitors from the liberated sugars. To investigate the binding of strain MF28 cells to insoluble materials, an adherence experiment was performed using strain MF28 grown in BCM1 medium containing insoluble xylan from beechwood. Results showed that strain MF28 cells could adhere to insoluble xylan at 67.2% and 60.9% in the exponential phase and in the decline phase (Fig. 5A), respectively, and exhibiting a relatively constant affinity to insoluble xylan during the growth phase. The binding of insoluble materials to cells plays an important role in the efficiency of the enzymatic hydrolysis of insoluble lignocellulosic biomass. Therefore, these results suggest that strain MF28 has an essential component that anchors cells to xylan, such as a xylan-binding domain (XBD) (Kulkarni et al., 1999). To further understand the binding domains, quantitative reverse transcription PCR was conducted to measure the expression of putative binding genes in strain MF28. The observed transcriptional up-regulation of members of the xylan-binding *xbd* gene family was consistent with the adherence of cells to insoluble substances (Fig. 5A and B). Furthermore, during the decline phase the transcription of one such gene, *xbd4* (encoding a separate carbohydrate-binding domain protein), was maintained at higher levels (Fig. 5B). The strong binding exhibited by the multienzyme complex produced by strain MF28 and the transcription data indicate the presence of XBDs in strain MF28 (a schematic diagram of a XBD linking between xylanase and xylan was shown in Fig. 5C). Naturally occurring “xylanosomes” are self-assembled complexes present on the cell surface, comprising a multimodular non-catalytic scaffold in protein and containing at least one carbohydrate-binding domain (e.g., XBD) which targets the catalytic domains toward saccharification of complex biomass substrates (Kulkarni et al., 1999). The high expression of members of the *xbd* gene family in strain MF28 strongly suggests that a xylan-binding domain/module contributes to carbohydrate-cell binding. This in turn leads to spatial enzyme proximity and enhanced efficiency of enzymatic hydrolysis of insoluble lignocellulosic substrates. This binding also minimizes the distance over which lignocellulose hydrolysis products must

diffuse, allowing efficient uptake of polysaccharides or other insoluble substrates by the cells, and facilitating production rate.

4. Conclusions

Clostridium sp. strain MF28 is for the first time discovered to possess unique hydrolyzing and solventogenic capabilities in simultaneous saccharification and fermentation of xylan to 3.2 g/L butanol in a consolidated process, and simultaneously consuming glucose, xylose and arabinose to produce butanol at yields of up to 11.9 g/L. Strain MF28 exhibits industrial potential due to several properties: (i) non-sporulating, (ii) no acetone/ethanol byproducts, and (iii) no carbon catabolite repression. Strain MF28 shows a great potential in overcoming barriers to utilizing lignocellulosic biomass for energy generation, which is a step forward in the path toward cost-effective biofuel production without addition of saccharolytic enzymes.

5. Abbreviations

Functional genes *ptsA*, *ptsB*, *ptsG* and *ptsD* encode enzyme IIA and IICBA components of glucose-specific phosphoenolpyruvate-dependent phosphotransferase system; *gidA* and *gidB* encode glucose-inhibited division protein A; *gltA*, *gltB* and *gltC* encode glucose transporter subunits IIA, IIB, and IIBC; *gk*, *xymA*, *xymB* and *xylT* encode glucokinase, xylose transporter, xylose isomerase, and xylulokinase. *spoinh*, *spo0A*, *spoIIP*, *spoIID*, *spoIVA*, *spoVS* and *spotr* encode sporulation initiation inhibitor protein, stage 0 sporulation protein A, stage II sporulation protein P, stage III sporulation protein D, stage IV sporulation protein A, stage V sporulation protein S, and putative sporulation transcription regulator, respectively. *xynA*, *xysB*, *bdhA*, *adc*, *adhA*, *ak* and *bkA* encode xylanase, β -xylosidase/ α -L-arabinofuranosidase, NADPH-dependent butanol dehydrogenase, acetoacetate decarboxylase, aldehyde dehydrogenase, acetate kinase and butyrate kinase, respectively. *xbd1*, *xbd2*,

Fig. 5. Binding capacity and qPCR analysis of xylan-binding domains in strain MF28. (A) Adhesion capacity of bacterial cells to insoluble xylan using strain MF28 growing in a BCM1 medium harvested at exponential and early decline phases. (B) Transcript expression analysis of selected genes involved in xylan-binding domains in strain MF28. Expression levels of related genes confirmed contribution of xylan-binding domain to overall uptake of xylan. Error bars represent standard deviation of biological triplicates. (C) Schematic diagram showing an active complex comprising a XBD linking between xylanase and xylan.

xbd4, *xbd7*, *xbd9*, *xbd10*, and *xbd16* encode xylan-binding domain family proteins. Enzymes are abbreviated as follows: phosphate acetyltransferase (PTA); acetate kinase (AK); thiolase (THL); β -hydroxybutyryl dehydrogenase (BHBD); crotonase (CRO); butyryl-CoA dehydrogenase (BCD); CoA transferase (CoAT); acetoacetate decarboxylase (AADC); butyrate kinase (BK); phosphate butyryltransferase (PTB); aldehyde dehydrogenase (ADH); butanol dehydrogenase I (BDHA); butanol dehydrogenase II (BDHB).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2016.07.138>.

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