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One-step production of lactate from cellulose as the sole carbon source without any other organic nutrient by recombinant cellulolytic *Bacillus subtilis*

Xiao-Zhou Zhang [a], Noppadon Sathitsuksanoh [a,b], Zhiguang Zhu [a], Y.-H. Percival Zhang [a,b,c,*]

[a] Department of Biological Systems Engineering, Virginia Tech, Blacksburg, VA 24061, USA
[b] Institute for Critical Technology and Applied Science, Virginia Tech, Blacksburg, VA 24061, USA
[c] BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

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ABSTRACT

Although intensive efforts have been made to create recombinant cellulolytic microorganisms, real recombinant cellulose-utilizing microorganisms that can produce sufficient secretory active cellulase, hydrolyze cellulose, and utilize released soluble sugars for supporting both cell growth and cellulase synthesis without any other organic nutrient (e.g., yeast extract, peptone, amino acids), are not available. Here we demonstrated that over-expression of *Bacillus subtilis* endoglucanase BsCel5 enabled *B. subtilis* to grow on solid cellulosic materials as the sole carbon source for the first time. Furthermore, two-round directed evolution was conducted to increase specific activity of BsCel5 on regenerated amorphous cellulose (RAC) and enhance its expression/secretion level in *B. subtilis*. To increase lactate yield, the alpha-acetolactate synthase gene (*alsS*) in the 2,3-butanediol pathway was knocked out. In the chemically defined minimal M9/RAC medium, *B. subtilis* XZ7(pBscel5-MT2C) strain (Δ*alsS*), which expressed a BsCel5 mutant MT2C, was able to hydrolyze RAC with cellulose digestibility of 74% and produced about 3.1 g/L lactate with a yield of 60% of the theoretical maximum. When 0.1% (w/v) yeast extract was added in the M9/RAC medium, cellulose digestibility and lactate yield were enhanced to 92% and 63% of the theoretical maximum, respectively. The recombinant industrially safe cellulolytic *B. subtilis* would be a promising consolidated bioprocessing platform for low-cost production of biocommodities from cellulosic materials.

1. Introduction

Lactate has a long history of uses for fermentation and preservation of human foodstuffs (Carr et al., 2002). The US Department of Energy also identified it as one of the top 30 potential building-block chemicals made from non-food lignocellulosic biomass (Werpy and Petersen, 2004). Lactate is commercially produced through bacterial fermentation based on corn starch or cane sugar (Carr et al., 2002; Jem et al., 2010). Cellulosic biomass is the most abundant renewable bioresource with great potentials in production of biocommodities for the sustainability revolution (Zhang, 2009). The economically viable production of low-value biocommodities from less costly cellulosic feedstock is an urgent need (Wyman, 1999; Zhang, 2010). Because current lactate fermentation starts from soluble sugars (Adsul et al., 2007), exogenously added cellulase is required to convert solid cellulosic material into soluble sugars. But high costs of cellulase remain an obstacle in biomass saccharification (Lynd et al., 2005; Taylor et al., 2008; Zhang et al., 2006a).

Consolidated bioprocessing (CBP) has been proposed to decrease cellulase use and increase volumetric productivity by integrating cellulase production, cellulose hydrolysis, and sugar fermentation into a single step (la Grange et al., 2010; Lynd et al., 2005; Zhang and Zhang, 2010). Intensive efforts have been made to introduce heterologous cellulase genes into non-cellulose-utilizing and industrially important microorganisms for decreasing cellulase use or even creating recombinant cellulolytic microorganisms (Arai et al., 2007; Den Haan et al., 2007; Fujita et al., 2004; la Grange et al., 2010; Tsai et al., 2009; Wen et al., 2010; Zhou et al., 2001). Among these microorganisms, few microorganisms can grow on cellulose as the sole carbohydrate source without the help of yeast extract or/and tryptone as carbon, nitrogen, and energy sources (e.g., 10 g/L) (Den Haan et al., 2007; Zhou and Ingram, 2001). Without such organic nutrients added, all of them cannot grow on cellulose by relying on their own recombinant secretory cellulase system

* Corresponding author at: Department of Biological Systems Engineering, Virginia Tech, Blacksburg, VA 24061, USA. Fax: +1 540 231 3199.
E-mail address: ypzhang@vt.edu (Y.-H. Percival Zhang).

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(Brenner et al., 2008; Zhang and Zhang, 2010). Since pretreated lignocellulosic materials are widely regarded as a nutrient-poor feedstock, the addition of costly organic nutrients is economically prohibited for the production of low-value biocommodities (Lawford and Rousseau, 1996; Wood et al., 2005; Zhang and Zhang, 2010). (Note: the sole carbon source is different from the sole carbohydrate source.)

Bacillus subtilis strains are widely used via aerobic fermentations for producing enzymes (e.g., amylase, protease), insecticides, antibiotics, purine nucleotides, poly-γ-glutamic acid, D-ribose, polyhydroxybutyrate (PHB), etc. (Harwood, 1992; Schallmey et al., 2004; Shi et al., 2009; Tannler et al., 2008; Zhang and Zhang, 2010). *B. subtilis* can grow on large ranges of carbohydrates, including monosaccharides (e.g., glucose, xylose), oligosaccharides (e.g., maltodextrins, cellodextrins), and polysaccharides (e.g., starch) but not cellulose (Deutscher et al., 2001). It is anticipated that *B. subtilis* will play an important role in the process of converting biomass into biocommodities (Prather et al., 2009; Stephanopoulos et al., 2008; Stulke et al., 2011; Zhang and Zhang, 2010). The genomic DNA sequence and physiological study indicate that *B. subtilis* 168 has one secretory glycoside hydrolase family 5 endoglucanase (BsCel5) and one intracellular β-glucosidase but no exoglucanase (Kunst et al., 1997). But very low expression level of the endogenous endoglucanse cannot support wild-type *B. subtilis* 168 to grow on cellulose. Under anaerobic conditions, *B. subtilis* produces lactate and 2,3-butanediol as major fermentation products as well as acetate (Cruz Ramos et al., 2000). The homo-lactate fermentation based on glucose and cellobiose has been demonstrated by using a metabolic engineered *B. subtilis* (Romero-Garcia et al., 2009).

In this work, we demonstrated that over-expression of *B. subtilis* endoglucanase BsCel5 enabled non-cellulose-utilizing *B. subtilis* to grow on amorphous cellulose and well-pretreated lignocellulosic biomass as the sole carbon source without any other organic nutrient. We further improved the specific activity and expression/secretion level of BsCel5 by two-round directed evolution. For enhancing lactate yield, the alpha-acetolactate synthase gene (*alsS*) in the 2,3-butanediol pathway was inactivated. One-step production of lactate from cellulose was achieved by the recombinant cellulolytic *B. subtilis*.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were reagent grade or higher, purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Microcrystalline cellulose – Avicel PH105 – was purchased from FMC (Philadelphia, PA). Regenerated amorphous cellulose (RAC) was prepared from Avicel as described elsewhere (Zhang et al., 2006b) with a minor modification: cellulose dissolution in H_3PO_4 at 50 °C for 6 h. Lignocellulosic biomass samples – switchgrass, corn stover, and common reed – were pretreated by cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) at 50 °C as described previously (Moxley et al., 2008; Sathitsuksanoh et al., 2009; Zhu et al., 2009a). Oligonucleotides were synthesized by Integrated DNA Technologies (Coraville, IA). All restriction enzymes used were purchased from New England Biolabs (Ipswish, MA).

2.2. Strains, plasmids, and media

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* JM109 was used as a host cell for all DNA manipulations and *E. coli* BL21 Star (DE3) (Invitrogen, Carlsbad, CA) was used as a host for recombinant protein expression. To avoid the histidine requirement in the defined medium, a competent *B. subtilis* 1A751 was transformed with chromosomal DNA from a prototrophic strain IH6140. The resulting prototrophic strain XZ3 was selected in an M9/glucose medium. Luria–Bertani (LB) medium was used for *E. coli* cell growth and regular recombinant protein expression (Sambrook et al., 1989). The M9 medium is a chemically defined synthetic minimal medium (contents per liter: $Na_2HPO_4 \cdot 7\,H_2O$ 12.8 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, $MgSO_4 \cdot 7\,H_2O$ 0.492 g, and $CaCl_2$ 0.111 g) with trace elements (Sambrook et al., 1989) plus the sole carbon source—0.5% (w/v) glucose, 0.5% (w/v) RAC, or 0.5% glucan from the pretreated biomass, unless otherwise noted. For *B. subtilis* 1A751, 50 μg/mL histidine was added in the M9 medium. Ampicillin (100 μg/mL) was added in the *E. coli* media;

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference
E. coli		
JM109	*recA*1, *supE*44 *endA*1 *hsdR*17 ($^{r-}$k, $^{m+}$k) *gyrA*96 *relA*1 *thi* (*lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ* ΔM15]	(Sambrook and Russel, 2001)
BL21 Star (DE3)	F$^-$ *ompT hsdS*B (rB$^-$mB$^-$) *gal dcm rne131* (DE3)	Invitrogen, Carlsbad, CA
B. subtilis		
168	*trpC2*	(Burkholder and Giles, 1947)
1A751	*his nprR2 nprE18* Δ*aprA3* Δ*eglS102* Δ*bglT bglSRV*	(Wolf et al., 1995)
IH6140	Prototrophic; derivative of *B. subtilis* Marburg strain 1A298; sporulation deficient and has reduced exoprotease activity	(Saris et al., 1990)
WB800	*trpC2 nprE aprE epr bpr mpr::ble nprB::bsr* Δ*vpr wprA::hyg*	(Cho et al., 2004; Wu et al., 2002)
XZ3	Prototrophic; 1A751 prototrophic mutant; transformed by genomic DNA of IH6140	This work
BS36	Prototrophic; WB700 (*trpC2* Δ*nprE* Δ*aprE* Δ*epr* Δ*bpf* Δ*mpr* Δ*nprB* Δ*vrpE* Ery^R Lin^R); *ldh::pdc-adhB* Cm^R, *alsS::spt*	
XZ7	XZ3 derivate, *alsS::spt*, transformed by *alsS::spt* PCR fragment from BS36	This work
SCK6	Erm^R, 1A751 derivate, *lacA*::P_{xylA}-*comK*	(Zhang and Zhang, 2011)
Plasmids		
pP43NMK	Amp^R, Km^R, *E. coli–B. subtilis* shuttle vector	(Zhang et al., 2005)
pP43N-Bscel5	Amp^R, Km^R, pP43NMK derivate with Bscel5 gene cloned	(Zhang and Zhang, 2011)
pP43N-Cpcel5c	Amp^R, Km^R, pP43NMK derivate with Cpcel5c gene cloned	This work
pP43N-Cpcel9	Amp^R, Km^R, pP43NMK derivate with Cpcel9 gene cloned	This work
pBscel5-WT	Cm^R, pNW33N derivate, with wild-type Bscel5 expression cassette cloned	(Zhang and Zhang, 2011)
pBscel5-MT2C	Cm^R, pBscel5-WT derivate, with Bscel5 mutant MT2C cloned	This work
pET20b	Amp^R, over-expression vector containing T7-dependent promoter	Novagen, Madison, WI

Erm^R, erythromycin-resistance; Amp^R, ampicillin-resistance; Km^R, kanamycin-resistance; Cm^R, chloramphenicol-resistance.

kanamycin (20 μg/mL), spectinomycin (100 μg/mL), or chloramphenicol (5 μg/mL) was used in the *B. subtilis* media.

2.3. DNA-manipulation techniques

The isolation and manipulation of recombinant DNA were performed by using standard techniques. *E. coli* transformation was performed as described by Sambrook et al. (1989). *B. subtilis* transformation was performed by the electrotransformation (Xue et al., 1999) or by using super-competent cells (Zhang and Zhang, 2011).

2.4. Construction of plasmids

Plasmids pP43N-Bscel5 and pBscel5-WT (Supplementary Fig. S1) were constructed previously (Table 1; Zhang and Zhang, 2011). For intracellular expression of newly generated BsCel5 mutants in *E. coli*, the mutated *Bscel5* genes were cloned into pET20b as previously described (Zhang and Zhang, 2011). For heterologous expression and secretion of endoglucanase from *Clostridium phytofermentans* ISDg (Zhang et al., 2010b), the glycoside hydrolase family 5 (CpCel5C, Genbank accession number: ABX42426) and family 9 (CpCel9, Genbank accession number: ABX43720) endoglucanase-encoding genes were cloned into pP43NMK by using the strategy, as described elsewhere (Zhang and Zhang, 2011).

2.5. Endoglucanase expression, purification, and assays

The expression, purification, and characterization of wild-type BsCel5 and its mutants were performed as previously described (Zhang and Zhang, 2011). For qualitative endoglucanase assays, *B. subtilis* WB800(pP43N-Bscel5) as recombinant cellulolytic strain and *B. subtilis* WB800(pP43NMK) as a control were grown on the LB or M9/glucose agar plates containing 0.2% low viscosity CMC, or M9 agar plates containing 0.5% RAC at 37 °C for 16 h. After the colonies were washed away, the plates were stained by a 0.2% Congo red solution. The clear halo zones were observed after de-staining by using 1 M NaCl solution.

2.6. Directed evolution of signal peptide SP_{nprB} and BsCel5

Directed evolution of the whole NprB signal peptide- and BsCel5-encoding sequence was performed by using error-prone PCR with the same primers as previously described (Zhang and Zhang, 2011). Briefly, the SP_{nprB}–BsCel5-encoding sequence was randomly mutagenized by error-prone PCR. The multimeric plasmids were prepared from the linearized pBscel5-WT and error-prone PCR product by using overlap extension PCR. The *B. subtilis* super-competent cells were transformed with plasmid multimers and followed with the library screening with RAC as the substrate (Zhang and Zhang, 2011). To check the expression and secretion levels of BsCel5, the *B. subtilis* strains were cultivated in a modified 2 × L-Mal medium (Ara et al., 2007) at 30 °C for 72 h. After centrifugation, the extracellular proteins in the supernatant were precipitated using the DOC-TCA method (Cold Spring Harbor Protocols, 2006). Protein samples were analyzed by using 12% (w/v) SDS-PAGE. The amount of target proteins in the SDS-PAGE gel was estimated with the densitometry analysis software Quantity One (version 4.4.0, Bio-Rad, Hercules, CA).

2.7. Knock-out of alsS

The DNA fragment of *alsS*::*spt* was PCR amplified from the chromosomal DNA of *B. subtilis* BS36 (Romero et al., 2007) by using primers P5 (5′- GCA TAC GTC GAC GTG TTG ACA AAA GCA ACA AAA GA -3′) and P6 (5′- TCC CCG AAT TCT TTC GGA AGC TTG TCA CT -3′). *B. subtilis* XZ3 was transformed with the PCR product and the transformants were selected on an LB plate supplemented with 100 μg/mL spectinomycin. The new strain was designated as XZ7 and the inactivation of *alsS* was verified by PCR.

2.8. Lactate fermentation

The strains stored at −80 °C in glycerol were revived by streaking on LB plates and incubated overnight at 37 °C. For preparing the seed cultures, these strains were inoculated in an LB medium and cultivated aerobically at 37 °C for ~14 h. Fermentations of cellulolytic *B. subtilis* XZ7 strains were conducted in 100 mL of the M9 medium containing RAC (glucose equivalent of ~7 g/L) in a 160 mL serum bottle with a rotary rate of 180 rpm at 37 °C. The bottle was sealed with rubber septa and aluminum cap. The inoculum size was 5% at an initial absorbency (600 nm) of ~0.15. Anaerobic conditions were achieved through consumption of oxygen by the inoculated bacteria. The samples were drawn by needle-syringes for measurement of residual glucan, cellular protein, lactate, 2,3-butanediol, and acetate.

2.9. Analytic methods

Cellular protein representing cell growth on RAC was measured by the ninhydrin assay after complete hydrolysis of cellular protein to amino acid by NaOH (Zhu et al., 2009b). The residual cellulose were quantified by the quantitative saccharification method (Moxley and Zhang, 2007; Zhang and Lynd, 2005). The concentrations of lactate, 2,3-butanediol, and acetate in the fermentation broth were measured by high-pressure liquid chromatography (HPLC) equipped with a Bio-Rad HPX-87H column (Richmond, CA) and a refractive index detector. HPLC was run with 5 mM H_2SO_4 as a mobile phase with a flow rate of 0.6 mL/min at 60 °C.

3. Results

3.1. Creation of recombinant cellulolytic B. subtilis

B. subtilis has an endogenous endoglucanase *Bscel5* gene in its chromosome, but its expression level is very low (Tjalsma et al., 2004). The recombinant BsCel5 expressed in *E. coli* had a maximum activity at pH 6.0 and 50 °C (Supplementary Fig. S2). The specific activities of BsCel5 on CMC, RAC, and Avicel were 96.4, 20.9, and 16.3 U/mg, respectively. The *Bscel5* gene was overexpressed under control of two strong constitutive promoters P_{HpaII} and P_{43} (Dartois et al., 1994; Wang and Doi, 1984), as well as the signal sequence of the *nprB* gene (Supplementary Fig. S1) in plasmid pP43N-Bscel5. *B. subtilis* WB800 harboring a negative plasmid pP43NMK exhibited small size halos on carboxymethyl cellulose (CMC) plates when growing on the LB or M9/glucose media (Fig. 1A), indicating that it produced a small amount of endogenous BsCel5. In contrast, *B. subtilis* WB800(pP43N-Bscel5) produced much larger halos, indicating the over-expression of secretory BsCel5 (Fig. 1A). When both strains grew on the M9 media containing regenerated amorphous cellulose (RAC) and yeast extract (YE), *B. subtilis* WB800(pP43N-Bscel5) hydrolyzed solid cellulose, resulting in a clear halo zone. But the halo zone was not observed for the negative control of WB800(pP43NMK) (Fig. 1A). When both strains were grown in the M9 liquid media containing 0.5% glucose, the secreted BsCel5 was the most abundant protein in the extracellular proteome for *B. subtilis* WB800(pP43N-Bscel5) (Fig. 1B). The BsCel5 expression levels can be checked through affinity adsorption by cellulose followed by SDS desorption. Fig. 1C suggested that *B. subtilis* WB800(pP43N-Bscel5) expressed ca. 50 times of BsCel5 of

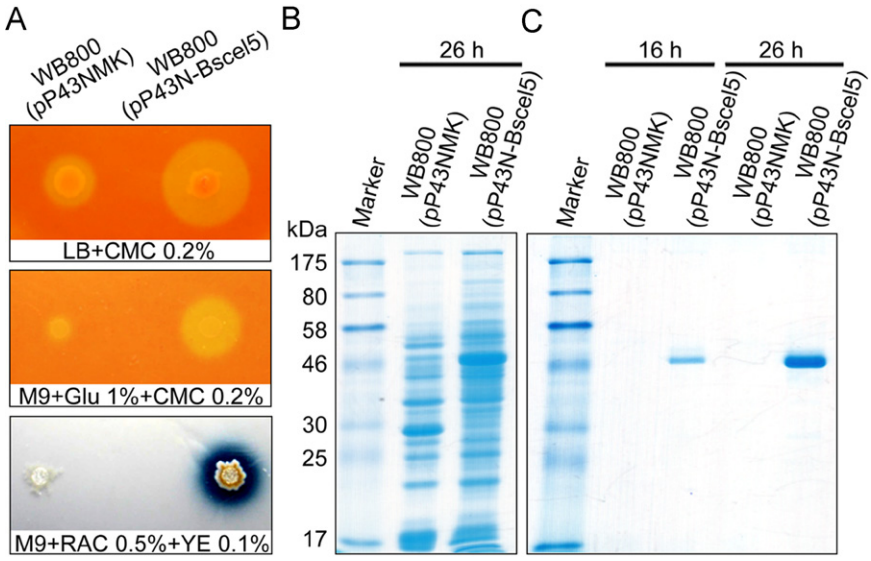

Fig. 1. Over-expression of BsCel5 in *B. subtilis* WB800 strains. (A) Recombinant strains harboring BsCel5 expression plasmid pP43N-Bscel5 or the negative control plasmid pP43NMK on the LB/CMC plate, M9/CMC supplemented with 0.5% glucose plate, and the M9/YE/RAC plate. After growing overnight at 37 °C, the colonies were washed off and the plates were stained with Congo red, and destained by 1 M NaCl solution. (B) Extracellular proteins profiles. Proteins in the supernatant of the cell cultures after 26 h cultivation were precipitated by trichloroacetic acid. Proteins from 100 μL of the cell culture were loaded for each lane. (C) The purified BsCel5 from the culture medium. BsCel5 from the supernatant of 1.5 mL cell culture was adsorbed by 1 mg of RAC and desorbed by SDS. *B. subtilis* strains were cultivated in the M9 medium supplemented with 0.5% glucose.

B. subtilis WB800(pP43NMK). But this ratio may be over-estimated since cellulose cannot capture low concentration cellulase as efficiently as that for high concentration cellulase (Hong et al., 2008). The extracellular cellulase ratio of WB800(pP43N-Bscel5) to WB800(pP43NMK) growing in the M9/glucose media was ca. 32 based on endoglucanase activity assay. The maximum BsCel5 concentration produced by WB800(pP43N-Bscel5) was ~14 mg/L, accounting for ~5.9% of the total cellular protein.

When the chemically defined minimal M9 medium was supplemented with RAC as the sole carbon source, *B. subtilis* WB800 (pP43N-Bscel5) grew with colonies, while no colony was observed for WB800(pP43NMK) (Fig. 2A). These results suggested that the over-expression of the endogenous endoglucanase enabled conversion of non-cellulose-utilizing *B. subtilis* to a cellulose utilizer that can produce enough cellulase and hydrolyze cellulose to support its growth and cellulase synthesis. Furthermore, the recombinant cellulolytic *Bacillus* strain grew well on the M9 plates containing the pretreated lignocellulosic biomass—switchgrass, corn stover, and common reed (Fig. 2B). The negative control did not grow on the M9 plates containing pretreated switchgrass.

To test whether over-expression of other endoglucanase genes resulted in cellulolytic *Bacillus* strains, the *Bscel5* gene was replaced by two *Clostridium phytofermentans* ISDg endoglucanase genes, *Cpcel5c* and *Cpcel9* (Liu et al., 2010; Zhang et al., 2010a). Both endoglucanases were functionally expressed according to the clear halo zones on a CMC-Congo red plate, but the hosts harboring heterologous endoglucanases did not grow on the minimal M9/RAC plates (Supplementary Fig. S3). This suggested that it was important to discover highly active and well expressed cellulases for creating recombinant cellulolytic microorganisms.

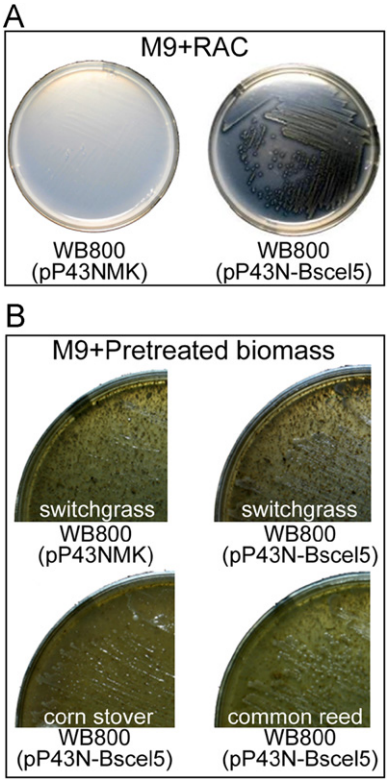

Fig. 2. Growth of *B. subtilis* strains WB800(pP43NMK) and WB800(pP43N-Bscel5) on the M9 plates containing 0.5% (w/v) RAC or pretreated biomass (5 g/L glucan) as the sole carbon source at 37 °C.

3.2. Performance improvement of BsCel5 by directed evolution

In our previous work, a new cellulase engineering platform based on *B. subtilis* was established for easy identification of better cellulase mutants based on the size of halo zones on insoluble RAC (Fig. 3A). Three positive mutants (MT1A, MT1B, and MT1C) featuring improved expression/secretion level and/or enhanced specific activity were previously obtained (Zhang and Zhang, 2011). In this work, a second round of random DNA mutagenesis was conducted based on the template of DNA mixtures containing the first-round three positive mutants. Three mutants (MT2A, MT2B, and MT2C) were screened from ~20,000 colonies, and they featured bigger and clearer halo zones as compared with the first-round three mutants (Fig. 3C).

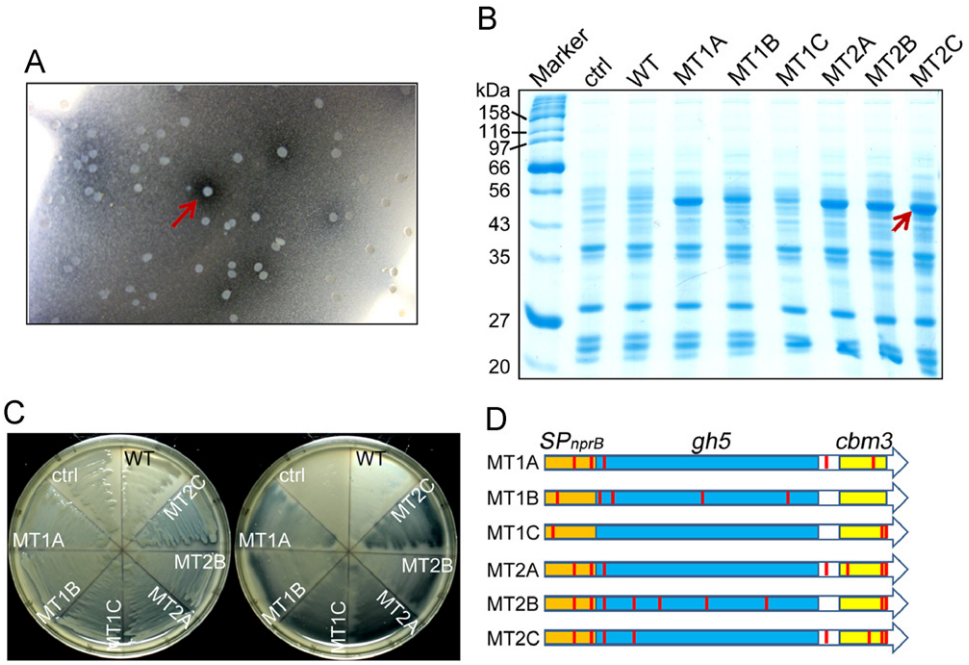

Fig. 3. Cellulase mutant library screening for *Bscel5* variants. (A) A positive mutant with bigger and clearer halo zone (red arrow) was screened. (B) Comparison of the performance of wild-type (WT) and mutants (MT1A, MT1B, MT1C, MT2A, MT2B and MT2C) based on the ability to degrade insoluble cellulose based on the formation of clear halo zones. The *B. subtilis* strains were streaked on an LB/RAC plate and incubated for 24 h at 37 °C. (C) BsCel5 expression and secretion profiles of wild-type and mutants. ctrl, negative control. Each lane was loaded by the proteins precipitated from 100 μL of the culture supernatant. The position of BsCel5 is indicated with an arrow. (D) Base mutations in *Bscel5* variants. Red bars indicate the base mutations in the variants. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The growth and cellulolytic ability of the strains harboring a negative control plasmid (ctrl), wild-type BsCel5 (WT), and six mutants were examined on an LB/RAC plate (Fig. 3C). After washing the cells from the plate, cellulase mutants from the second round screening had an enhanced ability of hydrolyzing RAC as compared to the mutants from the first-round and wild-type (Fig. 3C). As compared to the first-round mutants, all the second round positive mutants had further increases in secretory cellulase expression level (Fig. 3B). Among these mutants, MT2C had the highest expression level. In the whole cells fraction, no obvious intracellular accumulation of BsCel5 was found except mutant MT1B (Supplementary Fig. S4), suggesting that most of the mutated enzyme can be secreted efficiently across the cellular membrane.

The mutation sites in the three selected mutants from the second round screening are summarized in Table 2. Each had seven to nine mutations, which were distributed in the signal peptide-encoding region, catalytic module, linker, and carbohydrate-binding module (Fig. 3D). It was found that all three mutants had the same mutations within the signal peptide-encoding region (Fig. 3D). The wild-type BsCel5 and six mutants were over-expressed in *E. coli* and then were purified to homogeneity (data not shown). The specific activities of mutants MT2C and MT1C on RAC were the highest, ca. 46% higher than that of WT (Fig. 4). MT2C was expressed more efficiently than MT1C (Fig. 3B).

3.3. Knock-out of alsS in the 2,3-butanediol production pathway

Under anaerobic conditions, *B. subtilis* produces lactate and 2,3-butanediol as major products and acetate as a minor product (Fig. 5; Cruz Ramos et al., 2000; Goelzer et al., 2008; Romero et al., 2007). To eliminate 2,3-butanediol production and increase lactate yield, the *alsS* gene in the first step of the 2,3-butanediol synthesis pathway was attempted to be knocked out. First, a prototrophic *B. subtilis* XZ3 was selected on a minimal M9/glucose medium through the transformation of *B. subtilis* 1A751 (a histidine

Table 2
Amino acid substitutions in BsCel5 mutants from the second round screening and the corresponding base mutations.

Mutant	Amino acid substitution and corresponding base mutation
MT2A	T16I (ACA→ATA), S27P (TCA→CCA), K33I (AAA→ATA), I339T (ATT→ACT), G446E (GGA→GAA), K474E (AAA→GAA), K482E (AAA→GAA)
MT2B	T16I (ACA→ATA), S27P (TCA→CCA), K33I (AAA→ATA), V57E (GTA→GAA), S78G (AGC→GGC), N141[a] (AAT→AAC), Y280[a] (TAT→TAC), K474E (AAA→GAA), K482E (AAA→GAA)
MT2C	T16I (ACA→ATA), S27P (TCA→CCA), K33I (AAA→ATA), V57E (GTA→GAA), I339T (ATT→ACT), K439Q (AAA→CAA), K474E (AAA→GAA), K482E (AAA→GAA)

[a] Silent mutation.

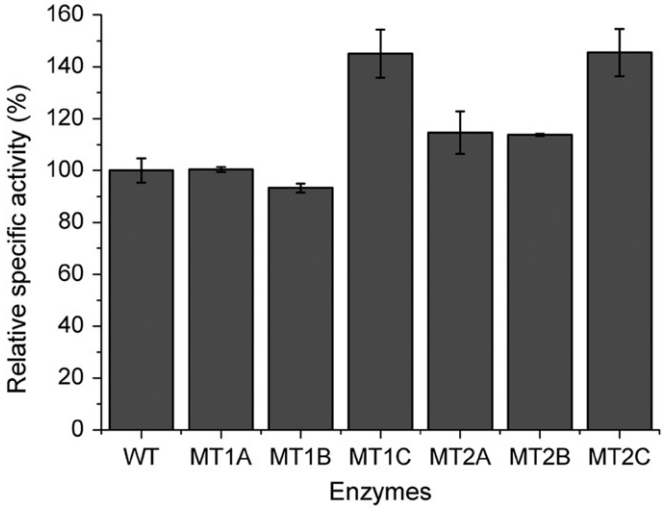

Fig. 4. Relative specific activities of wild-type BsCel5 (WT) and its mutants (MT1A, MT1B, MT1C, MT2A, MT2B, and MT2C). The specific activity of wild-type BsCel5 under the tested condition is ~698 U/μmol. The error bars represent the standard deviation of the triplicate measurements.

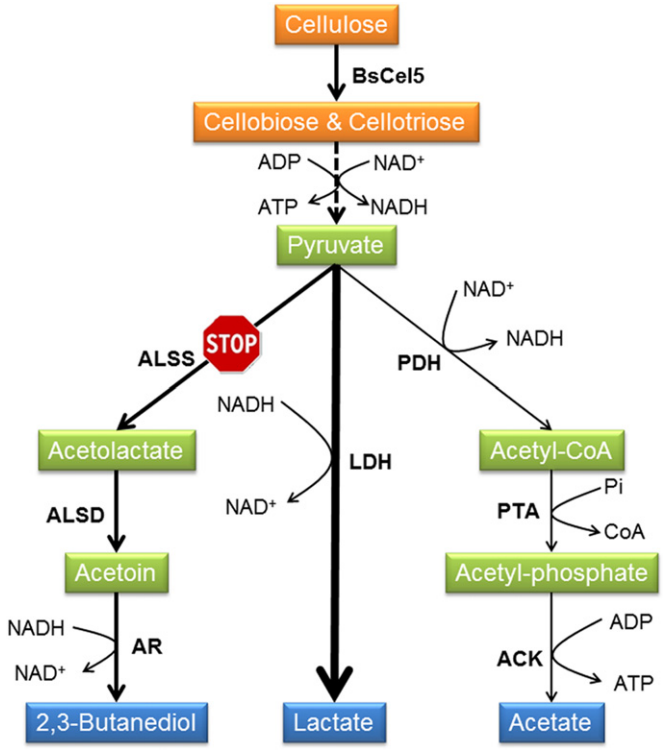

Fig. 5. Pathways for major anaerobic fermentative products in *B. subtilis*. The native pathway for ethanol is not shown because it is a minor product and usually undetectable by HPLC. "STOP" means the knock-out of the enzyme. ALSS, alpha-acetolactate synthase; ALSD, alpha-acetolactate decarboxylase; LDH, L-lactate dehydrogenase; PDH, pyruvate dehydrogenase; PTA, phosphotransacetylase; ACK, acetate kinase.

autotroph containing an inactivated *Bscel5* in the chromosome (Wolf et al., 1995)) with genomic DNA of a prototrophic strain IH6140. Second, the *alsS* gene of *B. subtilis* XZ3 was disrupted by the insertion of the spectinomycin resistance gene by a double cross-over recombination. The resulting strain XZ7 was selected on the LB plate supplemented with 100 μg/mL spectinomycin. The inactivation of the *alsS* gene in strain XZ7 was confirmed by PCR. When the fermentation products of strain XZ7 was checked by HPLC, there was no detectable 2, 3-butanediol in the fermentation broth. The knock-out of *alsS* in strain XZ7 affected neither the growth specific rate nor the maximum biomass (Supplementary Fig. S5). In the preliminary test, compared with the parent strain XZ3, it was shown that the knock-out of *alsS* in strain XZ7 resulted in about 16% increase in lactate volumetric productivity in M9 medium with RAC as the carbon source and supplemented with 0.1% yeast extract (Supplementary Fig. S5). This result was similar to the previous report on *alsS* knock-out in *B. subtilis* for improvement of lactate productivity (Romero-Garcia et al., 2009).

3.4. One-step lactate fermentation

B. subtilis XZ7 strains harboring plasmid pNW33N (negative control), pBscel5-WT encoding wild-type BsCel5, and pBscel5-MT2C encoding mutant MT2C were cultivated in the minimal M9 medium containing about 7 g/L RAC as the sole carbon source or supplemented with 0.1% yeast extract (Fig. 6). The negative control *B. subtilis* XZ7(pNW33N) did not hydrolyze cellulose (Fig. 6A,B), exhibited no cell growth (Fig. 6C), and produced no detectable lactate (Fig. 6D). Strain XZ7(Bscel5-WT), which can over-express wild-type BsCel5, slowly hydrolyzed cellulose to soluble sugars and then utilized soluble sugars for its growth, cellulase synthesis, and lactate production. At the end of six-day fermentation, XZ7(pBscel5-WT) consumed about 48% RAC and produced about 2.0 g/L lactate with a yield of 59.4% of the theoretical maximum. Strain XZ7(pBscel5-MT2C), which can produce a more active mutant MT2C with elevated expression level, exhibited much faster cellulose hydrolysis rates (Fig. 6B), had faster cell growth rates (Fig. 6C) and produced more lactate (Fig. 6D) as compared to strain XZ7(pBscel5-WT). At the end of fermentation for XZ7(pBscel5-MT2C), 74% RAC was consumed and about 3.1 g/L lactate was produced with a yield of about 60% of the theoretical maximum. When 0.1% (w/v) yeast extract was supplemented in the M9/RAC medium, both cell growth and cellulose hydrolysis rates of XZ7(pBscel5-MT2C) were enhanced significantly. At the end of fermentation, cellulose digestibility was increased to 92%, the titer of lactate reached about 4.1 g/L, and lactate yield was 63% of the theoretical maximum, indicating that even a small amount of yeast extract can greatly boost cellulose hydrolysis ability of the recombinant cellulolytic microorganism. Qualitative images clearly showed that the strain that can overexpress more active MT2C greatly decreased turbidity of the M9/RAC medium (Fig. 6A). The extracellular protein profiles showed that cellulase expression levels were strongly associated with cellulose hydrolysis digestibilities (Supplementary Fig. S6).

4. Discussion

The introduction of cellulolytic ability into industrially important and genetically tractable microorganisms is an important topic for low-cost production of biocommodities from cellulosic materials (la Grange et al., 2010; Zhang and Zhang, 2010). The creation of real cellulose-utilizing microorganisms on cellulose without yeast extract has not yet been successful (Brenner et al., 2008). This challenging task is attributed to three reasons: (i) heavy bioenergetic burden associated with over-expression of low turn-over number cellulase (Brenner et al., 2008; Zhang and Lynd, 2005); (ii) relatively low levels of secretory or cell-surface displayed active cellulase by most hosts, such as *E. coli* and *S. cerevisiae* (Den Haan et al., 2007; Shin and Chen, 2008); and (iii) complicated relationship and regulation between different modes of action of cellulases (Lynd et al., 2005; Zhang and Lynd, 2006). By utilizing high protein-secretion capacity *B. subtilis* (Zhang and Zhang, 2010), highly active endoglucanase BsCel5, plus highly reactive cellulosic materials pretreated by COSLIF (Moxley et al., 2008; Zhu et al., 2009a), we demonstrated for the first time that over-expression of BsCel5 through a good combination of endoglucanase expression/secretion plasmid and highly active BsCel5 enabled a non-cellulose-utilizing microorganism to grow on cellulose by relying on its own cellulase without the help of any other organic nutrient.

The addition of large amounts of yeast extract or other organic nutrients into nutrient-poor cellulosic materials was economically prohibited for producing low-value biocommodities (Lau and Dale, 2009; Lawford and Rousseau, 1996; Wood et al., 2005). Different from previous fermentations mediated by recombinant cellulolytic microorganisms, where a large amount of organic nutrients was added or a large inoculum (e.g., $A_{600}=\sim 50$) was used (Den Haan et al., 2007; Fujita et al., 2004; Tsai et al., 2010), here a small inoculum of recombinant *B. subtilis* strains ($A_{600}=0.15$) were able to utilize pretreated biomass without any other organic nutrient. This suggested great cost savings for future industrial fermentations. A small amount of yeast extract (e.g., 0.1%) significantly enhanced cell growth, cellulose hydrolysis, and product yield (Fig. 6). When high levels of organic nutrients were used in the medium, they provided carbon and energy sources for cell

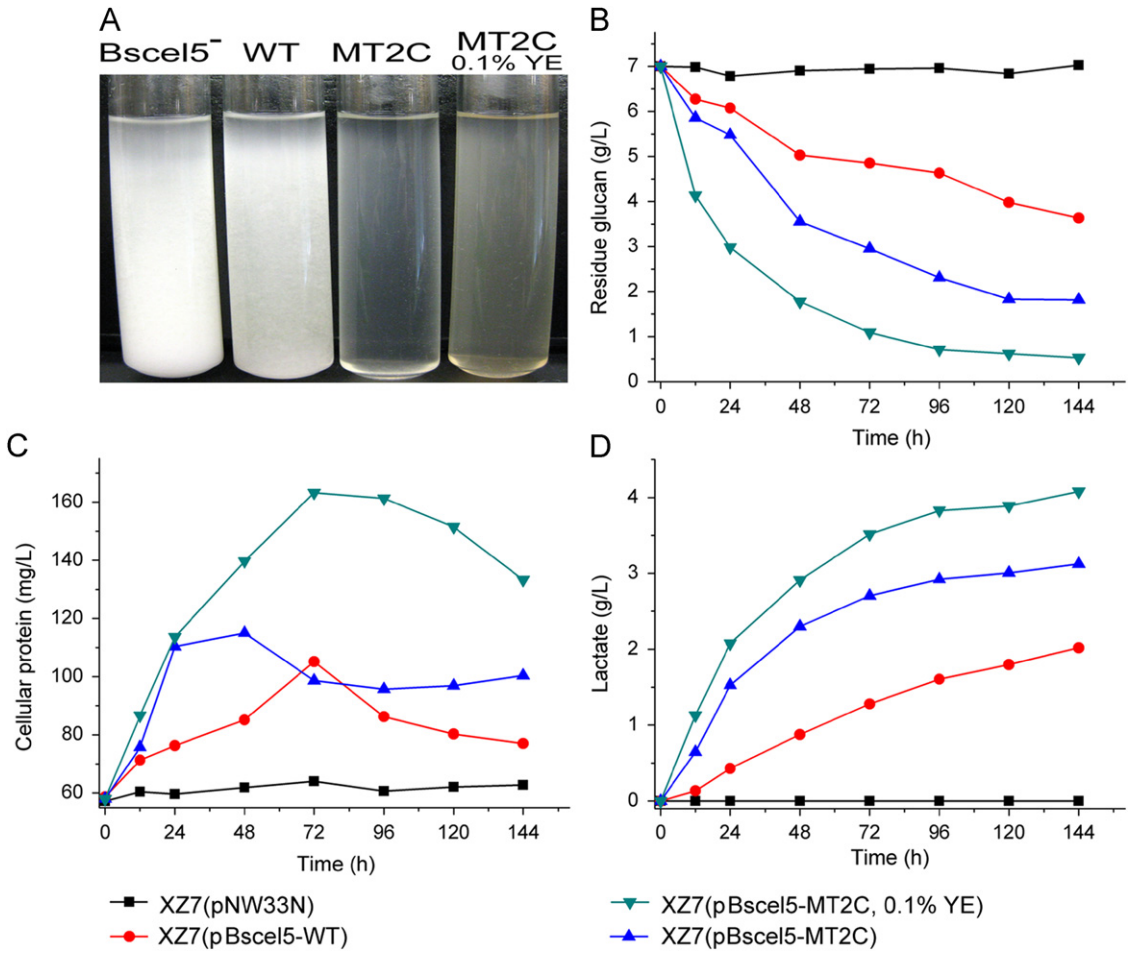

Fig. 6. Lactate fermentation based on RAC by a control strain XZ7(pNW33N), and two recombinant cellulolytic *B. subtilis* strains XZ7(pBscel5-WT) and XZ7(pBscel5-MT2C). Photos of the RAC-containing culture after 6 days of fermentation (A) as well as the profiles of residue glucan (B), cellular protein (C), and lactate (D).

mass synthesis and decreased energy burdens for the synthesis of proteins and amino acids. Therefore, high lactate yields (e.g., 95%) were usually obtained by metabolic engineered *B. subtilis* on a rich medium (Romero-Garcia et al., 2009). In the future, the addition of low-cost corn steep liquor or soy bean hydrolysate may be used for enhancing cellulose hydrolysis rates and biocommodity yields.

Directed enzyme evolution is becoming a widely accepted tool for enhancing enzyme performance. The greatest challenge of this technology is to correctly evaluate the performance of generated mutants (Zhang et al., 2006a). Most endoglucanase performances were examined based on the CMC/Congo red staining technology (Kim et al., 2000; Lin et al., 2009; Liu et al., 2010; Murashima et al., 2002; Nakazawa et al., 2009; Qin et al., 2008). Unfortunately there is no clear cellulase activity relationship on soluble substrate and insoluble substrate (Zhang et al., 2006a). By using a novel platform for identifying a more active endoglucanase on insoluble RAC (Zhang and Zhang, 2011), we further obtained a new mutant BsCel5-MT2C with a higher activity and better expression level than the previous ones (Zhang and Zhang, 2011). The recombinant *B. subtilis* strain harboring MT2C showed enhanced cell growth rates and RAC hydrolysis rates plus a higher cellulose digestibility (Fig. 6). This study demonstrated that cellulase engineering was vital to get more powerful recombinant cellulolytic microorganisms, which was suitable for consolidated bioprocess projects.

Recombinant cellulolytic *B. subtilis* strains have numerous features good for the production of biocommodities from low-cost cellulosic materials: (1) GRAS microorganisms without endotoxin, (2) low nutrient requirements for fermentation, (3) utilization of hexose and pentose sugars, (4) production of native hemicellulases, (5) strong tolerance to high concentration of salt or solvent, (6) rich knowledge of genome sequence, transcription, translation, protein folding, secretion mechanism, and large-scale fermentation, and (7) simple genetic manipulation (Zhang and Zhang, 2010, 2011). This work is the first step to develop potential industrially important *Bacillus* strains that can produce biocommodities from less costly cellulosic materials in a single step. Co-expression of several secretory glycoside hydrolase family 5, 9, and 48 cellulase components in one *Bacillus* strain or by consortium of *Bacillus* strains is under development. It is anticipated that future cellulolytic *Bacillus* strain or consortium would rapidly hydrolyze pretreated biomass and even non-pretreated less-recalcitrant genetically modified plant biomass with high glucan digestibility.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2011.04.003.

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