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Aerobic and anaerobic cellulose utilization by *Paenibacillus* sp. CAA11 and enhancement of its cellulolytic ability by expressing a heterologous endoglucanase

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ABSTRACT

For cost-effective lignocellulosic biofuel/chemical production, consolidated bioprocessing (CBP)-enabling microorganisms utilizing cellulose as well as producing biofuel/chemical are required. A novel strain *Paenibacillus* sp. CAA11 isolated from sediment was found to be not only as a cellulose degrader under both aerobic and strict anaerobic conditions but also as a producer of cellulosic biofuel/chemicals. *Paenibacillus* sp. CAA11 secreted cellulolytic enzymes by its own secretion system and produced ethanol as well as short-chain organic acids (formic acid, acetic acid, lactic acid) from cellulose. Cellulolytic activity of the strain was significantly enhanced by expressing a heterologous endoglucanase 168Cel5 from *Bacillus subtilis* under both aerobic and anaerobic conditions. The strain harboring the *168cel5* gene revealed 2-fold bigger halo zone on Congo-red plate and 1.75-fold more aerobic cellulose utilization in liquid medium compared with the negative control. Notably, under anaerobic conditions, the recombinant strain expressing 168Cel5 consumed 1.83-fold more cellulose (5.10 g/L) and produced 5-fold more ethanol (0.65 g/L) along with 5-fold more total acids (1.6 g/L) compared with the control, resulting 2.73-fold higher yields. This result demonstrates the potential of *Paenibacillus* sp. CAA11 as a suitable aerobic and anaerobic CBP-enabling microbe with cellulolytic production of ethanol and short-chain organic acids.

1. Introduction

Lignocellulosic biomass is the most abundant renewable resource for the production of biofuels and bio-based products. A major obstacle to use lignocellulosic biomass is its recalcitrance to hydrolysis into soluble sugars (Gong et al., 2017). Currently, chemical/physical pretreatment followed by hydrolyzing cellulose by adding high-cost cellulase externally is generally employed prior to bioconversion of lignocellulosic biomass through fermentation (Olson et al., 2012; Parisutham et al., 2014).

Alternatively, to lower the cost of exogenous cellulase by decreasing enzyme use, increasing attention has been given to consolidated bioprocessing (CBP) in which hydrolyzing cellulose and fermentation occur by a single microbe (Yıldırım and Çelik, 2017). Strain development for CBP has been pursued by two strategies: native cellulolytic strategy and recombinant cellulolytic strategy (Lynd et al., 2002; Olson et al., 2012). Native cellulolytic strategy starts with engineering naturally occurring cellulolytic microorganisms to produce desired valuable products or to improve the product yield and tolerance. This strategy has been investigated with anaerobic *Clostridium* bacteria which form cellulosomes, complexed multi-cellulolytic enzyme systems (Lynd et al., 2002). However, engineering cellulolytic *Clostridium* strains for CBP hosts has been hampered due to a lack of genetic tools and the strict anaerobic characteristics of *Clostridium*. After the development of gene transfer systems for *Clostridium cellulolyticum* and *C. thermocellum*, metabolic engineering and cellulosic biofuel production were investigated with those strains (Argyros et al., 2011; Brown et al., 2011; Gaida et al., 2016; Graham et al., 2014). Although engineering cellulolytic anaerobic microbes has shown promising results, limited genetic modification systems and low performance under industrial conditions are still

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remaining barriers (Olson et al., 2012).

Recombinant cellulolytic strategy has been investigated with model hosts such as *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Escherichia coli*, which are amendable to genetic manipulation with well-developed genetic tools and produce biofuel/chemicals. With the *S. cerevisiae* strains, ethanol production from cellulose has been attempted by expressing cellulolytic enzymes or by cell surface display of cellulosome components (Fitzpatrick et al., 2014; Fujita et al., 2004; Tsai et al., 2010). Another ethanol producer *Z. mobilis* was engineered to express a heterologous endoglucanase from *Enterobacter cloacae* (Thirumalai Vasan et al., 2011). However, there are problems with *Z. mobilis* and *E. coli* to secrete cellulases extracellularly because of the thick outer membrane (Jung et al., 2012). In addition, because the cellulolytic activity of recombinant cellulolytic microbes was not sufficient enough to support cell growth with cellulose, rich media containing plenty of yeast extract, peptone and/or casamino acids were required to get a desirable cell density (Fitzpatrick et al., 2014; Linger et al., 2010; Nakatani et al., 2013; Thirumalai Vasan et al., 2011).

For the development of suitable CBP microbes, it would be certainly advantageous to start with bacteria: i) having characteristics of native cellulolytic microorganisms (endogenous cellulase activity and secretion system) ii) producing biofuels/chemicals, and iii) being able to engineered with well-established available gene manipulation systems. Moreover, the utilization of various lignocellulose-derived sugars (glucose, xylose, and cellobiose) and the production of a broad range of metabolites would be additional desirable characteristics.

In this study, a novel strain of *Paenibacillus* sp. CAA11 isolated from foreshore sediments was investigated as a potential facultative CBP-enabling microorganism possessing the beneficial features mentioned above. Growth characterization of *Paenibacillus* sp. CAA11 was performed with respect to initial pH, temperature, sugars, and cellulose. Expression of heterologous endoglucanase was carried out to enhance the cellulolytic ability of *Paenibacillus* sp. CAA11 by applying *Bacillus* genetic tools. The effect of the enhanced cellulolytic ability on biofuel/chemical production was also evaluated under both aerobic and strict anaerobic conditions.

2. Material and methods

2.1. Chemicals and materials

Carboxymethyl cellulose (CMC, Mw ~90,000), Avicel PH-101, and Congo red were purchased from Sigma-Aldrich. Regenerated amorphous cellulose (RAC) was prepared from Avicel by dissolving it in H_3PO_4 at 50 °C for 6 h as described by Zhang et al. (2011). Oligonucleotides were synthesized by Macrogen (South Korea). All restriction enzymes were purchased from Thermo Scientific (USA).

2.2. Enrichment and isolation of cellulose-degrading bacteria

Sediment samples were collected from Dongmak foreshore in Republic of Korea and cultured in flasks with cellulose medium containing CMC 10 g/L for 6 days at 150 rpm and 30 °C. The cellulose medium contained (per liter): CMC 10 g, yeast extract 0.5 g, peptone 0.5 g, beef extract 0.3 g, K_2HPO_4 5 g, KH_2PO_4 3 g, $(NH_4)_2SO_4$ 2 g, $MgSO_4{\cdot}7H_2O$ 0.4 g, $CaCl_2{\cdot}2H_2O$ 0.1 g, and 1 ml trace element solution, pH 7.0. The trace element solution consisted of 0.07 g/L $ZnCl_2$, 0.1 g/L $MnCl_2{\cdot}4H_2O$, 0.06 g/L H_3BO_3, 0.2 g/L $CoCl_2{\cdot}6H_2O$, 0.02 g/L $CuCl_2{\cdot}2H_2O$, 0.02 g/L $NiCl_2{\cdot}6H_2O$, 0.04 g/L $NaMoO_4{\cdot}2H_2O$, and 1 ml/L hydrochloric acid. Liquid cultures were spread on agar plates containing CMC 5 g/L and cellulose-degrading colonies were screened by staining with 0.1% Congo red solution for 15 min followed by destaining with 1 M NaCl for 15 min to detect clear zones. Pure cultures of cellulose-degrading bacteria were isolated by several transfers in cellulose agar plates.

2.3. 16S rRNA gene sequence analysis

Genomic DNA of the isolate was purified using Wizard Genomic DNA purification kit (Promega Co., USA). The DNA was subjected to PCR with the universal primers 27F and 1492R. The PCR product was cloned into pGEM-T vector and plasmids were isolated using a plasmid extraction kit (Qiagen Co., Germany). Pairwise 16S rRNA gene sequence similarity comparison was achieved by using the EzTaxon server http://eztaxon-e.ezbiocloud.net/ (Kim et al., 2012). Phylogenetic analysis was performed by the Neighbor-joining method. Evolutionary distance matrices for the Neighbour-joining method were generated according to the model of Jukes and Cantor (Jukes and Cantor, 1969). The neighbor-joining tree topology was evaluated by bootstrap analyses based on 1000 resamplings. The phylogenetic tree was constructed using the MEGA 5 software.

2.4. Media and cell growth conditions

Luria-Bertani (LB) medium was used for *Escherichia coli* cell culture. The M9 medium was used for *Paenibacillus* sp. CAA11 and mutations. The M9 medium contained (per liter): Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 20 g, NH_4Cl 1 g, $MgSO_4{\cdot}7H_2O$ 0.492 g, $CaCl_2$ 0.111 g, yeast extract 1 g, and 1 ml trace element solution with a carbon source (glucose 5 g/L, xylose 5 g/L, cellobiose 5 g/L, CMC 5–10 g/L or RAC 10 g/L as indicated in the text). Chloramphenicol (25 μg/ml) was added to the LB medium for recombinant *E. coli* strains.

The seed cultures of *Paenibacillus* sp. strains were prepared using a modified LB medium containing NaCl 20 g/L and cultivated aerobically at 37 °C for 24 h. The seed cultures were centrifuged and inoculated into the M9 medium with an initial optical density at 600 nm (OD_{600}) of 0.1. Aerobic culture was performed in a 250 ml flask with a rotary rate of 200 rpm at 37 °C and anaerobic culture was conducted in 50 ml M9 medium containing cellulose in a 150 ml serum bottle with a rotary rate of 200 rpm at 37 °C. For anaerobic conditions, the medium was purged with argon gas for 20 min and the bottle was sealed with a butyl rubber stopper and aluminum crimp seal (Youn et al., 2016). Chloramphenicol (10 μg/ml) was used for the cultivation of the recombinant *Paenibacillus* sp. CAA11. The pH was adjusted to 7 with 5 M KOH or 5 M HCl unless otherwise stated.

2.5. Bacterial strains and plasmids

Description of the strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α was used as a host cell for all DNA manipulations and *Bacillus subtilis* 168 (Zhang et al., 2011) was used as a template for PCR of the target genes. To construct of the plasmids for *Paenibacillus* sp. CAA11, the pAD123 and pNW33N vectors were obtained from the Bacillus Genetic Stock Center because the vectors used for *Bacillus* have been found to also work for *Paenibacillaceae* in the previous studies (Poppinga and Genersch, 2012; Zarschler et al., 2009).

The primers used in this study are presented in Table S1. P_{43} promoter, a strong constitutive promoter in *Bacillus* (Zhang et al., 2005), was chosen. To test the intensity of P_{43} and the tandom promoter P_{43}-P_{43} in *Paenibacillus* sp. CAA11, the pAD123 containing a GFP reporter gene was used. P_{43} promoter was amplified with P7/P8 primers from chromosomal DNA of *Bacillus subtilis* 168 by PCR. The digested PCR products were inserted in front of the GFP reporter gene in pAD123 with *Sma*I and *BamH*I sites. To analyze the intensity of the tandem promoter, P_{43} was amplified with P1/P9, and cloned to pAD123-P_{43} at *BamH*I and *Xba*I sites, resulting in pAD123-P_{43}-P_{43}.

To construct pNW33N-P_{43}-P_{43} *nprB168cel5*, pNW33N-P_{43} was prepared by amplifying P_{43} with P7/P8, followed by cloning PCR products into pNW33N with *Sma*I and *BamH*I sites. The DNA sequences encoding P_{43} promoter, signal peptide of *B. subtilis nprB*, and mature *B. subtilis* 168 Cel5 (GenBank Accession No. CAA82317, 30–499 amino acids) were amplified with P1/P2, P3/P4, and P5/P6, respectively. The signal