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Short communication

Engineering *Clostridium beijerinckii* with the Cbei_4693 gene knockout for enhanced ferulic acid tolerance

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ABSTRACT

A mutant strain of *Clostridium beijerinckii* NCIMB 8052, *C. beijerinckii* M11, which exhibited ferulic acid tolerance up to 0.9 g/L, was generated using atmospheric pressure glow discharge and high-throughput screening. Comparative genomic analysis revealed that this strain harbored a mutation of the Cbei_4693 gene, which encodes a hypothetical protein suspected to be an NADPH-dependent FMN reductase. After disrupting the Cbei_4693 gene in *C. beijerinckii* NCIMB 8052 using the ClosTron group II intron-based gene inactivation system, we obtained the Cbei_4693 gene inactivated mutant strain, *C. beijerinckii* 4693::int. Compared with *C. beijerinckii* NCIMB 8052, 6.23 g/L of butanol was produced in P2 medium containing 0.5 g/L of ferulic acid by 4693::int, and the ferulic acid tolerance was also significantly increased up to 0.8 g/L. These data showed, for the first time, that the Cbei_4693 gene plays an important role in regulating ferulic acid tolerance in ABE fermentation by *C. beijerinckii*.

Butanol may play an important role in the overall success of the biofuel industry because its properties are more similar to those of gasoline than the properties of ethanol (Wang and Chen, 2011). For cost-effective biofuel production, the use of renewable lignocellulosic materials, such as corn fiber, wheat straw, and bagasse fiber, as substrates has been widely investigated. However, during the production of biofuels from these lignocellulosic materials, a range of lignocellulose-derived microbial inhibitory compounds (LDMICs), which are generated along with sugars during pretreatment (Wierckx et al., 2011), have been identified. LDMICs are known to partition into biological membranes, where they then increase cell fluidity, diminish proton motive force, reduce ATP levels, cause DNA mutagenesis, and inhibit the effects of essential enzymes on cell growth and metabolism (Baral and Shah, 2014; Ibraheem and Ndimba, 2013). In particular, phenolic compounds significantly inhibit growth and solvent production by solventogenic *Clostridium* species (Ezeji et al., 2007).

The antimicrobial activity of phenolic compounds is determined by their chemical structure (Adeboye et al., 2014; Sánchez-Maldonado et al., 2011). Ferulic acid (FA), one of the most common LDMICs, harbors a benzene ring, methoxy group, hydroxyl group, and double bond within its side chain and has the highest hydrophobicity of six model phenolic compounds (Table S1). Many reports have shown that FA has the strong antimicrobial properties in different bacterial strains at low concentrations (Ezeji et al., 2007; Guo et al., 2010; Lee et al., 2012). However, the exact mechanism of FA toxicity and tolerance to *Clostridium* are unclear.

Clostridium beijerinckii M11, which exhibits high FA tolerance, was obtained using atmospheric pressure glow discharge and high-throughput screening (Liu et al., 2015). From a comparative genomic analysis of *C. beijerinckii* M11 and its parental strain *C. beijerinckii* NCIMB 8052, **s**ingle nucleotide polymorphism (SNP) analysis showed that the mutated genes only contain 2 of SNPs in CDS, a synonymous substitution (Cbei_3182, alpha/beta fold family hydrolase) and a non-synonymous substitution (Cbei_4693, the reference base$_{5452204}$ G → A, site$_{157}$ W → L), then the mutated gene Cbei_4693 was identified. In this work, we hypothesize that this

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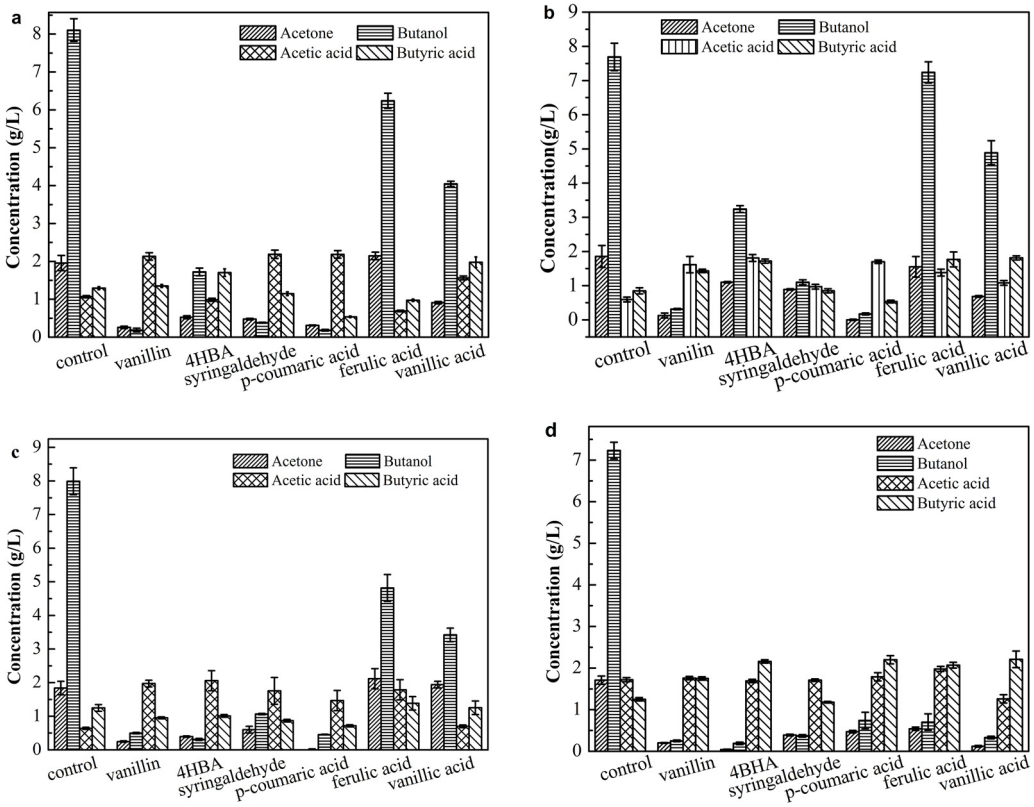

Fig. 1. ABE fermentation in P2 medium with 30 g/L glucose and 0.5 g/L of six model phenolic compounds by *C. beijerinckii* 4693::int (a), *C. beijerinckii* M11 (b), *C. beijerinckii* 4693::cp (c), and *C. beijerinckii* NCIMB 8052 (d) for 72 h.

Table 1
Ferulic acid tolerance of *C. beijerinckii* 4693::int and *C. beijerinckii* 4693::cp. The concentrations of the produced solvents were analyzed after 120 h for fermentation in P2 medium with 30 g/L of glucose, and the OD_{600} was measured at 48 h.

Strains	Ferulic acid (g/L)	Acetone (g/L)	Butanol (g/L)	Acetate (g/L)	Butyrate (g/L)	OD_{600}
4693::int	0	1.23 ± 0.21	8.15 ± 0.32	0.54 ± 0.03	0.87 ± 0.02	5.44 ± 0.41
	0.5	1.56 ± 0.11	6.21 ± 0.43	1.38 ± 0.31	1.77 ± 0.08	4.38 ± 0.32
	0.6	1.63 ± 0.12	5.02 ± 0.31	1.74 ± 0.31	1.79 ± 0.14	3.90 ± 0.31
	0.7	0.16 ± 0.02	0.69 ± 0.04	2.31 ± 0.42	1.61 ± 0.13	0.78 ± 0.02
	0.8	0.04 ± 0.01	0.30 ± 0.04	2.72 ± 0.45	1.25 ± 0.42	0.50 ± 0.02
	0.9	0	0	2.75 ± 0.41	0.89 ± 0.21	0.31 ± 0.01
4693::cp	0	1.83 ± 0.22	7.99 ± 0.41	0.64 ± 0.04	1.24 ± 0.02	5.38 ± 0.23
	0.5	2.11 ± 0.3	4.81 ± 0.40	1.78 ± 0.32	1.38 ± 0.18	4.41 ± 0.14
	0.6	0.18 ± 0.21	0.54 ± 0.03	2.13 ± 0.41	1.41 ± 0.31	0.66 ± 0.02
	0.7	0	0	2.21 ± 0.31	0.31 ± 0.11	0.38 ± 0.02
	0.8	0	0	2.51 ± 0.41	0.12 ± 0.06	0.32 ± 0.04
	0.9	0	0	2.49 ± 0.21	0.09 ± 0.04	0.29 ± 0.03

mutation in the Cbei_4693 gene plays an important role in increasing FA tolerance. The effects of the Cbei_4693 gene (encoding a hypothetical protein suspected to be an NADPH-dependent FMN reductase) on FA tolerance were studied.

The bacterial strains, plasmids, and primers used in this study are listed in Table S2. We chose the 335/336a position for insertion of the Cbei_4693 gene using the Clostron system (http://www.clostron.com). Subsequently, we synthesized group II intron fragments and constructed the plasmid pWJ1-4693 by inserting group II intron fragments into *Xho*I and *Bsr*GI restriction sites in the pWJ1 plasmid. The Cbei_4693 gene was inserted into the *Xho*I and *Nde*I restriction sites of the pWJ1 plasmid using an infusion one-step clone kit (Vazyme Biotech Inc., Nanjing, China), generating the express plasmid pWD1-4693.

Plasmids pWJ1-4693 and pWD1-4693 were methylated in *E.coli* TOP10 (pAN2) then transformation Colony polymerase chain reaction (PCR) was used for screening and isolating inactivated mutants (*C. beijerinckii* 4693::int) and complementation mutants (*C. beijerinckii* 4693::cp) (Xiao et al., 2012), followed by batch-fermentation in P2 medium (Guo et al., 2013).

As shown in Fig. 1a and d, we found that disrupting the Cbei_4693 gene significantly enhanced the FA tolerance of *C. beijerinckii*, 6.23 g/L butanol was produced in P2 medium containing 0.5 g/L FA, this was less than that produced by *C. beijerinckii* M11(Fig. 1b). This result strongly suggested that Cbei_4693 was not the only element contributing to FA tolerance. It is possible that there may be other mutated genes in *C. beijerinckii* M11 that we did not find in this study but that may also improve FA tolerance.

C. beijerinckii 4693::cp, we also investigated the tolerance of the strain to six model phenolic compounds (0.5 g/L) to confirm that the increased FA tolerance was attributed to disruption of the Cbei_4693 gene. However, the production of fermentation products was not significantly decreased in P2 containing 0.5 g/L ferulic acid by *C. beijerinckii* 4693::cp, and 4.82 g/L butanol was