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Molecular breeding of Clostridia for butanol production from rice straw

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Research aims

Biobutanol produced by *Clostridium* spp. can be used as a biofuel and/or chemical feedstock. For cost-effective and sustainable production of biobutanol, an untapped biomass such as rice straw could be used as a substrate. Rice straw, which is composed of cellulose, hemicellulose, and lignin, is cheap and abundant. However, the utilization of rice straw is limited because butanol-producing clostridia cannot degrade cellulose, which is the major carbon source in lignocellulose. To achieve butanol production from lignocellulose, cellulose needs to be hydrolyzed to di- or monosaccharides that can then be metabolized by *Clostridium* spp.

In this study, we developed a system for butanol production from rice straw using a co-culture of cellulose-degrading *Clostridium thermocellum* and butanol-producing *Clostridium saccharoperbutylacetonicum*. In addition, we showed that *β*-glucosidase enhances butanol production from rice straw, and identified promoters that may be used for high-level expression of the *β*-glucosidase gene to increase productivity.

Methods

Growth and co-culture of cellulose-degrading *C. thermocellum* and butanol-producing *C. saccharoperbutylacetonicum* were performed using optimized conditions as previously described.[1] Briefly, *C. thermocellum* cells were grown in National Institute of Technology and Evaluation Biological Resource Center (NBRC) medium 979 containing cellobiose at 60°C and collected by centrifugation. Cell pellets were washed and suspended in the same medium without any added carbon source and adjusted to an optical density at 600 nm ($OD_{600\,nm}$) of 2. Six microliter of the cell suspension was used to inoculate 6 mL of NBRC medium 979 containing rice straw treated with 0.1 N NaOH or non-treated rice straw (final $OD_{600\,nm}$=1) and cultured at 60°C. *C. saccharoperbutylacetonicum* cells were collected after 24 h by centrifugation, washed, and suspended in clostridial growth medium (CGM) containing 4% glucose and adjusted to $OD_{600\,nm}$=13. The cell suspension (1 mL) was then added to *C. thermocellum* cultures that had been incubated at 30°C (final $OD_{600\,nm}$=1). Co-cultures were incubated at 30°C. Cellulase and *β*-glucosidase enzymes were added to the co-culture medium at the same time as inoculation of *C. saccharoperbutylacetonicum*. Fermentation products were quantified by HPLC using an Aminex HPX-87H column (Bio-Rad Laboratories K.K., Japan) with a differential refractive index detector (Shimadzu Corporation, Japan) and an aqueous solution of 0.05 mM H_2SO_4 as the solvent. Enzyme activities for cellulase were measured according to the method described by Chundawat *et al.*[2]

Twelve promoter sequences resembling the consensus promoter sequence in *Clostridium* spp. (TTGACA-N(17)-TATAAT) were screened using in silico molecular cloning software. Promoters were ligated upstream of the AcGFP reporter gene in shuttle vector pNAK1. Recombinant plasmids were transfected into *C. saccharoperbutylacetonicum* strain N1–4 by electroporation.[3] AcGFP expression levels were compared using western blot analysis using an anti-AcGFP antibody.

Results

Butanol production from alkali-treated rice straw

Co-cultures of *C. thermocellum* and *C. saccharoperbutylacetonicum* produced very low levels of butanol (1.1± 0.1 g/L) from non-pretreated rice straw. The maximum $OD_{600\,nm}$ of co-culture was 2.7 after one day of cultivation and gradually decreased to 1.3 during cultivation, suggesting that *C. thermocellum* was not metabolizing cellulose to release usable sugars to sustain growth of *C. saccharoperbutylacetonicum*. The lack of butanol fermentation from cellulose in these culture conditions was likely because the rice straw contains lignin, which inhibits cellulase accessibility to the substrate.

In order to improve butanol production, the lignin in rice straw was removed by the alkaline-treatment and the pretreated rice straw was used as a substrate. As expected, butanol production from delignified rice straw—in which the lignin content was decreased to 70%—was 2.3-fold higher than that of non-pretreated rice straw (2.5±0.1 g/L) (Fig. 1). This result clearly indicates that delignification improves butanol production during co-culture of *C. thermocellum* and *C. saccharoperbutylacetonicum*. Although there was an increase in butanol production, the concentration was 67% lower than that achieved with crystalline cellulose, as previously reported.[1] This may be due to the presence of low levels of cellulase activity in the co-culture. Therefore, to confirm whether additional cellulase enhanced butanol production, commercial cellulase from *Aspergillus niger* was

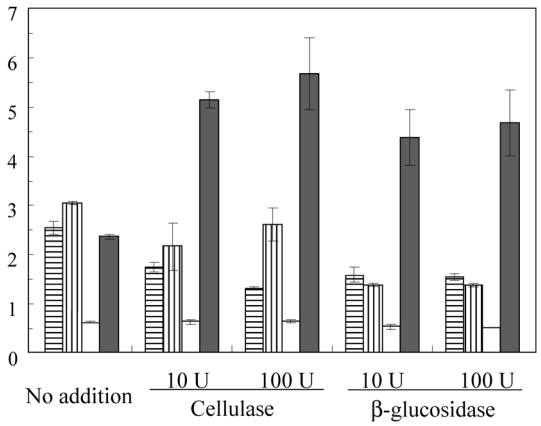

Fig. 1. Fermentation products (g/L) from alkali-treated rice straw *C. thermocellum* and *C. saccharoperbutylacetonicum* co-cultures. Cellulase or *β*-glucosidase (10 U and 100 U) were added to the co-culture medium. Bars indicate amounts of acetate (horizontal lines), butyrate (vertical lines), ethanol (solid white), and butanol (solid gray). All samples were assayed three times. Means±standard error are shown with arbitrary units.

Table 1. Comparison of enzyme activity related to lignocellulose degradation (U/mg).

	C. thermocellum	N1-4	Cellulase (10U)
Endoglucanase*	101.15±3.00	1.00±0.28	25.42±2.48
Exoglucanase*	4.51±0.40	0.47±0.08	10.79±0.24
β-glucosidase	0.07±0.00	0.35±0.02	17.38±1.94
xylanase	2.85±0.03	0.38±0.02	0.17±0.01

*Enzyme activity is expressed as milliunits/mg

added to the co-culture system. As expected, butanol production in the co-culture increased significantly with the addition of cellulase (Fig. 1). This result indicated that the lower yields of butanol in the co-culture system were caused by low activities of cellulase.

Delignified rice straw consists mainly of cellulose and hemicellulose, and the cellulase complex that degrades cellulose and hemicellulose contains endo-glucanase, exo-glucanase, *β*-glucosidase, and xylanase. The identification and expression of enzymes that contribute to high butanol production during co-culture is required for cost–effective butanol production. Therefore, cellulase activity in supernatants from single-cultures of *C. thermocellum* and *C. saccharoperbutylacetonicum* was compared to that of commercial cellulase (Table 1). Endo-glucanase and xylanase activities were the highest in *C. thermocellum*. By contrast, exo-glucanase and *β*-glucosidase activities were highest in the commercial enzyme (Table 1).

Interestingly, cellulase showed the highest *β*-glucosidase activity among the enzyme activities tested. Considering this result, we tested the effect of *β*-glucosidase in the co-culture and found that the addition of this enzyme significantly enhanced butanol production (Fig. 1). Butanol concentrations with 10 U and 100 U of *β*-glucosidase added per gram of biomass were 4.4 and 4.7 g/L, respectively. This result clearly indicates that the supplementation of *β*-glucosidase activity is important for butanol production from delignified rice straw during co-culture of *C. thermocellum* and *C. saccharoperbutylacetonicum*.

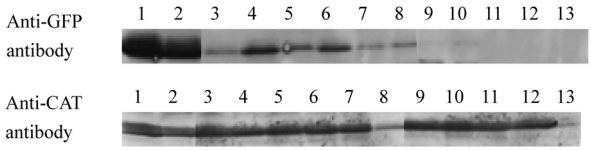

Fig. 2. Comparison of expression levels of AcGFP and Chloramphenicol acetyltransferase (CAT) by western blot analysis using anti-AcGFP and anti-CAT antibodies. Crude extracts from *C. saccharoperbutylacetonicum* strain N1–4 transformants harboring expression plasmids grown for 24 h were separated using SDS-PAGE. Lanes: 1, pNAK1/phosphotransferase systemIIC component promoter; 2, pNAK1/thiolase promoter; 3, pNAK1/hsp90 promoter; 4, pNAK1/rubrerythrin promoter; 5, pNAK1/ribosomal protein L13 promoter; 6, pNAK1/uncharacterized protein promoter; 7, pNAK1/butanol dehydrogenase promoter; 8, pNAK1/phosphate butyryltransferase promoter; 9, pNAK1/superfamilyII DNA and RNA helicases promoter; 10, pNAK1/adenine deaminase promoter; 11, pNAK1/ prolyl-tRNA synthetase promoter; 12, pNAK1/ Transcriptional antiterminater promoter 13, pNAK1 (control vector). Since the shuttle vector, pNAK1, contains CAT as a selective marker, maintenance of this vector was confirmed using an anti-CAT antibody (lower panel).

Screening of promoter sequences for β-glucosidase expression

Our data suggested that the expression of *β*-glucosidase by *Clostridium* spp. in co-cultures would be necessary to increase butanol production from rice straw. Therefore, promoter sequences that were similar to the consensus promoter sequence in *Clostridium* spp. were screened for activity in C. *saccharoperbutylacetonicum* using the reporter gene AcGFP. Among the promoter sequences selected from the whole genome sequence of strain N1–4, the strong expression of AcGFP was confirmed with promoters for phosphotransferase systemIIC component and thiolase genes. Such an expression of *β*-glucosidase using the promoters is required for high butanol production from rice straw.

Conclusion

We demonstrated that butanol production from rice straw by using a co-culture of cellulose-degrading *C. thermocellum* and butanol-producing *C. saccharoperbutylacetonicum* is increased by the addition of *β*-glucosidase. This result indicates that the molecular breeding of butanol-producing strains for secretion of *β*-glucosidase should increase butanol yield. In addition, we identified promoter sequences that upregulate the expression of *β*-glucosidase in *C. saccharoperbutylacetonicum*. In the future, we intend to develop an effective biobutanol production system involving clostridia by increasing *β*-glucosidase activity using promoters that we have identified.

References

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