Research Article

Expression of Heterologous Cellulases in Thermotoga sp. Strain RQ2

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The ability of Thermotoga spp. to degrade cellulose is limited due to a lack of exoglucanases. To address this deficiency, cellulase genes Csac1076 (celA) and Csac1078 (celB) from Caldicellulosiruptor saccharolyticus were cloned into T. sp. strain RQ2 for heterologous overexpression. Coding regions of Csac1076 and Csac1078 were fused to the signal peptide of TM1840 (amyA) and TM0070 (xynB), resulting in three chimeric enzymes, namely, TM1840-Csac1078, TM0070-Csac1078, and TM0070-Csac1076, which were carried by Thermotoga-E. coli shuttle vectors pHX02, pHX04, and pHX07, respectively. All three recombinant enzymes were successfully expressed in E. coli DH5α and T. sp. strain RQ2, rendering the hosts with increased endo- and/or exoglucanase activities. In E. coli, the recombinant enzymes were mainly bound to the bacterial cells, whereas in T. sp. strain RQ2, about half of the enzyme activities were observed in the culture supernatants. However, the cellulase activities were lost in T. sp. strain RQ2 after three consecutive transfers. Nevertheless, this is the first time heterologous genes bigger than 1 kb (up to 5.3 kb in this study) have ever been expressed in Thermotoga, demonstrating the feasibility of using engineered Thermotoga spp. for efficient cellulose utilization.

1. Introduction

Due to rising global energy demands, developing renewable forms of energy, such as solar, hydro-, and bioenergy, has become increasingly important. Traditionally, bioenergy is generated by fermenting the glucose derived from starch. Starch alone, however, accounts for too small a fraction of biomass to sustain a positive energy balance. The need to replace fossil fuels demands that cellulose, the most common and renewable organic material on Earth [1], must not be overlooked. Cellulose is a linear polymer of D-glucose units linked by 1,4-β-D-glycosidic bonds. It becomes useful as a food and energy source once it is broken down into soluble cellobiose (β-1,4 glucose dimer) and glucose, a process called hydrolysis because a water molecule is incorporated for each dissociated glycosidic bond. Effective hydrolysis of cellulose requires the cooperation of three enzymes, namely, endo-1,4-β-glucanase (EC 3.2.1.4), exo-1,4-β-glucanase (also called cellobiohydrolase) (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [2–5]. Endoglucanase randomly breaks down the β-1,4 linkages in the regions of low crystallinity, exoglucanase removes cellobiose units from the nonreducing ends of cellulose chains, and β-glucosidase converts cellobiose into glucose. In general, exoglucanases degrade cellulose more efficiently than endoglucanases.

Hyperthermophilic bacteria Thermotoga are attractive candidates for the production of biohydrogen and thermostable enzymes. Surveying of Thermotoga genomes in the CAZy database (http://www.cazy.org/) revealed dozens of carbohydrate-active enzymes, the molecular foundation that allows Thermotoga strains growing on a wide range of carbon sources such as glucose, xylose, semicellulose, starch, and carboxymethyl cellulose (CMC) [6–9]. Nevertheless, the apparent absence of exoglucanases suggests the limited ability of these organisms to use cellulose as their main carbon and energy source. Up to date, there are only two reports describing low levels of exoglucanase activities in Thermotoga [2, 4], a phenomenon probably caused by nonspecific reactions of endoglucanases [10–12].
This study aimed at introducing heterologous exoglucanase activities into *Thermotoga* by genetic engineering. *T.* sp. strain RQ2 was selected as the host strain, because its genome encodes the largest repertoire of carbohydrate-active enzymes among all published *Thermotoga* genomes (Table 1). Moreover, *T.* sp. strain RQ2 has recently been discovered to be naturally transformable, making the transformation procedure straightforward [13]. The selection of candidate cellulases was focused on *Caldicellulosiruptor* saccharolyticus, a Gram-positive anaerobe growing optimally at 70°C and can use cellulose as a sole carbon source [14]. *C. saccharolyticus* DSM 8903 have been experimentally characterized as multidomain proteins with both endo- and exoglucanase activities and are suitable candidates to be introduced into *T.* sp. strain RQ2. However, *Caldicellulosiruptor* are Gram-negatives and *Thermotoga* are Gram-negatives; CelA and CelB are unlikely to be secreted properly in *T.* sp. strain RQ2. The *Thermotoga* host would not benefit much from the heterologous cellulases unless the enzymes can be secreted to the extracellular environment. Signal peptides with a *Thermotoga* origin would be required to guide the transportation of foreign proteins in *T.* sp. strain RQ2. A literature search revealed that *T. maritima* TM1840 (amylase A, AmyA) [19–21] and TM0070 (xylanase B, XynB) [20] have been experimentally confirmed to be secretive proteins. The former is anchored on the “toga” part with catalytic domain facing outward, and the latter is secreted into the environment after the cleavage of its signal peptide. Therefore, the promoter regions and the signal peptide sequences of TM1840 (amyA) [19–21] and TM0070 (xynB) [20] were chosen to control the expression and transportation of the *Caldicellulosiruptor* cellulases in *T.* sp. strain RQ2.

### 2. Materials and Methods

#### 2.1. Strains and Cultivation Conditions

The bacterial strains and vectors used in this study are summarized in Table 2. All *E. coli* strains were cultivated in Luria-Bertani (LB) medium (1% tryptone, 1% NaCl, 0.5% yeast extract) at 37°C. *Thermotoga* strains were cultivated at 77°C, 125 rpm in SVO medium [22]. SVO plates were made with 0.25% (w/v) gelrite [23]. *Thermotoga* plates were put into Vacu-Quik Jars (Almore International Inc., Portland, OR, USA) filled with 96:4 N₂-H₂ and 4 g palladium catalyst (to remove oxygen) and incubated at 77°C for 48h. When needed, ampicillin was supplemented into LB medium to a final concentration of 100 μg mL⁻¹, and kanamycin was added into liquid SVO medium and SVO plates to a final concentration of 150 and 250 μg mL⁻¹, respectively.

#### 2.2. Construction of Vectors

All vectors were constructed by following standard cloning methods and verified by restrictive digestions. Primers used in this study are summarized in Table 3. The *Thermotoga*-*E. coli* shuttle vector pDH10 was used as the parent vector. Inverse PCR was performed with pDH10 [23] using primers DBs F and DBs R, and the amplicon was digested with BsiI followed by self-ligation to give rise to pDH26. With the same approach, pDH27 was generated based on pDH26 using primers DNd F and DNd R, and pHX01 was created from pDH27 using primers DLZ F and DLZ R. Compared to pDH10, pHX01 is 342 bp shorter and is free of the BsaI and NdeI recognition sites, which makes it a better cloning vector than pDH10. Based on pHX01, intermediate vectors pHX02.1, pHX04.1, and pHX07.1 were constructed. Vector pHX02.1 carries the promoter and signal peptide region of TM1840 (amyA), which was inserted immediately upstream of the Ap’ gene (Figure 1(a)); primers Amp P and Amp R were used to amplify the desired region from *T. maritima* chromosome, and the amplicon was digested with NotI and SacI. Vectors pHX04.1 and pHX07.1 both carry the promoter and signal peptide region of TM0070 (xynB), but one has the insert upstream of the Ap’ gene (Figure 1(b)) and the other has it downstream of the ori region (Figure 1(c)). Because the two insertions sites were recognized by different restriction enzymes, primers XyBPB F and XyBPB R were used to amplify the insert for pHX04.1, and the amplicon was digested with NotI and SacI; primers XyBPA F and XyBPA R were used to prepare the insert for pHX07.1, and the amplicon was digested by XhoI and PstI. As a result, a BsaI site was added to the vector.

#### Table 1: Number of predicted carbohydrate-active enzymes in *Thermotoga* genomes∗.

| Family categories | *T. sp. strain RQ2* | *T. maritima MSB8* | *T. neapolitana DSM 4339* | *T. petrophila RKU-1* | *T. naphthophila RKU-10* | *T. lettingae TMO* | *T. thermarum DSM 5069* |
|-------------------|--------------------|--------------------|--------------------------|----------------------|------------------------|---------------------|------------------------|
| Glycosidase       | 98                 | 94                 | 90                       | 88                   | 78                     | 66                  | 55                     |
| Hydrolase family  |                    |                    |                          |                      |                        |                     |                        |
| Glycosyltransferase family | 52         | 49                 | 49                       | 50                   | 40                     | 36                  | 32                     |
| Polysaccharide Lyase family | 20         | 22                 | 21                       | 17                   | 19                     | 20                  | 13                     |
| Carbohydrate Esterase family | 1            | 1                  | 0                        | 1                    | 0                      | 1                   | 0                      |
| Carbohydrate-binding module family | 5          | 5                  | 3                        | 5                    | 4                      | 5                   | 2                      |
| Total             | 98                 | 94                 | 90                       | 88                   | 78                     | 66                  | 55                     |

* Data collected from http://www.cazy.org/ on August 7, 2014.
### Strains and Vectors Used in This Study

| Strain or plasmid | Description | Reference |
|------------------|-------------|-----------|
| **E. coli**       |             |           |
| DH5α             | F− endA1 hsR17 (rk−, mk+) supE44 thy-1 λ− recA1 gyrA96 relA1 deoR Δ(lacZYA-argF)-U169Δ80lacZΔM15 | [24] |
| **Thermotoga**   |             |           |
| T. sp. strain RQ2| Isolated from geothermally heated sea sediment, Ribeira Quente, São Miguel, Azores. | [25] |
| T. maritima MSB8 | The first type strain of Thermotoga, isolated from geothermally heated sea sediments, Porto di Levante, Vulcano, Italy. | [25] |
| **Caldicellulosiruptor** |    |           |
| C. saccharolyticus DSM 8903 | Isolated from wood in the flow of geothermal spring, Taupo, New Zealand. | [14] |
| **Plasmids**     |             |           |
| pDH10            | Thermotoga-E. coli shuttle vector; Ap+, Kan− | [23] |
| pDH26            | pDH10-derived, with BsaI site erased; Ap+, Kan− | This study |
| pDH27            | pDH26-derived, with NdeI site erased; Ap+, Kan− | This study |
| pHX01            | pDH27-derived, having lacZ residual sequence removed; Ap+, Kan− | This study |
| pHX02.1          | Promoter and signal peptide region of TM1840 (amyA) were inserted into the NotI-SacI sites of pHX01; Ap+, Kan− | This study |
| pHX02            | Coding region (without the signal peptide) of Csac_1078 (celB) was inserted into BsaI-SacI sites of pHX02.1; Ap+, Kan− | This study |
| pHX04.1          | Promoter and signal peptide region of TM0070 (xynB) were inserted into the NotI-SacI sites of pHX01; Ap+, Kan− | This study |
| pHX04            | Coding region (without the signal peptide) of Csac_1078 (celB) was inserted into the BsaI-SacI sites of pHX04.1; Ap+, Kan− | This study |
| pHX07.1          | Promoter and signal peptide region of TM0070 (xynB) were inserted into the XhoI-PstI sites of pHX04.1; Ap+, Kan− | This study |
| pHX07            | Coding region (without the signal peptide) of Csac_1076 (celA) was inserted into the BsaI-PstI sites of pHX07.1; Ap+, Kan− | This study |

*Ap: ampicillin; Kan: kanamycin.

The transformation of E. coli was done with standard calcium chloride method, and the transformation of Thermotoga was done by natural transformation, as described previously [13]. The DNA substrates used to transform Thermotoga were in vitro methylated by methylase M. TnclDI [26, 27].

### 2.3. Detection of Endoglucanase Activity with CMC Plates.

Endoglucanase activities were evaluated using Congo red assays [28]. For preliminary screening, E. coli transformants were inoculated on CMC plates (1% NaCl, 0.5% yeast extract, 0.2% CMC, 1.5% agar) and incubated at 37°C for overnight.

The plates were then kept at 77°C for 8 h, stained with 0.1% Congo red (dissolved in water) at room temperature for 15 min, and washed with 1 M NaCl until clear halos showed up. After that, the plates were rinsed with 1 M HCl, which changed the background color into blue, providing a better contrast for the halos. To test liquid cultures, 40 μL of each normalized overnight culture was directly loaded onto CMC plates. To minimize the sizes of the loading spots, the liquid cultures were loaded through 8 times with 5 μL in each loading. The next round of loading only happened when the liquid from the previous loading had been completely absorbed. To localize the expression of the recombinant proteins, supernatants were collected from 1 mL of normalized overnight culture by centrifugation. Meanwhile, the cells were washed once with fresh medium and resuspended in 1 mL of the same medium.

### 2.4. Detection of Endoglucanase Activities with Zymogram.

Native polyacrylamide gel electrophoresis was modified from a previous report [29]. SDS (sodium dodecyl sulfate) was omitted from the gel (10%, w/v), but CMC was added to a final concentration of 0.08% (w/v). Protein samples were prepared in the absence of SDS, reducing agents, and the heat treatment. After electrophoresis, gels were rinsed with...
### Table 3: Nucleotidesequences of primers used in this study.

| Primer       | Sequence and restriction sites |
|--------------|--------------------------------|
| DBs F        | 5'-ATCATGGGTCTCGCGGTATCATGTGCACACTGGGGCCAGATGGTAAGC3' BsaI |
| DBs R        | 5'-ATCGTGCTCTCTACCCGCAAGAACCACGCTACCCGCTCCAGATTATCATGCG3' BsaI |
| DNd F        | 5'-ATCGTGCTCTCTACTATGCAATGTGACACCTTTGAGATTCGTGCTGGG3' BsaI |
| DNd R        | 5'-ATCGTGCTCTCTTATATGCAATGTGACACCTTTGAGATTCGTGCTGGG3' BsaI |
| DLZ F        | 5'-GTCTGACTAGTGCAAAGCAGATGGTCCTAGAGATTAGGGTGATGGTTCACGTAGTGG3' BsaI |
| DLZ R        | 5'-GACACGCATGCCGAGGCGCAGATGGGTAATTGTAATACGAC3' SphI |
| AmP F        | 5'-CGAGGAACGAACGCCGCGCCGCAACCTCCTCCTCGTCTGGACTTATGATG3' BsaI |
| AmP R        | 5'-GACTTAGCTGATGAGCAGGCTTCTGCCGCTGACTGGCTATCCTATGATGTG3' SacI |
| XyBPB F      | 5'-CGAGGAACGAACGCCGCGCCGCAACCTCCTCCTCGTCTGGACTTATGATG3' BsaI |
| XyBPB R      | 5'-GACTTAGCTGATGAGCAGGCTTCTGCCGCTGACTGGCTATCCTATGATGTG3' SacI |
| XyBPA F      | 5'-CGAGGAACGAACGCCGCGCCGCAACCTCCTCCTCGTCTGGACTTATGATG3' BsaI |
| XyBPA R      | 5'-CGAGGAACGAACGCCGCGCCGCAACCTCCTCCTCGTCTGGACTTATGATG3' SacI |
| CelBF        | 5'-GACGACGGTCTGCACTGGAGTATTCCAAGTTTATG3' BsaI |
| CelBR        | 5'-GACGACGGTCTGCACTGGAGTATTCCAAGTTTATG3' BsaI |
| CelAF        | 5'-GACGACGGTCTGCACTGGAGTATTCCAAGTTTATG3' BsaI |
| CelAR        | 5'-GACGACGGTCTGCACTGGAGTATTCCAAGTTTATG3' BsaI |
| CelBV F      | 5'-AGACGCGATGGGACATATCTATCCGGGTATG3' PstI |
| CelBV R      | 5'-AGACGCGATGGGACATATCTATCCGGGTATG3' PstI |

Deionized water for 3 times prior to immersion in 0.25 M Tris-HCl (pH 6.8) for 8 h at 77°C. Following the enzymatic reaction, gels were visualized with Congo red, as detailed above.

2.5. Detection of Exoglucanase Activity. MUC (4-methylum-belliferyl β-D-cellobioside) agar was used to detect exoglucanase activity [17, 30]. Under the hydrolysis of exoglucanase, MUC is converted to cellobiose and MU (4-methylumbelliferyl), which shows fluorescence under ultraviolet light. Forty microliters of normalized overnight culture of each Thermotoga transformant was spotted on MUC plates, incubated at 77°C for 8 h, and examined under UV light. The formation of fluorescent halos surrounding the loading spots indicates the activity of exoglucanase.

3. Results and Discussion

3.1. Expression and Localization of the Chimeric Enzymes in E. coli. In the endoglucanase activity screening experiment, all tested DH5α/pHX02 (Figure 2), DH5α/pHX04, and DH5α/pHX07 strains showed clear halos surrounding the overnight colonies, indicating functional expression of the recombinant cellulases in E. coli. To determine the localizations of the recombinant cellulases, cultures were normalized and supernatants and cell suspensions were tested separately. Because pHX07 and pHX04 share the same localization signal, experiments were carried out with just pHX04 and pHX02 transformants. On CMC plates, the pHX04 transformant demonstrated a higher endoglucanase activity than the transformants of pHX02 (Figure 3). For both constructs, most of the endoglucanase activity was associated with the cell suspensions (Figure 3). This is not surprising for pHX02, because its fusion protein is designed to be anchored on the outer membrane of a Gram-negative host. As for pHX04, since its fusion protein is meant to be released into the medium, these data suggest that the signal peptide of TM0070 (XynB) is not functional in E. coli, even though its promoter is.

3.2. Detection of Endoglucanase Activities in Thermotoga. As our main purpose is to express the recombinant cellulases in
Figure 1: Maps of the expression vectors pHX02 (a), pHX04 (b), and pHX07 (c). Gray region represents sequence of pRQ7. Unique restriction sites are shown.

Figure 2: Detection of endoglucanase activities in E. coli DH5α transformants (showing DH5α/pHX02 as an example). #1–#6, DH5α/pHX02 transformants; DH5α/pHX02.1, negative control.
Thermotoga, we next transformed these 3 expression vectors into T. sp. strain RQ2. Eleven T. sp. strain RQ2/pHX02, eleven RQ2/pHX04, and eight RQ2/pHX07 transformants were isolated and tested with Congo red assays. Wild type T. sp. strain RQ2 and C. saccharolyticus DSM 8903 were used as the negative and positive controls. Almost all transformants showed enhanced endoglucanase activities compared with the wild type strain (Figure 4), indicating the successful transformation and expression of the recombinant enzymes. Next we set out to validate the transformants with PCR and restriction digestions.

3.3. Validating Thermotoga Transformants. Three RQ2/pHX02 transformants (#2, #3, and #4) and three RQ2/pHX04 transformants (#3, #4, and #5) were picked up from SVO plates and grown overnight in liquid SVO medium with 150 μg kanamycin mL⁻¹. Plasmid extracts were prepared and used as the templates to amplify the exoglucanase domain of recombinant celB gene with primers celBV F and celBV R. One RQ2/pHX02 isolate (#4) and two RQ2/pHX04 isolates (#4 and #5) showed bands with the expected size (in addition to some nonspecific bands) (Figure 5). The positive bands from RQ2/pHX02 #4 and RQ2/pHX04 #5 were gel-purified, reamplified using the same primers, and digested with HaeIII (Figure 6). Both amplicons developed the expected digestion profile, demonstrating the authenticity of the two transformants, which were then selected to be further characterized in later studies. The attempts to amplify either exo- or endoglucanase domain from the pHX07 transformants failed. Since pHX07 carries the celA gene, instead of the celB as found in pHX02 and pHX04, further optimization of PCR conditions and/or primer selections may eventually allow one to validate the transformants of this vector. Nevertheless, pHX07 #2 was selected for further studies, because it at least displayed strong endoglucanase activity on CMC plates.

3.4. Detection of the Exoglucanase Activity in Thermotoga. The exoglucanase activity of the Thermotoga transformants was tested with MUC plates (Figure 7). Compared to the host strain, RQ2/pHX02 and RQ2/pHX04 demonstrated greatly enhanced exoglucanase activities, as bright fluorescent light emitted under UV light from the spots where their overnight cultures were loaded. This suggests that the exoglucanase domain of Caldicellulosiruptor CelB was successfully expressed and fully functional in T. sp. strain RQ2. However, the fluorescence level displayed by RQ2/pHX07 was at about the same level to the wild type strain. Because the recombinant enzymes carried by pHX04 and pHX07 share the same promoter and signal peptide, the low level of exoglucanase activity presented by RQ2/pHX07 indicates the exodomain of Caldicellulosiruptor CelA was either lost (which echoes the PCR results above) or not functional in T. sp. strain RQ2.

3.5. Localization of the Recombinant Cellulases in Thermotoga. Localization of the recombinant enzymes was carried with T. sp. strain RQ2/pHX02 #4, pHX04 #5, and pHX07 #2 by comparing the endoglucanase activity in supernatants versus cell suspensions. Unlike what happened in E. coli where the majority of the activity was associated with cell suspensions, the Thermotoga transformants had about half of the endoglucanase activity found in supernatants (Figure 8(a)). The endoglucanase activity in the supernatants was double-checked with native polyacrylamide gels followed by Congo red assay. All supernatants showed brighter bands than the wild type strain, indicating enhanced cellulases activities (Figure 8(b)). No protein bands were detectable in the supernatants by Coomassie brilliant blue staining. The cell suspensions retained the other half of the enzymatic activities, probably because of the cytoplasmic proproteins of the chimeric enzymes (Figure 8(a)).

3.6. Stabilities of the Recombinant Strains. Stabilities of the E. coli and T. sp. strain RQ2 recombinant strains were tested...
Figure 4: Screening of endoglucanase activities in *Thermotoga* transformants RQ2/pHX02 (a), RQ2/pHX04 (b), and RQ2/pHX07 (c). +, *C. saccharolyticus* DSM 8903, positive control; −, *T. sp.* strain RQ2, negative control.

Figure 5: Amplification of the exoglucanase domain in *T. sp.* strain RQ2 transformants.

Figure 6: Restriction digestion of PCR products of the exoglucanase domain of *T. sp.* strain RQ2 transformants.

by consecutively transferring corresponding cultures under the selection of antibiotics. After four transfers, all shuttle vectors were readily detected in the *E. coli* transformants (Figure 9(a)). The endo- and exodomains of *celA* and *celB* were also successfully amplified from the plasmid DNA extracts (data not shown). Congo red assays with DH5α/pHX02 showed that the enzyme was as active as before
and the expression level of the enzyme was not affected by the inclusion of 0.25% starch in the medium [21, 25] (Figure 9(b)). These results indicate that, in *E. coli* DH5α, the constructed vectors are stably maintained and the enzymes are constitutively expressed.

Unfortunately, in *T. sp.* strain RQ2, the vectors seemed to be gradually lost, as indicated by decreasing enzyme activities with each transfer. After the 3rd transfer, the activities of both endo- (Figure 10) and exoglucanase (data not shown) were at the same level as the negative control. Trying to induce the cultures with 0.25% starch or 0.25% xylose [21, 25, 31] did not result in improved expression of the enzymes, suggesting the diminishing of enzyme activities was a result of loss of genes rather than a lack of expression. Our previous study demonstrated that the *Thermotoga-E. coli* shuttle vector pDH10 is stably maintained in both *E. coli* and *Thermotoga* [23]. However, the vectors constructed in this study, which are derived from pDH10, were only stable in *E. coli*, but not in *Thermotoga*. This might be due to different genetics of the *Thermotoga* hosts. In the previous study, *T. sp.* strain RQ7 and *T. maritima* were used, and in this study, the host was *T. sp.* strain RQ2. Cryptic miniplasmids pRQ7 and pMC24 have been found in *T. sp.* strain RQ7 and *T. maritima* [32, 33], but no natural plasmids have ever been seen in *T. sp.* strain RQ2. Plasmids pRQ7 and pMC24 are only 846bp in length and encode just one apparent protein, which seems to play a role in plasmid replication but lacks the site-specific nuclease activity typical to a fully functional replication protein [34]. It is possible that the genomes of *T. sp.* strain RQ7 and *T. maritima* encode gene(s) essential to the replication of pRQ7-like plasmids, allowing the survival of pRQ7/pMC24-based vectors, whereas *T. sp.* strain RQ2 may lack such gene(s). As about half of the *Thermotoga* genomes encode uncharacterized proteins, finding such gene(s) requires thorough
4. Conclusions

This work demonstrated that it is possible to functionally express large heterologous proteins in *Thermotoga*. Transformed with the recombinant *Caldicellulosiruptor* cellulases, *T*. *sp*. strain RQ2 displayed increased endoglucanase activity with the expression of all three engineered enzymes, namely, TM1840 (AmyA)-Csac1078 (CelB), TM0070 (XynB)-Csac1078 (CelB), and TM0070 (XynB)-Csac1076 (CelA). Exoglucanase activity was also improved significantly in *T*. *sp*. strain RQ2 transformants expressing the chimeric enzymes TM1840 (AmyA)-Csac1078 (CelB) and TM0070 (XynB)-Csac1078 (CelB). However, the *Thermotoga* transformants lost their recombinant genes after three consecutive transfers. This study represents an important milestone in the effort of using *Thermotoga* to produce biohydrogen directly from cellulosic biomass. Future studies should be focused on improving the stability of the transformants.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

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