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Research Report

Identification of Cellulases from the Symbiotic Protists of Termites for Secretory Production in Yeast

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ABSTRACT For direct and efficient ethanol production from cellulosic materials, we screened cellulases from the symbiotic protists of termites through heterologous expression in the yeast *Saccharomyces cerevisiae*. To screen efficiently, we developed an original approach in which transgenic *S. cerevisiae* displaying *β*-glucosidase on the cell surface was used as a host strain, and used a screening medium containing cellulose as the sole carbon source. The 11 cellulases belonging to glycoside hydrolase family 5, 7, and 45 endoglucanases (EGs) have firstly been confirmed to produce in *S. cerevisiae*. Notably, recombinant yeast expressing SM2042B24 EG I (Genbank accession No. BAF57315) was more efficient at degrading carboxylmethyl cellulose than was *Trichoderma reesei* EG I, a major EG with high cellulolytic activity.

KEYWORDS Cellulase, Termite, Symbiotic Protists, *Saccharomyces cerevisiae*, Bioethanol

1. Introduction

As the primary polysaccharide of plant cell walls, lignocellulose has received considerable attention as a main feedstock for bio-refinery processes such as bio-fuel production. The enzymatic hydrolysis of lignocellulose to soluble sugars is considered to be the most important process in the production of environmentally friendly bio-ethanol. However, the spontaneous crystallization of cellulose due to the chemical uniformity of the glucosyl residues and the high degree of hydrogen bonding can result in densely packed microfibrils that are inaccessible to cellulolytic enzymes.[1] For the efficient utilization of cellulosic materials in biomass, there is a need to enhance the enzymatic saccharification of this biopolymer.

Termites play an important role in nutrient cycling within ecosystems by degrading lignocellulose.[2] Two separate degradation systems, endogenous cellulases and cellulases of symbiotic protist origin, are now known to coexist in phylogenetically lower termites.[3] This dual system seems to result in a high assimilation rate (in some cases, greater than 90%) of wood glucan;[4] therefore, it is expected that this symbiotic system contains effective genes for lignocellulose degradation.[5-7] However, because these symbiotic protists are anaerobic microorganisms, they are difficult to cultivate and use for cellulose degradation under aerobic conditions. To resolve this issue, heterologous expression of cellulase genes from the symbiotic protists of termites with other host cells has been used, and some success has been reported using *Escherichia coli* [8] and *Aspergillus oryzae.*[9,10] However, cellulases from these protists have not yet been expressed in yeast cells, which are widely used to produce ethanol. For consolidated bio-ethanol production using cellulosic materials,[11] if production of functional cellulases could be established in yeast, it would potentially be possible to produce ethanol directly from lignocellulose (**Fig. 1**).

To test the feasibility of this approach, we chose *Saccharomyces cerevisiae*, a typical alcohol producing microorganism, as the host yeast strain, and have attempted to identify functional cellulases through expression in the secretory pathway. In a previous study, we constructed a cDNA library from symbiotic protists of several kinds of lower termites to clarify the lignocellulose degradation process.[12] The contents of this cDNA library included abundant numbers of cellulase genes of the glycosyl hydrolase (GH) family orthologs (families 3, 5, 7, 8, 10, 11, 26, 43, 45, and 62). In this study, for direct and efficient ethanol production from cellulosic materials, we selected optimal cellulases for secretory production with

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S. cerevisiae using the previous cDNA library. In addition, to achieve efficient cellulase screening, we developed an original approach in which transgenic *S. cerevisiae* displaying β-glucosidase (BGL) on the cell surface was used as a host strain, and we used a screening medium containing cellulose as the sole carbon source.

2. Materials and Methods

2. 1 Microbial Strains, Media, and Growth Conditions

E. coli JM109 strain (Toyobo, Osaka, Japan) was used as a subcloning host strain for molecular cloning, and was routinely cultured in LB medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl, wt./vol., pH 7.0, Sigma-Aldrich, MO, USA) including 50 μg/ml ampicillin. The *S. cerevisiae* YPH499 strain (Stratagene, La Jolla, CA, USA) was used for secretory production of cellulase, and was routinely cultured using YPD medium (1% yeast extract, 2% peptone, and 2% D-glucose, wt./vol., Sigma-Aldrich).

2. 2 Cloning of Cellulase Genes from the cDNA Library

Cellulase genes from symbiotic protists of termites were isolated by Polymerase Chain Reaction (PCR) using a cDNA library that we constructed in our previous report[12] as a template. The site-specific oligonucleotide primer to amplify cellulase genes were designed using previous expressed sequence tag (EST) data for the symbiotic protists of lower termites. Each signal sequence of a cellulase gene was predicted by the PSORT program (http://psort.hgc.jp/), and the 5' end of the primer that deletes the signal sequence was designed to optimize gene expression in *S. cerevisiae*. In the PCR reaction, PrimeSTAR HS DNA polymerase (Takara Bio, Shiga, Japan) was used for DNA amplification. Each reaction was carried out with 25 cycles of 96°C for 30 sec., 53°C for 30 sec., 72°C for 60 sec. The amplified DNA fragments were cloned into the pESC-TRP-MO2 vector (see following 2.3) with two kinds of *Sfi* I sites.

2. 3 Construction of Plasmid Vector

A map of the expression vector 'pESC-TRP-MO2' is shown in **Fig. 2**. This vector, which was based on pESC-TRP (Stratagene), consists of a glyceraldehyde-3-phosphate dehydrogenase 3 (*TDH3*) promoter, an α-factor fragment (*MFα-1*) for use as a yeast secretory signal, and a cytochrome C (*CYC1*) terminator. Each fragment was isolated by PCR using genomic DNA of the *S. cerevisiae* YPH499 strain as a template, and then ligated to a pESC-TRP-MO2 vector. The ligase reaction was performed using a Liga-Fast Rapid DNA

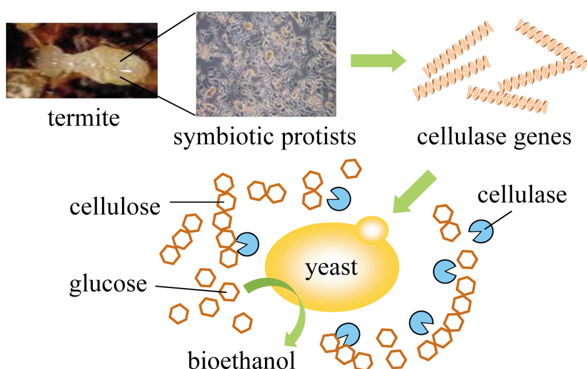

Fig. 1 Overview of this study. Screening of cellulase genes for cellulose degradation from symbiotic protists of termites that can potentiality degrade biomass with efficient expression in yeast.

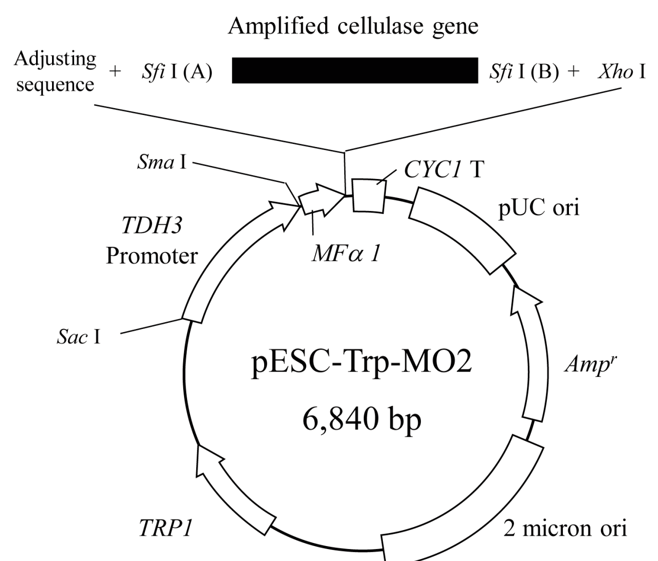

Fig. 2 A map of the plasmid vector used for cellulase screening. Two kinds of *Sfi* I restriction sites were added to the ends of each amplified fragment to ensure that it was inserted into the pESC-TRP-MO2 vector in the correct direction.

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Ligation System (Promega, Madison, WI, USA), with the competent *E. coli* JM109 strain used for transformation (Toyobo). Molecular cloning in this study was via standard protocol.[13]

2. 4 Yeast Transformation and Screening with Cellulose-containing Media

S. cerevisiae transformation was carried out with the Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA, USA). Transformants were cultured at 30°C for 4 days on SD-Trp selective plates (Clontech, Mountain View, CA, USA). For screening, recombinants were transferred to SD-Trp+CMC plates (without glucose) containing 10 g/l carboxymethyl cellulose (CMC, Sigma-Aldrich) or SD-Trp+PSC plates (without glucose) containing 10 g/l phosphoric acid swollen cellulose (PSC) using a Colony Picker PM-1 (Microtec, Chiba, Japan). After 5 days of cultivation at 30°C, each plate was washed with distilled water and stained using Congo-red solution (1 g/l Congo-red, 1 M Tris-HCl, pH 9.0, Wako Chemicals, Tokyo, Japan) to detect CMC degradation activity.

2. 5 Identified Cellulase Genes from Candidate Clones

Selected recombinant *S. cerevisiae* were cultured in 4 ml SD-Trp medium (30°C, 120 rpm, 18 h) and the plasmid DNA was extracted using a Zymoprep II Yeast Plasmid Minipreparation Kit (Zymo Research). To confirm cellulase genes in the plasmid vectors, the nucleotide sequences were determined with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2. 6 Carboxymethyl Cellulose Degradation Assay

Each recombinant was cultured at 30ºC for 48 h in YPD medium (Sigma-Aldrich). 20 μl of this supernatant was added to 1 ml of 1.0% CMC in a 0.1 M sodium acetate buffer (pH 5.5) and incubated at 37°C for 20 h. Then 40 μl of each reaction solution was incubated with 200 μl tetrazolium blue reagent (50 mM NaOH, 500 mM potassium sodium tartrate, 0.1% tetrazolium blue, Wako Chemicals) at 100°C for 10 min before absorbance was measured at 660 nm using a UV spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

2. 7 Phylogenetic Analysis

Sequence alignment was used to reconstruct a phylogenetic tree by the maximum likelihood (ML) distance method with PAUP[14] based on a GTR+G+I model. The rate-across-site variation was modeled on a discrete distribution with four rate categories. The credibility of each cluster in the tree was estimated on the basis of the bootstrap probability of the ML distance, Quartet puzzling, and Bayesian methods. Bootstrap resampling for each method was performed 100 times each.

3. Results and Discussion

3. 1 Cloning of Cellulase Genes from the cDNA Library of Symbiotic Protists

We had amplified 140 cellulase genes using the cDNA library as a template, and these were introduced into pESC-TRP-MO2 vectors with *Sfi* I. This cDNA library, i.e. multiple GH family enzymes by annotation of ESTs in a previous study,[12] contained the symbiotic protists of *Reticulitermes speratus* (Rhinotermitidae), *Hodotermopsis sjostedti* (Termopsidae), *Neotermes koshunensis* (Kalotermitidae), *Mastotermes darwiniensis* (Mastotermitidae), and *Cryptocercus punctulatus* (wood-feeding cockroach). To insert pESC-TRP-MO2 in the correct direction, two kinds of *Sfi* I restriction sequence were designed into the ends of each cellulase fragment: A) 5'-GGCC<u>TTGTT</u>GGCC-3' and B) 5'-GGCC<u>TATGT</u>GGCC-3' (Fig. 2). These construct mixtures were transformed into the transgenic *S. cerevisiae* that displayed *BGL* from *Aspergillus aculeatus* (Genbank accession No. D64088)[15] on the cell surface. Recombinant yeast expressing *BGL* leads to oligosaccharide resolution into glucose.[16] Even when cultured on a carboxymethyl cellulose medium without any glucose as a carbon source, the yeast was able to proliferate.

The filamentous fungus *Trichoderma reesei* secretes enzymes of high cellulolytic activity required for digestion of crystalline cellulose. The endoglucanase I (EG I) of *T. reesei* especially is a major endoglucanase enzyme with high glycosylasion activity, and it has already been used for heterologous expression with *S. cerevisiae*.[17] Thus, *T. reesei* EG I (Genbank accession No. M15665) was also ligated into pESC-TRP-MO2 and transformed into the host strain as a positive control in this study.

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3. 2 Screening of Cellulases Through Secretory Production of Yeast

Of the approximately 1,400 transformants that were screened by selective plating that included 1% CMC, 56 candidates exhibited clear halos (**Fig. 3**a).[18] By plasmid DNA sequence analysis of the 56 candidate strains, 11 cellulase genes belonging to glycoside hydrolase (GH) families 5, 7, and 45 EG from the symbiotic protists were eventually selected for secretory expression with *S. cerevisiae*. Each gene with its Genbank accession No. is listed in **Table 1**.[18] Via halo formation of candidate strains, the recombinant expressing SM2042B24 from *R. speratus* exhibited the greatest CMC degradation activity. In addition, its halo formation was clearer than that observed in the control strain expressing *T. reesei* EG I (Fig. 3b).[18]

Interestingly, cellobiohydrolase (CBH) secretion was not observed in any of the 140 cellulase genes that were screened. We consider this disparity in CBH secretion to have arisen because the cellulases that degraded non-crystalline cellulose had primarily been selected using a CMC medium. To isolate optimal CBH from the symbiotic protists of termites, we screened transformants using SD-Trp+PSC plates containing 1.0% PSC instead of glucose, but a target clone was not obtained. It has already been reported that the cellulase genes from symbiotic protists are GC (guanine - cytosine) rich.[9,12] Therefore, we considered that the difference of the codon usage between symbiotic protists and the yeast had a significant effect on the translation of mRNA and the synthesis of protein.

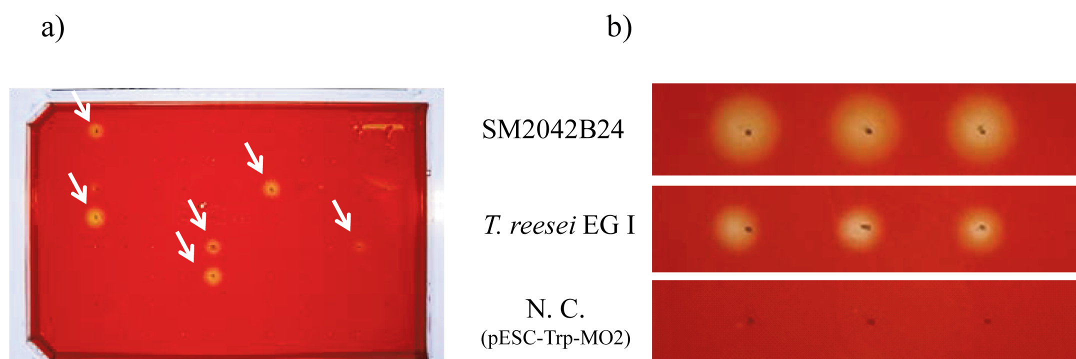

Fig. 3 a) Screening for the cellulase gene by observing CMC degradation activity on a plate without glucose. Arrows indicate halo formation. b) Comparison of degradation activity between SM2042B24 EG I and *T. reesei* EG I (n=3). The negative control (N.C.) was a transformed host strain containing the pESC-Trp-MO2 vector without a cellulase gene.[18]

Table 1 List of identified cellulase genes from symbiotic protists of termites expressed in transgenic *S. cerevisiae*.[18]

Gene name	GH family	Termite	Genbank No.	Positive clones
SM2039A92	GH 7 EG	*Reticulitermes speratus*	BAF57303	3
SM2040A65	GH 7 EG	*Reticulitermes speratus*	BAF57305	6
SM2042B24	GH 7 EG	*Reticulitermes speratus*	BAF57315	14
SM2038B84	GH 45 EG	*Reticulitermes speratus*	BAF57324	2
SM2042A70	GH 45 EG	*Reticulitermes speratus*	BAF57325	8
SM2632A27	GH 7 EG	*Neotermes koshunensis*	BAF57375	8
SM2637A29	GH 7 EG	*Neotermes koshunensis*	BAF57395	5
SM2760A28	GH 45 EG	*Mastotermes darwiniensis*	BAF57443	6
SM2765A61	GH 45 EG	*Mastotermes darwiniensis*	BAF57450	1
NT0285A72	GH 5 EG	*Hodotermopsis sjostedti*	BAF57328	2
NT0285A89	GH 45 EG	*Hodotermopsis sjostedti*	BAF57356	1

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3. 3 Analysis of Cellulose Degradation Activity

To compare the activity of CMC degradation, a tetrazolium blue (TZ) assay [19] based on the resolution ability of reducing sugar was conducted using the culture supernatants of the 11 transgenic strains. The CMC degradation activities of SM2042B24 EG I per culture supernatant of recombinant strain was observed to be 2.6 times higher compared with the *T. reesei* EG I (**Fig. 4**).[18] But the activities of the other EGs expressed in *S. cerevisiae* were lower than the positive control. Our previous study of GH family 7 EG expression by transgenic *Aspergillus oryzae* also showed that purified RsSym EG I (SM2038B11) protein had a higher specific activity and V_{max} value than *T. reesei* EG I.[10] Although RsSym EG I differs from the SM2042B24 EG I, this examination using transgenic yeast provides further evidence of the efficacy of cellulases from the symbiotic protists of termites.

We conducted a phylogenetic analysis with 123 GH family 7 cellulases based on the EST databases of filamentous fungus and symbiotic protests. The GH family 7 cellulases can be divided into CBHs and EGs based on the presence or absence of insertion sequences. As shown in **Fig. 5**, GH family 7 cellulases

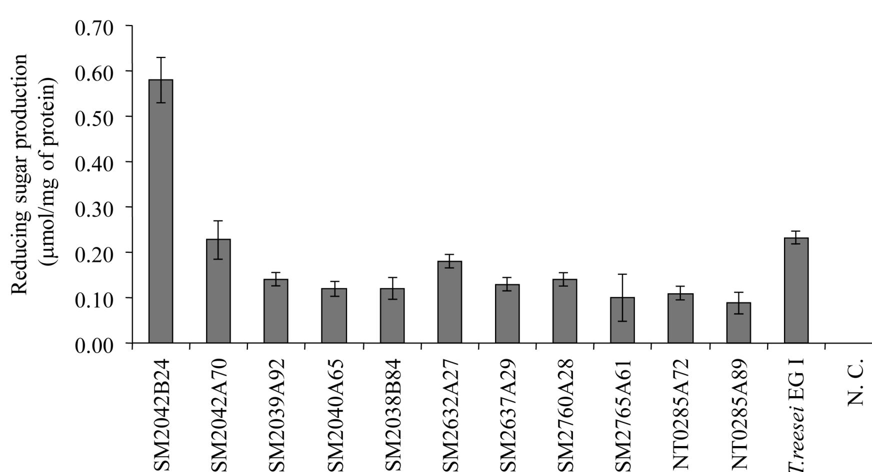

Fig. 4 CMC degradation activities in a TZ assay with 1% CMC solution. Values reflect the average and standard deviation (error bars) obtained for five independent experiments.[18]

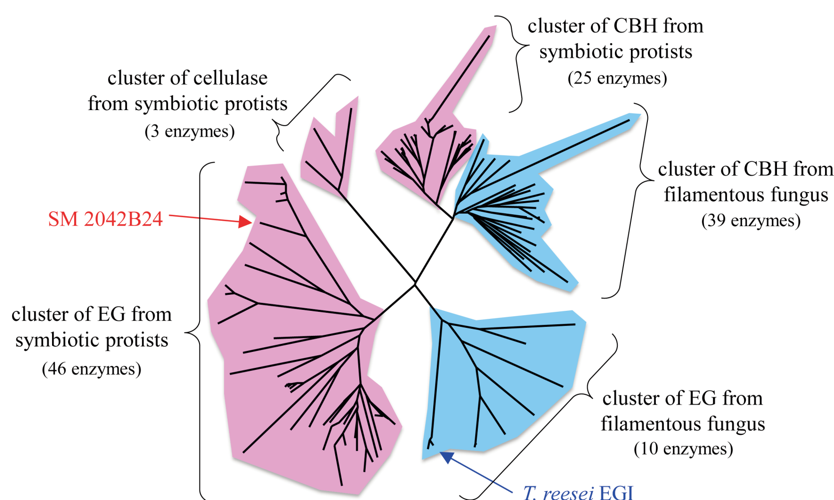

Fig. 5 Phylogenetic tree of 123 selected GH family 7 cellulases based on EST databases of symbiotic protists (red cluster) and filamentous fungus (blue cluster). The credibility (ML-distance/ Quartet puzzling /Bayesian) of each cluster is shown.

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of symbiotic protists clustered differently according to amino acid sequence than the general GH family 7 from filamentous fungus.[20] Additionally, cellulases from the symbiotic protists of termites do not have a carbohydrate-binding module family 1 (CBM1) domain,[12] and the amino acid identity of SM2042B24 EG I was low compared to *T. reesei* EG I (32.5% amino acid identity). The findings from our study suggest that EG I from symbiotic protists possibility act with a quite different mechanism than existing general cellulases. These considerations invite further empirical investigation into the protein structure.

Within the context of bioprocessing, developing an efficient screening method that can be applied to the secretory production of optimal cellulases by *S. cerevisiae* is highly beneficial. Only a single report has been published to date describing a similar screening process in transgenic *E. coli*[8]; however, the authors of that study employed a general medium containing glucose. To increase the efficiency of screening for useful genes, we examined recombinant *S. cerevisiae* expressing BGL as a host strain. This approach enabled us to conveniently select a functional cellulase gene, even in a medium containing cellulose as the only carbon source. Additionally, this screening method can also be applied to the selection of effective cellulase genes from other metagenomes, such as the marine and soil environments.

In this study, we confirmed that SM2042B24 from *R. speratus* is an optimal EG I for secretory expression with *S. cerevisiae*, and was confirmed to be more efficient at degrading carboxylmethyl cellulose than *T. reesei* EG I. To our knowledge, SM2042B24 EG I is the first functional cellulase of symbiotic protist origin confirmed to be expressed in *S. cerevisiae*. This finding shows that cellulases derived from symbiotic protists of lower termites can potentially be applied to biomass degradation,[2] and that further progress in the area of high performance cellulose saccharification can be expected using these characteristic ecosystems. Additionally, further methods and detailed analyses of these cellulases will contribute to the development of efficient degradation systems of plant biomass for industrial applications.

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Figs. 3, 4 and Table 1
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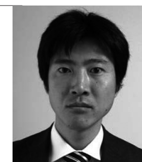

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