Cloning, Expression, and Characterization of Endoglucanase Gene egIV from *Trichoderma viride* AS 3.3711

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Endoglucanase gene egIV was cloned from *Trichoderma viride* AS 3.3711, an important cellulose-producing fungus, by using an RT-PCR protocol. The egIV cDNA is 1,297 bp in length and contains a 1,035 bp open reading frame encoding a 344 amino acid protein with an estimated molecular mass of 35.5 kDa and isoelectric point (pI) of 5.29. The expression of gene egIV in *T. viride* AS 3.3711 could be induced by sucrose, corn straw, carboxymethylcellulose (CMC), or microcrystalline cellulose, but especially by CMC. The transcripts of egIV were regulated under these substrates, but the expression level of the egIV gene could be inhibited by glucose and fructose. Three recombinant vectors, pYES2-xegIV, pYES2Mα-egIV, and pYES2Mα-xegIV, were constructed to express the egIV gene in *Saccharomyces cerevisiae* H158. The CMCase activity of yeast transformants IpYES2Mα-xegIV was higher than that of transformant IpYES2-xegIV or IpYES2Mα-egIV, with the highest activity of 0.18 U/ml. These properties would provide technical parameters for utilizing cellulase in industrial bioethanol production.

**Keywords:** *Trichoderma viride*, endoglucanase EGIV, gene cloning, yeast expression

The utilization of bioethanol for transportation has the potential of contributing to a cleaner environment. It is expected that the bioethanol industry will benefit from the efficient utilization of lignocellulosic wastes [28]. The conversion of cellulose to glucose is the rate-limiting step in the conversion of lignocellulose into biofuel. Cellulases catalyze the hydrolysis of cellulose, which are mainly of three types: endoglucanases (E.C. 3.2.1.4), cellobiohydrolases (E.C. 3.2.1.91), and β-glucosidases (E.C. 3.2.1.21). Many microorganisms, both bacteria and fungi, are capable of efficient cellulase breakdown by secreting cellulase-degrading enzymes [13, 35]. The filamentous fungus *Trichoderma viride* has been especially famous for producing cellulolytic enzymes with relatively high enzymatic activity [19]. Seven cellulase components [i.e., endoglucanases Cel7B (EG I), Cel12A (EG III), and Cel61A (EG IV); cellobiohydrolases Cel7A (CBH I), Cel6A (CBH II), and Cel6B (CBH IIb); and β-glucosidase from *T. viride*] have been separated [38]. Of these genes, egI, egIII, egVIII, cbhl, and cbhII have been researched in different hosts [10, 17, 18, 30]. However, the heterologous expression of egIV has not been reported.

The yeast *Saccharomyces cerevisiae*, which is a traditional good ethanol producer, is widely used as a heterogeneous host to express filamentous fungi cellulase genes for bioethanol production [25, 37]. Endoglucanases as well as a variety of additional saccharolytic enzymes have been successfully expressed in *S. cerevisiae* [21, 24]. However, the expression level and activity of recombinant enzyme are usually lower than that in the native strains [23, 31]. One reason for this phenomenon is that the signal peptide of an exogenous gene could not be recognized by the host yeast efficiently. Therefore, it is a good way that the signal peptide of an exogenous gene could be replaced by that of the host yeast. The α-mating factor (MFα) signal peptide was used widely to enhance secretion of recombinant protein in yeast [9].
To increase production of the enzyme β-1,4-endoglucanase, the egIV gene from *T. viride* AS3.3711 was isolated and heterologously expressed in yeast *S. cerevisiae*. To increase secretion of the enzyme, three expression vectors were constructed using MFα signal peptide from *S. cerevisiae*. Furthermore, the biological characteristics of recombinant EGIV enzyme are shown in this paper.

**Materials and Methods**

**Strains, Cultivation Conditions, and Plasmids**

*T. viride* AS 3.3711 was routinely maintained on potato dextrose agar (PDA; Difco, Detroit, MI, USA) slant at 4°C and subcultured periodically. The culture was reactivated by transferring onto fresh PDA plates and cultured at 28°C for 5–7 days. Mycelial and spores of *T. viride* AS 3.3711 were scraped from the PDA plate, suspended in sterile distilled water, filtered with swab, and collected with a Pasteur’s capillary. The spores were centrifuged at 7,000 × g for 7 min, the supernatant was discharged, and the spores were suspended again in distilled water. The conidia concentration was adjusted to 1×10⁸ conidia per milliliter, and inoculated into 50 ml of potato dextrose broth (Difco, Detroit, MI, USA) in a 250 ml Erlenmeyer flask with 1% (v/v) conidial suspension. The cultures were incubated at 28°C in a rotary shaker at 200 rpm for 48 h as seed liquid.

*S. cerevisiae* H158 was used as the host and cultured in YPD broth medium (0.67% YNB, 0.01% each of adenine, arginine, lysine, histidine, isoleucine, methionine, phenylalanine, proline, serine, threonine, and tryptophan, and 0.005% each of aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, valine, and tyrosine) [1]. The pYES2 vector (Invitrogen, USA) was used for the expression of gene egIV in *S. cerevisiae* H158.

**Cloning of Endoglucanase Gene egIV from T. viride AS 3.3711**

Mycelia of *T. viride* AS 3.3711 were collected by filtration, thoroughly washed with sterile water, lyophilized, and stored at −80°C. Total RNA was isolated using an RNA mini kit (Invitrogen, USA) and treated with DNaseI using a DNA-free kit (Ambion, USA) to remove DNA contamination. According to the manufacturer’s recommendations, the first-strand cDNA was synthesized from total RNA with a reverse transcription kit (TaKaRa, Japan) for egIV gene cloning.

**Expression Response of Gene egIV to Different Induced Substrates**

To study the egIV gene expression in *T. viride* AS 3.3711, Mandels’ medium (MM) supplied with different substrates was used. The main component of MM included (NH₄)₂SO₄ 1.4 g/l, arbamide 0.3 g/l, KH₂PO₄ 2.0 g/l, CaCl₂ 0.3 g/l, MgSO₄·7H₂O 0.3 g/l, peptone 1.0 g/l, Tween 80 1.0 g/l, FeSO₄·7H₂O 5.0 mg/l, ZnSO₄·7H₂O 1.4 mg/l, MnSO₄·H₂O 1.6 mg/l, and CoCl₂ 2.0 mg/l, pH 5–6, added with 2% substrate. The substrates were 2% each of contained glucose, fructose, sucrose, carboxymethylcellulose (CMC), microcrystalline cellulose (MCC), and corn straw, respectively. Corn straw was dried at 45°C for 48 h in a stove and then milled. One hundred ml of medium with 10⁶ conidia per ml was inoculated into 500 ml Erlenmeyer flasks, and cultivated at 200 rpm and 28°C for 48 h as

**Bioinformatics Analysis of Endoglucanase Gene egIV**

Homology of endoglucanase gene egIV was identified using BlastX against the NCBI non-redundant database. The open reading frame (ORF) was identified using the ORF program (http://www.ncbi.nlm.nih.gov/orf/gorf/gorf.htm). The signal peptide was predicted using the SignalP3.0 server (http://www.cbs.dtu.dk/services/SignalP/), and the theoretical molecular mass and isoelectric point of the protein were calculated using the Anthe5.0 program. The conserved domain of the EGIV protein was identified by InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). The N-glycosylation and O-glycosylation sites were predicted by the NetNGlyc v1.0 and OGPET v1.0 programs (http://us.expasy.org/tools/, respectively. EGIV amino acid sequences from different fungi (including *T. viride* AS 3.3711) were aligned by the ClustalX program [32].

**Table 1. PCR primers used for gene isolation and plasmid construction.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence (5’–3’) (restriction sites are underlined)</th>
<th>GenBank Acc. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGIVF</td>
<td>TGCGAATTCATGATCCAGAAGCTTTCCAA (EcoRI)</td>
<td>Y11113</td>
</tr>
<tr>
<td>EGIVR</td>
<td>CGCTTTAGACTATCTAAAGCGGAATGGAAGGA (XbaI)</td>
<td>Y11113</td>
</tr>
<tr>
<td>NEGIVF</td>
<td>TGCTTTCCACCCGACGCTACC</td>
<td>EU518929</td>
</tr>
<tr>
<td>NEGIVR</td>
<td>CCTGAGACCGCAATGTTGAAGC</td>
<td>EU518929</td>
</tr>
<tr>
<td>18SF</td>
<td>GCGAAATGCGGATAATGATAAGT</td>
<td>DQ315456</td>
</tr>
<tr>
<td>18SR</td>
<td>AGAAGTTGGGTTGTATGCGG</td>
<td>DQ315456</td>
</tr>
<tr>
<td>MaF</td>
<td>CGGGATCCATGAGATTCTCCCTTC (BamHI)</td>
<td>J01340</td>
</tr>
<tr>
<td>MaR</td>
<td>CGGGAATTCAGTCTACGCTCTCTC (EcoRI)</td>
<td>J01340</td>
</tr>
<tr>
<td>xeGIVF</td>
<td>TGCGAATTTCCATGGACATATTAAATGACATTGT (EcoRI)</td>
<td>EU518929</td>
</tr>
</tbody>
</table>
Construction of the Yeast Expression Vector
Initially, the coding region of the egIV gene was amplified from T. viride AS 3.3711 with primers EGIVF and EGIVR. The egIV gene fragment was digested with EcoRI and Xhol, and inserted into vector pYES2, yielding pYES2-egIV.

In addition, for enhancing the secreted level of gene egIV in S. cerevisiae, a new vector (pYES2-α) was constructed based on pYES2. According to the sequence information of vector pPIC9 (Invitrogen, USA), primers M6aF and Mrα were designed to amplify the M6α sequence. The conditions of total RNA isolation and PCR cycling were the same as that of egIV gene cloning. Firstly, the M6α sequence and pYES2 plasmid were digested with BamHI and EcoRI, then ligated with T4 DNA ligase, and the resulting plasmid pYES2-α was transformed into E. coli DH5α competent cells. E. coli transformation and plasmid extract experiments were performed as described by Sambrook et al. [27]. Based on vector pYES2-α, plasmids pYES2Mo-egIV and pYES2Mo-egIV were constructed. Gene egIV (without signal peptide sequence) was amplified by PCR using the primers xEGIVF and EGIIVR. The amplified fragment was digested with EcoRI and Xhol, and then ligated into pYES2-α, yielding pYES2Mo-egIV. Similarly, the egIV gene fragment was digested with EcoRI and Xhol, and inserted into vector pYES2-α, yielding pYES2Mo-egIV. Recombinant plasmids (pYES2-egIV, pYE2Mo-egIV, and pYE2Mo-egIV) and control plasmid pYES2 were transformed into S. cerevisiae H158 by using the lithium acetate method, as described by Krautwurst et al. [16]. Yeast transformants were designated IpYES2-egIV, IpYES2Mo-egIV, IpYES2Mo-xegIV, IpYES2Mo-egIV, and IpYES2, respectively, and stored in SC-U glycerol medium at −80°C.

Northern Blot Analysis of egIV Gene Expression in Yeast
To study expression of gene egIV in transformants, the yeast transformants were cultured in SC-U medium containing 2% galactose and were collected at inducing for 12, 24, 36, 48, and 60 h, respectively. Total RNA (15 µg) was fractionated on a formaldehyde agarose gel (1%) and blotted onto a Nylon membrane. The Digoxigenin High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) was used for probe preparation and detection of the transcripts of gene egIV. The relative expression levels of gene egIV in S. cerevisiae H158 were compared based on the hybridization signal on Northern blot membrane.

Assay of Cellulase Activity in Recombinant Yeast
The CMCase activity was measured according to the method described by Jabbar et al. [12]. The transformants of S. cerevisiae H158 were grown on SC-U medium. The expression of egIV in S. cerevisiae was induced by 2% β-1,3-galactose and repressed by glucose (SC-U-G) as control. Yeast cells were collected and centrifuged at inducing for 12, 24, 36, 48, 60, 72, 84, 96, and 108 h, and then supernatants (enzyme solutions) were used for measuring CMCase activity. The supernatant was boiled at 100°C for 20 min and used as a control.

Properties of Recombinant EGIV from IpYES2Mo-xegIV
The yeast transformant IpYES2Mo-xegIV was induced by 2% β-1,3-galactose at 30°C for 6 days. Yeast cells were centrifuged, and supernatants were used in the following experiments. The supernatant was boiled at 100°C for 20 min and used as a control. To determine the optimum temperature for enzyme activity, the reaction mixtures (pH=4.5) were incubated at 30°C to 90°C for 30 min at 5°C intervals. To determine the optimum pH for enzyme activity, the pH value of the reaction mixture was adjusted between 3 and 9 at an interval of 0.5 pH unit. The pH value of the reaction mixture was adjusted using a series of buffers: Citric acid–sodium citrate buffer (pH 3.0 to 6.6), NaHPO4–NaH2PO4 buffer (pH 5.8 to 8.0), and Tris buffer (pH 8.14 to 9.10).

To measure the thermal stability of recombinant EGIV enzyme, supernatants (enzyme solutions) were incubated at 30°C to 90°C for 1 h at 5°C intervals (pH=4.5) and then chilled on ice; the remaining activity was then measured. To determine the pH tolerance of recombinant EGIV enzyme, the enzyme solution was adjusted to 3–9 and kept at 4°C for 48 h, then the solution was adjusted to the optimum pH for enzyme activity and measured.

To investigate the effect of metal ions, enzymatic activity was measured in the reaction buffer that was supplemented with 0.75 mM of a metal ion in the form of a chloride or sulfate salt. Several different buffer solutions were prepared, with different titers of metal salt, namely, MgSO4, CuSO4, FeSO4, ZnSO4, MnSO4, Al(SO4)3, KCl, NaCl, CaCl2, BaCl2, CoCl2, FeCl3, and AgNO3.

To analyze the decomposition ability of the recombinant EGIV enzyme to different substrates, bran, corn stalk, rice straw, cotton straw, filter paper, CMC and MCC were used in this study. Samples of the reaction solution were collected then used to measure EGIV enzyme activity.

All the experiments were completed in triplicate, and average values were calculated based on results from each independent experiment.

RESULTS

Sequence Analysis of Gene egIV
The length of the egIV gene coding region was 1,297 bp, including the 3 untranslated region of 262 bp and ORF of 1,035 bp. The mature EGIV protein could encode 344 amino acids with a theoretical isoelectric point of 5.29 and molecular mass of 35.5 kDa. The cDNA sequence of egIV was deposited into the GenBank database with the accession number of EU518929.

SignalP prediction showed that there was a signal peptide (21 amino acids) in the N-terminal sequence of EGIV amino acid sequence (ACD36973). The signal peptides cleavage site of the EGIV amino acid sequence was between positions G21 and H22, indicating that the predicted EGIV protein is an extracellular protein. Glycosylation site prediction showed that only one potential N-glycosylation
site was found at position Asn158, and two possible O-glycosylation sites were predicted at positions Thr41 and Thr279. InterProScan analysis revealed that the EGIV protein had a catalytic domain of glycoside hydrolase family 61 (InterPro Acc. No. IPR005103), indicating that it was an endoglucanase. The EGIV protein from *T. viride* AS 3.3711 was structurally similar to fungal endoglucanases of glycoside hydrolase family 61 and it also has a modular structure, containing a cellulose-binding domain (CBD) of 37 amino acids at its C-terminal, a linker peptide of 51 amino acids and a catalytic domain (CD) of 235 amino acids [22].

Multiple sequence alignment of the EGIV protein from different fungal species was carried out using ClustalX. The EGIV amino acid sequence identities between ACD36973 from *T. viride* AS 3.3711 and other fungal species ranged

![Fig. 1. Aligned amino acid sequences of endoglucanase EGIV from 7 different fungal species.](image)

CHARACTERIZATION OF RECOMBINANT ENDOGLUCANASE GENE egIV FROM TRICHODERMA VIRIDE 394

from 43% to 100%, demonstrating that the amino acid sequences of the EGI V protein from different fungi were not highly conserved. There were two conserved domains containing “QCGG” in CBD and “GNYLRHE” in CD, respectively (Fig. 1).

Expression Analysis of egIV Gene Response to Different Substrates

The expression patterns of gene egIV in T. viride AS 3.3711 were analyzed under different substrates. No transcript signal was detected when glucose (2%) or fructose (2%) was used as the substrate (Fig. 2a; Fig. 2f). When induced by sucrose, corn straw, carboxymethylcellulose (CMC), or microcrystalline cellulose (MCC), the transcript of the egIV gene reached the highest level at 48 h. The transcript level of gene egIV in CMC was higher than that in the other substrates all the time, indicating that CMC was the optimal induced substrate.

Transcript of Gene egIV in Yeast Recombinant

To demonstrate the transcript level of gene egIV in recombinant yeast, Northern blot analyses were performed (Fig. 3). When using gene egIV as a probe, no hybridization signal was observed from transformant IpYES2 RNA. The transcript properties were similar between IpYES2-egIV and IpYEMα-xegIV. The mRNA level of egIV detected for 12 h after inoculation remained low, increased rapidly between 24 and 36 h, and then decreased when the time was longer than 36 h. The highest expression of gene egIV was observed from transformant IpYES2Mα-xegIV at 36 h.

Assay of CMCase Activity of Yeast Transformants

The CMCase activities of the transgenic yeast IpYES2-egIV, IpYES2Mα-egIV, and IpYES2Mα-xegIV showed a
peak value at 48 h under galactose induction (Fig. 4). The recombinant EGIV activity of the transgenic yeast IpYES2Mα-xegIV (0.13 U/ml) was higher than that of IpYES2-egIV and IpYES2Mα-egIV, indicating that the MFα signal peptide played an important role in the secretion of the recombinant EGIV. Additionally, CMCase activity was not detected in the fermentation liquid of yeast transformant IpYES2 after galactose induction, indicating that the enzyme activity of recombinant EGIV displayed in transgenic yeast cells was due to expression of the exogenous egIV gene.

Recombinant EGIV Properties from Yeast Transformant IpYES2Mα-xegIV

To determine properties of recombinant EGIV from transgenic yeast IpYEMα-xegIV, yeast cells were centrifuged, and supernatants were used for measuring the CMCase activity. CMCase activity increased slowly from 30 to 35°C (0.0736 to 0.0767 U/ml), and reached its peak at 55°C (0.1398 U/ml), and then decreased when the temperature was higher than 55°C, and enzyme activity was lost when the temperature reached 90°C (Fig. 5A). Therefore, the optimal reaction temperature for CMCase in the yeast transformant was 55°C. The enzyme activity had a peak of activity at pH 5 with the activity value of 0.1454 U/ml. Enzyme activity was reduced rapidly when the pH exceeded 6.0 and the enzyme activity was very low (0.025 U/ml) when the pH reached 9.0 (Fig. 5B). Enzyme activity of the yeast transformant was stable when incubated between 35°C and 65°C for 1 h and decreased rapidly when temperature exceeded 65°C. The CMCase was inactivated when incubated at 85°C for 1 h (Fig. 5C). Enzyme activity of recombinant EGIV was stable when the pH was at 3.5–7.5 and decreased rapidly when the pH exceeded 7.5 (Fig. 5D).

The effect of metal ions on the enzyme activity was examined by measuring the activity in the presence of 0.75 mM of each metal ion. The analysis of variance showed that CMCase activity of EGIV was significantly different (F=132.38, df=13, P<0.01) in the presence of different metal ions. The activity of recombinant enzyme was stimulated by Ba^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Na^{+}, and Mg^{2+}, but was inhibited by Ag^{+}, Al^{3+}, Fe^{3+}, and Ca^{2+}. The effect of K^{+}, Co^{2+}, and Zn^{2+} on the recombinant enzyme was not obvious. In particular, the enzyme activity of recombinant EGIV reached 0.1818 U/ml when activated by Ba^{2+} (Fig. 6).

The activity of recombinant EGIV from transformant IpYES2Mα-xegIV toward different substrates was measured. The analysis of variance showed that the activity of CMCase toward different substrates was significantly different (F=59.70 df=6, P<0.01), with activities ranging from 0.126 to 0.047 U/ml. The recombinant EGIV showed the highest activity toward CMC substrate (Fig. 7).

The β-1,4-endoglucanase EGIVs have been detected and isolated from many different fungal strains, but there is no report about egIV gene cloning from T. viride. This is the first report about the cloning and expression of gene egIV from T. viride AS 3.3711. The EGIV protein from T. viride AS 3.3711, consisting of 344 amino acids, contained a CBD at the C-terminus and a linker in the middle region. The putative catalytic region showed homology with endoglucanase belonging to the glycoside hydrolase family 61 [15]. The EGIV amino acid sequence similarity between T. viride

**Fig. 3.** Northern blot analysis of egIV gene expression of yeast transformants. Total RNA was extracted from transformants. a: RNA expression of transformant IpYES2; b: RNA expression of transformant IpYES2-egIV; c: RNA expression of transformant IpYES2Mα-xegIV. Induction times are listed at the top.

**Fig. 4.** CMCase activity of recombinant EGIV from transgenic yeast. Circle: EGIV activity from transformant IpYES2; Triangle: EGIV activity from transformant IpYES2Mα-egIV; Square: EGIV activity from transformant IpYES2-egIV; Diamond: EGIV activity from transformant IpYES2Mα-xegIV.
and other fungal species ranged from 43% to 53%, demonstrating that the amino acid sequences of EGIV from different fungi were not highly conserved. However, the EGIV protein from Trichoderma species was much conserved. The similarity of EGIV between T. viride and T. reesei was as high as 99%. Comparing with the EGIV from the other fungi, there were two conserved domains containing “QCGG” in CBD and “GNYVLRHE” in CD (Fig. 1), respectively. The function of the CBD is to bring the cellulose catalytic domain in close proximity to the cellulose surface and maintain it there for the course of hydrolysis. Endoglucanase with a CBD could increase the ...
reactivity of pulp and decrease the viscosity [11]. The fungal CBDs have either four or six cysteine residues that form two or three disulfide bridges, respectively [8]. The EGIV CBD of *T. viride* contains four cysteines and thus it is probably stabilized by two disulfide bridges. Transcription levels of *cel61A* (eglIV) from *Phanerochaete chrysosporium* were relatively high in crystalline cellulose, and glucose could induce the *cel61A* expression [36]. The eglIV gene from *T. viride* AS 3.3711 could also be induced by cellulose but was inhibited by glucose and fructose (Fig. 2); the same result was obtained from *T. reesei eglIV* expression [7]. Moreover, gene eglIV from *T. reesei* was also transcribed when using lactose, sophorose, Solka Floc, sorbose, and cellobiose as the carbon sources [34].

A signal peptide plays a very important role to direct the secretion of a protein. The native signal sequence of the endoglucanase I (eglI) gene replaced by the mating pheromone α-factor (MFα) from *S. cerevisiae* was characterized with respect to gene expression and growth on cellulose substrate. Increased enzyme activity and cellulose utilization were observed. The enzyme activity of the transformants containing the MFα secretion signal was 61.5% higher than that of the transformants containing native secretion signal. The MFα signal peptide was more efficient than the native signal peptide of gene eglI, suggesting that signal peptide replacement was an efficient way to enhance the cellulase expression level in yeast [39]. Glucan degradation was enhanced through co-expression of EXG1, BEG1, and END1 in *S. cerevisiae* fused to the secretion signal sequence of the yeast MFα [33]. Similarly, the MFα signal peptide could increase the heterologous protein expression in *Kluyveromyces cicerisporus* [4]. In this study, the CMCase activity of transformant IpYEMα-xeglIV with MFα signal peptide from *S. cerevisiae* was 2-fold higher than that of EGIIV with native signal peptide (Fig. 4), illustrating that the signal peptide of *S. cerevisiae* could regulate exogenous gene expression more effectively in *S. cerevisiae*.

Saha [26] summarized the characteristics of EG from different fungi, and the EG protein was generally stable over pH 4–6 and at temperatures 50–60°C. The CMCase of yeast recombinant IpYEMα-xeglIV was stable over a wide pH range (3.5–7.5) and optimal temperature of 55°C and pH of 5.0 (Fig. 5), which was consistent with the character of fungal endoglucanase enzymes. The EGIIV enzyme was suitable for utilization in cellulase saccharification at moderate temperatures and pH levels. Endoglucanase, exoglucanase, and cellobiohydrolase showed different responses to metal ions and trace elements. The endoglucanase activity could be stimulated by Na⁺, Ba²⁺, or Mn²⁺ and was inhibited by Fe³⁺, Pb²⁺, Ag⁺, Zn²⁺, Cu²⁺, or Al³⁺ [29]. In the present study, we found that the EGIIV was stimulated to a high degree by Ba²⁺, Mn²⁺, Cu²⁺, Fe³⁺, Na⁺, or Mg²⁺ and strongly inhibited by Ag⁺, Al³⁺, Fe³⁺, or Ca²⁺. The effect of K⁺, Co²⁺, and Zn²⁺ on the recombinant enzyme was not obvious (Fig. 6). However, the enzymatic activity of recombinant EGVIII from *T. viride* AS 3.3711 was activated by Zn²⁺ [10]. Cel61A from *T. reesei* homologous expression research showed that it had low activity on CMC, β-glucan, and lichenan and had no detectable activity on any other studied substrates [14]. Furthermore, Cel61A from *T. reesei* expressed in *S. cerevisiae* showed activity against Avicel, amorphous cellulose (PASC), CMC, and β-glucan [22]. It has been reported that family 61 endo-β(1,4)-glucanase (AN1602.2) from *Aspergillus nidulans* expressed in *Pichia pastoris* also exhibits a low hydrolytic activity toward soluble CMC [3]. The AkCel61 proteins from *Aspergillus kawachii* had a poor hydrolytic activity toward soluble CMC, similar to the *A. nidulans* endo-β(1,4)-glucanase [15]. In contrast, CEL1 of *Agaricus bisporus* has been reported to show no detectable glucanase activity [2]. EGIIV from *T. viride* AS 3.3711 showed a strong hydrolytic activity toward soluble CMC and CCM (Fig. 7). However, the rAkCel61 protein did not exhibit hydrolytic activity toward CCM [15]. Although the eglIV gene sequence from *T. viride* AS 3.3711 had a 100% identity against *Trichoderma*, the activities toward various substrates were different. The CMC activities of recombinant EGIIV in *P. pastoris* (2.4 U/ml) was higher than that in the *S. cerevisiae* (0.18 U/ml) (Fig. 6). Therefore, we proposed that endoglucanase activity was affected by the expression system [20]. Furthermore, the difference in genetic background of host strains did affect significantly on the cell growth, and expression and secretion of protein [5].

Many hydrolyzing enzymes, including endoglucanases, exoglucanases, and β-glucosidases, took part in a complicated biomass-degrading process. Some of glycolytic enzymes were constitutively expressed in glucose, sorbohydrate, and lactose, so expression of these genes were not affected by the added carbon sources. However, some of them were repressed by glucose. Initially, as glucose levels increased in culture, the degrading enzyme activity was similarly increased because glucose was used for cell division, mycelia growth, and cell mass accumulation [7]. However, when the glucose concentration reached higher levels, the degrading enzyme genes were repressed, consequently decreasing degrading enzyme activity and the endoglucanase induction time was prolonged in *T. viride* [6]. The endoglucanase EGIIV is a small part of several endoglucanases found in *T. viride* [38], so its activity was not assayed with the exception of enzyme purification or heterologous gene expression. In this study, we observed that when the glucose concentration in cultures was very high during prolonged culture, the recombinant enzyme activity was low. This phenomenon was likely because high glucose concentration repressed the endoglucanase gene expression. Similarly, the influence on the amount of enzyme produced was also noticeable in *Sporotrichum pulverulentum* [6].
In summary, endoglucanase gene egIV was cloned from *T. viride* AS 3.3711 and successfully expressed in *S. cerevisiae*. The CMCase properties of transformant showed that the optimal reaction system appeared to occur when Ba\(^+\) was present at 0.75 mM, the temperature was at 55°C, the pH was 5.0, and CMC was used as reaction substrate. The transformant IpYES2Mex-xegIV would be a good applicant strain for industry bioethanol production.

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