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A N I N T E R N A T I O N A L J O U R N A L

Coexpression of rumen fungal xylanase and bifunctional cellulase genes in *Escherichia coli*

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ABSTRACT

Rumen fungi inhabit the gastro-intestinal tract of ruminants and the most non-ruminant herbivores. Rumen fungi produce highly active plant cell wall degrading enzymes, therefore they have gained scientific interest. In this study, genes encoding xylanase (xynA-7) and cellulase (celA-5) were amplified from Neocallimastix sp. GMLF7 and Orpinomyces sp. GMLF5, respectively, and expressed in Escherichia coli. XynA-7 was found to be active only on xylan, however CelA-5 had activity both on carboxymethyl cellulose and lichenan. Lichenase activity of CelA-5 was found to be higher than carboxymethyl cellulase activity. The optimal conditions were at pH 6.0 and 40 °C for CelA-5 and at pH 6.5 and 50 °C for XynA-7. A coexpression vector was constructed to coproduce the XynA-7 and CelA-5 and then transformed into E. coli. The ability of the transformed E. coli strain to produce CMCase, xylanase and lichenase was evaluated. The transformed E. coli strain acquired the capacity to degrade CMC, xylan and lichenan.

Key words: Rumen fungi, xylanase, cellulase, coexpression, *E. coli*

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INTRODUCTION

The major components of a plant cell wall are cellulose and xylan [1]. Cellulose is composed of insoluble linear chains of β-D-1,4-linked glucose units and composed of crystalline and amorphous regions [2]. Xylan is a main component of the hemicellulose, and it is a complex polymer consisting of a β-D-1,4-linked xylopyranoside backbone which is partially substituted with acetyl, arabinosyl, and glucuronosyl side chains [3]. Many microorganisms are capable of degrading and utilizing cellulose and xylan as carbon and energy sources [4]. Cellulase (endo-1,4-β-glucanase, EC 3.2.1.4) randomly attacks the internal O-glycosidic bonds in cellulose, resulting in glucan chains of different lengths [5]. Xylanase (endo-1,4-β-xylanase, E.C.3.2.1.8) acts on homopolymeric backbone of 1,4-linked β-D-xylopyranose producing xylooligomers [6]. Cellulases and xylanases are receiving considerable attention because of their potential applications in several industrial processes.

The rumen contains a complex microbial ecosystem, which extracts nutrients from lignocellulosic agricultural by-products [7]. Rumen fungi are obligate anaerobic microorganisms and constitute a significant portion of the rumen microbial biomass [8]. Rumen fungi rapidly colonize plant cell walls [9]. They degrade cell wall carbohydrates such as cellulose and hemicellulose from the plant parts where they attached [10]. This degradation is performed by various highly active cellulases, xylanases, glycosidases and xylosidases of rumen fungi [11]. The cellulolytic and xylanolytic capacity of rumen fungi was also studied in detail by characterizing the enzyme encoding genes. Several cellulase and xylanase encoding genes have been cloned from *Neocallimastix* sp., *Orpinomyces* sp. and *Piromyces* sp. [12]. Recently, 357 glycoside hydrolases, 24 polysaccharide lyases and 92 carbohydrate esterases were reported in the genome of *Orpinomyces* sp. C1A [13].

Neocallimastix spp. and *Orpinomyces* spp. are well studied microorganisms and they have been reported as excellent cellulase and xylanase producers. In this study, we describe the cloning, coexpression and secretion of the xylanase encoding gene *xynA-7* from *Neocallimastix* sp. GMLF7, and cellulase encoding bifunctional gene *celA-5* from *Orpinomyces* sp. GMLF5 in *Escherichia coli*. We also conducted the partial characterization and examined the activity of the recombinant enzymes.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

The rumen fungi were grown in anaerobic medium at 39 ^{o}C. Anaerobic medium was prepared according to Orpin [14] and dispensed into Hungate tubes under strictly anaerobic conditions. Wheat straw was used as energy source for the maintenance media, and glucose was used as energy source to obtain relatively higher amounts of cells for DNA extraction [15]. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth in a shaking incubator (150 rpm) at 37 ^{o}C. The list of fungal and *E. coli* strains is given in Table 1.

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant features	Source or reference
Strains		
Neocallimastix sp. GMLF7	Xylanase coding gene (*xynA-7*) source	[16]
Orpinomyces sp. GMLF5	Bifunctional cellulase and lichenase coding gene (*celA-5*) source	[16]
E. coli EC1000	Plasmid free strain, Km^r, cloning host	[17]
E. coli MC1022	Plasmid free strain, Str^r, cloning host	[18]
E. coli pCTX7	*E. coli* EC1000 carrying pCTX7	This study
E. coli pGEMC	*E. coli* EC1000 carrying pGEMC	This study
E. coli pCTXC-EC	*E. coli* EC1000 carrying pCTXC	This study
E. coli pCTXC-MC	*E. coli* MC1022 carrying pCTXC	This study
Plasmids		
pCT	Amp^r, cloning vector	Favorgen (Taiwan)
pGEM-T Easy	Amp^r, cloning vector	Promega (USA)
pCTX7	Amp^r, derivative of pCT vector containing *xynA-7*	This study
pGEMC	Amp^r, derivative of pGEM-T Easy vector containing *celA-5*	This study
pCTXC	Amp^r, derivative of pCTX7 containing *celA-5*	This study

Genomic DNA Extraction and Polymerase Chain Reaction

Rumen fungi were grown for 2 days and the fungal biomass was harvested by centrifugation at 1250 *g* for 10 min. Biomass was frozen using liquid nitrogen and immediately broken down using a Mixer Mill (Retsch MM301). Genomic DNA extraction was performed according to Chen et al. [19]. The xylanase and cellulase encoding genes were amplified from *Neocallimastix* sp. GMLF7 and *Orpinomyces* sp. GMLF5, respectively, by using xylanase primers (F: 5'ACTGTTGCTAAGGCCCAATG-3' and R: 5'-ACCCCATTTACCATCGTCATC-3') and the cellulase primers (F: 5'-ATTATATTCATATTCACTGGTTGAA-3' and R: 5'-TTAGAATGGTGGGTTAGCATTT-3').

Plasmids and Cloning Strategy

The pCT (Favorgen, Taiwan) and pGEMT-Easy (Promega, USA) vectors were used for construction of xylanase and cellulase expression vectors, respectively. The list of constructed vectors was given in Table 1. The constructed vectors were transformed into *E. coli* EC1000 and by using $CaCl_2$ method [20]. Plasmid isolations were carried out with Plasmid Isolation Kit (Vivantis, Malaysia). Cellulase gene digested from pGEMC with EcoRI and ligated into pCTX7. The resultant plasmid (pCTXC) was transformed into *E. coli* EC1000 and *E. coli* MC1022. Xylanase and cellulase positive colonies were screened according to Teather and Wood [21].

Enzyme Assay

For investigation of the cell-associated and extracellular enzyme activities, transformant *E. coli* strain was transferred to LB broth containing ampicillin (50µg/ml) and incubated at 37 ^oC for 12h in a shaking incubator (150 rpm). The culture was centrifuged at 6000 rpm for 10 min at 4 ^oC. The supernatant was collected and used as extracellular enzyme. The cell pellet washed twice with 50

mM sodium-phosphate buffer (pH 6.0) and then broken down by using ball-mill dismembranator (Retsch, Germany) and resuspended in the same buffer. Cellular debris was subsequently removed by centrifugation and the clarified extract used as cell-associated activity. Extracellular and cell-associated enzyme fractions were stored at -20 ^{o}C until required. Xylanase, cellulase, avicelase and lichenase activities were determined by measuring the amount of reducing sugar released by enzyme fractions incubated with 0.5% birchwood xylan (Sigma), 0.5% carboxymethyl cellulose (Sigma), 0.2% Avicel (Merck), and 0.1% lichenan (Megazyme), respectively, in 50 mM sodium phosphate buffer (pH 6.0). Reducing sugars released were measured by the dinitrosalicyclic acid reagent [22]. One unite of enzyme activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute in the reaction mixture under the assay conditions. Optimum pH and temperature, pH and temperature stabilities were determined according to Comlekcioglu et al. [23].

RESULTS

Cloning of xynA-7 and celA-5

The endo-β-1,4-xylanase (*xynA-7*) and endo-β-1,4-glucanase (*celA-5*) genes were amplified from *Neocallimastix* sp. GMLF7 and *Orpinomyces* sp. GMLF5, respectively, using the genomic DNA as the template. The *xynA-7* and *celA-5* fragments were about 800 and 1500 bp and cloned into pCT and pGEMT-Easy vectors, respectively. The resultant plasmids were named as pCTX7 and pGEMC, and transferred to *E. coli* EC1000. Recombinant *E. coli* pCTX7 and *E. coli* pGEMC strains were produced XynA-7 and CelA5 enzymes, respectively.

Characterization of XynA-7 and CelA-5 activities

XynA-7 and CelA-5 enzymes were produced from recombinant *E. coli* strains. The substrate specificities of the recombinant enzymes for xylan, CM-cellulose, lichenan and avicel were determined. The activities were determined for both cell associated and supernatant fraction. The major enzymatic activities of XynA-7 and CelA-5 were found to be cell associated in *E. coli*. More than 90% of the XynA-7 and CelA-5 activities were recovered from cellular biomass. Therefore, characterization of the enzymes was carried out using the cell associated enzyme in further studies. XynA-7 was found to be active only on xylan. CelA-5 had activity both on CMC and lichenan, however activity of CelA-5 against lichenan was much higher than CMCase activity. Avicelase activity was also tested for CelA-5, however no avicelase activity was detected in CelA-5.

The activities of the two enzymes at various temperatures and pH values are shown in Figure 1. XynA-7 and CelA-5 displayed their highest activities at 50 and 40°C, respectively. XynA-7 retained 70% of its activity after preincubation at 50°C for 3 h in the absence of substrate. The maximum thermal stability was obtained at 40 ^{o}C for CelA-5 and 59% of activity was still remained after 3 h incubation, although the CelA-5 activity diminished at 50°C after 1 h. The maximum activities were at pH 6.0 for CelA-5 and at pH 6.5 for XynA-7. Enzyme activities greater than 60% of the highest activity were attained at pH 5.0 to 7.0 for CelA-5 and at pH 4.0 to 8.0 for XynA-7. After preincubation at different pH values at optimum temperature for 30 min, both enzymes lost more than 60% of the maximal activity above pH 7.0.

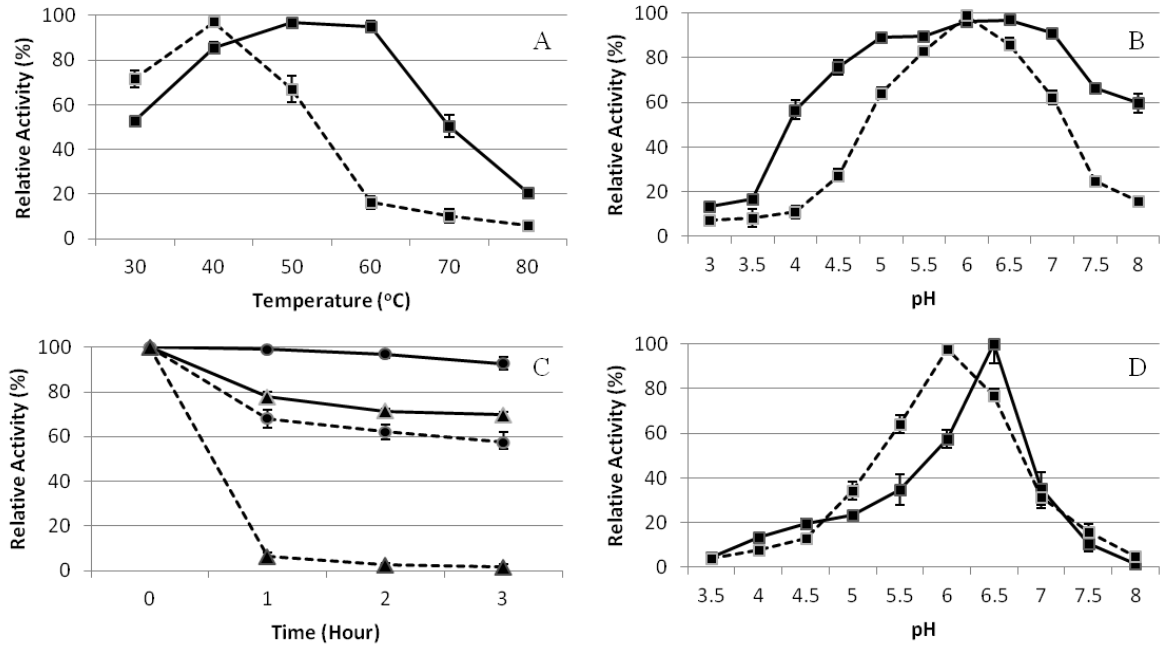

Figure 1. Effects of temperature (A) and pH (B) on the activities of XynA-7 (solid line) and CelA-5 (dashed line). (C) Thermal stability at 40 °C (●) and 50 °C (▲). (D) pH stability after incubation for 30 min at optimum temperature.

Construction of co-expression vector

To coexpress the *xynA-7* gene of *Neocallimastix* sp. GMLF7 (800 bp fragment) and *celA-5* gene of *Orpinomyces* sp. GMLF5 (1500 bp fragment), *celA-5* were cloned into the vector pCTX7, generating pCTXC. *celA-5* fragment was obtained by digestion from the pGEMC vector using EcoRI, and ligated to a similarly digested pCTX7 (Fig. 2). The resultant plasmid (pCTXC) was then transformed into *E. coli* EC1000 and *E. coli* MC1022 as hosts. The presence of the pCTXC in the *E .coli* transformants was investigated by screening the CMCase activity using Congo red plate assay. A CMCase positive colony was selected and the recombinant plasmid pCTXC was isolated. pCTXC was verified by EcoRI digestion, which produced the *celA-5 fragment* (1500 bp) (Figure 3). A Congo red plate assay was also used to confirm the xylanase and lichenase activities of the selected CMCase positive transformant. The cell lysates of the *E. coli* pCTXC strains produced yellow halo zones on the substrate containing plate, indicating that *xynA-7* and bifunctional *celA-5* were functionally coexpressed by pCTXC containing *E. coli* transformants (Fig. 4). Enzyme production of *E. coli* EC1000/pCTXC was found to be higher than *E. coli* MC1022/pCTXC, hence pCTXC harboring *E. coli* EC1000 was used in further studies.

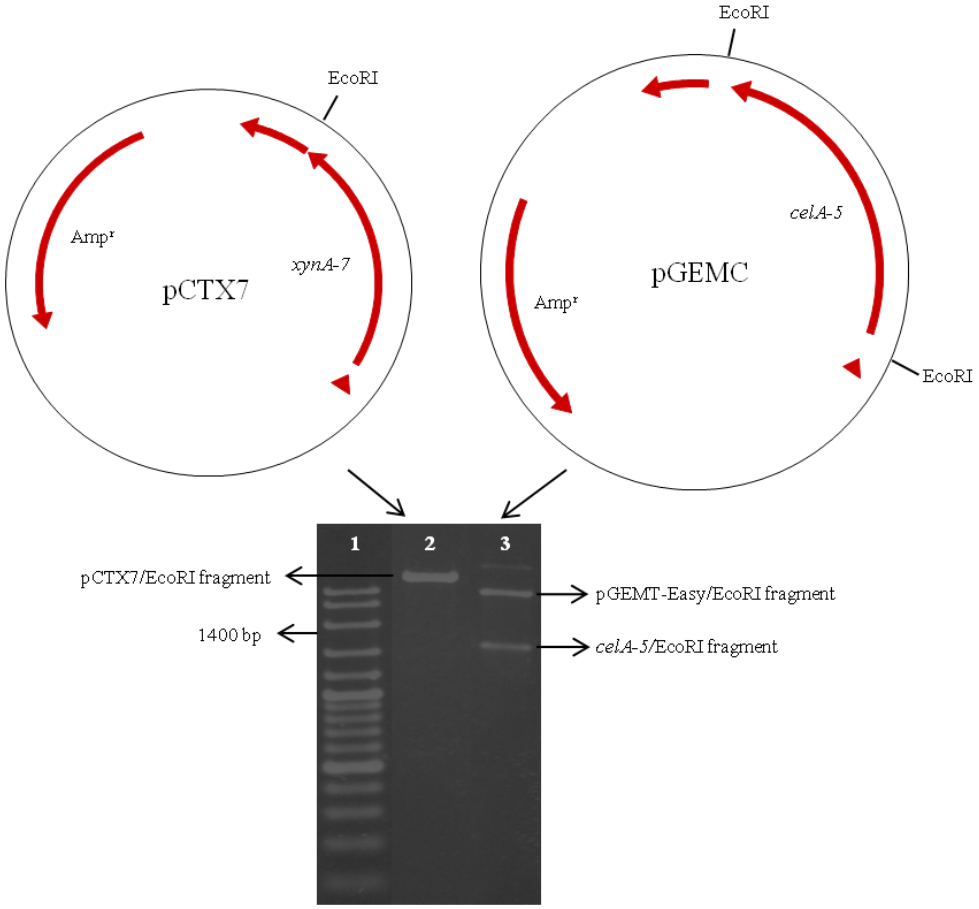

Figure 2. Schematic diagram of xylanase gene containing pCTX7 and bifunctional gene containing pGEMC. pCTX7 and pGEMC were digested with EcoRI, *celA-5*-EcoRI fragment was isolated from the gel and ligated to pCTX7-EcoRI fragment. Lane 1, 100 bp ladder; lane 2, EcoRI digestion of pCTX7; lane 3, EcoRI digestion of pGEMC.

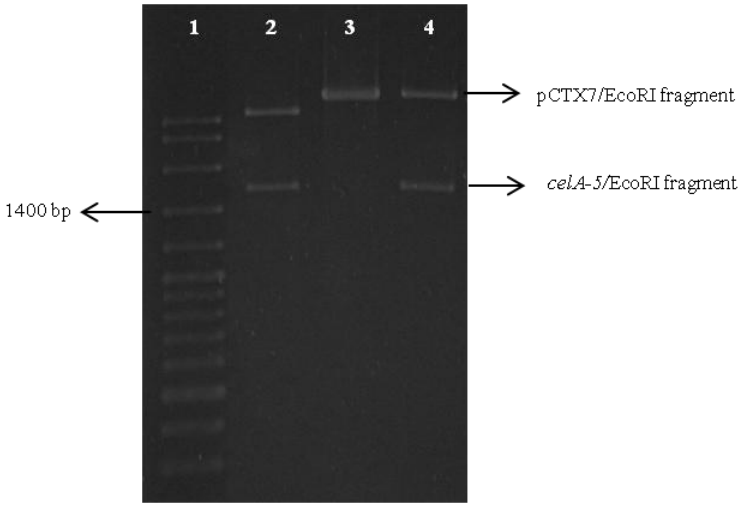

Figure 3. Confirmation of *celA-5* gene in pCTXC by EcoRI digestion. Lane 1, 100 bp ladder; lane 2, EcoRI digestion of pGEMC; lane 3, EcoRI digestion of pCTX7; lane 4, EcoRI digestion of pCTXC.

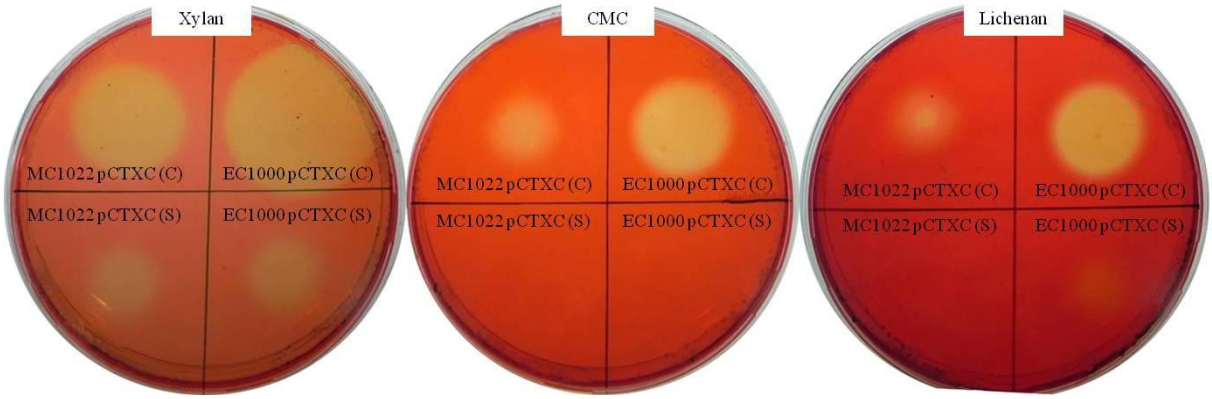

Figure 4. Plate tests for xylanase, CMCase and lichenase activities of pCTXC harboring *E. coli* EC1000 and MC1022. Extracellular (S) and cell associated (C) enzyme fractions were pipetted onto xylan, CMC and lichenan containing agarose plates, allowed to incubate and stained with Congo red.

Enzyme production of E. coli pCTXC strains

When cultured in the LB medium at 37°C, *E. coli* pCTXC reached stationary growth after 12 h, which was similar to that observed for E. coli EC1000 (data not shown). The specific activities of *xynA-7* and *celA-5* were determined in the LB medium. The host strain *E. coli* EC1000 did not show any activity. The highest activities observed for xylanase, CMCase and lichenase of *E. coli* pCTXC were 30.88, 1.83 and 11.48 U ml^{-1}, respectively. Xylanase and lichenase activities of pCTXC were similar with XynA-7 and CelA-5 expressed from separate promoters. As shown in Table 2, the xylanase and lichenase activity in the extracellular fraction of the *E. coli* pCTXC culture were much higher than that of the cell associated fraction. However, CMCase activity of cell associated fraction of *E. coli* pCTXC was lower from the extracellular fraction.

Table 2. The activity of fibrolytic enzymes of *E. coli* strains

Strain	Enzyme activity (U ml^{-1})					
	Xylanase		CMCase		Lichenase	
	Cell Associated	Supernatant	Cell Associated	Supernatant	Cell Associated	Supernatant
E. coli EC1000	Nd*	Nd	Nd	Nd	Nd	Nd
E. coli pCTX7	29.89 ± 0.91	3.23 ± 0.06	Nd	Nd	Nd	Nd
E. coli pGEMC	Nd	Nd	2.14 ± 0.35	1.38 ± 0.15	10.62 ± 0.27	1.52 ± 0.73
E. coli pCTXC	30.88 ± 0.76	4.20 ± 0.13	0.93 ± 0.18	1.83 ± 0.14	11.48 ± 0.28	1.50 ± 0.13

*Not detected

DISCUSSION

Enzyme supplementation to improve the feed utilization by ruminants has an increasing attention. Supplementing dairy cow and feedlot cattle with fibrolytic enzymes has potential for improving the efficiency of feed utilization and animal performance [24]. Fibrolytic enzyme mixture is reported to enhance the milk yield of cows in early lactation without changing feed intake [25]. Complete degradation of cellulose and hemicellulose in feeds requires a number of enzymes [26]. Recently,

recombinant DNA technology has enabled the bacteria to coexpress the specific genes. Genes encoding superior genes can be combined in a vector and transferred into well characterized microbial hosts.

Rumen fungi produce a wide range of plant cell wall degrading enzymes that enable them to utilize fibrous plant biomass efficiently. Studies on the functional transfer of rumen fungal genes to tractable bioprocessing hosts are rapidly emerging[27]. In this study, xylanase encoding *xynA-7* and cellulase and lichenase encoding bifunctional *celA-5* were cloned into *E. coli* and we performed partial characterization of XynA-7 and CelA-5. Both enzymes showed maximum activity in the range of 40-50°C and pH 6 to 6.5. Optimum pH and temperature values are found to be similar with other rumen fungal enzymes [28, 29, 11]. The similar optimum activities of XynA-7 and CelA-5 are a prominent property for the simultaneous production of both enzymes in the same host.

E. coli has a number of benefits for recombinant protein expression, such as low production cost, rapid growth and high yields to produce desired products [30]. While numerous cellulose and xylan degrading enzymes have been cloned in *E. coli*, to the best of our knowledge, there are few reports representing the co-expression of cellulase and xylanase in *E. coli* [31]. In this study, two *E. coli* strains, *E. coli* EC1000 and MC1022, were tested for the coexpression of *xynA-7* and *celA-5*, and *E. coli* EC1000 harboring the pCTXC plasmid had more enzyme activity than that of *E. coli* MC1022 containing the pCTXC. These results indicate that difference in *E. coli* strains are a important factor for high production of coexpressed enzymes. Simultaneous expression of genes can be conducted using either single or multiple plasmids in *E. coli*, and in the case of a single plasmid, genes can be controlled by single or seperate promoter [30].

CONCLUSION

We have established a coexpression system for the production of XynA-7 and bifunctional CelA-5 in *E. coli*. Coexpressed xylanase and bifunctional cellulase enzymes exhibited properties similar to those of the individually expressed enzymes. In addition to XynA-7 and CelA-5, other polysaccharide degrading enzymes used in animal nutrition, waste treatment, paper manufacturing and fuel production could also be combined in co-expression systems. The use of a single host cell for the production of enzymes may allow for cost-effective enzyme formulations. Additionally, coexpression vectors could also be designed with different strategies, such as using other genes, various promoters and selection markers in order to obtain high production and specifity.

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