



Polycistronic cellulase gene expression in *Pichia pastoris*

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Abstract

Cellulases are among the most important groups of industrial enzymes that are widely consumed in biofuel production, pulp and paper, textile, and detergent industries. The methylotrophic yeast *Pichia pastoris* was used for heterologous expression of a thermophilic cellulase collection. *P. pastoris* cells were transformed by the codon-optimized polycistronic EBG construct. This construct included *exxA* gene (from *Ampullaria crosseana*, with endo- and exoglucanase activities), *cglT* gene (from *Thermoanaerobacter brockii*, with β -glucosidase activity), and *zsgreen* (a fluorescent marker). Gene expression was examined at mRNA level using RT-PCR technique. The results indicated successful transcription of all transgenes. CglT and ZsGreen recombinant proteins were respectively detected by enzymatic assay and fluorescent microscope, while endo- and exoglucanase activities were not determined by enzymatic assays. The highest β -glucosidase activity was measured at 65 °C and pH 5.5. CglT is a good candidate for completing cellulase collections with low β -glucosidase activity. These cellulase sets could be used in biofuel production because of the high glucose tolerance property of CglT.

Keywords CglT protein · *Pichia pastoris* · Thermophilic cellulase enzymes · 2A sequence

1 Introduction

Classic cellulase enzyme system consists of three main enzyme groups, exoglucanases or cellobiohydrolases (EC 3.2.1.91), endoglucanases or CMCase (EC 3.2.1.4), and β -glucosidases (EC 3.2.1.21). Synergistic cooperation of these enzymes could decompose cellulose (homopolymer of glucose units) to a hexacarbon sugar, glucose. First, endoglucanase and exoglucanase enzymes act on cellulose, and their activity for β -1,4-glycosidic bonds hydrolyzing leads to production of cellobiose molecules which are then cleaved by β -glucosidases into glucose [1]. Cellulases have a very

wide range of applications in different industries (such as food and animal feed, textile, and biofuel industries) individually or in combined groups [2–5]. They could be applied for industrial and agricultural waste management and environmental conservation [6–8].

The thermal stability of cellulase enzymes is an important parameter for many utilizations due to biotechnological processes and enzymes' storage conditions. It is suggested that the possibility of conducting the processes at higher temperatures would lead to reduced enzyme requirements, increased reaction rate, and a decrease in the risk of contaminations with mesophilic microorganisms. Besides, thermophilic enzymes can be transferred and stocked easier than mesophilic enzymes [9].

Cellulase enzymes could be provided by cellulase-producing microorganisms or recombinant protein technology. We planned to have a complete set of recombinant cellulases; and therefore, a gene cassette was designed including *exxA*, *cglT*, and *zsgreen* genes which encoded endoglucanase and exoglucanase (EGXA protein) [10], β -glucosidase (CglT protein), and a green fluorescent marker (ZsGreen protein), respectively. *exxA* gene was obtained from *Ampullaria crosseana* snail encoding a 560 amino acid thermophilic enzyme with endoglucanase and exoglucanase activities, while *cglT* gene was provided from *Thermoanaerobacter brockii*

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bacterium that encodes a 450 amino acid thermophilic β -glucosidase enzyme [10, 11].

Pichia pastoris (*Komagataella phaffii*) is a suitable eukaryotic host for secreted recombinant protein production at academic or industrial levels due to obtaining high cell density and secreting low levels of endogenous proteins into culture media [12, 13]. A strong and tightly regulated promoter P_{AOX1} is widely used for foreign protein production in *P. pastoris* which could separate the growth phase from the production phase; therefore, biomass density could reach proper levels before initiating the expression of gene of interest [13]. Furthermore, codon optimization was applied for improving cellulase gene expression in this host [14].

There are several strategies for co-expression of multiple genes in eukaryotic cells including introducing different plasmids into the host cell, using a plasmid with multiple promoters, designing proteolytic sites, internal ribosome entry sites, or self-processing 2A sequences between genes. 2A sequences are short peptides that allow co-translation of all open reading frames (ORFs) presented on a polycistronic mRNA by ribosomal skipping. It terminates the translation at the carboxyl terminus of the first 2A peptide and releases the polypeptide chain, while ribosome starts the translation of the second ORF. GDVEXNPGP is the conserved oligopeptide among different 2A sequences, and cleavage occurs before the last proline. 2A peptide is normally added to the C-terminal of the first and P at N-terminal of the next polypeptide chain. The main advantages of this technique are relatively high cleavage efficiency and almost equal expression levels of different ORFs of the construct [15–17]. 2A sequences have been used for heterologous protein production in fungi such as *Trichoderma reesei* [18], *Saccharomyces cerevisiae* [19, 20], and *Pichia pastoris* [21]. We used this technique for the co-expression of three genes (two cellulase genes and a fluorescent marker) in this study. We aimed to have a cellulase enzyme set consisting of EGXA and CglT proteins, investigated the expression of these genes in *P. pastoris* for the first time, and compared the ability of *P. pastoris* in expression of these genes with other hosts. Although *P. pastoris* is a suitable host for expression of many heterologous genes, it is necessary to examine its possible efficiency in new cases.

2 Materials and methods

2.1 Microorganism strains and vector

Escherichia coli JM109 was used for pPICZ α A vector amplification, and *P. pastoris* GS115 (Invitrogen) was utilized as the host for foreign protein production. The construct EBG including *egxA* coding sequence (CDS) (GenBank accession number, FJ183727.1) from *Ampullaria crosseana* encoding

endoglucanase and exoglucanase enzymes, *cglT* CDS from *Thermoanaerobacter brockii* (GenBank accession number, Z56279.1) encoding β -glucosidase enzyme, and *zsGreen* CDS (GenBank accession number, AF168422.1) encoding fluorescent marker protein was synthesized by Genray company (China) (Fig. 1). It should be mentioned that these three genes were connected by two 2A sequences (P2A and T2A), and a signal peptide sequence (MF4I) was placed before *cglT* CDS. A nine-nucleotide segment (GGATCTGGT) was added before 2A sequences to improve their functions [17, 22, 23]. The company exerted the codon optimization (proper to *P. pastoris* codon usage) and cloned it into the expression vector pPICZ α A using *EcoRI* and *NotI* restriction enzymes.

2.2 Culture media

Escherichia coli (*E. coli*) JM109 were cultured in lysogeny broth (LB) medium (Sigma-Aldrich). Different media including yeast extract-peptone-dextrose (YPD; 1% yeast extract, 2% peptone, 2% dextrose or glucose) (Sigma-Aldrich), yeast extract-peptone-dextrose-sorbitol (YPDS; 1% yeast extract, 2% peptone, 2% dextrose or glucose, 1 M sorbitol), buffered glycerol complex (BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% yeast nitrogen base w/o amino acids, 4×10^{-5} % biotin, 1% glycerol), and buffered methanol complex (BMMY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% yeast nitrogen base w/o amino acids, 4×10^{-5} % biotin, 0.5%

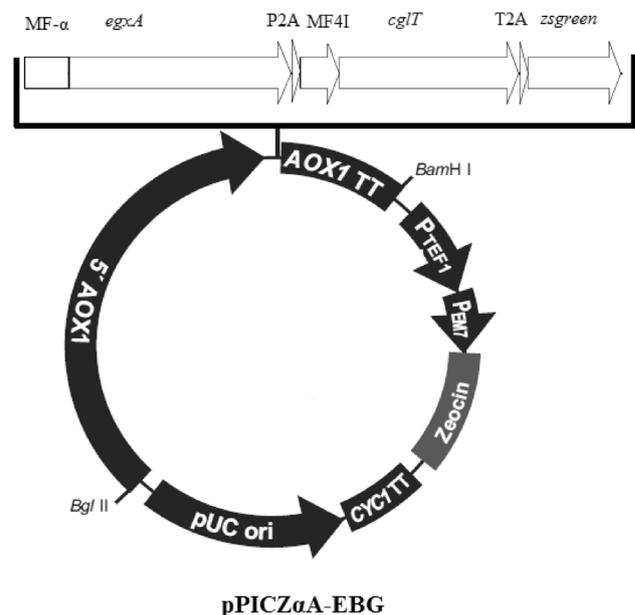


Fig. 1 Scheme of EBG construct. EBG construct was designed and cloned in pPICZ α A vector to produce a recombinant cellulase enzyme set by yeast *P. pastoris* cells

methanol) were used for various phases of experiments. YPDS, BMGY, and BMMY media were prepared according to EasySelect *Pichia* Expression Kit Manual. All required chemicals were purchased from Merck company.

2.3 Plasmid verification, linearization and microorganism transformation

Recombinant plasmid (pPICZ α A-EBG) was digested by two restriction enzymes (*EcoRI* and *NotI*, NEB) for verification of EBG fragment insertion in pPICZ α A plasmid. DNA fragments obtained from enzymatic double digestion were examined on agarose gel (0.7%) by gel electrophoresis [24].

To propagate the original and recombinant plasmids (pPICZ α A and pPICZ α A-EBG, respectively), competent *E. coli* JM109 cells were prepared and transformed following Inoue method [25]. Transformed colonies were selected on LB-Agar medium containing 25 μ g/ml Zeocin antibiotic. GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) was applied for plasmid extraction from *E. coli* and *P. pastoris* cells.

Original and recombinant plasmids were linearized by *SacI* restriction enzyme (Thermo Fisher Scientific) and transferred to *P. pastoris* GS115 cells by transformation technique [24]. *P. pastoris* electrocompetent cells were prepared based on EasySelect *Pichia* Expression Kit Manual and were transformed using manufacturer instructions of the BioRad electroporation system. After transformation, the cells were incubated in YPDS medium for 60 min and then transferred on YPDS plates containing 100 μ g/ml Zeocin antibiotic and incubated at 30 °C for 4–5 days. Appeared colonies were used for protein expression cultures.

2.4 *P. pastoris* transformants cultivation

To investigate recombinant protein expression, transformed colonies were cultured in BMGY medium (at 30 °C and 230 rpm) overnight (OD₆₀₀ = 2–6), then yeast cells were pelleted by centrifugation at 1500 g for 5 min (room

temperature). Pellets were resuspended in BMMY medium (10 ml and OD₆₀₀ = 1), transferred into 100-ml flasks, and incubated at 30 °C and 230 rpm for 6 days. To induce the AOX1 promoter, methanol (500 μ l, 0.5% final concentration) was added to each flask every 24 h.

2.5 Detecting the expression of recombinant genes at mRNA level

Our construct included three genes; two of which (*egxA* and *cglT*) encoding enzyme proteins, and *zsgreen* gene that encodes a green fluorescent protein. Recombinant colonies of *P. pastoris* were cultured in BMGY and then BMMY media. Cell pellets were obtained by centrifugation (8000 g and 4 °C for 10 min), rinsed with PBS, and grinded in liquid nitrogen for 5 min. Thermo Fisher Scientific Kits were used for RNA extraction, DNase treatment, and cDNA synthesis. Forward and reverse primers for recombinant genes and *act1* gene from *P. pastoris* (as a control gene) were designed by Allel ID v.6 software (Table 1). mRNA expression of *egxA*, *cglT*, *zsgreen*, and *act1* genes (GenBank accession numbers: FJ183727.1, Z56279.1, AF168422.1, and AF216956.1, respectively) was examined using RT-PCR technique.

2.6 Detecting the expression of recombinant genes at protein level

Transgenic *P. pastoris* colonies were grown in BMGY medium overnight and then cultured in BMMY medium (50 ml containing 0.5% methanol). After 6 days, the cells were separated by centrifugation at 10,000 g and 4 °C for 10 min. The supernatants were utilized for enzyme assays. Solid ammonium sulfate was dissolved in supernatants (85% saturation) and kept overnight at 4 °C. Centrifugation at 10,000 g and 4 °C for 10 min pelleted the proteins which were then dissolved in 1 ml citrate buffer (100 mM) and used for recombinant enzyme activity measurements [26]. Extraction of intracellular proteins of pelleted yeast cells was performed using glass beads and lysis buffer (NaCl,

Table 1 List of primers designed for RT-PCR

Gene	Accession number	Forward and reverse primers	Amplified fragment length
<i>egxA</i>	FJ183727.1	F 5' GTTCTTTACGGTCATCACGCTGTC 3' R 5' AATTGTGGGTTTCGTCTGTCATCC 3'	178 bp
<i>cglT</i>	Z56279.1	F 5' AAGGAAATCGGTGTCAAGGCATAC 3' R 5' TTTGTCGTAAGCCATTGAGGAAG 3'	186 bp
<i>zsgreen</i>	AF168422.1	F 5' TGTCCAAGAAAGATGCCTGATTG 3' R 5' GCGGAACAGATGCGATAGC 3'	120 bp
<i>act1</i>	AF216956.1	F 5' TCTCCTTACCACACGCTATTTTCG 3' R 5' CCTTGATGTCACGGACGATTTC 3'	135 bp

100 mM., 2-mercaptoethanol, 10 mM., EDTA, 1 mM) [27]. Extracted proteins were used for enzyme assays.

2.6.1 ZsGreen protein, fluorescent marker

P. pastoris samples (containing recombinant vector as main samples and parental vector as control samples) were taken from BMMY media and used for preparing yeast slides. These slides were observed by a fluorescent microscope (Olympus EX51 microscope).

2.6.2 Endo- and exoglucanase assays

Carboxymethyl cellulose (CMC) (2% w/v) (Sigma-Aldrich) in citrate buffer (50 mM) and avicel PH 101 (2% w/v) (Merck) in citrate buffer (100 mM) were used as substrates for endoglucanase and exoglucanase activity detection, respectively. Reaction mixtures including substrate solution and enzyme solution (1:1) were prepared with various pHs (3–9) and incubated in different temperatures (30–60 °C) for several durations (30 min to overnight). Then, enzyme activities were assayed by dinitrosalicylic acid (DNS) method, and glucose concentrations were determined by measuring the absorbances at 540 nm [28–30].

2.6.3 β -glucosidase assay

The β -glucosidase activity assay was performed by 4-nitrophenyl- β -D-glucopyranoside (pNPG) (Sigma-Aldrich) as a substrate of the enzyme. Substrate solutions (in phosphate buffer, 100 mM) and enzyme solutions were mixed (1:9) in 100 μ l final volume and incubated at 75 °C for 30 min. Sodium bicarbonate (1 M, 100 μ l, Merck) was utilized for stopping the enzyme reactions. Then, optical densities were read at 410 nm [29, 31].

Different pHs (3.5–8.5) and temperatures (30–75 °C) were tested to find conditions that showed the highest enzyme activity of the recombinant protein. Enzyme activities were calculated based on the standard curve of *p*-nitrophenol, and one unit of enzyme activity was defined as the amount of enzyme required to produce 1 μ mol *p*-nitrophenol within 1 min under the reaction conditions.

2.6.4 Optimum methanol concentration for induction

The induction of the BMMY medium could be done by using different concentrations of methanol. Among the recombinant colonies, the GR1 colony, with the highest β -glucosidase activity, was selected for optimizing methanol concentration. Cultures of GR1 colonies in BMMY medium were prepared and then induced daily with 0.5%, 1%, and 2%

methanol. The GR1 colony (transgenic *P. pastoris* colony which had received pPICZ α A-EBG vector), GRP (transgenic *P. pastoris* colony that received parental pPICZ α A vector), and GS115 (control colony of *P. pastoris*) were grown in these media for 6 days. Then, yeast cells were pelleted, and the supernatants were used for β -glucosidase assays.

Each concentration of methanol and each enzyme assay were tested three times.

2.6.5 SDS-PAGE analysis

Samples used for SDS-PAGE analysis included concentrated culture media proteins (provided by solid ammonium sulfate, 85%). Stacking (5%) and running gels (12%) were prepared at pH 6.8 and pH 8.8, respectively, and protein bands were stained with Coomassie Brilliant Blue R-250 [32, 33].

2.6.6 Possibility of recombinant enzymes digestion by Kex2 protease

The dipeptides Lys-Arg or Arg-Arg (as the recognition sites of Kex2 enzyme) were searched throughout EGXA and CglT proteins. According to [34] study, an octapeptide sequence including these recognition sites was scored for possible digestion of recombinant proteins by Kex2 enzyme.

3 Results

3.1 Plasmid verification and linearization

Electrophoresis of double digestion (by *Eco*RI and *Not*I restriction enzymes) of pPICZ α A-EBG plasmid showed two DNA fragments related to pPICZ α A plasmid (3.6 kb) and EBG construct (4.2 kb). This observation confirmed that EBG construct was correctly inserted in pPICZ α A plasmid. Linearization of recombinant plasmid (by *Sac*I restriction enzyme) is also confirmed by agarose gel electrophoresis (Fig. 2).

3.2 Expression of recombinant genes at mRNA level

We utilized the RT-PCR technique to examine the proper transcription of recombinant genes (*egxA*, *cglT*, and *zsGreen*) in yeast *P. pastoris* GS115 strain. The RT-PCR was done for recombinant yeast cells that received pPICZ α A-EBG vector, and those cells receiving parental vector pPICZ α A (as control). The cells were cultured in two different non-induced BMGY medium and induced BMMY medium. Electrophoresis of RT-PCR products showed that recombinant genes

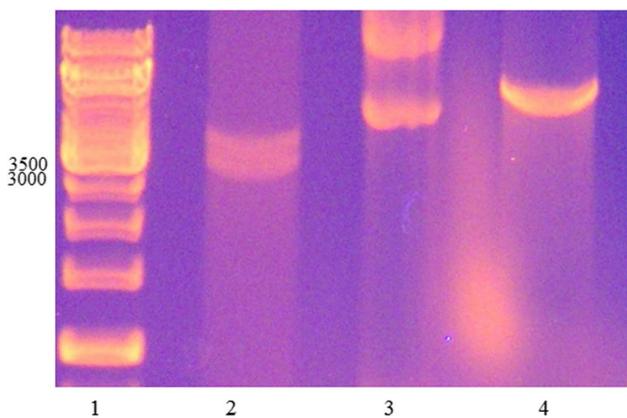


Fig. 2 Enzymatic digestions of recombinant plasmid pPICZαA-EBG. Enzymatic digestion of recombinant plasmid was performed by *EcoRI* and *NotI* restriction enzymes. Two DNA fragments (3.6 and 4.2 kb) were observed on 0.7% agarose gel. Digestion of recombinant plasmid with *SacI* restriction enzyme resulted in linear plasmid. DNA size marker (1 kb) (lane 1), products of double enzymatic digestion (lane 2), circular recombinant vector pPICZαA-EBG (relaxed and supercoiled forms) (lane 3), linear recombinant vector pPICZαA-EBG (lane 4)

were transcribed in recombinant yeast cells that received pPICZαA-EBG vector and cultured in induced medium. Negative controls indicated no transcription of recombinant genes (Fig. 3).

3.3 Expression of recombinant genes at protein level

3.3.1 Fluorescent marker protein

Slides of yeast cells that received recombinant and parental vectors (pPICZαA-EBG and pPICZαA vectors, respectively) were observed by fluorescent microscope. The former cells expressed bright green ZsGreen protein (Fig. 4).

3.3.2 Endoglucanase and exoglucanase activities

Endo- and exoglucanase assays using culture media and intracellular extracted proteins of genetically engineered yeast cells (cultures in induced BMMY medium) led to no detection of enzyme activities. Enzyme assays were performed under different reaction conditions (various pHs, temperatures, and reaction times) and repeated for concentrated culture media (prepared by solid ammonium sulfate) as well; absorbances of reaction mixtures were almost 0 at 540 nm. Therefore, enzyme activities were considered 0.

3.3.3 β-Glucosidase activity

Recombinant colonies obtained from yeast transformation with pPICZαA-EBG vector were cultured in induced BMMY medium. After 6 days, culture media were harvested and tested for β-glucosidase activity. Enzyme reactions were performed at 75 °C and pH 5.5 (optimum temperature and pH for CglT activity from *T. brockii*

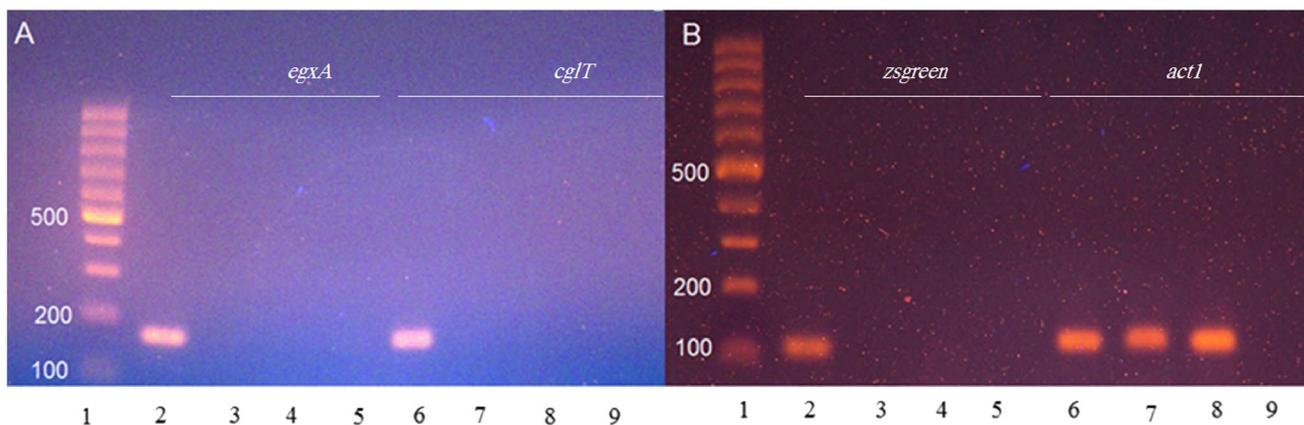


Fig. 3 Expression of recombinant cellulase and marker genes in transgenic *P. pastoris* GS115 cells at mRNA level. DNA fragments with 178, 186, 120, and 135 bp lengths were obtained from RT-PCR which related to recombinant *egxA*, *cglT*, and *zsgreen* genes, as well as host *act1* gene (as positive control), respectively. DNA size marker, 100 bp (lanes 1), *P. pastoris* cells which received pPICZαA-EBG

vector and cultured in induced BMMY medium (lanes 2 and 6), *P. pastoris* cells which received pPICZαA-EBG vector and cultured in non-induced BMMY medium (lanes 3 and 7), *P. pastoris* cells which received parental pPICZαA vector and cultured in induced BMMY medium (lanes 4 and 8), and negative controls (lanes 5 and 9)

Fig. 4 Expression of fluorescent ZsGreen protein in *P. pastoris* GS115 cells. The yeast cells that had received recombinant pPICZ α A-EBG vector expressed the marker protein (ZsGreen) and were seen as bright green dots on the slide (A). The yeast cells that had received parental vector (pPICZ α A vector) were used as negative control (B). Scale bar represents 200 μ m

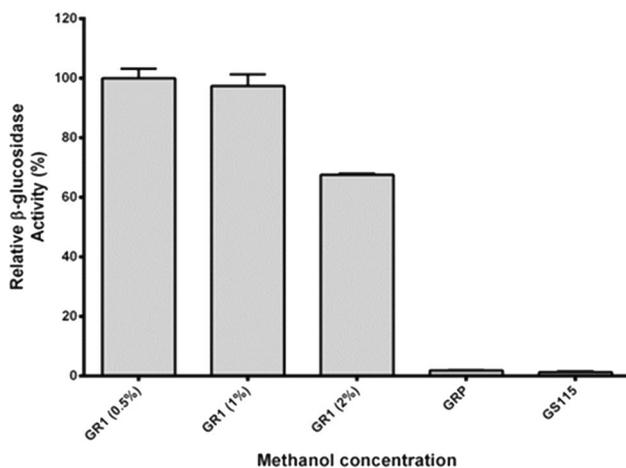
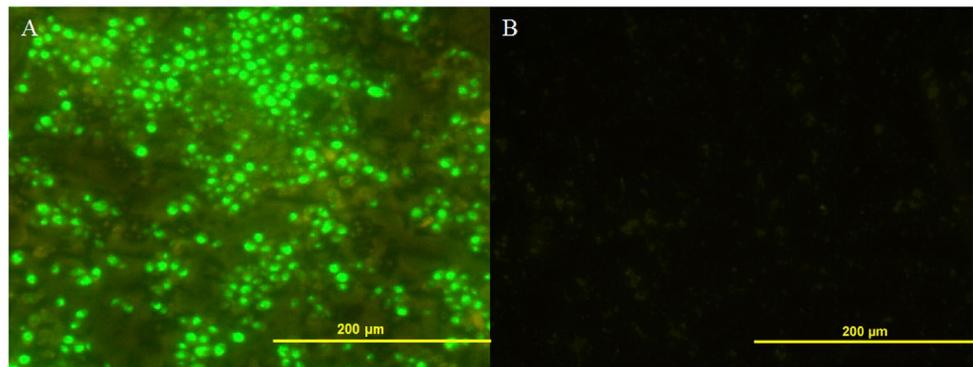


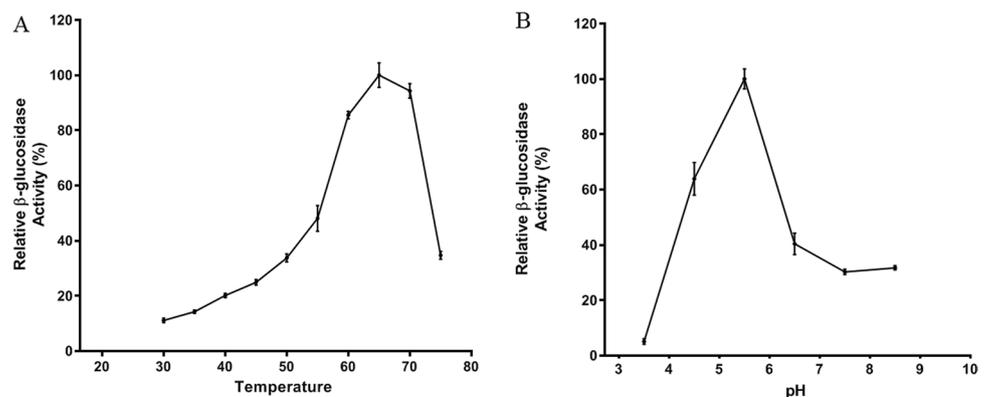
Fig. 5 Effect of methanol concentrations on recombinant CglT production by yeast *P. pastoris*. Comparing the relative activities of recombinant β -glucosidase enzymes produced by GR1 yeast colonies in BMMY media with different concentrations of methanol (0.5%, 1%, and 2%). Reaction enzymes were carried out at 75 $^{\circ}$ C and pH 5.5. GR, recombinant *P. pastoris* GS115 yeasts received pPICZ α A-EBG vector, GRP, recombinant *P. pastoris* GS115 colonies containing parental pPICZ α A vector, GS115, non-recombinant *P. pastoris* GS115 colonies

bacterium) [11]. The best colony was selected based on *p*-nitrophenol production during enzyme assays and used for the optimization phase. It was observed that most of the colonies produced *p*-nitrophenol in the same range and only two colonies (GR6 and GR7) indicated significantly lower levels of *p*-nitrophenol production during the β -glucosidase reaction. However, the GR1 colony was chosen for the next phases of the experiment because it showed the highest level of *p*-nitrophenol production.

3.3.4 Optimization of methanol concentration in BMMY medium

Different concentrations of methanol (0.5%, 1%, and 2%) were used for BMMY media preparation. GR1 colonies were cultured in these media for 6 days, and then culture media were used for assessment of β -glucosidase activity. Recombinant CglT activities did not show significant differences between media with 0.5% and 1% methanol, whereas 2% methanol in BMMY medium led to a significant reduction of β -glucosidase activity (Fig. 5). Induced medium (BMMY medium) with 0.5% methanol was selected for the next phases.

Fig. 6 Optimum temperature and pH for recombinant CglT enzyme activity. The graphs indicate the relative β -glucosidase activities of recombinant CglT enzyme at temperature range, 30–80 $^{\circ}$ C (A) and various pHs from 3.5 to 8.5 (B)



3.3.5 Optimum temperature and pH for recombinant CglT enzyme

GR1 colonies were cultured in BMMY medium (with 0.5% methanol) for 6 days. Then, culture media were harvested and used for β -glucosidase assays at different temperatures

(30–80 °C) and various pHs (3.5–8.5). CglT enzyme activities were compared in varied reaction conditions and indicated that 65 °C temperature and pH 5.5 were the best conditions for this recombinant β -glucosidase activity (Fig. 6A, B).

3.3.6 SDS-PAGE analysis of recombinant proteins production by *P. pastoris*

SDS-PAGE analysis was utilized for examination of recombinant protein production by *P. pastoris*. Samples were prepared from concentrated induced culture media of transformed yeasts which had received pPICZ α A-EBG and parental pPICZ α A vectors. A comparison of these two samples showed two extra bands for yeast cells containing pPICZ α A-EBG. These two bands were possibly related to CglT protein. It seems that the polypeptide chain of recombinant β -glucosidase has been hydrolyzed in *P. pastoris* cells or culture media because we expected a 52-kD protein band for this enzyme, instead of the two observed protein bands (about 39 and 37 kD) by SDS-PAGE (Fig. 7). These protein bands are in agreement with recognition of two potential cleavage sites (PGKRTEMG and LLKRLDRE) on CglT polypeptide chain by Kex2 enzyme (Table 2). It seems that these truncated proteins keep their enzyme activities, because we detected β -glucosidase activities in recombinant yeast culture media.

3.3.7 Possibility of recombinant enzymes digestion by Kex2 protease

Scoring EGXA and CglT proteins for likely digestion by Kex2 is summarized in Table 2. Five and six potential cleavage sites were observed for CglT and EGXA polypeptide chains, respectively. Two cleavage sequences of each protein obtained a high score. It seems that the Kex2 function is the strong potential cause of these two proteins digestion. It appears that this possible digestion did not influence on

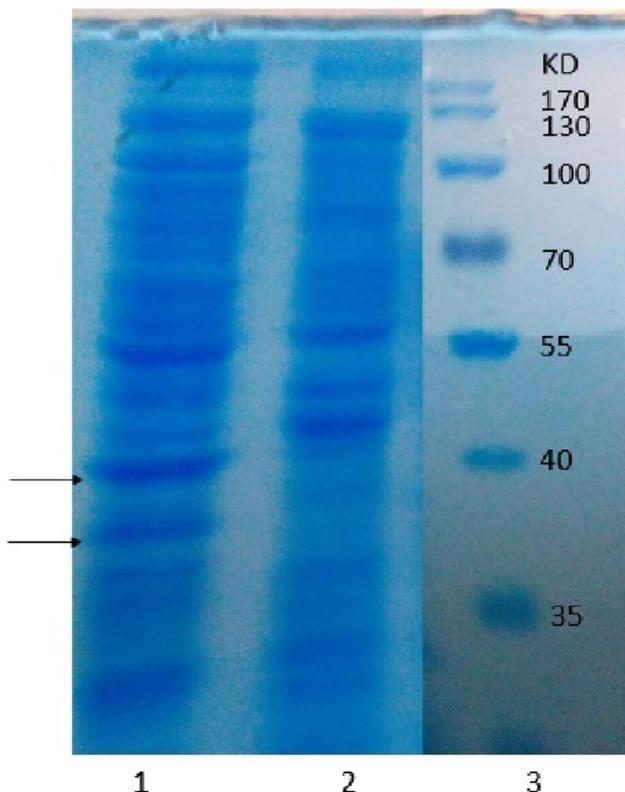


Fig. 7 SDS-PAGE analysis of recombinant proteins produced by *P. pastoris* GS115. Total protein presented in culture media of recombinant yeast *P. pastoris* cells containing pPICZ α A-EBG vector (lane 1) and parental pPICZ α A vector (lane 2). Two protein bands related to recombinant proteins are shown with arrows. Molecular weight marker (lane 3)

Score	Expect	Method	Identities	Positives	Gaps
345 bits(886)	2e-124	Compositional matrix adjust.	164/165(99%)	165/165(100%)	0/165(0%)
Query 4	LAKFPRDFVWGTATSSYQIEGAVNEDGRTPSIWDTFSKTEGKTYKGHTGDVACDHYHRYK				63
Sbjct 1	MAKFPRDFVWGTATSSYQIEGAVNEDGRTPSIWDTFSKTEGKTYKGHTGDVACDHYHRYK				60
Query 64	EDVEILKEIGVKAYRFSIAWPRIIFPEEGKYNPKGMDFYKLLIDELQKRDIVPAATIIYHWD				123
Sbjct 61	EDVEILKEIGVKAYRFSIAWPRIIFPEEGKYNPKGMDFYKLLIDELQKRDIVPAATIIYHWD				120
Query 124	LPQWAYDKGGGWLNRRESIKWVVEYATKLFEEELGDAIPLWITHNPE			168	
Sbjct 121	LPQWAYDKGGGWLNRRESIKWVVEYATKLFEEELGDAIPLWITHNPE			165	

Fig. 8 Protein blast of CglT protein and a β -glucosidase from *Thermoanaerobacter* sp. Protein blast of *Thermoanaerobacter brokii* CglT (β -glucosidase, 450 amino acids, GenBank accession number: CAA91220.1) and a β -glucosidase from *Thermoanaerobacter*

sp. (GenBank accession number: HBW59855.1, 165 amino acids) showed a 99% identity between amino acids 4–168 of CglT and amino acids 1–165 of this enzyme

Table 2 Scoring possible Kex2 recognition sites on EGXA and CglT proteins

Protein	Amino acid sequence	P4	P3	P2	P1	P1'	P2'	P4'	Potential of digestion	Calculated MW of fragments resulted from likely digestion (kD)
CglT	LQKRDIVP 108–115	0.09	0.71	0.50	1.00	+	+			12.7
CglT	PGKRTEMG 320–327	0.2	0.70	0.50	1.00		+		Almost strong	39.3
CglT	LLKRLDRE 338–345	0.09	0.90	0.50	1.00		+	+	Strong	36.8
CglT	YSKRFGIV 414–421	0.47	0.64	0.50	1.00			+	Strong	15.2
CglT	TQKRILKD 427–434	0.17	0.71	0.50	1.00	+		+		13.0
EGXA	QYKRVASQ 110–117	0.41	0.85	0.50	1.00	+	+		Strong	47.8
EGXA	SVKRMPSG 162–169	0.17	0.91	0.50	1.00					4.2
EGXA	LQKRKAFP 207–214	0.09	0.71	0.50	1.00	-	+			2.6
EGXA	RLRRSDIT 181–188	1.00	0.90	0.50	1.00		+		Strong	49.4
EGXA	ENRRADFY 441–448	0.05	0.69	0.50	1.00	+	+			2.6
EGXA	AGRRVLEL 493–500	0.25	0.70	0.50	1.00					49.4
										50.6
										18.2
										44.8
										23.0
										40.0
										20.1
										42.9
										49.6
										13.4
										55.4
										7.6

enzyme activity of some CglT copies, while EGXA proteins lost their activity as a result of polypeptide chain cleavage.

There is a possibility for secretory recombinant proteins to be hydrolyzed by internal or external proteases of the host cells, partially or completely. It seems that partial hydrolysis may have occurred on the recombinant CglT protein in our study. To further clarify, a β -glucosidase enzyme was found in *Thermoanaerobacter* sp. (GenBank accession number: HBW59855.1, 165 amino acids), and a protein blast of *Thermoanaerobacter brokii* CglT (β -glucosidase, 450 amino acids, GenBank accession number: CAA91220.1) with this protein showed 99% identity between amino acids 4–168 of CglT with amino acids 1–165 of this enzyme (Fig. 8). Therefore, it seems that the existence of N terminal fragment of CglT is enough for its β -glucosidase activity. Thus although some copies of recombinant CglT are hydrolyzed partially, they could keep their catalytic activity.

4 Discussion

Cellulase enzymes play roles as critical components of various industries such as paper, detergents, food, and feed industries [35]. Besides, these enzymes can degrade biomass and could be utilized for renewable biofuel production [36].

Therefore, they have the potential to be converted to the largest group of industrial enzymes [37].

Researchers follow two pathways to produce cost-effective cellulase enzymes. (1) They design investigations to increase fungal cellulase production levels in natural hosts and (2) conduct experiences intending to optimize the recombinant expression systems of microorganisms. Heterologous hosts permit the development of microbial strains that are capable of producing a set of synergistic cellulase enzymes. These enzyme sets could be produced by a single cell [38] or by different strain combination [39].

4.1 Recombinant β -glucosidase enzyme activity

We designed the EBG construct that included *egxA* and *cglT* cellulase genes and a fluorescent marker gene (*zsgreen*) and examined the expression of this cellulase set in *P. pastoris*. These three genes were connected by two 2A sequences (P2A and T2A). Therefore, we expected the whole construct to be transcribed as a polycistronic mRNA and two cellulase genes to be translated equally. We anticipated that the two signal peptides (MF- α and MF4I) would conduct the two cellulase polypeptides towards the endoplasmic reticulum (ER) and Golgi apparatus and thus the final recombinant proteins could be secreted to the culture medium.

We detected recombinant β -glucosidase (CglT) activity in the culture medium of *P. pastoris* and performed optimization for its better production and activity. The highest CglT activity (821 mU/ml) was obtained in induced culture medium containing 0.5% methanol while the reaction enzyme was performed at 65 °C and pH 5.5. Primary studies on this enzyme were done with the intracellular recombinant expression of *cglT* gene in bacterial hosts (*E. coli* and *Bacillus subtilis*). The recombinant enzyme activity was measured as 873 mU/ml (mU activity per ml of cell extract). This β -glucosidase enzyme is classified in the GH1 family of glycoside hydrolase enzymes. Two main advantages of this enzyme are high tolerance against glucose inhibition ($k_i = 200$ mM), and its activity at high temperatures [11]. Therefore, CglT, as a good candidate, could be applied to compensate for deficiency of β -glucosidase activity in cellulase sets during biomass decomposition. This enzyme was successfully applied for improving cellulase activities of *Clostridium thermocellum* S14 cellulosomes. When the bacterial cellulosomes were used for lignocellulosic material decomposition, cellobiose aggregation occurred and led to endoglucanase and exoglucanase inhibition. Adding recombinant CglT to these cellulase sets (cellulosomes) resulted in cellobiose degradation and removing the inhibition of endoglucanase and exoglucanase enzymes. On the other hand, CglT showed high tolerance against glucose and remained active at high glucose concentrations. It was indicated that improved *C. thermocellum* S14 cellulosomes functioned more efficiently (10 times) than commercial cellulase collection (Celluclast 1.5 L and Novozyme-188) in biomass material degradation [40]. Another research entered CglT into the cellulase enzyme sets of *C. thermocellum* (CeID, CBHA, CBH48Y) and was succeeded to find a cellulase combination with optimum activity at 60 °C which degraded 70% of corn stover to glucose after fermentation for 24 h [41]. Such cellulase sets could be used in enzymatic digestion of biomass for biofuel production. Besides, there are various applications for β -glucosidase enzymes in the food industry. These enzymes hydrolyze bitter compounds of fruit juices especially citrus juices [42, 43], release flavors from glycosylated materials presented in plant tissues such as tea [44], and function in extraction pathways of medical compounds (for example isoflavones and genistein) from plant materials [45, 46]. β -Glucosidase enzymes are also applied in the feed industry and help better digestion of cellulosic nutrition by animals and poultry [47, 48].

4.2 Recombinant EGXA protein

We placed *egxA* gene in EBG construct to obtain endoglucanase and exoglucanase activities and complete cellulase activities of this construct. RT-PCR analysis showed that this gene was transcribed in yeast host cells successfully,

whereas we could not detect expected enzyme activities related to EGXA protein in yeast cells and BMMY culture medium. This might be caused by the following reasons:

(1) MF- α signal peptide sequence positioned before *egxA* gene (as a part of pPICZ α A vector) was expected to conduct EGXA protein towards ER to be finally secreted from host cells. MF- α pre-pro signal peptide sequence is the most extensively used secretion signal for recombinant expression of proteins in *P. pastoris* [49], including fungal proteins [50–52], green fluorescent proteins [53], human insulin [54], or human C-reactive protein [55]. The MF- α signal peptide consists of a pre-region (19 amino acids), a pro region (64 amino acids), and a spacer (6 amino acids). The pre region guides the polypeptide chain towards the ER and is deleted by signal peptidase [56–58]. Pro region and spacer are required for the final processing of protein in Golgi apparatus and its secretion to the culture medium [56, 59]. Kex2 protease cleaves the pro region after the Lys84-Arg85 sequence [60], four extra amino acids remain at the N-terminal of protein which are deleted by STE13 protease, and mature protein can be secreted to the extracellular space [61]. This signal peptide acts with a post-translational translocation mechanism [62, 63]. Hsp40/Hsp70 chaperons are very important in this mechanism and maintain complete polypeptide chains at a loosely folded state which is a translocation state of polypeptide chains [64]. The expression of recombinant proteins could load additional stress on the translocation pathway of ER and reduce its efficiency. Misfolded recombinant proteins in the cytosol would be sent to the proteasome for degradation.

Because MF- α signal peptide directs post-translational translocation of the related protein into the ER, it might not be suitable for recombinant proteins with intracellular native position, because these proteins are prone to fold in cytosol completely, and their translocation into the ER and subsequently secretion may be performed inefficiently. However, this possibility is weak about EGXA recombinant protein, because it natively is a secretory protein and its folding is done in the lumen of ER. MF- α factor might result in aggregation of EGXA protein at a high-level in the ER lumen. However, an investigation was performed for recombinant expression of E2-Crimson by MF- α factor and a mutated MF- α signal peptide of pPICZ α vector (replacement of Leu42 with Ser42) in *P. pastoris*. It showed that although MF- α factor caused E2-Crimson aggregation in the ER lumen, mutated MF- α factor removed this problem, and no aggregation was observed [65]. We utilized pPICZ α A vector, and this mutated signal peptide for EGXA expression, given that E2-Crimson is a dimeric protein and more complex than EGXA protein, we expected that recombinant EGXA could be translocated to the Golgi apparatus efficiently, and it seems that the probability of EGXA aggregation in the ER lumen is weak. However, replacement of the

MF- α sequence with another signal peptide such as Ost-1 might improve *egxA* gene expression in *P. pastoris*. We will explain that protein digestion by the Golgi proteases is a stronger possibility than wrong signal peptide for EGXA protein.

(2) The secretion of some recombinant proteins could be affected by improper folding in yeast ER [66, 67]. EGXA polypeptide might have been translocated to the ER properly, but misfolding in the ER lumen led to its degradation by endoplasmic reticulum-associated degradation (ERAD) mechanism [68, 69]. In some cases, misfolded proteins were transferred to the yeast Golgi apparatus and then to the vacuole and were degraded by the vacuolar protease complex [70, 71]. Yeast cells possess a quality control system that detects the misfolding proteins by vacuolar protein sorting receptors (VPSRs) at the Golgi apparatus and targets them to the vacuole [72]. It was indicated that these VPSRs could distinguish recombinant proteins with suitable folding and direct them towards vacuole [53, 73]. This proposed that these receptors extended their ligand range to proteins which their conformation was different from normal protein conformation of the yeast secretory pathway [53]. Yeast cells without VPSRs secreted foreign proteins more efficiently [53, 74, 75]. It must be mentioned that VPSR deletion led to vacuolar hydrolases to be presented in culture medium of yeast cells [76]. These hydrolases might contribute to secreted recombinant protein degradation in culture medium.

(3) We attempted to investigate the likely digestion of EGXA by *P. pastoris* proteases. Most likely candidates which could influence recombinant EGXA are the Golgi apparatus proteases (including Kex2 and Yapsin 1). Kex2 is responsible for cleavage of the pro part of α -factor signal peptide and produces a mature secretory protein in the trans-Golgi saccules. In the absence of the Kex2 enzyme, Yapsin 1 can carry out this function. These enzymes identify Lys-Arg or Arg-Arg residues and hydrolyze the peptide bond after Arg. Therefore, a mature secretory protein could be targeted out of the cell [77–79]. These enzymes can potentially identify the Lys-Arg or Arg-Arg residues that exist throughout polypeptide chains. Of course, other residues surrounding the dipeptide (two residues before and three residues after the Lys-Arg) are important for recognition. Bader et al. [34] scored four positions of X-X-Lys-Arg or X-X-Arg-Arg (P4, P3, P2, and P1, respectively) according to Kex2 substrates. They tried to predict the potential substrates of this enzyme. In addition to this scoring, they stated that the presence of small or acidic amino acids (Ala, Val, Ile, Asp or Glu) at positions P1', P2', and P4' (first, second, and fourth residues after Lys-Arg or Arg-Arg dipeptide) increased the chance of dipeptide recognition by Kex2 enzyme and presence of basic amino acids (Arg, Lys, or His) at these positions decreased the possibility of digestion site recognition by the enzyme. It seems that proline presence at P1' position inhibits the

enzyme activity [80]. We utilized this information to predict the possible cleavage of our recombinant proteins which were expressed in *P. pastoris*. Six potential cleavage sites for EGXA polypeptide chain were detected from which two cleavage sequences obtained a high score (Table 2). It seems that the Kex2 function is the strongest potential cause of EGXA protein digestion. It appears that EGXA proteins lost their activities as a result of polypeptide chain cleavage.

Although enzyme activities of EGXA were not detected in culture media and intracellular-extracted proteins of recombinant *P. pastoris* cells that had received pPICZ α -EBG vector, insect Tn5 cells [81] and mammalian cells including cell lines PK15, HEK293A, 3T3, and CHO 1–15 [82] were suitable hosts for the heterologous expression of this protein.

5 Conclusion

The expression of polycistronic EBG construct (including cellulase activities combination) partly succeeded in yeast *P. pastoris* cells and led to secretion of recombinant β -glucosidase (CglT) enzyme (from *T. brockii*) to the culture medium. It seems that *P. pastoris* is not a suitable host cell for recombinant expression of *egxA* gene from *Ampularia crossean*, and its expression is forced with some post-translational problems and degradation in *P. pastoris* cells. Recombinant CglT is a good candidate for enforcing thermophilic cellulase enzyme sets which could not function properly because of inefficient β -glucosidase activity.

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Declarations

Conflict of interest The authors declare no competing interests.

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