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Enhancement of *Escherichia coli* cellulolytic activity by co-production of beta-glucosidase and endoglucanase enzymes

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Abstract Cellulase is a group of enzymes (endoglucanase, exoglucanase and beta-glucosidase) required for cellulosic feedstock hydrolysis during bioethanol production. The use of recombinant cellulase is a strategy to reduce the enzyme cost. In this context, the present work describes the construction of a cellulase expression vector (pEglABglA), which allowed constitutive co-expression of endoglucanase A (EglA) from an endophytic *Bacillus pumilus* and the hyperthermophilic β-glucosidase A (BglA) from *Fervidobacterium* sp. in *Escherichia coli*. When compared to the non-modified strain DH5α, the recombinant *Escherichia coli* DH5α (pEglABglA) reduced fivefold the viscosity of the carboxymethylcellulose medium (CMC-M). Also, it presented almost 30-fold increase in reducing sugar released from CMC-M, enabling the recombinant strain to grow using CMC as the sole carbon and energy source. When cultivated in rich media, specific growth rates of recombinant *E. coli* strains BL21, JM101 and Top10 were higher than those of DH5α and DH10B strains. The constructed plasmid (pEglABglA) can be used as backbone for further cellulase gene addition, which may enhance even more *E. coli* cellulolytic capacity and growth rate.

Keywords: beta-glucosidase, cellulase cassette, cellulose bioconversion, endoglucanase, heterologous expression

INTRODUCTION

The use of cellulosic residue as a substrate for bioethanol synthesis represents one of the main strategies for petroleum substitution and greenhouse effects reduction (Hahn-Hägerdal et al. 2006). However, its economical viability is directly dependent to the efficiency of cellulose hydrolysis to glucose, which may be carried out by a group of three kinds of cellulases: endoglucanases, exoglucanases and β-glucosidases. Endoglucanases hydrolyze internal β (1→4) bonds of cellulose leaving cello-oligosaccharides. These cello-oligosaccharides are further hydrolyzed by exoglucanases releasing mainly cellobiose and shorter cello-oligosaccharides, which are hydrolyzed to glucose by β-glucosidases (Percival Zhang et al. 2006). Strategies to enhance the cellulose degradation includes: bioprospection of cellulolytic

microorganisms, classical strain breeding, protein and metabolic engineering, as well as heterologous expression of cellulases (Kumar et al. 2008).

Genetically modified microorganisms able to express heterologous cellulases have been successfully described for bacteria, filamentous fungi and yeast. Examples of heterologously expressed cellulases include the characterization of new cellulases in traditional hosts, such as *Escherichia coli* (Lima et al. 2005), as well as its use in protein engineering programs through site mutagenesis or directed evolution (Wang et al. 2005). Moreover, the use of cellulase expression cassettes has proved to be efficient to enable non-cellulolytic microorganisms to consume cellulosic substrates. As an example, a recombinant *Saccharomyces cerevisiae* modified to produce β-glucosidase I from *Aspergillus aculeatus*, endoglucanase II and cellobiohydrolase II from *Trichoderma reesei* was able to synthesize ethanol using amorphous cellulose as carbon source (Fujita et al. 2004). Similar results were achieved using the bacteria *Klebsiella oxytoca* expressing two endoglucanases from *Erwinia chrysanthemi* and genes for ethanol production from *Zymomonas mobilis* (Zhou and Ingram, 2001). Despite the fact that multiple cellulases expression systems are efficient in conferring cellulolytic capacity to microorganisms, just a few vectors are available for bacteria. In this context, we evaluated if a bacterial cellulase expression cassette encoding the endophytic endoglucanase A (EglA) from *Bacillus pumilus* (Lima et al. 2005) and the hyperthermophilic β-glucosidase A (BglA) from *Fervidobacterium* sp. (Lima et al. 2009) would enable *E. coli* to grow using carboxymethylcellulose as sole source of carbon and energy. The effect of different strains of *E. coli* carrying the constructed plasmid on specific growth rate was also determined.

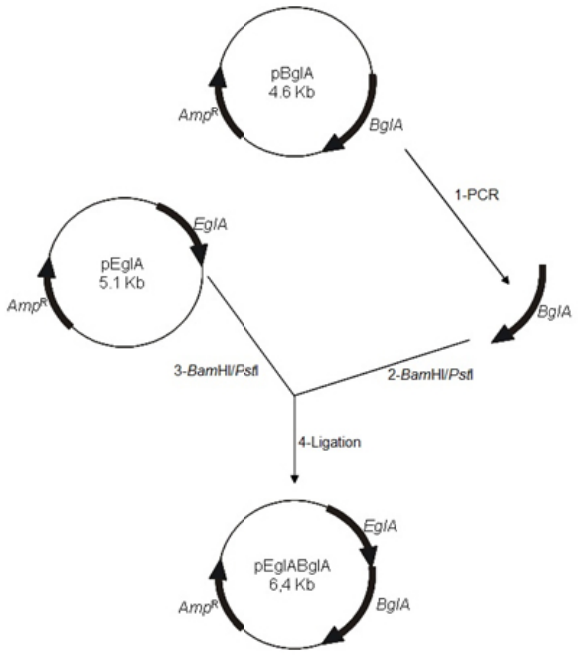

Fig. 1 Scheme of pEglABglA construction. 1. PCR amplification of *Bgl*A gene. 2. Digestion (*Bam*HI/*Pst*I) of *Bgl*A Amplicon. 3. Digestion (*Bam*HI/*Pst*I) of pEglA vector.4. Ligation of *Bgl*A amplicon into *Bam*HI/*Pst*I site of pEglA.

MATERIALS AND METHODS

Plasmids and cloning procedure

The plasmid pEglABglA was obtained in this study by combining DNA segments from plasmids pEglA and pBglA (Figure 1). The vector pEglA, described previously by Lima et al. (2005), harbors the endoglucanase A gene (GenBank accession number AY339624) from *Bacillus pumilus*. The plasmid pBglA was kindly provided by Dr. Douglas E. Eveleigh (Rutgers-USA) and contains the β-glucosidase A gene (AY151267) from *Fervidobacterium* sp. Both plasmids confer resistance to ampicillin. *Escherichia coli* DH5α was used as a host for the plasmids and expression of cellulases by culturing the different recombinant strains in LB medium supplemented with 100 µg/ml ampicillin (Sambrook and Russell, 2001). The *Bgl*A gene was PCR amplified using the forward 5'AACA***GG***A*TC****C***AATCAAACCAG3' and reverse 5'AGAAC***CTGC***A*G*CTCACCTAA3' primers. As the necessary restriction sites for cloning were not present in pBglA, it was required to insert them through the modification of the 5' region of the primers designed. The mutations inserted in the primers are shown in bold. Restriction sites for enzymes *Bam*HI and *Pst*I are shown in italic in the forward and reverse primers, respectively. The PCR reaction conditions consisted of a 4 min denaturation step at 94ºC, 35 cycles of 15 sec at 94ºC, 30 sec at 54ºC, and 2.5 min at 72ºC, followed by a final extension step at 72ºC for 6 min. The PCR amplicon (*Bgl*A - RBS and ORF) and pEglA were double digested with *Bam*HI/*Pst*I restriction enzymes. The *Bgl*A amplicon was subcloned downstream of the *Egl*A gene in pEglA resulting in the plasmid pEglABglA. Gene *Bgl*A was inserted in pEglA such that it is transcribed as a single mRNA with gene *Egl*A. Therefore, the transcription of both genes was under control of the constitutive promoter of gene *Egl*A. Competent *E. coli* DH5α cells were transformed by heat shock (Sambrook and Russell, 2001). It was described previously that the enzyme BglA has activity against X-gal (20 µg/ml) (Lima et al. 2009) thus forming blue colonies when expressed in solid medium supplemented with this compound. This feature together with the ability of EglA to produce a degradation halo when cultured on solid LB medium supplemented with 5 g/l CMC and stained with Congo red were used to identify transformants containing the plasmid pEglABglA. It should be pointed out that no functional *lac*Z gene was present in the plasmid construction used. In addition, the presence of the *Bgl*A gene in plasmid pEglABglA was confirmed by PCR amplification of this gene using plasmid pEglABglA extracted from transformants presenting BglA and EglA activities. PCR conditions and primers used were the same employed for *Bgl*A amplification, as described above.

Growth on cellulosic substrates

In order to evaluate the growth of the strains on cellobiose and CMC, inocula were prepared by normalizing the cell densities (λ = 595 nm) of overnight LB cultures, followed by medium removal by centrifugation/resuspension (three times) in 8.5 g/l NaCl. The bacterial strains were then grown (120 hrs, 37ºC, 150 rpm) in shake flasks containing 10 ml of a modification of the minimal Mops Medium (Neidhardt et al. 1974), hereafter referred to as MM medium. The cultures of the strains harboring plasmids were supplemented with 100 µg/ml ampicillin. The MM medium was composed of 1.6 mM K_2HPO_4, 9.52 mM NH_4Cl, 1 mM $MgSO_4$, 0.52 mM $MgCl_2$, 10 µM $FeSO_4$, 0.5 µM $CaCl_2$, 50 mM NaCl, 50 mM Tris(hydroxymethyl)aminomethane, 3 nM $(NH_4)_6(Mo_7)_{24}$, 0.4 µM H_3BO_3, 30 nM $CoCl_2$, 40 nM $CuSO_4$, 80 nM $MnCl_2$, 10 nM $ZnSO_4$, at pH 7.2 adjusted with 500 mM HCl. The carbon and energy sources used

were either 5 g/l carboxymethylcellulose (CMC) or 5 g/l cellobiose. After cultivation, the cell dry weights were determined based on a cell optical density (λ = 595 nm) standard curve. Additionally, the number of colony-forming units (CFU) was assessed by spreading the cells on agar-solidified LB medium and culturing at 37ºC for 24 hrs. The differences among dry cell weights and CFU were analyzed using ANOVA ($P \leq 0.05$) and the Tukey's test ($P \leq 0.05$).

Enzymatic assays and polymer degradation

Enzymatic activities of BglA and EglA were quantified using the chromogenic substrates *p*-nitrophenyl-β-d-glucopyranoside (PNPG, Sigma) and Remazol Brilliant Blue carboxymethylcellulose (RBB-CMC, Loewe), respectively. Enzyme production was achieved by cultivation (24 hrs, 37ºC, 200 rpm) of *E. coli* DH5α (pEglABglA) in LB medium (20 ml) supplemented with 100 µg/ml ampicillin. Intracellular proteins were obtained by cell centrifugation (14,000 x *g* for 2 min), followed by disruption (three rounds of vortexing with glass beads for 1 min) in 0.5 ml of Tris-HCl buffer (200 mM, pH 7, 2 mM PMSF) and supernatant recovery (14,000 x *g* for 6 min), which was diluted twice in the same buffer. Non-transformed *E. coli* DH5α was used as control. The evaluation of β-Glucosidase (BglA) activity was based on a modification of a method described previously (Lima et al. 2009). BglA reactions were carried out with 25 µl enzyme, 25 µl PNPG (6 mM), and 50 µl Tris-HCl buffer (200 mM, pH 7). After incubation for 1 h at 85ºC, the reaction was stopped with 100 µl of glycine-NaOH buffer (200 mM, pH 10.5), and the optical density was measured at 405 nm. The evaluation of endoglucanase (EglA) activity was based on a modification of a method described previously (Lima et al. 2005). For quantification of EglA activity, the reaction mix contained 25 µl CMC-RBB, 25 µl protein extract, and 50 µl Tris-HCl buffer (200 mM, pH 7). The reaction was carried out for 1 h at 55ºC and stopped by adding 25 µl of 2 M HCl. The reaction mix was then incubated for 10 min at 0ºC, centrifuged (14,000 x *g* for 5 min), and the optical density (λ = 595 nm) of the supernatant determined. Enzyme activities were normalized by the protein content which was determined by the Bradford method (Bradford, 1976). CMC depolymerization was assessed by viscosity reduction and release of reducing sugars. *E. coli* DH5α (pEglABglA) and the parent strain were cultured (120 hrs, 37ºC, 150 rpm) in MM medium with 5 g/l CMC (MM-CMC medium). The viscosities of the cultures were determined with a rotational viscosimeter (Haake, Viscotester VT 550, Sensor SVDIN) at 25ºC using a shear rate of 80 s^{-1}. Determination of reducing sugars released into the medium containing CMC was carried out by the Nelson-Somogyi method adapted by Lima et al. (2005). Differences among treatments were analyzed using ANOVA ($P \leq 0.05$) and the Tukey's test ($P \leq 0.05$).

Specific growth rate in rich media

Specific growth rate was determined for five different *E. coli* strains (BL21, DH5α, DH10B, JM101, Top10) carrying either pEglABglA or pEglA. Inoculums (5 ml culture in LB/100 µg ampicillin, 24 hrs, 37ºC, 150 rpm) were diluted 2X in 8.5 g/l NaCl and 300 µl of suspension was transferred into each well of 24-well microplate containing 1.2 ml LB medium supplemented with 100 µg/ml ampicillin and 0.1% (w/v) of l-arabinose (4 replicates/treatment). The cultures were performed for 24 hrs at 37ºC and 150 rpm. The optical density (λ_{600nm}) was measured periodically (1.5-2 hrs intervals) for 24 hrs cultivation time and data was converted to viable cells/ml using a specific linear regression ($y = 0.0268 \times -0.0003$; $R^2 = 0.9979$; data not shown). Finally, the specific growth rate (µ) was calculated considering the angular coefficient

obtained from a linear regression between log of viable cell density and cultivation time.

RESULTS AND DISCUSSION

Recombinant *E. coli* DH5α (pEglABglA) was identified by its ability to grow under ampicillin restrictive conditions and its activities of both BglA and EglA enzymes on X-Gal and CMC substrates, respectively. In addition, the presence of the *Bgl*A gene in plasmid pEglABglA was confirmed by PCR amplification, which yielded a band with the corresponding length of *Bgl*A. When quantitatively assayed, the enzymatic activities for both proteins were significantly higher ($P < 0.0002$) than the enzymatic activity of the parent strain *E. coli* DH5α (Table 1), indicating that both enzymes were constitutively expressed by the construct.

The amount of reducing sugars released by *E. coli* strains harboring the plasmids pEglA or pEglABglA was significantly ($P = 0.0001$) superior to that of strains harboring the plasmid pBglA and the parent strain *E. coli* DH5α (Table 1). This occurrence was due to the activity of EglA on CMC releasing cello-oligosaccharides which will increase the number of reducing ends (Cohen et al. 2004). As cello-oligosaccharides could not be used by *E. coli*, these sugars accumulated in the medium. Shorter cello-oligosaccharides was not the principal end product of the EglA activity, hence the amount of substrate available for BglA was not enough to provide a greater growth of *E. coli* DH5α (pEglABglA) compared to that of *E. coli* DH5α (pEglA). It was observed that the viscosity (Table 1) of the MM medium supplemented with CMC was significantly ($P < 0.0001$) reduced in the cultures where the *Egl*A gene was expressed. It was due to the degradation of the CMC polymer by the action of the EglA enzyme present in plasmids pEglA and pEglABglA.

Table 1. Enzymatic activities of EglA and BglA and polymer degradation.

Strainsa	Specific Activityb		Polymer Degradationc	
	BglA (nmol/µg·h)	EglA (nmol/µg·h)	Reducing sugars (µg/ml)d	Viscositye (mPAs)
E. coli DH5α	0.023	45.91	3.11 A	51.79 A
E. coli DH5α (pBglA)	Ndf	Nd	3.15 A	60.04 A
E. coli DH5α (pEglA)	Nd	Nd	81.17 B	10.87 B
E. coli DH5α (pEglABglA)	3.001*	353.17*	87.50 B	11.17 B

[a] *E. coli* DH5α is the parental strain. *E. coli* DH5α (pBglA) and *E. coli* DH5α (pEglA) express BglA and EglA enzymes, respectively. *E. coli* DH5α (pEglABglA) express both EglA and BglA enzymes. [b] Specific activities of BglA and EglA were defined as the amount (nmoles) of p-nitrophenol (PNP) or Remazol Brilliant Blue (RBB) released, respectively, per µg protein extract per hour (pH 7, 85ºC for BglA and 55ºC for EglA). Means were calculated for three (BglA) and five (EglA) replicates.[c] Values followed by the same letters in the columns are not significantly different at 5% probability level. Means were calculated for three replicates.[d] Reducing sugars were analyzed by the Somogy-Nelson method, using supernatants of MM CMC medium after the growth of the *E. coli* strains.[e] Rheological analysis were carried out with rotational viscosimeter to determine the viscosity of MM CMC medium after the growth of the *E. coli* strains.[f] Nd: not determined. * The values in the column are significantly different at 5% probability level.

The strains harboring plasmids that contain the *Bgl*A gene (Table 2) were able to grow significantly (P = 0.001) more in MM medium with cellobiose than the strains that do not contain this gene. This was possibly due to the activity of BglA on releasing glucose from cellobiose for growth (González-Candelas et al. 1989). The growth of *E. coli* DH5α (pEglABglA) in MM medium supplemented with CMC was significantly (P = 0.01) superior to that of *E. coli* DH5α (pBglA) and *E. coli* DH5α (pEglA) (Table 2). There was a significant difference between the CFU of the strains *E. coli* DH5α (pEglABglA) and *E. coli* DH5α cultured in MM-CMC medium. The number of CFU of the *E. coli* DH5α (pEglABglA) was significantly (P < 0.025) higher than that of the parent strain *E. coli* DH5α. This suggests that the action of both BglA and EglA released more glucose molecules for growth on CMC than the action of these enzymes separately. Partial saccharification of CMC by both EglA and BglA enzymes released cello-oligosaccharides of different lengths. The action of BglA on shorter cello-oligosaccharides released by EglA could make glucose available for growth, enabling *E. coli* to use CMC as carbon and energy source (Srivastava et al. 1995).

Considering the specific growth rate among different *E. coli* strains and plasmids (Figure 2), the highest values were observed from BL21, JM101 and Top10 carrying either pEglABglA or pEglA vectors. The numbers of cells were roughly doubled in two hours. On the other hand, the lowest performances were presented by DH5α and DH10B strains, which specific growth rate varied statistically depending on the plasmid carried. The strains Top10 and DH10B are genotypically similar, as described by the manufacturer (Invitrogen). Despite the similarity, it was observed that Top10 (pEglABglA) grows 1.5-fold faster than DH10B (pEglABglA) and Top10 (pEglA) grows

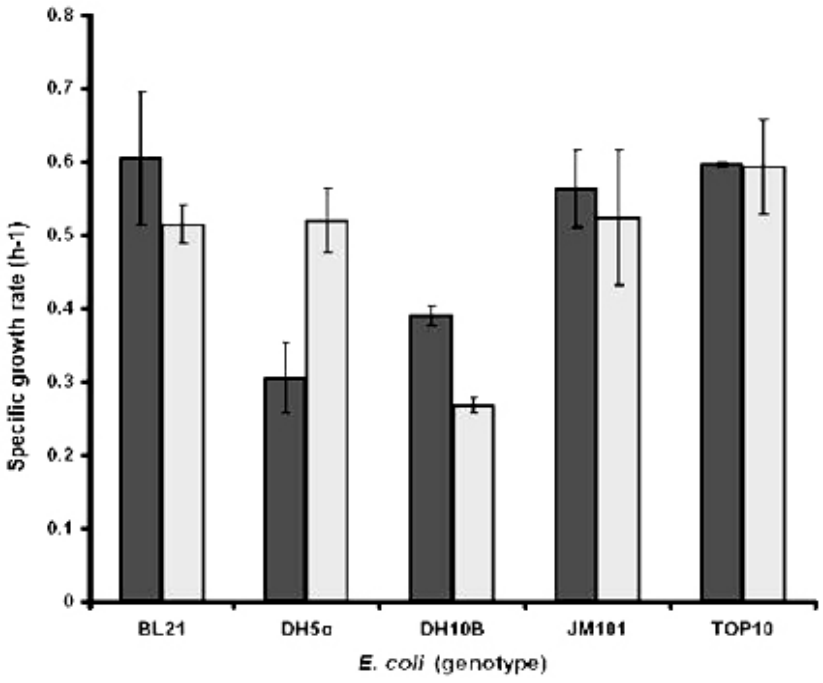

Fig. 2 Specific growth rate of recombinant *Escherichia coli* expressing cellulases. Strains carrying either pEglA/BglA (gray column) or pEglA (white column).

2-fold faster than DH10B (pEglA), probably due to some additional genetic mutation. As described earlier (Chou et al. 1999), different strains may present distinguished growth rate even carrying the same plasmid. Also, here we describe that a strain may present diverse growth kinetics when carrying different vectors.

The expression of the *Bgl*A and *Egl*A genes enabled *E. coli* DH5α (pEglABglA) to partially degrade CMC. A portion of the sugars released was used as carbon and energy sources for growth. Specific growth rate analysis of recombinant *E. coli* carrying pEglABglA, evidentiated BL21, JM101 and Top10 as the fast-growing strains among the strains tested. It is considered that pEglABglA can be further modified in order to receive an exoglucanase gene and potentially confer to *E. coli* the ability to completely hydrolyse cellulosic substrates. There are many works describing the construction of cellulolytic microorganisms for saccharification of cellulosic residues due to the availability of this low-cost material that can be used to produce high-value products (Haan et al. 2007). The trend for biofuel production will make large amounts of cellulosic residues available, such as sugarcane bagasse (Pandey et al. 2000). Therefore, approaches regarding the use of cellulosic wastes will take advantage of the availability of this material and, consequently, will contribute to minimizing the negative impacts of this waste on the environment.

Table 2. Analysis of the growth of *Escherichia coli* strains on cellulosic substrates.

Strains	Growth on cellulosic substrates[a]		
	MM Cellobiose (mg DW/ml)	MM CMC (mg DW/ml)	MM CMC (CFU·10^4/ml)
E. coli DH5α	21.33 A	20.08 A	2.16
E. coli DH5α (pBglA)	26.36 B	19.78 A	Nd[b]
E. coli DH5α (pEglA)	20.5 A	20.51 A	Nd
E. coli DH5α pEglABglA)	25.7 B	30.23 B	26.93*

[a]Strains were cultured on MM medium supplemented with cellobiose (MM Cellobiose medium) or CMC (MM CMC medium) for 120 hrs at 37ºC and 150 rpm. Values followed by the same letters in the columns are not significantly different at 5% probability level. Means were calculated for three replicates.[b] Nd: not determined. * The values in the column are significantly different at 5% probability level

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