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BIOENERGY/BIOFUELS/BIOCHEMICALS

Cell surface display of a β-glucosidase employing the type V secretion system on ethanologenic *Escherichia coli* for the fermentation of cellobiose to ethanol

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Abstract We used the autodisplay system AIDA-I, which belongs to the type V secretion system (TVSS), to display the β-glucosidase BglC from *Thermobifida fusca* on the outer membrane of the ethanologenic *Escherichia coli* strain MS04 (MG1655 Δ*pflB*, Δ*adhE*, Δ*frdA*, Δ*xylFGH*, Δ*ldhA*, P*pflB*::pdc_{Zm}-$adhB_{Zm}$). MS04 that was transformed with the plasmid pAIDABglCRHis showed cellobiase activity (171 U/g_{CDW}) and fermented 40 g/l cellobiose in mineral medium in 60 h with an ethanol yield of 81 % of the theoretical maximum. Whole-cell protease treatment, SDS-PAGE, and Western-blot analysis demonstrated that BglC was attached to the external surface of the outer membrane of MS04. When attached to the cells, BglC showed 93.3 % relative activity in the presence of 40 g/l ethanol and retained 100 % of its activity following 2 days of incubation at 37 °C with the same ethanol concentration. This study shows the potential of the TVSS (AIDA-I) and BglC as tools for the production of lignocellulosic biocommodities.

Keywords Cellobiose · Bioethanol · *Escherichia coli* · Type V secretion system AIDA-I · *Thermobifida fusca* β-glucosidase

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Introduction

Oil depletion and the pollution that is associated with burning fossil fuels are driving research to explore environmentally friendly renewable fuels. Ethanol can be used as a fuel, and at present, "flexible-fuel vehicles" that can run with any blend of gasoline and ethanol are on the market [2]. Agricultural residues are rich in lignocellulosic material that can be employed as a potential low-cost source of fermentable sugars that does not compete with food crops [10, 11]. Lignocellulose has a complex structure, and to release the fermentable sugars, a pretreatment process is needed to permit the hemicellulose hydrolysis, alter the crystalline structure of cellulose and/or to break the lignin [29]. Subsequently, the cellulose can be depolymerized into glucose through the enzymatic action of cellulases and β-glucosidases [10, 11, 29]. Lignocellulose hydrolysis produces primarily hexoses, such as glucose, mannose and galactose, and pentoses, such as xylose and arabinose [10, 13, 29]. To convert all of these different sugars to ethanol, a microorganism is required that is capable of fermenting pentoses and hexoses [10, 11, 13, 30]. *Escherichia coli* has this ability and has been the target of metabolic engineering to generate homo-ethanologenic strains [6, 9, 12, 31, 39].

The lignocellulose depolymerization process is one of the most expensive steps in lignocellulosic ethanol production [10, 11, 29]. As stated by Somerville [35]: "although the cost of cellulases per gallon of ethanol has been reduced ten times approximately in the last decade, that is still 20 times higher than the enzyme costs for a gallon of corn ethanol". To reduce costs, it is necessary to construct strains capable of secreting enzymes that can hydrolyze some lignocellulose fractions [10, 18]. Despite the fact that *E. coli* can ferment a wide variety of sugars, it

is unable to ferment some oligosaccharides, such as cellobiose (a glucose dimer formed during cellulase mediated cellulose hydrolysis). Therefore, introduction of the genes that code for hydrolases is required. The insertion of the cellobiose phosphotransferase genes (*casAB*) from *Klebsiella oxytoca* has been employed for the transport and hydrolysis of cellobiose in *E. coli* [7, 28]. Recently, the introduction of a constitutive promoter in the cryptic *E. coli* operons *asc* and *chb* permitted its growth on cellobiose [38].

Secretion systems have been employed for delivering heterologous hydrolases out of the cell. The type II secretion system (TIISS), specifically, the *out* genes from *Erwinia chrysanthemi,* was used to secrete an endoglucanase (EGZ) or a pectate lyase (PelE) and an oligogalacturonide lyase (Ogl) in *E. coli* [7, 40]. However, the TIISS comprises approximately 15 genes, which must be expressed heterologously, and the genes that complete the extracellular secretion are highly species-specific [40]. A different strategy is that of a cell surface display that employs some outer-membrane proteins as anchors, thus "immobilizing" enzymes to the cell surface. Recently, the membrane protein poly-γ-glutamate synthase (PgsA) from *Bacillus subtilis* was used for attaching cellulases from *Clostridium cellulolyticum* [33], and the bacterial lipocalin (Blc) from *E. coli* was used for *Thermobifida fusca* (former *Thermomonospora fusca*) β-glucosidase display [37].

Among Gram-negative bacteria secretion systems, the type V is considered the simplest [5], and the adhesin that is involved in diffuse adherence (AIDA-I) of the diarrheagenic *E. coli* strain has been employed for heterologous protein secretion [14–17, 24]. The complete information for protein secretion is contained within a single gene that produces a multidomain pre-protein consisting of a signal peptide (SP), a passenger protein, and a translocation unit (TU) [5, 14]. The amino-terminal region codes for the SP that sends the pre-protein into the periplasmic space where it is cleaved by a signal peptidase [5, 14]. This action releases the passenger domain that is translocated into the extracellular space by the TU, which is formed by a linker region and a β-barrel (at the C terminus) [5, 14]. The linker region anchors the passenger protein to the cell surface. To display a heterologous protein, the native sequence of the passenger protein can be replaced by a heterologous protein sequence [14]. The TVSS has never been employed for the secretion of enzymes involved in the lignocellulose depolymerization for bio-fuels production.

In the present study, we report the fermentation of cellobiose into ethanol using the ethanologenic *E. coli* MS04 strain (MG1655: Δ*pflB*, Δ*adhE*, Δ*frdA*, Δ*xylFGH*, Δ*ldhA*, P*pflB*::pdc_{Zm}-$adhB_{Zm}$; [9, 23]). To achieve this goal we used the AIDA-I system, which is also known as the autodisplay system [14], to attach the *T. fusca*-derived β-glucosidase (BglC) to the outer membrane. BglC was selected because it has an optimum hydrolysis pH of 7, and even though BglC shows its maximum activity at 50 °C, the activity reported at 25 °C is acceptable [36]. These BglC characteristics are well matched with *E. coli* growth conditions (pH 7 and 37 °C). Furthermore, BglC cellobiase activity is inhibited by glucose only at concentrations above 50 g/l [8].

Materials and methods

Strains and plasmids

Escherichia coli DH5α was used for plasmid propagation during plasmid construction. When required, *E. coli* JM110 (*dam*$^-$) was employed for non-methylated plasmid production. Cell surface display analysis and fermentations were performed using the ethanologenic strain *E. coli* MS04 (MG1655: Δ*pfl*B, Δ*adh*E, Δ*frd*A, Δ*xyl*FGH, Δ*ldh*A, *Ppfl*B::*pdc-adh*B [9, 23]).

Plasmids and primers used for this study are shown in Table 1. Standard procedures were employed for plasmid preparation, restriction-enzyme digestions, transformations, and gel electrophoresis [34]. Each plasmid construction was verified by its restriction pattern in an agarose gel and by sequencing. Polymerase chain reaction (PCR) was performed using Elongase Enzyme Mix (Invitrogen, Carlsbad, CA, USA). During *bglC* gene amplifications, PCR was supplemented with dimethyl sulfoxide (DMSO, 10 %) because of its high percentage of GC (68 %). DNA manipulations using kits (PCRs, site-directed mutations and ligations) were conducted according to supplier protocols.

The plasmid pTrc99A was used for protein expression [1]. Using site-directed mutagenesis, we modified the *Nco*I site (CCATGG) at position 265 to *Nde*I (CATATG) and deleted the *Nde*I site at position 2699, thus obtaining plasmid pTrc99A2. These changes were performed using the Quik-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The *Nco*I site was modified using D1Ptr and R1Ptr primers, whereas the *Nde*I site was modified using D2Ptr and R2Ptr primers (see Table 1 for primer sequences).

The SP and the TU of AIDA-I were PCR-amplified using the pJM7 plasmid as a template [24, 25]. Amplification of the *bglC* gene was conducted using the pNS6 plasmid as a template [36]. The SP was amplified using the DPSNde and RPSpol primers, amplification of *bglC* was performed using the DBglCBgl and RBglCSac2 primers, and the TU was amplified using the DAidaSac and RAI-Xba1 primers. Due to the sequences coding for the SP, the TU and *bglC* have the majority of the restriction sites that are present in the pTrc99A2 polylinker, the reverse primer for SP amplification introduced the *Bgl*II and the *Sac*I sites,

Table 1 Plasmids and primers used in this study

Plasmid	Description	Source
pTrc99A	Designed for IPTG-inducible expression of proteins under hybrid *trp/lac* promoter. Amp^r	[1]
pTrc99A2	pTrc99A derivative modified for *Nde*I cloning instead of *Nco*I	This study
pJM7	Designed for heterologous secretion of cholera toxin B subunit using the autotransporter AIDA-I	[24]
pNS6	pET-26b(+) derivative employed for BglC heterologous production in *E. coli*	[36]
pAIDABglC	Designed for heterologous secretion of BglC using the autotransporter AIDA-I	This study
pAIDABglCRHis	pAIDABglC derivative that introduce a His-tag between BglC and AIDA-I linker	This study

Primer	Sequence[a]
D1Ptr	5′ CAC ACA GGA AAC AGC ATA TGG AAT TCG AGC TCG 3′
R1Ptr	5′ CGA GCT CGA ATT CCA TAT GCT GTT TCC TGT GTG 3′
D2Ptr	5′ CGG TAT TTC ACA CCG CAC ATG GTG CAC TCT C 3′
R2Ptr	5′ GAG AGT GCA CCA TGT GCG GTG TGA AAT ACC G 3′
DPSNde	5′ TAA GGA TGA CAT ATG ATT AAA TTA AAA TTT GGT GTT TTT 3′
RPSpol	5′ CAA ATC GAG CTC AGA TCT AGG TGT TCC ATG TGC GTA TGC TG 3′
DBglCBgl	5′ GAG ATA GAT CTA TGA CCT CGC AAT CGA CG 3′
RBglCSac2	5′ CTG GCG GAG AGC TCC TGT CCG AAG ATT CCC C 3′
DAidaSac	5′ CCT GGT GAG CTC AAT CCT ACA AAA GAA AGT GCA G 3′
RAIXba1	5′ GCC GGC TCT AGA TCA GAA GCT GTA TTT TAT CCC 3′
RBglCHis[b]	5′ TGC GAG CTC **GTG GTG GTG GTG GTG GTG** CTG TCC GAA GAT TCC CCC GTT GC 3′

[a] Restriction sites employed during plasmid construction are underlined

[b] His-tag sequence is indicated with *bold*

thus making it easier to clone *bglC* subsequently. Furthermore, because the pJM7 plasmid has an *Nde*I site at position 50, which is inside the SP (5′-GCA TAT GCA CAT GGA-3′) and 14 bases prior to the signal peptidase site, the reverse primer introduced a silent mutation that eliminated *Nde*I (5′-GCA TAC GCA CAT GGA-3′).

Following PCR amplifications, each domain was cloned sequentially. First, the SP was digested using NdeI and SacI and cloned into pTrc99A2. Next, employing BglII and SacI, *bglC* was cloned into this plasmid. Finally, the TU was cloned into this derivative plasmid using SacI and XbaI to obtain the pAIDABglC plasmid (Fig. 1). A version of pAIDABglC with a His-tag sequence between *bglC* and the TU was also constructed.

Using pAIDABglC as a template, *bglC* was PCR-amplified using the DBglCBgl and RBglCHis primers. The endogenous *bglC* gene in pAIDABglC was then removed using the endonucleases BglII and SacI. Next, the digested plasmid was agarose-gel purified. Finally, the PCR product was digested using the same restriction enzymes and cloned to produce the pAIDABglCRHis plasmid (Fig. 1).

Whole-cell protease treatment and OMP purification

Whole-cell protease treatment was adapted from a protocol originally described by Maurer et al. [24], and outer membrane proteins (OMP) were prepared as a Triton X-100 insoluble fraction as described by Puente et al. [32]. Cells were grown overnight in 5 ml of Luria broth (LB) at 37 °C and 300 rpm. The overnight culture was used to inoculate 25 ml of LB, bringing to 0.05 OD_{600}, and the cells were induced with 10 μM isopropyl *β*-D-1-thiogalactopyranoside (IPTG; Sigma, St. Louis, MO, USA) when they reached 0.5 OD_{600}. When the cells reached 2.5 OD_{600}, 4 ml of the culture was concentrated in a 1.5-ml microcentrifuge tube at room temperature (RT), and the cell pellet was washed twice with 1 ml phosphate-buffered saline (PBS). The cell pellet was then resuspended in 950 μl PBS and 50 μl 50 mg/l trypsin (Sigma) was added. Following 10-min incubation at 37 °C, the reaction was stopped by washing the cells twice with 1 ml PBS.

To proceed to OMP purification, trypsin-treated and non-treated cell pellets were resuspended in 500 μl 10 mM Na_2HPO_4, pH 7.2, and disrupted using sonication. Cell debris and intact bacteria were removed by centrifuging for 2 min at 13,400×*g* in a microfuge at room temperature. The membrane fraction was recovered by centrifuging the supernatant at 13,400×*g* for 30 min in a microfuge at 4 °C and then resuspended in 500 μl 2 % Triton X-100/10 mM Na_2HPO_4, pH 7.2. After incubating at 37 °C for 30 min, the Triton-X-100 insoluble fraction was recovered by centrifuging at 13,400×*g* for 30 min in a microfuge at

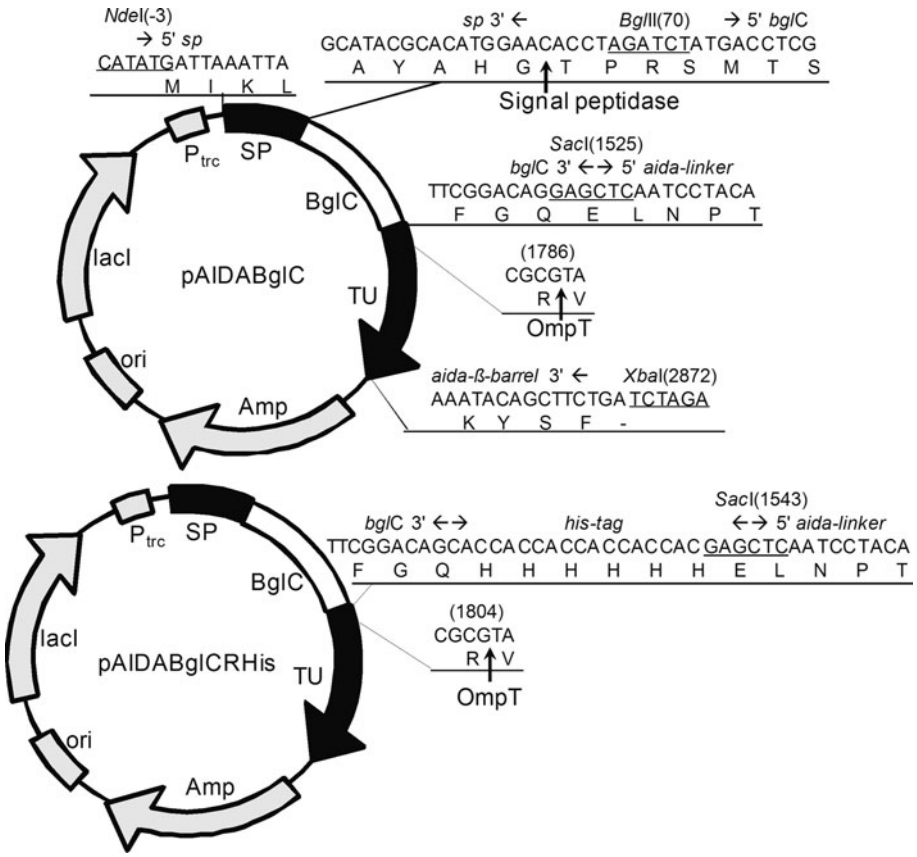

Fig. 1 Structure of the AIDA-BglC system. The borders between each domain are given as sequences, and are indicated by *horizontal arrows*. Restriction sites used for plasmid construction are *underlined*. Protease cleavage site for OmpT (in the linker region) and the one for signal peptidase is indicated with *vertical arrows*. SP, signal peptide; BglC, *T. fusca* *β*-glucosidase; TU, translocation unit, which is formed by a linker region and a *β*-barrel. The passenger protein (BglC) is attached to the cell surface by the linker region

4 °C. This insoluble fraction was washed with 500 µl 10 mM Na_2HPO_4, pH 7.2, and recovered by centrifuging at 13,400×*g* for 30 min in a microfuge at 4 °C. Finally, the OMPs were resuspended in 50 µl PBS pH 7.4 for SDS-PAGE analysis.

Western-blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12 % gels, as described by Laemmli [19]. Samples were mixed with 2× loading buffer, heated in boiling water for 5 min, and subjected to electrophoresis. Gels were electroblotted onto a nitrocellulose membrane (Amersham Hybond-ECL, GE Healthcare Waukesha, WI, USA) and probed using a Penta-His horseradish-peroxidase-conjugated antibody (Penta-His HRP Conjugate, Qiagen, Hilden, Germany).

Culture conditions

For stock culture preparation, *E. coli* MS04 was transformed with the plasmid and grown in a plate of LB agar. Later, a single colony was selected and grown overnight in a flask containing LB. Finally, the cells were stored in glycerol (40 % final) at −70 °C. During all cultivation steps, plasmid-carrying *E. coli* MS04 were grown in 100 mg/l carbenicillin.

Batch fermentations were conducted in mineral AM1 medium [22] employing glucose (J.T. Baker, Avantor Performance Materials, Phillipsburg, NJ, USA) and/or cellobiose (Sigma) as carbon sources and supplemented with 0.1 g/l citrate (J.T. Baker) and 2 g/l sodium acetate (J.T. Baker; [9]). Cellobiose was filter-sterilized to avoid hydrolysis. Culturing was performed in Fleakers mini-fermentors [4] containing 200 ml of mineral AM1 medium, without aeration, at pH 7, 37 °C, and 150 rpm. Automatic additions of 2 N KOH preserved a constant pH.

Prior to fermentations, the cells were adapted to grow in mini-fermentors. First, a sample from a stock culture was grown overnight in a 125-ml flask containing 25 ml LB at 37 °C and 300 rpm. Later, a mini-fermentor with 20 g/l of carbon source was brought to 0.1 OD_{600} with the overnight and the cells were grown until 1 OD_{600}. Finally, the culture was aliquoted into cryotubes containing glycerol (40 % final) to a final volume of 1.4 ml and stored at −70 °C.

To develop the inoculum for fermentation, one cryotube containing cells adapted to mini-fermentors was seeded into a mini-fermentor with 20 g/l of carbon source. When the cells reached an OD_{600} of almost 0.5, they were centrifuged for 10 min at 4,000×*g* and inoculated, at an initial OD_{600} of 0.1, into mini-fermentors containing 40 g/l of

carbon source. When cellobiose was employed, the cells were induced from the beginning using 10 μM IPTG. The inoculum for fermentations employing a mix of glucose (20 g/l) and cellobiose (20 g/l) was developed using glucose.

Enzyme assays during fermentation

Cell surface-attached *β*-glucosidase was assayed using *p*-nitrophenyl-*β*-*d*-glucopyranoside (pNPG; Sigma) and cellobiose. Cells were cultivated in mini-fermenters with 40 g/l of carbon source using the same conditions as described above, and they were allowed to grow until they reached an OD_{600} of 1. During pNPG assays, 3.75 ml cells was recovered by centrifuging in a microfuge at 9,300×*g* (2 min, RT). The cell pellet was washed twice (RT) with 50 mM phosphate buffer (pH 7) and resuspended in 3.65 ml of the same buffer. The cell suspension was then allowed to equilibrate to the assay temperature (37 °C) for 5 min. The reaction was initiated with the addition of 100 mM pNPG (100 μl), and the kinetics were followed by taking samples (of 750 μl each) at regular time intervals. The reaction was terminated by adding 2 M Na_2CO_3 (250 μl to each sample). The absorbance, at 400 nm, was measured after cells were centrifuged in a microfuge at 9,300×*g* (2 min, RT), and enzyme activity was calculated with the extinction coefficient for *p*-nitrophenol (18.5 mM^{-1}, [36]). One pNPGase unit was defined as the amount of BglC attached to the cells that catalyzes the formation of 1 μmol pNP per minute.

During cellobiose assays, 2 ml of cells was recovered by centrifuging in a microfuge at 9,300×*g* (2 min, RT). The cell pellet was washed as described previously and resuspended in 920 μl 50 mM phosphate buffer (pH 7). The cell suspension and cellobiose stock were allowed to equilibrate to the assay temperature (37 °C) for 5 min. The reaction was initiated by adding 50 g/l cellobiose (80 μl), and the kinetics were followed by sampling 200 μl at regular time intervals. To stop the reaction, samples were centrifuged in a microfuge at 13,400×*g* (2 min, RT), and the supernatant was then boiled in a water bath for 5 min. Finally, the glucose concentration in the supernatant was measured to calculate activity. One cellobiase unit was defined as the amount of BglC attached to the cells that catalyzes the formation of 1 μmol of glucose per minute.

Effect of ethanol on the activity and stability of MS04-attached BglC

Escherichia coli MS04 cells carrying the pAIDABglCRHis plasmid were grown overnight in 5 ml LB at 37 °C and 300 rpm. The overnight culture was then used to bring 50 ml of LB to 0.05 OD_{600}, and when the culture reached 0.5 OD_{600}, the cells were induced using 10 μM IPTG. When cells reached an OD_{600} of 2.5, they were harvested by centrifuging at 4,000×*g* (10 min, RT) and the cell pellet was washed twice with 10 ml of 50 mM phosphate buffer (pH 7). Finally, the cell pellet was concentrated in 5 ml of 50 mM phosphate buffer (pH 7), and the cellobiase and pNPGase activities were measured as described above after appropriate dilution. The cell protein was estimated using a standard curve of protein concentration versus OD_{600} (1.0 OD_{600} corresponds to 0.27 mg of cell protein per milliliter).

The effect of ethanol on BglC activity was measured using cellobiose as a substrate. The reactions were performed as described previously and employed 0.1 U (cellobiase) of the cell concentrate per milliliter (0.37 U/mg of cell protein or 0.27 U/mg of dry cell weight) with different ethanol concentrations in 1 ml of total volume. To measure the stability of BglC in the presence of ethanol, 0.4 U (pNPGase) of the cell concentrate per milliliter (1.48 U per mg of cell protein or 1.08 U per mg of dry cell weight) mixed with different ethanol concentrations in 1.5 ml of total volume was incubated at 37 °C. Samples of 50 μl were taken at regular intervals for measurements of residual activity, employing pNPG as a substrate and using the same conditions as described previously. In both cases, the activities are shown as relative activities, with the control without ethanol set to 100 %.

Analyses

Growth was determined spectrophotometrically as optical density at 600 nm (DU-70, Beckman Instruments, Inc., Fullerton, CA, USA) and converted to dry cell weight (DCW) per liter using a calibration curve (1 optical density = 0.37 g_{DCW}/l). Samples were centrifuged (RT) and the cell-free culture broth was frozen until analysis. The concentration of glucose was measured with an enzymatic analyzer (Model 2700, YSI Inc., Yellow Springs, OH, USA), whereas the cellobiose concentration was measured employing the 3,5-dinitrosalicilic acid (DNS) method as described by Miller [27]. When fermentations were carried out with a mix of sugars, their concentrations were measured with an HPLC system consisting of 600E quaternary bomb, 717 automatic injector, and 2410 refractive index (Waters, Millipore Co. Milford, MA, USA) employing an Aminex HPX-87P column (300 × 7.8 mm; 9 μm) (Bio-Rad, Hercules, CA, USA) with H_2O as mobile phase with a flow of 0.6 ml/min at 85 °C. The ethanol was analyzed by gas chromatography using *n*-butanol (1 %) as internal standard (6850 Series GC System, Agilent, Wilmington, DE, USA). Concentrations of cell biomass, ethanol, and sugars were corrected for fermentation volume changes caused by the addition of base during the cultures.

Specific growth rates (μ) were calculated as the slope of linear regressions of the cell mass natural log versus time during the exponential growth phase. The ethanol yield (g ethanol/g carbon source) was estimated as a percentage of the theoretical maximum, taking into account that the theoretical yields with glucose and cellobiose are 0.51 and 0.54, respectively. All cultures and enzyme assays were carried out at least in triplicate (average and standard deviations are shown in plots and tables). Graphpad Prism software (Graphpad, San Diego, CA, USA) was used to perform an ANOVA (analysis of variance) and a Student's *t* test for comparison of data presented in Tables 2 and 3, respectively. Means were judged significant different when *p* values were ≤ 0.05.

Results

Construction of the AIDA-BglC secretion system

To modulate the secretion of BglC using AIDA-I, the pTrc99A plasmid that contains the IPTG-inducible *trc* promoter was chosen as the expression vector; this vector is a medium copy number plasmid with about 30 copies per chromosome [1]. The polylinker has the *Nco*I (CCATGG) site for cloning at its 5′ end. However, the G nucleotide, after the ATG, modifies the codon for the second amino acid of the SP (5′ATG ATT AAA…). Because the amino acid sequences of SPs are very important targets during protein transport to the periplasmic space, it is necessary to retain their intact sequences. For this reason, *Nco*I was substituted with *Nde*I (CATATG) using site-directed mutagenesis. Consequently, the *Nde*I site, localized at position 2699 in pTrc99A, had to be eliminated using a site-directed mutation, thus obtaining the derivative plasmid pTrc99A2. Figure 1 depicts the chimeric proteins and the important restriction sites employed during the construction of the pAIDABglC and pAIDABglCRHis plasmids. The figure also shows the sequences at the borders between each domain, the position of the His-tag in pAIDABglCRHis, and the OmpT protease-cleavage site. The calculated molecular weight of the chimeric protein, without the SP, is 103 kDa, and it was named P103.

Whole-cell protease treatment analysis

Trypsin cleaves proteins on the C-terminal side of lysine and arginine amino acid residues. By taking advantage of this characteristic, proteins attached and exposed externally on the cell surface can be removed using trypsin. The linker region of the AIDA-I system and BglC have many sites that are susceptible to trypsin hydrolysis. To demonstrate that BglC is displayed on the cell surface, whole cells of pAIDABglCRHis plasmid-transformed *E. coli* MS04 were treated with trypsin. Cells carrying pTrc99A2 were used as a negative control. After growing, the cells were harvested, "shaved" with trypsin and subjected to OMPs purification.

Figure 2a shows a polyacrylamide gel with the OMPs purified from trypsin-treated or untreated cells. In contrast to the negative control, the cells expressing the autodisplay system without the proteolytic treatment showed a band at the level of the 100-kDa protein marker. When the cells were treated with trypsin, this P103 protein band disappeared. The OMPs were transferred to a nitrocellulose membrane and probed with an anti-His antibody and a signal that corresponded to protein P103 was revealed (Fig. 2b). The bands observed in Fig. 2a at approximately 35 kDa correspond to the OmpF and OmpA. OmpC, which migrates together with OmpF, is not found in the figure because during the xylose growth adaptation, the parental *E. coli* strain JU15 [23] lost a 25,335-bp region of the genome that contains the *ompC* gene (unpublished data).

Cellobiose fermentation and BglC activity

To test whether the BglC attached to the surface was functional and that the introduction of the His-tag to

Table 2 Growth and kinetic parameters of *E. coli* MS04 and derivatives

Plasmid	Carbon source	μ (h^{-1})	Ethanol yield (% of the maximum theoretical)	Cell mass[a] (g_{CDW}/l)
Without plasmid	Glucose	0.306 (0.003)	93 (1.6)	1.87 (0.17)
pTrc99A2	Glucose	0.265 (0.006)	84 (1.9)	1.47 (0.21)
pAIDABglCRHis	Glucose	0.159 (0.012)	82 (1.4)	0.93 (0.41)
pAIDABglCRHis	Glucose and cellobiose	0.167 (0.004)	80 (0.8)	1.28 (0.11)
pAIDABglCRHis	Cellobiose	0.121 (0.004)	81 (1.2)	1.14 (0.02)
pAIDABglC	Cellobiose	0.127 (0.011)	84 (1.8)	1.02 (0.16)

Values in *parenthesis* indicate SD

[a] Cell mass was obtained at the onset of the stationary phase

Table 3 BglC activity with whole cells during their growth in fermentation conditions

Plasmid	MS04 grown in	pNPGase activity (U/g_{CDW})	Cellobiase activity (U/g_{CDW})
pAIDABglCRHis	Glucose	4.6 (0.4)	ND
pAIDABglCRHis	Cellobiose	42.4 (3.3)	171 (17)
pAIDABglC	Cellobiose	47.9 (2.2)	173 (6.3)

Values in *parenthesis* indicate SD

ND not determined

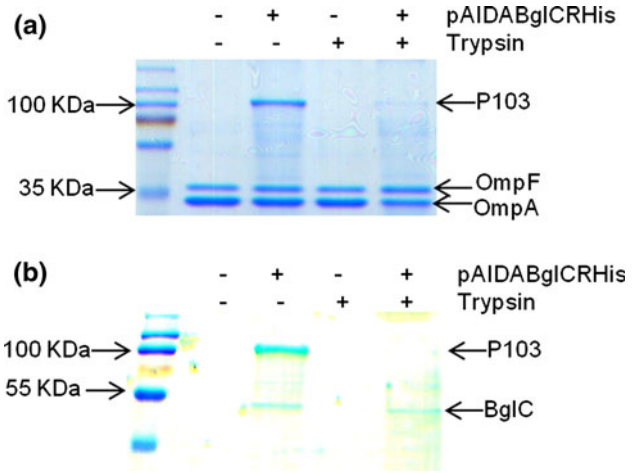

Fig. 2 Protease treatment analysis of *E. coli* MS04 transformed with pAIDABglCRHis. **a** SDS-PAGE and **b** Western blot showing protease accessibility of BglC attached to the surface of the outer membrane. PM is the protein marker; P103 is the protein product of pAIDABglCRHis without the SP, and represents the sum of AIDA-I TU and BglC. The plasmid pTrc99A2 was employed as a negative control. The symbol *minus* in the *line* of pAIDABglCRHis means proteins from cultures carried out with MSO4/pTrc99A2

pAIDBglCRHis did not affect BglC properties, we fermented 40 g/l cellobiose as the sole carbon source. Figure 3a shows cell growth, cellobiose consumption, and ethanol production during the fermentation of *E. coli* MS04 cells carrying pAIDABglC or pAIDABglCRHis. The specific growth rate (μ), ethanol yield and maximum cell biomass reached are shown in Table 2. During these fermentations, we observed that MS04 transformed with either of the plasmids displayed almost identical kinetics (Fig. 3a), with a μ of 0.121–0.127 h^{-1} (Table 2). Similarly, both cultures reached a similar maximum cell mass, depleted the cellobiose at almost the same time, and produced similar titers of ethanol (Fig. 3a; Table 2). These results prove that the TVSS, specifically the AIDA-I system, permits the display of BglC in an active form and that *E. coli* MS04/pAIDABglC or pAIDABglCRHis can ferment cellobiose into ethanol.

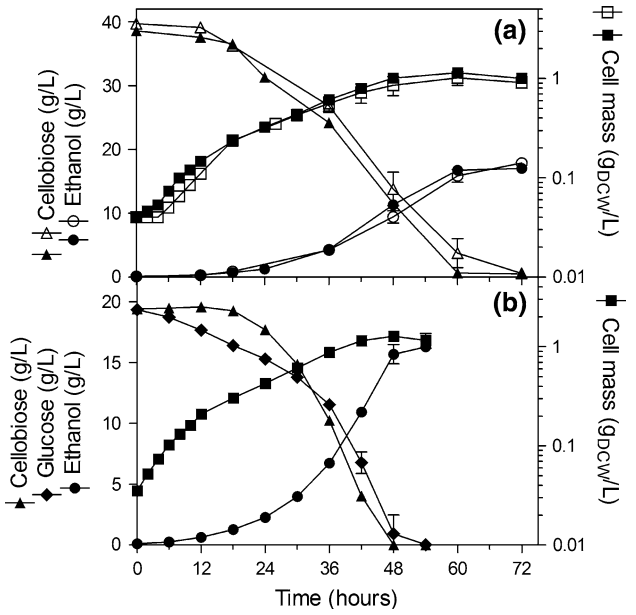

Fig. 3 **a** Cellobiose fermentation kinetics by MS04/pAIDABglC (*empty symbols*) and MS04/pAIDABglCRHis (*filled symbols*). **b** Fermentation kinetic of a mix of cellobiose and glucose by MS04/pAIDABglCRHis

Taking advantage of the fact that BglC cellobiase activity is not inhibited in the presence of 20 g/l glucose [8], we cultured cells using a mix of cellobiose (20 g/l) and glucose (20 g/l). The cell growth, cellobiose and glucose consumption, and ethanol production during the fermentation are shown in Fig. 3b. During the first 18 h of fermentation, glucose was consumed in preference to cellobiose; subsequently, the rate of cellobiose consumption increased dramatically and cellobiose was depleted, prior to glucose, within 48 h of fermentation. The μ, ethanol yield, and maximum cell biomass reached are shown in Table 2.

To determine whether the expression level of the AIDA-BglC system hampered cell growth, cultures in 40 g/l glucose of cells carrying either pAIDABglCRHis, the empty vector (pTrc99A2), or no plasmid were performed. Using data from the cultures of *E. coli* MS04/pAIDABglCRHis in cellobiose for comparison, Fig. 4 shows the cell growth, carbon source consumption, and ethanol production during the fermentations. Table 2 shows the μ, ethanol yield and maximum cell biomass reached during these cultures. The cells without plasmid displayed the highest rate of exponential growth, followed by *E. coli* MS04/pTrc99A2. The cells with plasmid pAIDABglCRHis, in glucose, displayed a significant higher rate of exponential growth than the cells grown in cellobiose, but a significant lower rate than MS04 cells transformed with pTrc99A2.

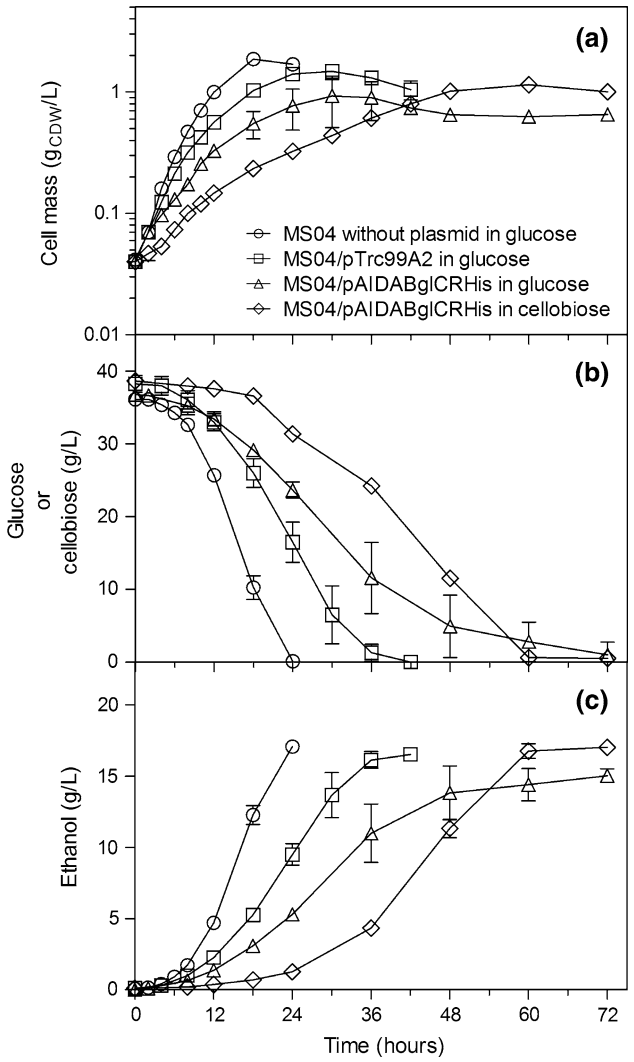

Fig. 4 Glucose fermentation by MS04-carrying plasmid pTrc99A2, pAIDABglCRHis, or without plasmid. The data from the cultures with *E. coli* MS04/pAIDABglCRHis in cellobiose are included for comparison

As expected, *E. coli* MS04 without plasmid had the highest carbon source consumption rate, consuming all glucose within 24 h (Fig. 4b). The cells carrying pTrc99A2 required 18 further hours to consume the glucose, and cells carrying pAIDABglCRHis in glucose or cellobiose required two further days to consume the carbon sources. Importantly, even though the cells carrying pAIDABglCRHis and grown in glucose showed a higher μ than the cells grown in cellobiose, both required the same time to consume the carbon source. MS04 without plasmid reached a titer of 17.1 g/l ethanol with a yield of 93 % of the theoretical maximum (Table 2). The cells carrying plasmids reached titers of 15–17 g/l (Fig. 4c), but their ethanol yields were 81–84 % of the theoretical maximum (Table 2).

To determine the extent of BglC activity in the cells during fermentation, the cells carrying pAIDABglC or pAIDABglCRHis were harvested when they reached 0.37 g_{CDW}/l (1 DO_{600}) and their activities measured using pNPG and cellobiose as substrates. During cellobiose fermentation, the cells were induced from the beginning with IPTG to consume the carbon source. The BglC kinetic parameters obtained with whole cells are shown in Table 3. The cells grown in cellobiose and carrying any of the plasmids had no significant differences in BglC activity, and in both cases, a three-fold higher cellobiase activity was observed than pNPGase activity. Although the cells grown in glucose were not induced, they displayed pNPGase activity because the AIDA-BglC system is controlled by the strong promoter *trc*, which has a high basal level of transcription [1]. However, the cellobiase activity of MS04/pAIDABglCRHis grown in glucose was not determined because it was too low, and the glucose released from the cellobiose during the assay did not accumulate because the cells consumed it.

Effect of ethanol on the activity and stability of MS04-attached BglC

To determine the inhibition and stability of BglC in the presence of ethanol, MS04/pAIDABglCRHis cells were grown in a flask containing LB, and the cells were induced using IPTG for BglC production. The cellobiase activity of BglC was measured in the presence of different concentrations of ethanol. As shown in Fig. 5a, the relative activity decreased slightly, in a linear trend, with increasing ethanol concentration. To measure BglC stability, the cells were incubated at 37 °C with different concentrations of ethanol, and the residual activity was measured using pNPG. As shown in Fig. 5b, BglC was very stable in the presence of 40 g/l of ethanol after 2 days of incubation and even 120 g/l ethanol had little effect on BglC stability.

Discussion

Cellobiose plays an important role during cellulose hydrolysis because it inhibits directly the action of endo- and exocellulases [3]. Although the inhibition can be alleviated by adding β-glucosidases [3], it would be more advantageous to develop an ethanologenic *E. coli* strain with the capacity to consume cellobiose. This would be one step in the consolidated bioprocessing (CBP) where the fermentation and depolymerization process could be conducted in a single reactor without adding β-glucosidases and cellulases.

The characteristics of the enzymes that would be employed in CBP have to be well matched with the

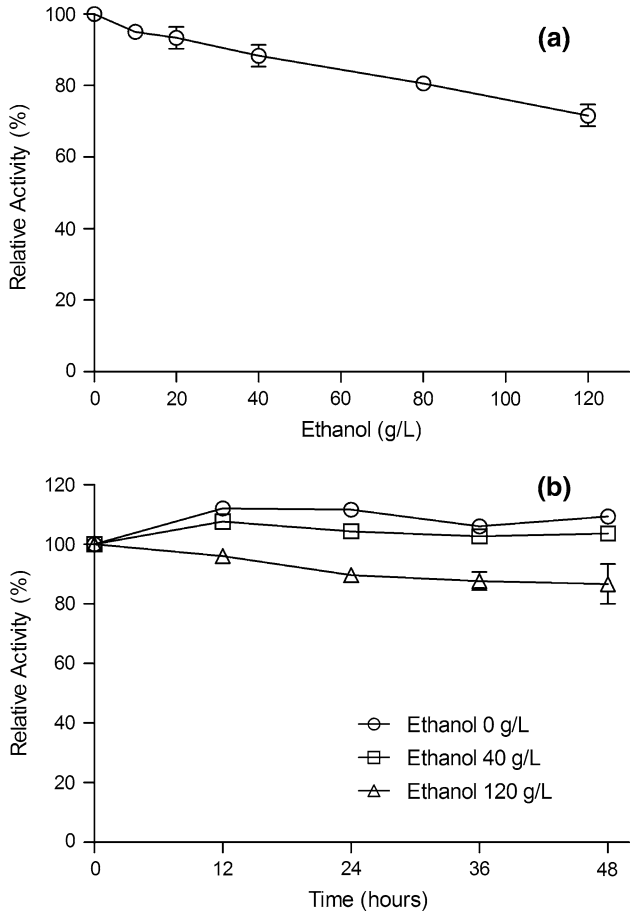

Fig. 5 Effect of ethanol at different concentrations on the **a** activity and **b** stability of BglC attached to the outer membrane of MS04/pAIDABglCRHis

microorganism growth conditions. For that reason, in this study, the ethanologenic *E. coli* strain MS04 was provided with the ability to consume cellobiose by secreting BglC from *T. fusca*. During cellobiose fermentation experiments, the cells carrying the AIDA-BglC system were induced with 10 μM IPTG; previously, in flask cultures with glucose as the carbon source, it was found that induction with higher IPTG concentration the cell growth was hindered.

After constructing the AIDA-BglC system, the OMPs of MS04 cells transformed with pAIDABglCRHis were analyzed using SDS-PAGE revealing the protein P103. This protein disappeared following protease treatment and MS04 cells that carried this plasmid fermented cellobiose to ethanol efficiently. Together, these data demonstrate that an active form of BglC was attached to the external surface of the outer membrane. To confirm that the P103 band corresponded to the chimeric protein formed from the TU and BglC, we performed a Western blot. A signal was observed in the lane corresponding to the cells that expressed the AIDA-BglC system that were not treated with trypsin (Fig. 2b). Additionally, a weak protein band is observed below the 55-kDa marker, which corresponded to BglC (53.4 kDa) that detached from the outer membrane [36]. OmpA has a trypsin-susceptible periplasmic moiety; hence, OmpA from cells with a disrupted outer membrane becomes sensitive to trypsin and can be used to evaluate outer membrane integrity [20, 24]. A comparison of the OMPs following trypsin treatment does not reveal any change in OmpA, proving that the anchored BglC did not affect the integrity of the cell envelope.

The first attempt to produce ethanol from cellobiose using an ethanologenic *E. coli* was performed by Moniruzzaman et al. [28] and by expressing the *Klebsiella oxytoca casAB* operon in plasmid, *E. coli* K011 fermented 90 g/l cellobiose and produced 45.4 g/l of ethanol with a theoretical yield of greater than 94 %. Notably, in this study, we obtained a yield of 84 %, but we used a mineral medium supplemented with 40 g/l cellobiose instead of a rich medium (LB) as was used by Moniruzzaman et al. [28]. Moreover, a major disadvantage of employing rich medium is the increase in process cost. Recently, Vinuselvi and Lee [38] introduced a constitutive promoter on the cryptic *chb* and *asc* operons of *E. coli* MG1655 and adapted this strain for cellobiose consumption. The new strain, CP12CHBASC30, was capable of growth under aerobic conditions and used 4 g/l cellobiose in mineral medium, consuming 3 g/l and reaching 1.7 DO_{600} in 24 h, whereas in this study, *E. coli* MS04 fermented approximately 7 g/l cellobiose directly to ethanol within 24 h. Furthermore, in our opinion, the attachment of a *β*-glucosidase to the cell surface, such as BglC, which has the capacity to hydrolyze a wide range of cellooligosaccharides [36, 37], is more efficient than a simple capacity to metabolize one disaccharide (cellobiose) only.

The potential of *T. fusca*-derived BglC for metabolizing cellobiose by *E. coli* has been demonstrated recently by Tanaka et al. [37]. BglC was attached to the cell surface of *E. coli* JCM20137, and subsequently, the cells were grown in mineral medium containing 2 g/l cellobiose under aerobic conditions and reached 1 DO_{600} in 20 h. In our study, using the AIDA-I system to induce BglC surface display, the fermentation of 40 g/l cellobiose into ethanol was performed in a mineral medium by an *E. coli* strain that was previously metabolically engineered to produce ethanol as the sole fermentation product [9, 23]. Within 24 h, the strain MS04/pAIDABglCRHis reached approximately 0.9 DO_{600} (0.33 g_{CDW}/l), and within 60 h, the maximum cell biomass was 2.8 DO_{600} (1 g_{CDW}/l, Fig. 3). The cells required 72 h to consume 40 g/l of cellobiose (Fig. 3) and produced 17.0–17.9 g/l of ethanol (Fig. 3) with a yield that was 81–84 % of the theoretical maximum (Table 2). To our knowledge, this is the first report that shows the fermentation of cellobiose into ethanol by an ethanologenic *E. coli* strain using a mineral medium. Furthermore, these

results demonstrate that BglC is compatible with *E. coli* growth conditions, as reported by Tanaka et al. [37], and demonstrate the great potential of the TVSS (AIDA-I) as a tool for depolymerase secretion in the area of lignocellulosic bio-commodities production.

It is well known that commercial β-glucosidases, including the widely employed β-glucosidases from *Aspergillus niger*, are inhibited profoundly by glucose [3]. However, Ferchak and Pye [8] reported that BglC was not inhibited during cellobiose hydrolysis until 50 g/l of glucose was present in the reaction. This characteristic can be employed, for example, in a pre-saccharification and subsequent simultaneous saccharification and fermentation process (SSFP): i.e., the glucose released during the pre-saccharification step will not inhibit BglC attached to MS04, permitting the hydrolysis of the cellobiose released by the cellulases during the SSFP and eliminating the endo and exo-cellulases inhibition by cellobiose. To study the performance of BglC attached on the cells using a mix of glucose and cellobiose, fermentations with 20 g/l of each sugar were performed. As shown in Fig. 3b, BglC hydrolyzed the cellobiose and allowed the co-fermentation of both sugars within 48 h resulting in a yield of 80 % (Table 2). MS04/pAIDABglCRHis cells grown in the mixture of sugars consumed both carbon sources 12 h before the cells that were grown in cellobiose (Fig. 3a) and showed almost 40 % higher μ (Table 2) due to the presence of glucose in the mix. These results show the potential of BglC for use in pre-saccharification and subsequent SSFP, because BglC eliminated the cellobiose-induced inhibition of the endo- and exocellulases more efficiently than commercial β-glucosidases.

Plasmid synthesis and maintenance impose a metabolic burden to cells. For example, Martinez et al. [21] reported that the addition of pUC19 to the ethanologenic *E. coli* LY01 reduced the growth rate dramatically and reduced the ethanol yield by approximately 50 % compared to the unmodified LY01. To verify whether the introduction of plasmids for BglC secretion hampered MS04 growth, fermentations in glucose with pAIDABglCRHis or pTrc99A2 were performed. As expected, the transformation with the empty plasmid pTrc99A2 significantly reduced the μ and ethanol yield by 13 and 10 %, respectively, compared to MS04 without plasmid (Table 2). For this reason, the decrease in the ethanol yield observed with the cells carrying the AIDA-BglC system grown in cellobiose compared with MS04 without plasmid in glucose cannot be ascribed to expression and activity of BglC only. Indeed, the growth of MS04 with pAIDABglCRHis (a larger plasmid than pTrc99A2) in glucose significantly reduced the μ by 40 % compared with MS04 carrying pTrc99A2. Additionally, a comparison of the cell biomass (Table 2), shows that cells with the AIDA-BglC system grown in either glucose, cellobiose or the mix of sugars reached between 30 and 50 % significant less cell biomass than MS04 without plasmid.

During the BlgC activity assays, the cells carrying pAIDABglC or pAIDABglCRHis showed almost identical cellobiase and pNPGase activities, indicating that insertion of the His tag does not reduce BglC activity. Although we did not find more examples of β-glucosidase attachment in *E. coli* in the literature, this approach has been employed widely in *Saccharomyces cerevisiae*. McBride et al. [26] displayed the *Saccharomycopsis fibuligera*-derived β-glucosidase BGL1 on *S. cerevisiae*, thus obtaining the strain Y294[ysFI], which had an pNPGase activity of 112 U/g_{CDW} during exponential cell growth (0.155 h^{-1}) on cellobiose in anaerobic conditions. Interestingly, in our study, MS04 carrying pAIDABglC or pAIDABglCRHis and grown in cellobiose showed a μ of 0.121 and 0.127 h^{-1}, respectively, which are similar to those of Y294[ysFI] [26]. In the case of the pNPGase activities shown by MS04 carrying the AIDA-BglC system (Table 3), we observed 60 % lower activities than *S. cerevisiae* Y294[ysFI]. It is important to mention that the pNPGase activity of *S. cerevisiae* Y294[ysFI] was obtained during the exponential cell growth phase, whereas pNPGase activities shown in our study were obtained after exponential cell growth when the cells reached 0.37 g_{CDW}/l (1 DO_{600}; Fig. 3). Another important note is that *E. coli* MS04 possesses the outer membrane protease OmpT, which is reported to cleave surface-exposed passenger proteins [24]. In our case, the presence of OmpT did not interfere with the cell-surface display of BglC because the BglC provided sufficient activity to allow *E. coli* MS04 to grow on cellobiose.

Cellulases and β-glucosidases employed in CBP or SSFP must tolerate the presence of ethanol and other inhibitors to function. For this reason, and as a first approach, the study of BglC in the presence of ethanol was performed. Although MS04 produced less than 20 g/l of ethanol in our study, the inhibition and stability of cell-attached BglC by ethanol was evaluated at up to 120 g/l. The activity of BglC in the presence of 10 and 20 g/l of ethanol was decreased to 95 and 93 %, respectively (Fig. 5a), and the enzyme was stable when it was incubated at 37 °C for 48 h in medium containing 40 g/l ethanol (Fig. 5b), which is consistent with the results obtained during cellobiose fermentations because BglC activity and stability must be retained to hydrolyze cellobiose during culturing. At the maximum concentration of ethanol evaluated (120 g/l), BglC retained a relative activity of 72 % (Fig. 5a) and was quite stable (Fig 5b). Notably, during the stability experiments, the cells treated with or without ethanol lysed during the incubation. This finding could underlie the slight increase in relative activity in control- or 40 g/l ethanol-treated cells (Fig. 5b). It is possible that the cells retained sufficient intracellular BglC,

which provided the increase in activity when it was released into the medium following cell lysis. Although cells were lysed, the control BglC activity did not decrease during the experiment, indicating that the enzyme was stable even in the presence of proteases. Thus, the decrease in relative activity during the incubation with 120 g/l ethanol can be ascribed to the ethanol and not to proteolysis.

In conclusion, the TVSS (AIDA-I) allowed the secretion of *T. fusca*-derived BglC in *E. coli* MS04, thus permitting the fermentation of cellobiose into ethanol without adding external β-glucosidases and allowing the use of simple mineral media. Furthermore, the AIDA-I system attached BglC without affecting the outer membrane integrity. This approach is one important step in developing CBP. An important part of CBP is the use of cellulases that possess a pH and hydrolysis temperature that is compatible with the growth conditions of the ethanologenic microorganism. In addition, cellulases must retain activity and be stable in the presence of ethanol during fermentation. Because *E. coli* has an optimum pH and temperature for growth of 7 and 37 °C, respectively, suitable cellulases for *E. coli*-mediated CBP, such as BglC, are required. Finally, ethanologenic *E. coli* strain MS04 displaying BglC permits to carry out fermentations with a mixture of glucose and cellobiose, showing its potential for pre-saccharification and subsequent SSFP.

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