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Fermentation of cellobiose to ethanol by industrial *Saccharomyces* strains carrying the β-glucosidase gene (*BGL*1) from *Saccharomycopsis fibuligera*

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ABSTRACT

Constructs carrying the *Saccharomycopsis fibuligera* β-glucosidase gene (*BGL*1) under the control of a constitutive actin or a galactose-inducible promoter were introduced into eleven Saccharomyces strains. In ten of these recombinant strains, *BGL*1 expression driven by the actin promoter was between 1.6- and 18-fold higher than that obtained with the galactose-inducible promoter. Strains carrying the actin promoter yielded ethanol concentrations from cellobiose of between 0.5% and 14%, depending on their ability to accumulate Bgl1 (between 30 and 250 mU/mL) but also on their genetic background. Comparative analysis of a *S. cerevisiae* strain and its corresponding petite version showed similar ethanol yields, despite a 3-fold lower β-glucosidase production of the latter, suggesting that respiratory activity could be one of the factors influencing ethanol production when using carbon sources other than glucose. This study provides a selection of strains that may be good candidates as hosts for ethanol biosynthesis from cellulosic substrates.

1. Introduction

Bioethanol obtained from waste cellulosic materials is a promising renewable fuel source (Ragauskas et al., 2006; Sánchez and Cardona, 2008). Large-scale ethanol production involves the hydrolysis of cellulose into fermentable sugars, a process carried out with high efficiency by the synergistic cooperation of enzymes, including endoglucanases (hydrolyzing the internal bonds of cellulose), cellobiohydrolases (cleaving cellulose from reducing or non reducing ends to release cellobiose) and β-glucosidases (hydrolyzing cellobiose to glucose) (Fujita et al., 2004; Wen et al., 2010; Yanase et al., 2010a). β-Glucosidases are not only required for this last transformation, but also increase the efficiency of the previous steps when the three enzymes are combined together, since cellobiose is a potent inhibitor of endoglucanases and cellobiohydrolases (Bezerra and Dias, 2005; Du et al., 2010; Shen et al., 2008). Although β-glucosidases also undergo product inhibition by glucose (Andric et al., 2010), this problem can be overcome by simultaneous saccharification and fermentation (SSF) processes. Some recent advances in the field have shown that the expression of trifunctional minicellulosomes containing the three types of enzymes assembled through dockerin–cohesin interactions, are more efficient for ethanol production than minicellulosomes lacking β-glucosidases (Tsai et al., 2010; Wen et al., 2010). Moreover, β-glucosidase synthesis may be limiting for ethanol production, depending on the expression system (du Plessis et al., 2010).

Sequences encoding β-glucosidases from different origin have been cloned and expressed in yeast for SSF, including those from *Saccharomycopsis fibuligera* (du Plessis et al., 2010; Gundllapalli et al., 2007; Jeon et al., 2009; Shen et al., 2008; van Rooyen et al., 2005), diverse filamentous fungi (Fujita et al., 2004; Kitagawa et al., 2010; Kotaka et al., 2008; Tsai et al., 2010; Ueda and Tanaka, 2000; van Rooyen et al., 2005; Yanase et al., 2010a) and bacteria (Rajoka et al., 2003), all belonging to the glycosyl hydrolase family GH3 (Cantarel et al., 2009), although enzymes pertaining to other structural groups, as that from *Candida wickerhamii*, classified in family GH1, have also been tested (van Rooyen et al., 2005). Besides exploring the natural diversity of β-glucosidases, some effort has been directed towards the improvement of their characteristics by enzyme engineering. This has been achieved either by combining the catalytic core with a carbohydrate binding domain to increase the hydrolytic activity towards cellulosic substrates (Gundllapalli et al., 2007), by attaching a C-terminal dockerin to construct minicellulosomes (Wen et al., 2010), by addition of a cell-surface anchoring tag (van Rooyen et al., 2005) or by directed evolution to improve thermostability (Hardiman et al., 2010).

The β-glucosidase from *S. fibuligera* (Bgl1) has been successfully synthesized heterologously in *S. cerevisiae* conferring the transformant strain with cellobiose fermentation capabilities more efficiently than with enzymes from other origins (van Rooyen et al., 2005). Bgl1 from *S. fibuligera*, as other β-glucosidases from

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the GH3 family synthesized by *S. cerevisiae* transformants, is secreted and remains mostly cell-associated. The fibronectin-like C-terminal domain of Bgl1 has been proposed to mediate the cell-adhesion properties of the enzyme (Marin-Navarro et al., 2011), probably through some interaction with the carbohydrate matrix of the cell wall. The expression of *BGL*1 in previous studies was driven by the *GAL1/GAL10p* (Jeon et al., 2009), *GAL10/CYCp* (Marin-Navarro et al., 2011), and *PGK1p* (Gundllapalli et al., 2007; Shen et al., 2008; van Rooyen et al., 2005) promoters, and the secretion of the protein was directed by the α-mating-factor (Gundllapalli et al., 2007; Jeon et al., 2009), *Trichoderma reesei xyn2* (van Rooyen et al., 2005), or the *S. cerevisiae* var. *diastaticus STA1* (Marin-Navarro et al., 2011) signal factors. Most of the β-glucosidase encoding genes have been expressed in *Saccharomyces* laboratory strains, but industrial strains (Kotaka et al., 2008; Saitoh et al., 2008; Shen et al., 2008) or even yeasts from other genera, like *Kluyveromyces marxianus* (Yanase et al., 2010b) or *Issatchecnkia orientalis* (Kitagawa et al., 2010) have also been assayed for ethanol production. However, little is known about the performance of different host strains harbouring recombinant β-glucosidase genes in SSF. Factors such as ethanol tolerance or respiratory performance of the host strain may affect the final ethanol yield (Panoutsopoulou et al., 2001). Acetaldehyde derived from glycolysis is located at the metabolic crossroad of two competing pathways (fermentation and respiration) and can be either reduced to ethanol through a reversible reaction or oxidized to acetate that will be finally incorporated in the TCA cycle in the form of acetyl-CoA (van Dijken et al., 1993, Schüller, 2003). Therefore, respiratory metabolism may diminish ethanol production by diverting part of the acetaldehyde into the TCA cycle, or by acting as an electron sink for re-oxidized ethanol, which may be used as an alternative energy source.

The objectives of the current study were to compare *BGL*1 expression in different *Saccharomyces* strains when the gene was under the control of a galactose-inducible promoter or an actin promoter and to measure ethanol yields in different recombinant strains expressing *BGL*1 with cellobiose as a substrate. This information may be valuable to select strains as optimal hosts for further engineering experiments directed to convert cellulosic substrates into ethanol.

2. Methods

2.1. Yeast strains and transformation procedure

Saccharomyces strains used in this study as hosts for transformation are listed in Table 1. Yeast transformation was carried out by the lithium acetate procedure, as previously described (Gietz and Woods, 2002), with minor modifications. After heat shock for 40 min at 42 °C, cells were sedimented by centrifugation, resuspended in YPD (1% yeast extract, 2% peptone, 2% glucose) and incubated at 30 °C for 1 h. Transformants were selected on YPD-agar (2% bacteriological agar in YPD) plates supplemented with 50 mg/L geneticin (G-418).

2.2. Plasmid construction

The kanMX4 module, conferring resistance to geneticin in yeast, was excised with *Sma*I/*Sac*I from plasmid pFA6KanMX4 (Wach et al., 1994) and subcloned in the episomal vector Yeplac195 (Gietz and Sugino, 1988), generating plasmid pMCG3. The actin promoter *pACT1* was recovered from plasmid YEpACT-AMY (Rández-Gil et al., 1995) by digestion with *Sma*I/*Xba*I and cloned in the corresponding sites of plasmid pMCG3. The resulting construct pACT-Kan was used as a vector to insert the sequence encoding the β-glucosidase from *S. fibuligera* fused to the signal peptide of *S. cerevisiae STA1*. This sequence (SPS-Bgl1) was amplified from plasmid pSPS-Bgl1 (Marin-Navarro et al., 2011) by PCR using Pfu DNA polymerase (Fermentas) with primers JM697 (CGTCTGTCTAGAATGGTAGGCCT CAAAAATCCATATAC) and JM698 (GCTACCGTCGACTCAAATAGTAA ACAGGACAGATGTC), which contain the restriction sites (underlined) *Xba*I and *Sal*I, respectively. The oligonucleotide JM698 includes a stop codon (bold) at the end of the coding region of Bgl1, without specific transcription terminator sequences downstream. The PCR product was cut with *Xba*I/*Sal*I and cloned in pACT-Kan generating the construct pACT-SPS-Bgl1-Kan (Fig. 1a), which was checked by restriction analysis and by sequencing the entire SPS-Bgl1 region.

In order to express the *BGL*1 gene under the control of a galactose-inducible promoter, the pKS2 plasmid containing both the *STA1* gene under the control of the GAL10/CYC promoter and the

Table 1
β-Glucosidase activity obtained from the different transformants generated in this work.

Transformants[a]	Host strain	Source	Total β-glucosidase activity[b] (mU/mL of culture) [% cell attached][c]	
			GAL promoter	Actin promoter[d]
T500 g/T500	*S. cerevisiae* BY4741 (MATa *his3 leu2 met15 ura3*)	Lab strain	105 ± 5* [73 ± 2]	250 ± 30‡ [68 ± 3]
T74 g/T74	*S. cerevisiae* FJF135	Sherry wine[e]	<10	77 ± 11 [62 ± 3]
T75 g/T75	*S. cerevisiae* FJF305 ("flor" yeast)	Sherry wine [e]	<10	180 ± 14 [82 ± 1]
T370 g/T370	*S. cerevisiae* Y370	Bread[f]	68 ± 6 [75 ± 2]	139 ± 2‡ [78 ± 1]
T491 g/T491	*S. cerevisiae* LA1	Wine[g]	230 ± 20 [84 ± 1]	243 ± 5 [85 ± 1]
T499 g/T499	*S. cerevisiae* LA1 ρ^0	Wine[h]	<10	73 ± 7 [74 ± 2]
T111 g/T111	*S. cerevisiae* subtype *boulardii* Y111	Probiotic[i]	72 ± 3* [86 ± 2]	230 ± 20‡ [81 ± 2]
T101 g/T101	*S. bayanus* NYCY1324	Beer[j]	25 ± 2 [79 ± 1]	140 ± 7‡ [79 ± 1]
T28 g/T28	*S. pastorianus*	Beer[k]	33 ± 3 [62 ± 5]	73 ± 20† [69 ± 8]
T92 g/T92	*S. pastorianus* NYCY512	Beer[j]	21 ± 3 [72 ± 7]	33 ± 4† [54 ± 3]
T93 g/T93	*S. pastorianus* NYCY519	Beer[j]	38 ± 4 [75 ± 3]	190 ± 20‡ [68 ± 2]

[a] The transformants expressing *BGL*1 under the GAL promoter are denoted by the same code as those under the control of the actin promoter, but adding a final "g".
[b] Standard deviation of duplicates (*) or triplicates (all the rest) is indicated with ±.
[c] Percentage of the total β-glucosidase activity measured in the cell fraction.
[d] β-glucosidase activities significantly higher than those obtained with the GAL promoter are indicated with † (95% confidence) and ‡ (99% confidence). Student's *t*-test was not applied to T74, T75 and T499.
[e] Benitez et al. 1983.
[f] Isolate from sourdough, a gift from Dr. J.A. Prieto, IATA collection.
[g] Del Castillo 1985; Del Castillo 1992.
[h] Mitochondrial petite version of T491.
[i] Edwards-Ingram et al. 2007.
[j] NCYC: National Collection of Yeast Cultures, Norwich, UK (website address: http://www.ncyc.co.uk).
[k] *S. carlsbergensis* isolate CC3/2 from Cruzcampo breweries (Nilsson-Tillgren et al. 1981; Hansen et al., 1994; Børsting et al. 1997).

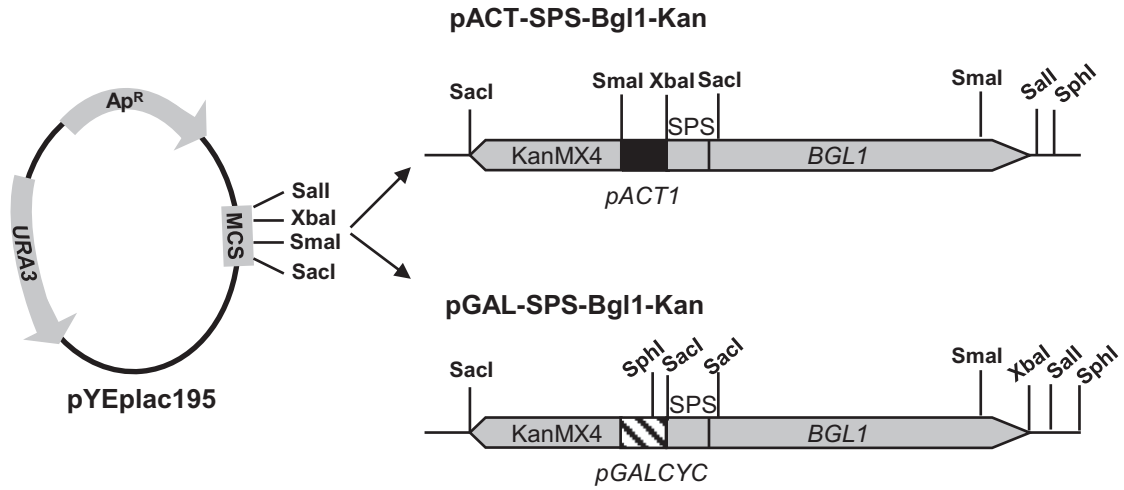

Fig. 1. Schematic restriction map of the constructs pACT-SPS-Bgl1-Kan and pGAL-SPS-Bgl1-Kan, both inserted in the multiple cloning site (MCS) of Yeplac195 (Gietz and Sugino 1988). SPS indicates the sequence encoding the signal peptide of *STA1* from *S. cerevisiae var. diastaticus*. pACT1 and pGALCYC represent the actin promoter from YEpACT-AMY (Rández-Gil et al. 1995) and the GAL10/CYC promoter from pKS2 (Latorre-García et al. 2008), respectively. ApR represents the gene conferring ampicillin resistance in *E. coli*. *URA3* is the allele encoding orotidine 5-phosphate decarboxylase, involved in the synthesis of uracil.

KanMX4 casette (Latorre-García et al., 2008) was used as the recipient vector. The *STA1* gene was removed from pKS2 by digestion with *Sph*I, and substituted by the *Sph*I fragment encoding the β-glucosidase from *S. fibuligera* fused to the signal peptide of *S. cerevisiae STA1*, obtained from pSPS-Bgl1. The construct with the right orientation of the *Sph*I fragment respect to the GAL10/CYC promoter was selected by analysis of the restriction pattern, and named pGAL-SPS-Bgl1-Kan (Fig. 1a).

2.3. Enzymatic assays

β-Glucosidase activity was assayed using the substrate analogue *p*-nitrophenyl glucopyranoside, as previously described (Arrizubieta and Polaina, 2000). One unit of activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute. Three different colonies obtained from each transformation (or two, in the cases specified in Table 1) were inoculated in YPD with 50 mg/L geneticin and, in the case of the strains expressing *BGL*1 under the control of the GAL promoter, supplemented with 0.5% galactose. Cultures were grown with agitation at 250 rpm at 30 °C for 72 h and cells were separated from the culture medium by centrifugation at 10,400 $g \times 2$ min. This supernatant was directly used to assay the activity released from the cell. A sample of cells resuspended in H_2O was used to test the β-glucosidase activity bound to the cell-surface. Total activity was calculated as the sum of the values (expressed in mU of enzyme per mL of culture) corresponding to the two fractions.

2.4. Protein analysis

S. cerevisiae transformants and the corresponding parental strains were grown in YPD with or without 50 mg/L geneticin, respectively, at 30 °C with agitation at 250 rpm. After 40 h these cultures were centrifuged (1500 $g \times 10$ min) to separate the cell pellet from the culture media. The supernatant (150 mL) was frozen at −80 °C prior to lyophilization during 2 days in an equipment VIRTIS genesis 35EL, with the vacuum set at 1mTorr and the condenser temperature at −85 °C. The resulting powder was resuspended in 7.5 mL of 50 mM citrate buffer pH 5.4, dialysed against the same buffer and further concentrated to 0.2 mL by ultrafiltration through a membrane with a molecular mass cut-off of 20 kDa (Pierce). An aliquot of this sample was treated with 8 U/L EndoH (New England Biolabs) including a protease inhibitor cocktail (Complete EDTA-free, Roche) and 140 mM β-mercaptoethanol, at 37 °C for 16 h. A control sample was incubated in the same conditions without EndoH. These samples were mixed afterwards with SDS 2× loading buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 700 mM β-mercaptoethanol, 0.05% bromophenol blue) at 95 °C for 6 min. SDS–PAGE was performed using 10% polyacrylamide gels that were stained with Coomassie R250. Standards used for molecular mass determination were Page Ruler from Fermentas (cat# SM0661) and Spectra Multicolor from Fermentas (cat# SM1841).

2.5. Fermentation assays

YP cultures with 7% cellobiose were inoculated with the diverse strains and grown in a screw-capped tube with 30% empty space at 30 °C without agitation. The 7% cellobiose concentration was chosen as a practical limit that initially allowed easy dissolution of the sugar in the medium. As fermentation proceeded and the carbon source was consumed, cellobiose powder was serially added (7 g/100 mL culture each time). The progress of the fermentation was followed by the observation of CO_2 bubbling, by the production of ethanol and by the solubilisation of the cellobiose powder. A minimum concentration of ethanol was allowed to evolve before each cellobiose addition, which was 6 g/L for the first one, 30 g/L for the second one and 70 g/L for the third one. For those strains unable to accumulate any of these amounts of ethanol, no further cellobiose additions were carried out. For assays under total anaerobic conditions a similar protocol was followed but the cultures were grown in a system that sustained constant nitrogen bubbling into the media and cellobiose additions where carried out when indicated. Alternatively, YP cultures with 20% glucose and 50 mg/L geneticin were inoculated and grown in an equivalent way, without further additions. At different time points a 200 μL aliquot was taken and the culture medium was separated from the cells by centrifugation at 10,400 $g \times 2$ min. The ethanol concentration was determined in the supernatant with the Enzytec™ fluid ethanol kit (Scil Diagnostic GmbH).

3. Results and discussion

3.1. Analysis of the efficiency of the diverse strains to synthesize β-glucosidase

A set of *Sacharomyces* strains was selected mainly among those involved in processes related to ethanol synthesis (isolated from wines or beer), since they are expected to produce and tolerate high ethanol concentrations. The petite version of one of these, strains with biotechnological importance (probiotic) or related to other industrially relevant applications (bread making), and a laboratory strain, were also included in this study for comparison of relatively diverse genetic backgrounds (Table 1).

The GAL promoter has been successfully used to direct the expression of the *S. fibuligera BGL*1 gene upon induction with galactose of mid-logarithmic phase yeast cultures grown in YPD (Marin-Navarro et al., 2011). However, since the final goal of the current study was to obtain transformants able to perform SSF using cellobiose as a carbon source, a constitutive expression of the β-glucosidase gene was mandatory. Thus, we attempted to compare *BGL*1 expression driven by a *sensu stricto* constitutive promoter (the actin promoter) with that obtained with the GAL promoter induced from the time of inoculum. The expression of *BGL*1 under the control of the actin or GAL promoter was analyzed both in the cell-attached fraction and in the culture medium. In all cases β-glucosidase synthesis controlled by the actin promoter was significantly higher than that achieved with the GAL promoter, except for the pair T491/T491 g which showed similar values with both promoters (Table 1). The lower efficiency obtained with the GAL promoter may rely just on a weaker transcription initiation power, but also on the rate of consumption of galactose in the culture media, and subsequently, on the stability of the *BGL*1 mRNA within the cell. Based on these results, strains expressing *BGL*1 under the control of the actin promoter were selected for further analysis. Among those strains producing Bgl1 under the control of the actin promoter, their efficiency was quite variable ranging from those producing more than 200 mU/mL (T500, T491 and T111) to those synthesizing less than 100 mU/mL (T499, T92, T28 and T74) with intermediate values (T75, T93, T101 and T370). Although not all strains grew to the same optical densities (with OD values ranging from 3 to 10), the β-glucosidase synthesis was not correlated to the OD values (data not shown). The differences in β-glucosidase production may arise from divergences in cell growth combined with other endogenous factors involved in any step between transcription and post-translational events, including protein glycosylation and stability.

3.2. Cell-adhesion properties and glycosylation of β-glucosidase synthesized by different Saccharomyces strains

Bgl1 from *S. fibuligera* contains a fibronectin-like domain on the C-terminal end that has been related to its cell-attachment properties (Marin-Navarro et al., 2011). Expression of *BGL*1 in diverse genetic backgrounds also yielded a protein which was mostly cell-associated but with a variable degree of affinity (Table 1). These differences might arise from changes in the glycosylation properties of the heterologous protein that change the cell-wall binding properties of the enzyme. In order to investigate this possibility, *S. cerevisiae* strains with highest Bgl1 synthesis yields (above 150 mU/mL) were selected to study the glycosylation of the protein secreted to the media. Among these, the three industrial strains T75, T491 and T111 showed a slightly but significantly (p value <0.02) higher fraction of cell-attached enzyme (around 80–85%) compared to the laboratory strain T500 (around 70%). The glycosylation state of Bgl1 released by these strains was analyzed by SDS–PAGE. The protein pattern of each transformant matched that of its corresponding parental strain except for one single differential band (Fig. 2). The only potentially secretable proteins encoded by the plasmid used for the yeast transformations (pACT-SPS-Bgl1-Kan, Fig. 1) are Bgl1 and β-lactamase (the enzyme conferring resistance to ampicillin in *E. coli*). However, the gene encoding β-lactamase is under the control of a prokaryotic promoter and therefore it is not likely to be expressed at high levels in yeast. Moreover, β-lactamase has a molecular mass of 31.5 KDa, with no potential *N*-glycosylation sites and therefore it is out of the size range analyzed here. Bgl1 has an expected size of 96 KDa, with six potential *N*-glycosylation sites predicted by the program NetNGlyc from the Expasy server (data not shown). Thus, the differential bands shown in Fig. 2 are very likely to represent Bgl1.

In all cases Bgl1 migrated as a poorly stained, dispersed band, as is characteristic of hyperglycosylated proteins (Fig. 2). Treatment with EndoH, which removes accessible *N*-linked glycosyl moieties, yielded an intense, sharp band with a lower molecular weight (around 110 KDa in all cases) that is quite similar to the expected theoretical mass of the monomeric protein (96 KDa). The apparent molecular weight of the glycosylated protein ranged from 200 KDa (T500, T75) to 260 KDa (T491, T111), indicating that there is no correlation between the protein glycosylation and the cell-attachment properties. Therefore, the differences in the cell-attachment properties of Bgl1 synthesized in diverse strains should have other cause. The cell wall strength or composition has been reported to show some versatility depending on the growth state or external

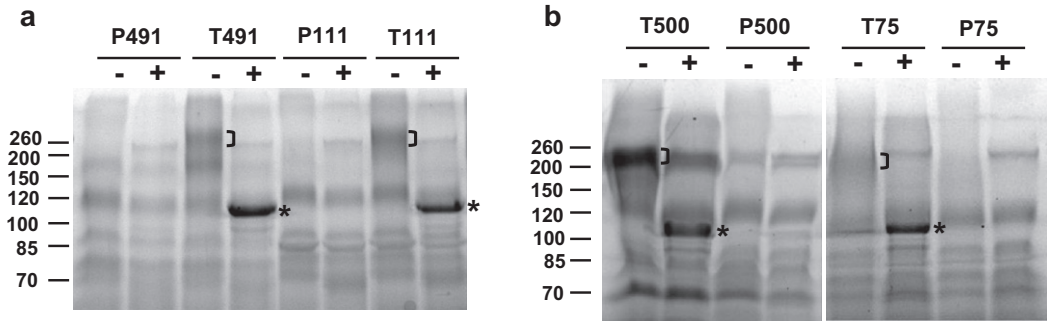

Fig. 2. Electrophoretic pattern of secreted proteins from diverse *S. cerevisiae* strains expressing *BGL*1 from *S. fibuligera* (Bgl1). A concentrated sample from the culture media of yeast transformants (T491, T111, T500 and T75) and the corresponding parental strains (P491, P111, P500 and P75, respectively) was incubated with (+) or without (−) EndoH for 16 h at 37 °C. Differential bands compared to the parental strain and assigned to the heterologous protein (Bgl1) in each case, are indicated with asterisks for the EndoH-treated samples and with brackets for the non-treated samples. The migration of protein standards and their sizes in KDa is shown at the left of each panel.

conditions (Lesage and Bussey, 2006; Stateva, 1998). Thus, it could be hypothesized that different strains have variations in the carbohydrate cell-wall composition or thickness that may alter the Bgl1 binding affinity.

3.3. Analysis of the fermentative performance of the different strains using glucose as a carbon source

The efficiency for converting glucose into ethanol was analyzed for the different transformants as an initial control of the intrinsic fermentative capabilities arising from the host strains. Theoretical maximum ethanol production can be calculated from the stoichiometry of this methabolic pathway, with 1 mol of glucose rendering 2 mol of ethanol, i.e. 0.511 g ethanol/g glucose (Bai et al., 2008). Six of the strains (T500, T75, T370, T491, T499 and T111) achieved a value close to the theoretical maximum ethanol production whereas the remaining five (T74, T101, T28, T92 and T93) produced ethanol up to 40–60% of the expected maximum (Fig. 3). In each case, 30 h after inoculation the transformants had achieved 50% and after 70 h, 100% of the maximum ethanol production.

3.4. Fermentative capabilities of the transformants using cellobiose as a carbon source

To assess the fermentative performance of the transformants with cellobiose as a carbon source, the disaccharide was initially added at a concentration of 7%. Because of the low solubility of cellobiose (12% in H_2O), the transformants were challenged to produce highest ethanol concentrations by serial additions of cellobiose as it was hydrolyzed by Bgl1. This was accompanied by an increase in the ethanol concentration and by gas bubbling, as a result of CO_2 release. The substrate additions were stopped when the ethanol production reached a stable value below the expected theoretical maximum for the sugar source concentration in each case (0.538 g ethanol/g cellobiose) (Fig. 4). In contrast with glucose fermentations, cellobiose fermentations assays revealed a broad range of ethanol synthesis yields, producing more than 100 g/L ethanol in four strains (T500, T491 and T499), around 70 g/L in T75, between 30 and 50 g/L in six strains (T370, T111, T101, T92 and T93) and less than 7 g/L in two strains (T28 and T74) (Fig. 4). In all cases the kinetics of ethanol production from cellobiose was slower than that obtained directly from glucose, with a longer lag phase: only around 125 h after inoculation the transformants reached 50% of their respective maximum ethanol productions, which were achieved around 175 h (Fig. 4). Durham-tests with parental strains showed no CO_2-bubbling during 170 h after inoculum. This result shows that, at least for strains T500, T491 and T499, the hydrolysis of cellobiose would not be a bottleneck for the production of relatively high (>100 g/L) ethanol concentrations from cellulose.

3.5. Relationship between cellobiose fermentation, β-glucosidase synthesis and genetic background

The cellobiose fermentative capability of the yeast strains might depend on the levels of expression of β-glucosidase if the conversion of cellobiose to glucose is a limiting step to the transformation of glucose to ethanol. To evaluate possible correlations between cellobiose fermentation and β-glucosidase expression in the different strains, the ethanol production from cellobiose was normalized by the intrinsic capability of converting glucose into ethanol (Fig. 5). Among the *S. cerevisiae* strains, a positive dose–response relationship can be seen between the efficiencies of the strains to synthesize β-glucosidase and to ferment cellobiose, with the exception of T499 (the petite mutant of T491) and T111 (*S. cerevisiae* subtype *boulardii*), which show relatively high and low cellobiose fermentative capabilities, respectively (Fig. 5). The *S. bayanus* strain and two of the *S. pastorianus* strains (T28 and T93) show the same tendency as most of the *S. cerevisiae* strains whereas T92, with much less β-glucosidase than T93 has a similar fermentative performance from cellobiose than its *S. pastorianus* counterpart.

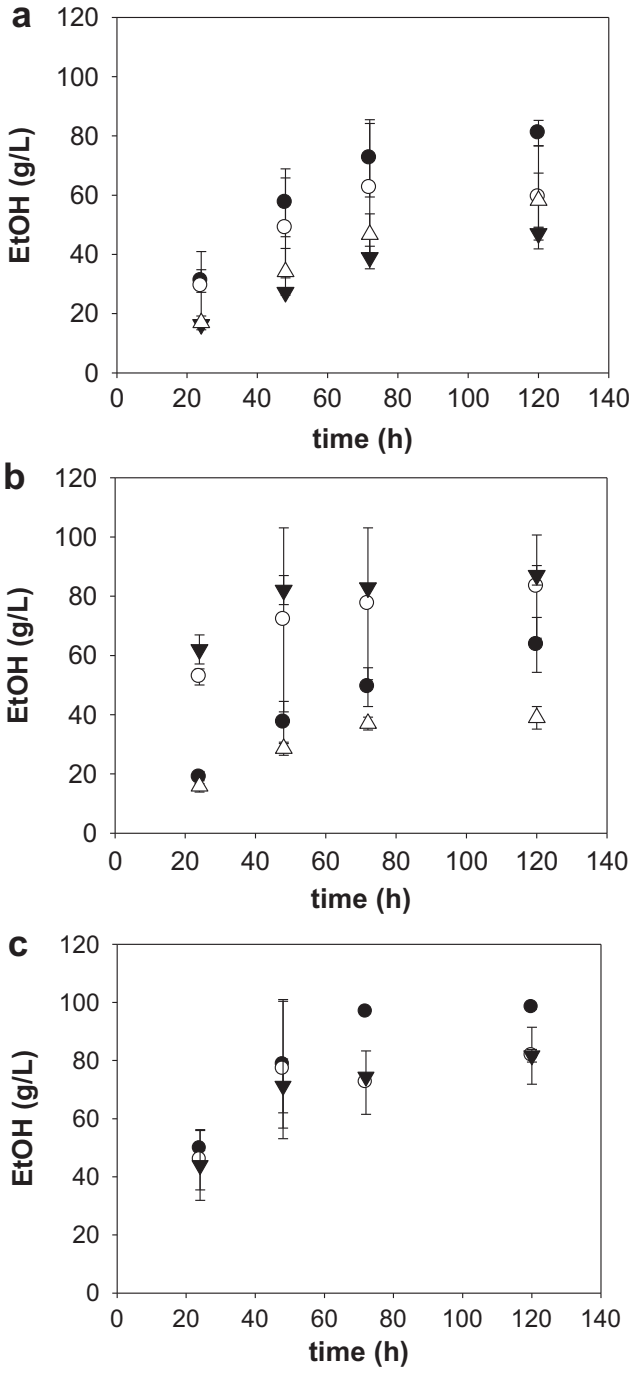

Fig. 3. Ethanol production from glucose (20%) as a carbon source in different transformants: (a) T75 (closed circles), T101 (open circles), T92 (inverted, closed triangles), T74 (open triangles); (b) T93 (closed circles), T111 (open circles), T370 (inverted, closed triangles), T28 (open triangles); (c) T500 (closed circles), T491 (open circles), T499 (inverted, closed triangle). Error bars represent the standard deviation of duplicates.

Therefore, the different efficiencies in converting cellobiose to ethanol seem to rely on the β-glucosidase expression level and on the genetic background of each strain. Diverse endogenous factors

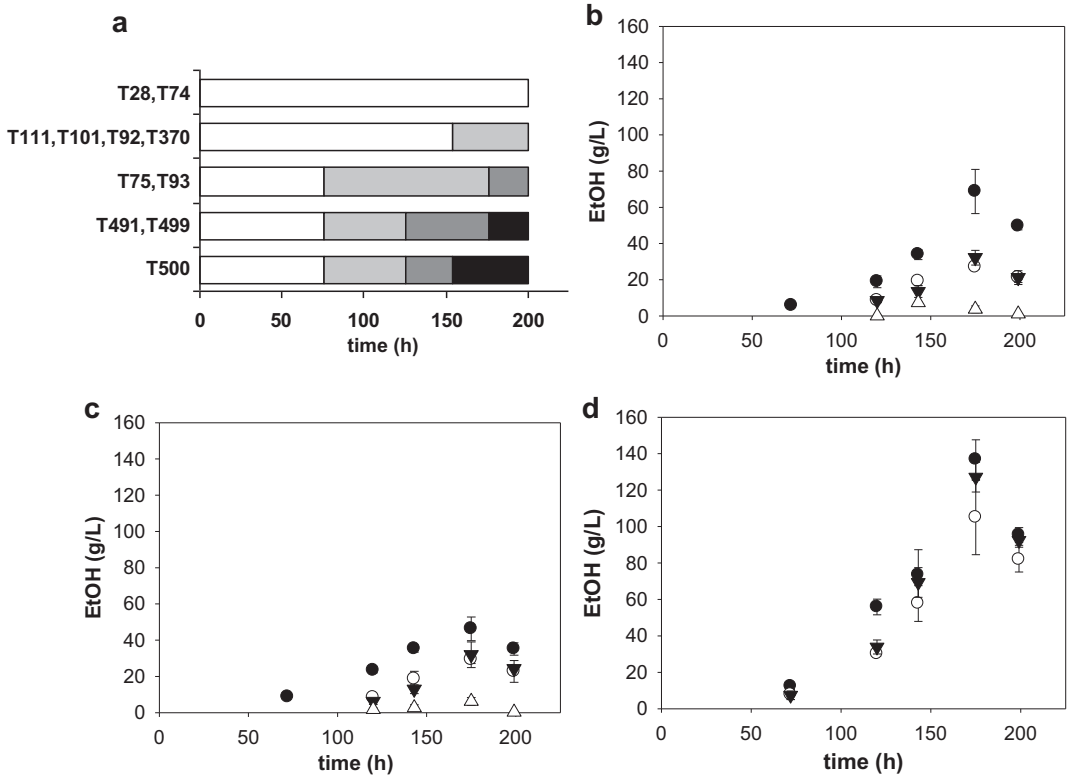

Fig. 4. Ethanol production from cellobiose as a carbon source in different transformants: (a) temporal pattern of serial cellobiose additions. Strains were initially inoculated in YP with 7% cellobiose, and further cellobiose additions were performed as fermentation progressed in each case. The time of each cellobiose addition (7 g/100 mL of culture) is shown by a change of colour in a grey-scale bar; (b) T75 (closed circles), T101 (open circles), T92 (inverted, closed triangles), T74 (open triangles); (c) T93 (closed circles), T111 (open circles), T370 (inverted, closed triangles), T28 (open triangles); (d) T500 (closed circles), T491 (open circles), T499 (inverted, closed triangle). Error bars represent the standard deviation of duplicates.

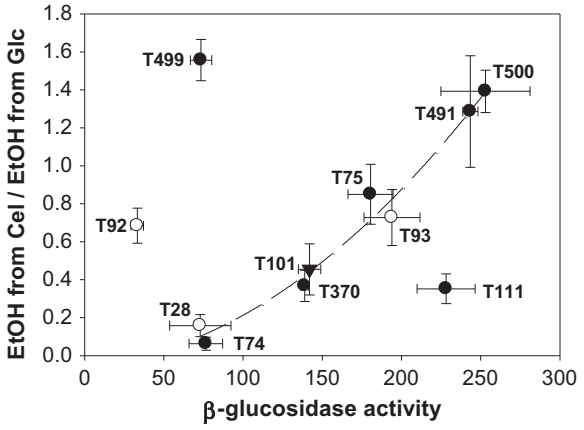

Fig. 5. Correlation between the ethanol production from cellobiose (corrected by the ethanol production from glucose) and β-glucosidase expression in the different strains: *S. cerevisiae* (closed circles), *S. pastorianus* (open circles), *S. bayanus* (inverted, closed triangles). Error bars on abscises (β-glucosidase activity) show standard deviation of triplicates, as determined in Table 1. Ordinates represent maximum ethanol production from cellobiose (Ec) divided by maximum ethanol production from glucose (Eg), obtained from the data shown in Figs. 4 and 3, respectively. Error bars on ordinates show the standard deviation of each quotient (Q = Ec/Eg). This was calculated following usual error propagation calculus, from the formula $(s(Q)/Q)^2 = (s(Ec)/Ec)^2 + (s(Eg)/Eg)^2$, where s(Ec), s(Eg) and s(Q) are the standard deviations of Ec, Eg and Q, respectively.

may be involved, but an interesting clue about one of these arises from the comparison of the phenotype of T491 and T499, which show similar cellobiose fermentation with a three-fold difference in β-glucosidase synthesis. Because T499 is the petite version of T491, mitochondrial activity seems a key factor in the efficiency of cellobiose to ethanol transformation. Although ethanol yields from glucose fermentation were similar in the grande and mitochondrial petite strain, a major difference between the two fermentation assays is the effective glucose concentration. When glucose is used directly as a carbon source, mitochondrial respiratory enzymes are expected to be repressed (Crabtree effect). However, when cellobiose is used as a carbon source, glucose concentration is probably much lower. In this situation, mitochondrial activity (if present) might be responsible not only for diverting glucose to the respiratory pathways but also for ethanol consumption. Previous studies have pointed out the potential of respiration-deficient nuclear petite mutants for ethanol production based on glucose or starch fermentation (Hutter and Oliver, 1998; Toksoy Öner et al., 2005). Thus, under total anaerobic conditions it would be expected that T491 shows a faster ethanol production rate than T499, according to the higher β-glucosidase synthesis of the former. Indeed, when fermentation experiments with cellobiose as a carbon source were carried out under constant nitrogen bubbling to ensure oxygen removal, ethanol production of T499 was initially (up to 100 h) delayed compared to that of T491 (Fig. 6a). Because such a delay was not observed when fermentation was performed without nitrogen bubbling (Fig. 4a) this result supports that respiratory activity has a detrimental effect on ethanol production. However, it is also remarkable that the petite strain, after this slow-kinetics period, switches to a second phase with faster kinetics of ethanol production (Fig. 6a), alongside a steady synthesis of β-glucosidase (Fig. 6b). This may indicate that once the β-glucosidase synthesis surpasses certain threshold, the conversion of cellobiose to glucose is no longer a rate-limiting step in the fermentation process.

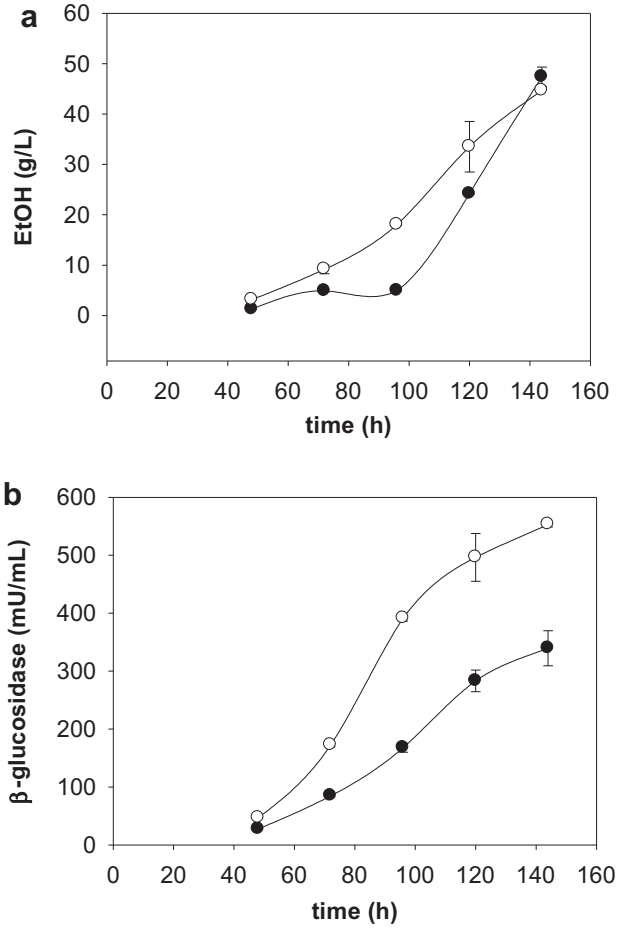

Fig. 6. Fermentation of cellobiose under anaerobic conditions with constant nitrogen bubbling. Transformants T491 (open circles) and T499 (closed circles) were inoculated in duplicate in YP supplemented with 7% cellobiose. Further additions of 7 g cellobiose/100 mL of culture were performed after 56 and 79 h. Ethanol production (a) and β-glucosidase activity (b) were assayed at different time points. Error bars represent the standard deviation of duplicates.

Following this line of discussion, a putative endogenous lower mitochondrial activity under low glucose concentrations (and a continuous accumulation of β-glucosidase) might explain the relatively high cellobiose fermentation efficiency of T92, despite its initially low β-glucosidase synthesis. Although T92, T93 and T28 are *S. pastorianus* strains, within this hybrid species (*S. bayanus* × *S. cerevisiae*) major differences have been found regarding genome rearrangements, ploidy variations and DNA sequence polymorphisms, leading to the classification of two groups of different origin (Dunn and Sherlock, 2008). On the other hand, *S. cerevisiae var. boulardii* (T111) shows a trisomy in chromosome IX (Edwards-Ingram et al., 2007), which contains some genes encoding mitochondrial proteins related to the respiratory pathway, such as *KGD1*, *COX5B* or *MAM33*, among others. It is tempting to speculate that overexpression of such proteins might cause an enhanced respiratory metabolism that hampers cellobiose fermentation despite the relatively high β-glucosidase expression levels of the strain T111. Alternatively, other endogenous factors affecting the induction of the fermentative pathway under low glucose concentrations or the kinetics of β-glucosidase synthesis may have an impact on the final yield of ethanol production from cellobiose.

Overall, the results obtained in the present study provide a selection of strains that, because of a high β-glucosidase synthesis capacity and/or their intrinsic fermentative performance under low glucose concentrations, are good candidates for further manipulations directed to transform cellulosic substrates into ethanol.

4. Conclusion

The selection of a strain for ethanol production from glycosidic substrates other than glucose should take in account both its efficiency for heterologous gene expression and its intrinsic proficiency to perform fermentation under low glucose concentrations. Ethanol biosynthesis from cellobiose fermentation can be visualized as a two-phase process, with the first one converting cellobiose into glucose and the second one transforming glucose into ethanol. Thus, β-glucosidase synthesis needs to be optimized over the limit that makes cellobiose hydrolysis a rate-limiting step. A low respiratory activity together with other endogenous factors determine the efficiency of the second step.

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