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BIOENERGY/BIOFUELS/BIOCHEMICALS

Fungal β-glucosidase expression in *Saccharomyces cerevisiae*

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Abstract Recombinant *Saccharomyces cerevisiae* strains expressing β-glucosidases from *Thermoascus aurantiacus* (*Tabgl1*) and *Phanerochaete chrysosporium* (*PcbglB* and *Pccbgl1*) were constructed and compared to *S. cerevisiae* Y294[SFI], previously identified as the best β-glucosidase-producing strain. The *PcbglB* was also intracellularly expressed in combination with the *lac12* lactose permease of *Kluyveromyces lactis* in *S. cerevisiae* Y294[PcbglB + Lac12]. The recombinant extracellular β-glucosidases indicated maximum activity in the pH range 4–5 and temperature optima varying from 50 to 75 °C. The *S. cerevisiae* Y294[Pccbgl1] strain performed best under aerobic and anaerobic conditions, producing 2.6 times more β-glucosidase activity than *S. cerevisiae* Y294[SFI] and an ethanol concentration of 4.8 g l^{-1} after 24 h of cultivation on cellobiose as sole carbohydrate source. *S. cerevisiae* Y294[Tabgl1] was unable to grow on cellobiose (liquid medium), whereas *S. cerevisiae* Y294[PcbglB + Lac12] exhibited limited growth.

Keywords Cellulose degradation · Cellobiose · β-glucosidase · Bio-ethanol · *S. cerevisiae*

Introduction

Lignocellulosic biomass has potential as a renewable alternative to fossil fuels due to its low cost and availability [6]. Cellulose is the major component of plant biomass, reaching levels of up to 55 %. Complete cellulose hydrolysis requires a combination of endoglucanase (E.C. 3.2.1.4), cellobiohydrolase (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21) enzymes. Endoglucanases randomly hydrolyze glycosidic bonds within internal amorphous regions in the cellulose chain, generating oligosaccharides of various lengths [7]. Cellobiohydrolases degrade the oligosaccharides from the reducing- or non-reducing ends, cleaving off cellobiose units. These cellulases work synergistically to efficiently degrade cellulose to soluble cellobiose [17]. The β-glucosidases cleave cellodextrins and cellobiose in the final step of cellulose hydrolysis, releasing glucose molecules. Accumulation of cellobiose results in feedback inhibition of endoglucanases and cellobiohydrolases [2, 5]. Therefore, the β-glucosidases play an important role in cellulose degradation, resulting in higher efficiency of the cellulase system.

One of the major challenges in lignocellulose-ethanol production is the development of low-cost technology [19]. A single-step strategy (consolidated bioprocessing—CBP) has been proposed that involves the production of cellulolytic enzymes, biomass hydrolysis, and conversion of fermentable sugars to desirable products via a cellulolytic micro-organism [13]. The development of a suitable micro-organism would require the heterologous expression of cellulases in a non-cellulolytic micro-organism that exhibits high product yields and titers [4] and has the ability to convert biomass to desired products without requiring further additional enzymes [14].

Saccharomyces cerevisiae has been the focus of several research groups due to its long fermentation history, high ethanol tolerance, and GRAS (Generally Regarded as Safe) status [4]. Although *S. cerevisiae* is a good choice for CBP, one of its few drawbacks is the limited range of polysaccharides it can utilize. Therefore this yeast has been used as a platform for the development of improved industrial micro-organisms whose substrate range has been expanded

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through genetic engineering [14]. Several studies have demonstrated β-glucosidase-expressing *S. cerevisiae* strains growing on various carbohydrate sources [4, 5, 8, 16, 21]. Van Rooyen et al. [21] successfully constructed recombinant *S. cerevisiae* strains that utilize cellobiose as sole carbon source. The recombinant *S. cerevisiae* strain expressing the β-glucosidase encoding gene (*bgl1*) of *Saccharomycopsis fibuligera* was identified as the most promising strain. In further studies by Du Plessis et al. [5], the *S. fibuligera bgl1* was co-expressed with *Trichoderma reesei cbhI* (cellobiohydrolase) and *egII* (endoglucanase). Cellobiose accumulated in the medium, indicating inadequate recombinant β-glucosidase (*S. fibuligera* BGL1) activity, resulting in hampered growth on cellobiose (and ultimately cellulose) as the sole carbon source.

In this study, we expressed codon-optimized genes of the thermostable *TaBgl1*β-glucosidase of *Thermoascus aurantiacus* [10] and the two GH1 β-glucosidases (*PcBglB* and *PccBgl1*) of *Phanerochaete chrysosporium* [20] in *S. cerevisiae* Y294 to determine the efficiency of heterologous expression and levels of extracellular activity displayed by this host. This study demonstrated growth on cellobiose, with ethanol production under anaerobic conditions. The *S. cerevisiae* strain expressing the *bgl1* gene of *S. fibuligera* [21] was used as a benchmark strain and was therefore included in all experiments. Galazka et al. [8] introduced the cellodextrin transport system of *Neurospora crassa* into *S. cerevisiae* to allow its transport, while cellobiose transport had been demonstrated by Sadie et al. [16] introducing the *Kluyveromyces lactis* lactose permease (*lac12*) gene into *S. cerevisiae*. Therefore, the feasibility of using an intracellular β-glucosidase together with a cellobiose transport system in *S. cerevisiae* was also investigated.

Materials and methods

Media and cultivation

All chemicals used were of analytical grade. *Escherichia coli* DH5α (Takara Bio Inc.) was the host strain for recombinant DNA manipulations and plasmid propagation. Bacterial cultivation (37 °C) took place in Luria–Bertani liquid medium or agar (5 g l^{-1} yeast extract, 10 g l^{-1} NaCl and 10 g l^{-1} tryptone) supplemented with 100 μg ml^{-1} ampicillin for selection [18]. All *S. cerevisiae* strains were aerobically cultivated on a rotary shaker (250 rpm) at 26 °C in 125-ml Erlenmeyer flasks containing 30 ml of synthetic complete (SC^{-URA}) medium (1.7 g l^{-1} yeast nitrogen base (Difco Laboratories), 5 g l^{-1} $(NH_4)_2SO_4$, 10 g l^{-1} glucose/10 g l^{-1} cellobiose and supplemented with appropriate amino acids. Anaerobic fermentations were carried out in rubber-stoppered glass serum bottles containing 100 ml of synthetic complete (SC^{-URA}) medium. The *S. cerevisiae* Y294 transformants were selected and maintained on agar plates of the same composition.

Strains and plasmids

The relevant genotypes of all the bacterial and yeast strains, along with the plasmids used in this study, are listed in Table 1.

Plasmid construction

Standard protocols were followed for DNA manipulations [18]. The enzymes used for restriction digests and ligations were purchased from Roche Molecular Biochemicals and used according to the manufacturer's instructions. Digested DNA was eluted from agarose gel using the Zymoclean Gel Recovery Kit (Zymo Research Corporation, USA). Polymerase chain reactions (PCR) were carried out with a Perkin Elmer GeneAmp® PCR System 2400 (Perkin Elmer, USA) using TaKaRa *Ex-Taq* DNA polymerase (TaKaRa Bio Inc., Japan) according to the supplier's specifications. Details of the primers used in this study are provided in Table 2.

The *Tabgl1, Pcbgl1B*, and *Pccbgl1* genes were synthetically made and codon-optimized (GeneArt, Germany) for expression in *S. cerevisiae*, with however, the native secretion signal sequences omitted. The 2,521-bp *T. aurantiacus Tabgl1*, the 1,623-bp *P. chrysosporium Pcbgl1B* and 2,401-bp *Pccbgl1* were amplified through the PCR and cloned into plasmid yBBH4 by means of yeast-mediated ligation [3] resulting in vectors yTabgl1, yPcbgl1B, and yPccbgl1. The yBBH4 vector contains the coding region of the *T. reesei xyn2* secretion signal, which works well in *S. cerevisiae* [12]. The *iPcbgl1B* gene (no secretion signal encoded) was amplified with Pcbgl1B-L and Pcbgl1B-R primers. The PCR product was digested with *Eco*RI and *Xho*I and cloned between corresponding sites on yBBH1, resulting in yBBH1-iPcbgl1B. The relevant plasmid maps are shown in Fig. 1a and b. The $ENO1_P$–*lac12*-$ENO1_T$ region was retrieved from yBBH-Lac12 [16] as a *Bgl*II-*Bam*H1 fragment and cloned into the *Bgl*II site of plasmid yBBH1–iPcbgl1B, yielding plasmid yBBH1–iPcbgl1B + Lac12.

Yeast transformations

Saccharomyces cerevisiae Y294 strains were transformed with the recombinant plasmids using the lithium acetate/DMSO method described by Hill et al. [9]. Transformants were selected for growth on SC^{-URA} plates and the presence of the genes confirmed by PCR.

Table 1 Microbial strains and plasmids used in this study

Strains or plasmids	Genotype	Source/reference
Strains:		
E. coli DH5α	*supE44ΔlacU169 (∅80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lacZ*53	[18]
S. cerevisiae Y294	*α leu2*-3,112 *ura3*-52 *his3 trp1-289*	ATCC 201160
S. cerevisiae Y294[SFI]	*URA3 PGK1$_P$- XYNSEC-BGL1*-PGK1$_T$*	[21]
S. cerevisiae Y294[BBH1]	*URA3 ENO1$_P$-ENO1$_T$*	This study
S. cerevisiae Y294[BBH4]	*URA3 ENO1$_P$-XYNSEC-ENOI$_T$*	This study
S. cerevisiae Y294[Tabgl1]	*URA3 ENO1$_P$-XYNSEC-Tabgl1-ENO1$_T$*	This study
S. cerevisiae Y294[Pcbgl1B]	*URA3 ENO1$_P$-XYNSEC-Pcbgl1B-ENO1$_T$*	This study
S. cerevisiae Y294[iPcbgl1B]	*URA3 ENO1$_P$-iPcbgI1B-ENO1$_T$*	This study
S. cerevisiae Y294[Pccbgl1]	*URA3 ENO1$_P$-XYNSEC-Pccbgl1-ENO1$_T$*	This study
*S.cerevisiae*Y294[iPcbgl1B + Lac12]	*URA3ENO1$_P$-iPcbgl1B-ENOI$_T$*; *ENOI$_P$-lac12-ENOI$_T$*	This study
S. cerevisiae Y294[Lac12]	*URA ENO1$_P$-lac12-ENO1$_T$*	[16]
Plasmids:		
YENO1	*bla URA3 ENO1$_P$-ENO1$_T$*	[4]
ySFI	*bla URA3 PGK1$_P$- XYNSEC-BGL1*-PGK1$_T$*	[21]
yBBH1	*bla URA3 ENO1$_P$-ENO1$_T$*	[16]
yBBH4	*bla URA3 ENO1$_P$-XYNSEC-ENO1$_T$*	This laboratory
yTabgl1	*bla URA3 ENO1$_P$-XYNSEC-Tabgl1-ENO1$_T$*	This study
yBBH1-iPcbgl1B	*bla URA ENO1$_P$-iPcbgl1B-ENO1$_T$*	This study
yPcbgl1B	*bla URA ENO1$_P$-XYNSEC-Pcbgl1B-ENO1$_T$*	This study
yPccbgl1	*bla URA3 ENO1$_P$-XYNSEC-PccbgI-ENO1$_T$*	This study
yBBH-iPcbgl1B + Lac12	*bla URA3ENO1$_P$-iPcbg1B-ENO1$_T$*, *ENO1$_P$-lac12-ENO1$_T$*	This study
yBBH-Lac12	*bla URA3 ENO1$_P$-lac12-ENO1$_T$*	[16]

* BGL1 was derived from *Saccharomycopsis fibuligera* [21]

Plate assays

Extracellular β-glucosidase enzyme activity was confirmed using the esculin (esculetin 6-O-glucoside) hydrolysis test [15]. Recombinant strains were transferred to esculin (SC^{-URA} containing 1 g l^{-1} esculin and 0.5 g l^{-1} ferric citrate) plates. The active extracellular β-glucosidase enzyme cleaves the glucoside group from esculin, releasing esculetin [15]. The esculetin reacts with the ferric citrate, resulting in the formation of black zones around the colonies. Recombinant strains were also evaluated for growth on SC^{-URA} (cellobiose) plates incubated at 30 °C for 72 h.

Liquid enzyme activity assays

For enzymatic assays, pre-cultures were used to inoculate SC^{-URA} medium to approximately 1×10^5 cells per ml. Samples were taken periodically to determine the optical density and dry cell weight (DCW). The β-glucosidase activity was measured by incubating diluted cell cultures with 2 mM of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG, Sigma-Aldrich, Germany) in 50 mM citrate buffer at various temperatures and pH for 5 min [21]. The *p*-nitrophenol released from *p*NPG was detected at 400 nm after addition of 1 M Na_2CO_3. All enzymatic assays were performed in triplicate and enzyme activities expressed as nkatals [1]. Little or no activity was detected when *p*-nitrophenyl-β-D-cellobiose (*p*NPC, Sigma-Aldrich, Germany) or *p*-nitrophenyl-β-D-mannoside (*p*NPM, Sigma-Aldrich, Germany) were used as substrates.

Anaerobic fermentations and HPLC analysis

All *S. cerevisiae* strains were evaluated for growth in liquid SC^{-URA} cellobiose medium under anaerobic conditions in rubber-capped serum bottles at 30 °C with shaking at 200 rpm. High-performance liquid chromatography (HPLC, Waters, Germany) was conducted on a Surveyor Plus liquid chromatograph from Thermo Scientific consisting of a LC pump, auto-sampler, and RI detector. Samples were filtered through 0.22-μm syringe filters prior to separation on the Rezex RHM polymer-based column

Table 2 K_ms of the native β-glucosidases used in the study and sequences of the PCR primers used for amplification of their respective genes

Gene and source	Protein Accession nr	K_m (μM) on *p*NPG	Primer sequence (5′–3′)* with relevant restriction sites underlined
Tabgl1	AAZ95588	260[a]	Tabgl1-L:GAACCCGTGGCTGTGGAGAAGCGCTCGCGAAAGATGATTTGGCTTATTCTCCA
T. aurantiacus			Tabgl1-R:GGACTAGAAGGCTTAATCAAAAGCTCTCGAGTTAATATGGTGGCAATGGAGCTTGCAAAT
iPcbgl1B	BAE87009	614[b]	Pcbgl1B-L:TGCTTATCAACACACAAACACTAAATCAAAGAATTCTCGCGAATGTCTGCTTCTGCTGCT
P. chrysosporium			Pcbgl1B-R:GGACTAGAAGGCTTAATCAAAAGCTCTCGAGTTACAAACCCAAGAAAGCAGAAATATAAC
Pcbgl1B	BAE87009	–	xynPcbgl1B-L:GAACCCGTGGCTGTGGAGAAGCGCTCGCGATCTGCTTCTGCTGCTCCACCAAACAAATTG
P. chrysosporium			xynPcbgl1B-R:GGACTAGAAGGCTTAATCAAAAGCTCTCGAGTTACAAACCCAAGAAAGCAGAAATATAAC
Pccbgl1	AAC26481	117[c]	Pccbgl-L: GAACCCGTGGCTGTGGAGAAGCGCTCGCGACAATCTGGTTTGTATCAACAATGT
P. chrysosporium			Pccbgl-R:GGACTAGAAGGCTTAATCAAAAGCTCTCGAGTTAGTTTGTAATAGAACCTTCAATCTCA
SFI		251[d]	No primer made for this study
S. fibuligera			
lac12	PO7921		Lac12-L: ACTCGAGGGTTAAACAGATTCTGC
K. lactis			Lac12-R:CGATATCAAATGGCAGATCATTCGA

* Primer sequences were based on the codon optimized gene sequences with the exception of the *lac12* primers, (a) [10], (b) [20], (c) [11], and (d) [21]

(300 × 7.8 mm). The column temperature was maintained at 60 °C using a Gecko 2000 column heater. The mobile phase, 5 mM H_2SO_4, was set at a flow rate of 0.6 ml min^{-1}.

Results

Functional expression of β-glucosidases in *S. cerevisiae*

The *Tabgl1* of *T. aurantiacus* (2,521 bp), *Pcbgl1B* of *P. chrysosporium* (1,623 bp) and *Pccbgl1* of *P. chrysosporium* (2,401 bp) were cloned in frame with the *T. reesei xyn2* secretion signal [12] for constitutive expression under the transcriptional control of the *S. cerevisiae ENO1* (enolase I) promoter and terminator (Fig. 1a and b). The Kex2 protease recognition site (Lys–Arg) was introduced between the XYNSEC and mature β-glucosidase protein moieties, resulting in the removal of the secretion signal upon secretion. The *iPcbgl1B* (no secretion signal) and *lac12* (containing native secretion signal) genes were cloned into the yBBH1 vector. The presence of extracellular β-glucosidase enzyme activity by recombinant *S. cerevisiae* strains was indicated by the formation of black zones surrounding the colonies upon cultivation on esculin plates (Fig. 1c). The successful expression of the extracellular enzymes was demonstrated by the *S. cerevisiae* Y294[Tabgl1], Y294[Pccbgl1], and Y294[Pcbgl1B] strains (Fig. 1c). *S. cerevisiae* Y294[iPcbgl1B] and *S. cerevisiae* Y294[iPcbgl1B + Lac12] did not demonstrate activity on esculin plates due to the intracellular nature of the enzyme.

Cellobiose utilization by recombinant *S. cerevisiae* strains

To test the ability of recombinant strains to sustain growth on cellobiose, the strains were streaked onto SC^{-URA} medium containing cellobiose as sole carbon source and incubated at 30 °C for 72 h (Fig. 1d). All extracellular β-glucosidase-producing strains grew well on cellobiose. Intracellular β-glucosidase-expressing *S. cerevisiae* Y294[iPcbgl1B] showed no growth on cellobiose nor did *S. cerevisiae* Y294[BBH4] and Y294[Lac12] strains. *S. cerevisiae* strain Y294[iPcbgl1B + lac] was also able to grow, suggesting that the Lac12 permease was able to facilitate the transport of cellobiose into the cell allowing the breakdown of cellobiose by iPcbgl1B.

Characterization of recombinant β-glucosidase activity

The activity of extracellular β-glucosidases was determined using *p*-Nitrophenyl-β-D-glucopyranoside (*p*NPG) as substrate. The optimal conditions for enzyme activity

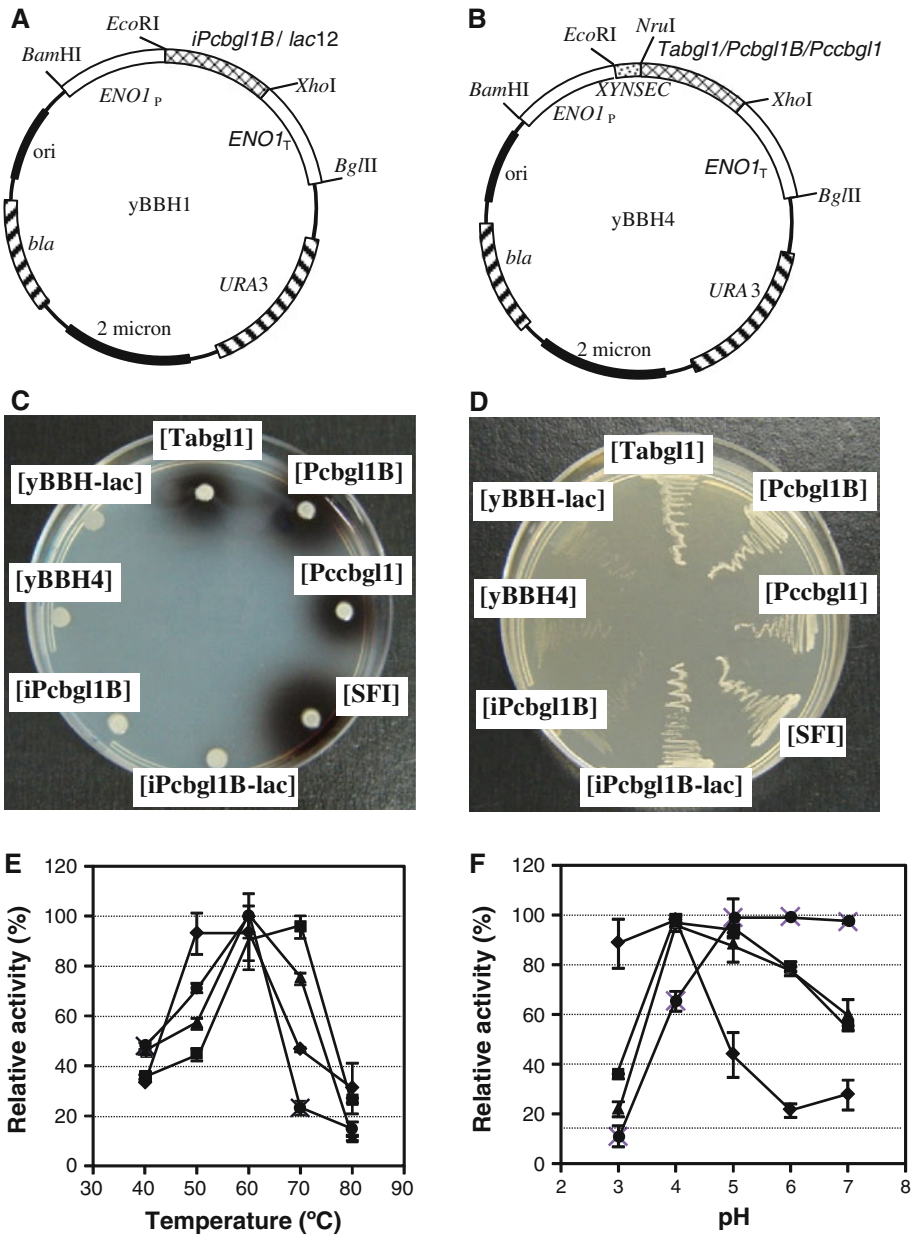

Fig. 1 Schematic representation of the vectors used in this study. The *S. cerevisiae ENO1* (enolase I gene) promoter and terminator sequences were used for the expression of **a** *iPcbgl1B* and *lac12* (lactose permease) on yBBH1; and **b** *Tabgl1, Pcbgl1B* and *Pccbgl* on the yBBH4 plasmid. **c** Extracellular β-glucosidases resulted in the formation of a dark halo on SC^{-URA} plates containing esculin. **d** Strains *S. cerevisiae* Y294[SFI], Y294[Tabgl1], Y294[Pcbgl1B], Y294[Pccbgl1], and Y294[iPcbgl1B + Lac12] displayed growth on cellobiose as sole carbon source. Temperature **e** and pH **f** activity profiles of *filled diamond* Tabgl1, *filled square* PcbglB, *filled triangle* Pccbgl1 and *filled circle* SFI on pNPG as substrate. Activity is expressed as a percentage of the highest value. Activity assays were performed in triplicate with the error bars representing the standard deviation

(temperature and pH) are depicted in Fig. 1e and f. Tabgl1 displayed optimal activity at pH 4 and 55 °C, Pcbgl1B had optimal activity at pH 4 and 70 °C, Pccbgl1 at pH 4 and 60 °C, and SFI at pH 5 and 60 °C. The data are consistent with those reported by [2] and [21].

Enzyme activity measurement

Recombinant β-glucosidase activity was quantified using *p*NPG at 60 °C and pH 4.5. The β-glucosidase of *S. cerevisiae* Y294[Pccbgl1] displayed maximum specific activity of 7.2 nkat mg^{-1} dry cell weight (DCW) (equal to 55 nkat ml^{-1}) after 48 h of growth in glucose containing SC medium (Fig. 2a–c). The highest specific enzyme activity of *S. cerevisiae* Y294[Pccbgl1] was 2.6–3.0 times higher than that of Y294[Pcbgl1B] and *S. cerevisiae* Y294[SFI], previously identified as the *S. cerevisiae* recombinant strain with highest β-glucosidase activity [21]. However, the three strains *S. cerevisiae* Y294[Pccbgl1], Y294[Pcbgl1B], and *S. cerevisiae* Y294[SFI] produced similar levels of activity when cultivated in either cellobiose or glucose. Maximum β-glucosidase activity per DCW was obtained after approximately 64 h of growth on cellobiose, compared to 48 h on glucose. The recombinant *S. cerevisiae* strains Y294[iPcbgl1B + Lac12] and Y294[iPcbgl1B] express the intracellular β-glucosidase, hence no extracellular activity was detectable. Although the activity level of the recombinant Tabgl1 enzyme was

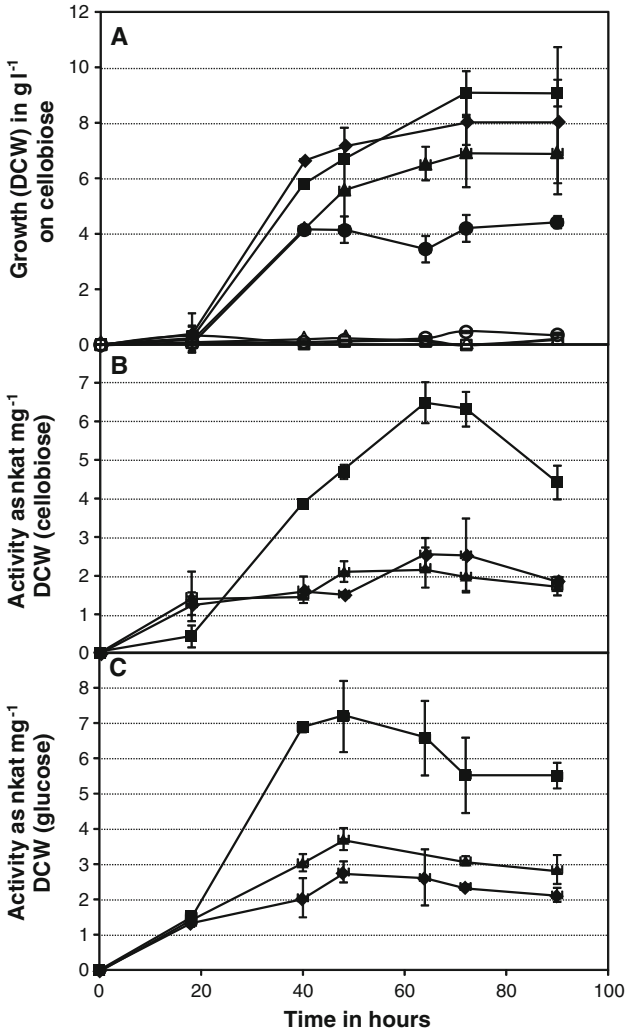

Fig. 2 Biomass yield (**a**) was determined for *filled square S. cerevisiae* Y294[Pccbgl1], *filled diamond S. cerevisiae* Y294[SFI], *filled triangle S. cerevisiae* Y294[Pcbgl1B], *filled circle S. cerevisiae* Y294[iPcbgl1B + lac], *empty circle S. cerevisiae* Y294[iPcbgl1B], *empty triangle S. cerevisiae* Y294[Lac12] and *empty square S. cerevisiae* Y294[BBH4]. Extracellular β-glucosidase activity was determined as nkat g^{-1} DCW (dry cell weight) on cellobiose (**b**) and glucose (**c**) as carbon source using *p*NPG as substrate. Assays were performed in triplicate at pH 5 and 60 °C with the *error bars* representing the standard deviation. The *S. cerevisiae* Y294[BBH4], Y294 [iPcbgl1B] and Y294[Lac12] strains were omitted from graphs (**b**) and (**c**) since no extracellular β-glucosidase activity was detected

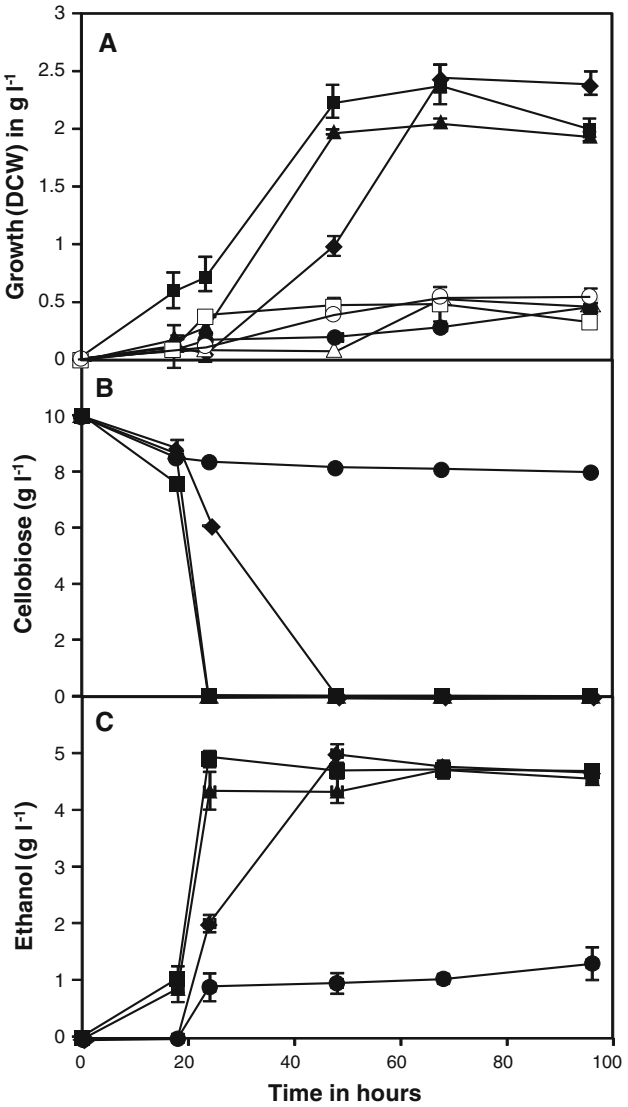

Fig. 3 Comparison of biomass yield (a) by *filled square S. cerevisiae* Y294[Pccbgl1], *filled diamond S. cerevisiae* Y294[SFI], *filled triangle S. cerevisiae* Y294[Pcbgl1B], *filled circle S. cerevisiae* Y294[iPcbgl1B + lac], *empty circle S. cerevisiae* Y294[iPcbgl1B], *empty triangle S. cerevisiae* Y294[Lac12] and *empty square S. cerevisiae* Y294[BBH4] under anaerobic conditions on cellobiose as carbon source. The residual cellobiose (**b**) and extracellular ethanol (**c**) was determined. The *S. cerevisiae* Y294[BBH4], Y294[iPcbgl1B], and Y294[Lac12] strains were omitted from **b** and **c** since they were unable to utilize cellobiose

high enough to be detected using *p*NPG, the level was too low to support growth on cellobiose.

Anaerobic fermentations of cellobiose to ethanol

The recombinant *S. cerevisiae* strains were inoculated in liquid cellobiose containing SC^{-URA} medium and incubated anaerobically in rubber-stoppered serum bottles. *S. cerevisiae* Y294[Pccbgl1], Y294[SFI], and Y294[Pcbgl1B] showed significant growth (Fig. 3a), whereas *S. cerevisiae* Y294[iPcbgl1B], Y294[Lac12], Y294[BBH4], and Y294[Tabgl1] showed feeble growth. Glycerol, cellobiose, and ethanol concentrations present in the supernatant of the different cultures were determined by HPLC (Fig. 3b–d). Acetic acid concentrations were similar for all the strains tested, reaching 0.3 g l^{-1} after 48 h (data not shown). The *S. cerevisiae* Y294[SFI] strain consumed all the cellobiose, producing 4.88 g l^{-1} ethanol after 48 h; *S. cerevisiae* Y294[Pccbgl1] and *S. cerevisiae* Y294[Pcbgl1B] consumed all the cellobiose yielding 4.80 and 4.30 g l^{-1} ethanol,

respectively, after 24 h. *S. cerevisiae* Y294[iPcbgl1B + lac12] consumed 2.1 g of cellobiose producing 1.41 g l^{-1} ethanol after 96 h of cultivation.

Discussion

β-Glucosidases are a diverse cellulase group responsible for the final step of cellulose hydrolysis. They convert cellobiose and soluble cellodextrins, generated by endo- and exoglucanases, to glucose. The β-glucosidases have been shown to play an important role in cellulose saccharification by eliminating the feedback inhibition of cellobiose on cellobiohydrolases [5, 13]. Therefore, insufficient supply of β-glucosidase activity (intracellular or extracellular) is detrimental and can be addressed by increasing the enzyme production or using a more efficient enzyme.

An array of β-glucosidase enzymes from various fungi have been independently isolated by several research groups [2]. Two β-glucosidase genes from *P. chrysosporium* and one from *T. aurantiacus* were selected for this study [10, 11, 20]. The resulting *S. cerevisiae* strains have been evaluated for their ability to utilize cellobiose as sole carbohydrate source and subsequent ethanol production. The recombinant *S. cerevisiae* Y294[SFI] strain, constructed by [21], was used as a benchmark.

Recombinant strains expressing extracellular β-glucosidases displayed growth on cellobiose as sole carbohydrate source (Fig. 1d). Although unable to produce extracellular β-glucosidase, the *S. cerevisiae* Y294[iPcbgl1B + Lac12] strain grew on cellobiose, suggesting that the Lac12 permease was able to facilitate the transport of cellobiose across the plasma membrane into the cell where it was utilized by the intracellular β-glucosidase. The use of intracellular β-glucosidases minimizes the risk of contamination since no glucose is released into the extracellular medium [16].

The β-glucosidase enzymes in general display hydrolytic activity towards various glycosidic compounds including cellobiose, cello-oligosaccharides, different aryl and alkyl β-D-glucosides [20], and show a similar substrate recognition pattern for the *p*NP-glycosidic substrates. In this study, all the β-glucosidases released *p*-nitrophenol when incubated with *p*NPG. No activity was detected on *p*-nitrophenyl-β-D-cellobiose (*p*NPC) or *p*-nitrophenyl-β-D-mannoside (*p*NPM), indicating that the enzymes were not true β-glucosidases, but rather cellobiases.

Strains were cultivated in liquid SC^{-URA} for evaluation. Similar growth profiles were obtained for all the yeast strains cultivated on glucose, indicating that the additional genes do not result in a metabolic burden on the cells. Recombinant *S. cerevisiae* Y294[Pccbgl1] proved to be the most promising strain yielding maximum activity of > 6 nkat mg^{-1} (CDW) after 60 h on cellobiose as carbon source (Fig. 2b). The activity produced by *S. cerevisiae* Y294[Pccbgl1] was more than two times higher than that produced by the benchmark strain, *S. cerevisiae* Y294[SFI]. When cultivated anaerobically in SC^{-URA} (cellobiose) medium, *S. cerevisiae* Y294[Pccbgl1], Y294[Pcbgl1B], and Y294[SFI] were able to grow (Fig. 3a). The *S. cerevisiae* Y294[SFI] strain grew to a slightly higher biomass yield than *S. cerevisiae* Y294[Pccbgl1], but maintained a longer lag phase. *S. cerevisiae* Y294[SFI], Y294[Pccbgl1], and Y294[Pcbgl1B] consumed all the cellobiose, producing > 4.68 g l^{-1} ethanol.

Sadie et al. [16] recently reported the construction of a *S. cerevisiae* strain capable of intracellular cellobiose utilization. The lactose permease (Lac12) functioned well in transporting the cellobiose across the cell membrane under aerobic conditions. The limited growth of the *S. cerevisiae* Y294[iPcbgl1B + Lac12] strain (using the same permease) on cellobiose under aerobic conditions, indicates that the problem lies with the iPcbgl1B. The strain was unable to grow under anaerobic conditions, which might suggest that the constitutive expression of the *lac12* in *S. cerevisiae* is problematic in the absence of oxygen. This is the first report of the attempted use of the *lac12* for ethanol production from cellobiose. Much still needs to be done in terms of evaluation including testing of the *S. cerevisiae* Y294[iPcbgl1B + Lac12] strain in a bioreactor where the oxygen levels can be monitored more stringently.

This study contributes to the repertoire of β-glucosidase producing *S. cerevisiae* strains capable of utilizing cellobiose as sole carbohydrate source. The rate of bio-ethanol production is directly influenced by the level of β-glucosidase activity and rate of glucose release. Therefore, β-glucosidase activity needs to be high enough to prevent cellobiose hydrolysis from being the rate-limiting step. The *S. cerevisiae* Y294[Pccbgl1] strain was identified as the strain which performs best, thus providing an alternative β-glucosidase candidate to the well-studied Bgl1 β-glucosidase of *S. fibuligera* for efficient cellobiose conversion by recombinant *S. cerevisiae* strains. The *S. cerevisiae* Y294[Pccbgl1] strain might be further improved upon by the random mutagenesis of the yPccbgl1 vector. To our knowledge, this is the first report of the expression of these β-glucosidases in *S. cerevisiae* and the first reported use of the genes for the production of bioethanol. This is also the first report of a β-glucosidase-producing *S. cerevisiae* strain that outperforms the *S. cerevisiae* Y294[SFI] strain originally constructed by Van Rooyen et al. [21]. The *S. cerevisiae* Y294[Pccbgl1] is a good candidate for further manipulation directed towards the conversion of cellulosic substrates to ethanol.

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References

1. Bailey MJ, Biely P, Poutanen K (1992) Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 23:257–270
2. Baldrian P, Valášková V (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 32:501–521
3. Cho K, Yoo YJ, Kang HS (1999) Novel SSF process for ethanol production from microcrystalline cellulose using the δ-integrated recombinant yeast, *L2612δGC*. J Microbiol Biotechnol 9:340–345
4. Den Haan R, Rose SH, Lynd LR, Van Zyl WH (2007) Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. Metab Eng 9:87–94
5. Du Plessis L, Rose SH, Van Zyl WH (2009) Exploring improved endoglucanase expression in *Saccharomyces cerevisiae* strains. Appl Microbiol Biotechnol 6:1503–1511
6. FitzPatrick M, Champagne P, Cunningham MF, Whitney RA (2010) A biorefinery processing perspective: treatment of lignocellulosic materials for the production of value-added products. Bioresour Technol 101:8915–8922
7. Fujita Y, Ito J, Ueda M, Fukuda H, Kondo A (2004) Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. Appl Environ Microbiol 70:1207–1212
8. Galazka JM, Tian C, Beeson WT, Martinez B, Glass NL, Cate JHD (2010) Cellodextrin transport in yeast for improved biofuel production. Science 330:84–86
9. Hill J, Ian KA, Donald G, Griffiths DE (1991) DMSO-enhanced whole cell yeast transformation. Nucleic Acids Res 19:5791
10. Hong J, Tamaki H, Kumagai H (2007) Cloning and functional expression of thermostable β-glucosidase gene from *Thermoascus aurantiacus*. Appl Microbiol Biotechnol 73:1331–1339
11. Kawai R, Yoshida M, Tani T, Igarashi K, Ohira T, Nagasawa H, Samejima M (2003) Production and characterization of recombinant *Phanerochaete chrysosporium* β-glucosidase in the methylotrophic yeast *Pichia pastoris*. Biosci Biotechnol Biochem 67:1–7
12. La Grange DC, Pretorius IS, Van Zyl WH (1996) Expression of a *Trichoderma reesei* beta-xylanase gene (*XYN2*) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 62:1036–1044
13. Lynd LR, Weimer PJ, Van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol 66:506–577
14. McBride JE, Zietsman JJ, Van Zyl WH, Lynd LR (2005) Utilization of cellobiose by recombinant β-glucosidase-expressing strains of *Saccharomyces cerevisiae:* characterization and evaluation of the sufficiency of expression. Enzyme Microb Technol 37:93–101
15. Quadri SMH, Desil VAMI, Zubairi S (1980) Rapid test for determination of esculin hydrolysis. J Clin Microbiol 12:472–474
16. Sadie CJ, Rose SH, Den Haan R, Van Zyl WH (2011) Co-expression of a cellobiose phosphorylase and lactose permease enables intracellular cellobiose utilisation *Saccharomyces cerevisiae*. App Microbiol Biotechnol 90:1373–1380
17. Sanchèz C (2009) Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 27:185–194
18. Sambrook J, Fritsch EF, Miniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
19. Tsai S-L, Oh J, Singh S, Chen R, Chen W (2009) Functional assembly of minicellulosomes on the *Saccharomyces cerevisiae* cell surface for cellulose hydrolysis and ethanol production. Appl Environ Microbiol 75:6087–6609
20. Tsukada T, Igarashi K, Yoshida M, Samejina M (2006) Molecular cloning and characterization of two intracellular β-glucosidases belonging to glycoside hydrolase family 1 from the basidiomycete *Phanerochaete chrysosporium*. Appl Microbiol Biotechnol 73:807–814
21. Van Rooyen R, Hahn-Hägerdal B, La Grange DC, Van Zyl WH (2005) Construction of cellobiose growing and fermenting *Saccharomyces cerevisiae* strains. J Biotechnol 120:284–295