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ORIGINAL PAPER

Efficient expression of a *Paenibacillus barcinonensis* endoglucanase in *Saccharomyces cerevisiae*

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Abstract The endoglucanase coded by *celA* (GenBank Access No. Y12512) from *Paenibacillus barcinonensis*, an enzyme with good characteristics for application on paper manufacture from agricultural fibers, was expressed in *Saccharomyces cerevisiae* by using different domains of the cell wall protein Pir4 as translational fusion partners, to achieve either secretion or cell wall retention of the recombinant enzyme. Given the presence of five potential *N*-glycosylation sites in the amino acid sequence coded by *celA*, the effect of glycosylation on the enzymatic activity of the recombinant enzyme was investigated by expressing the recombinant fusion proteins in both, standard and glycosylation-deficient strains of *S. cerevisiae*. Correct targeting of the recombinant fusion proteins was confirmed by Western immunoblot using Pir-specific antibodies, while enzymatic activity on carboxymethyl cellulose was demonstrated on plate assays, zymographic analysis and colorimetric assays. Hyperglycosylation of the enzyme when expressed in the standard strain of *S. cerevisiae* did not affect activity, and values of 1.2 U/ml were obtained in growth medium supernatants in ordinary batch cultures after 24 h. These values compare quite favorably with those described for other recombinant endoglucanases expressed in *S. cerevisiae*. This is one of the few reports describing the expression of *Bacillus* cellulases in *S. cerevisiae*, since yeast expressed recombinant cellulases have been mostly of fungal origin. It is also the first report of the yeast expression of this particular endoglucanase.

Keywords Endoglucanase · *Paenibacillus barcinonensis* · *Saccharomyces cerevisiae* · Secretion of recombinant proteins · Pir4 cell wall protein

Introduction

Cellulose is, together with xylan, the major component of plant biomass; it is a polymer of β-D-1,4-linked glucose units that is the main constituent of plant cell walls and one of the most abundant organic compounds in the biosphere. The degradation of cellulose that occurs in nature is carried out mainly by microorganisms, and many fungal and bacterial species able to use cellulose as a carbon source have been identified. Most cellulolytic microorganisms produce a battery of cellulases that act synergistically to solubilize crystalline cellulose [5, 33]. Efficient hydrolysis of cellulose depends on the action of two enzymes. Endo-1,4-β-glucanases (E.C. 3.2.1.4) catalyze the endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, hydrolyzing cellulose to cellooligosaccharides. Simultaneously, exo-1,4-β-glucanases (E.C. 3.2.1.91), cellobiohydrolases, remain attached to the substrate and processively release cellobiose from one (reducing or non-reducing) of the two chain ends [6, 56].

Baker's yeast, *Saccharomyces cerevisiae*, is an attractive host for the production of recombinant proteins. It is a GRAS (generally regarded as safe) organism that has been used in the food industry and grown at the industrial scale for centuries. More recently, *S. cerevisiae* has been applied

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to the production of bioethanol used as an alternative renewable transportation fuel. Bioethanol represents an attractive alternative, with lignocellulosic biomass receiving considerable interest as a resource for production of bioethanol due to its abundance and low cost [16, 30, 33–35, 62, 64]. *S. cerevisiae* is known to produce ethanol from fermentable sugars, has a high ethanol tolerance, and it is very robust in industrial processes. However, it cannot naturally utilize cellulosic material and the production of ethanol from this source requires preliminary pre-treatment steps to yield fermentable sugars [35, 64]. This limitation has led to the development of strains of *S. cerevisiae*, expressing recombinant cellulases, which posses the ability to enzymatically degrade lignocellulose and ferment the resulting sugars to ethanol in a single step, in a process that has been named consolidated bioprocessing (CBP) [35]. Synergistic expression of cellulase enzymes for degradation of cellulosic substrates has been demonstrated by several studies [15–18, 26, 28, 63], with some of the strains created being able to grow on acid-swollen cellulose and some others requiring reduced amounts of added external cellulases in the pretreatment step.

Other potential, and more traditional, fields of application of *S. cerevisiae* strains expressing recombinant cellulases are those of wine-making [12, 31, 49, 61] and beer brewing [4, 21, 23, 32]. The expression of an endo-β-1,4-glucanase, among other enzymes, in a recombinant wine yeast strain, decreased turbidity after maceration and increased free-flow wine, showing the potential of recombinant cellulase-secreting wine yeast strains in the commercial-scale processing and clarification, color extraction and stabilization and aroma enhancement of wine [31, 61]. Similarly, the expression of endo-β-1,4-glucanase *egl1* gene from *Trichoderma reesei* in a brewer's yeast strain led to a reduction of the content of barley β-glucans in the beer wort, enhancing filterability as a result [32].

In this paper, we present the expression of the *celA*-coded endo-β-1,4-glucanase from *Paenibacillus barcinonensis* in *S. cerevisiae*. *P. barcinonensis* [48] is a newly identified species, originally isolated from a rice field, which shows a multiple enzyme β-glycanase system correlated with its high polysaccharide degrading potential [8–10, 43, 47]. Endoglucanase A (CelA) has been previously characterized, showing the ability to modify cellulose fibers and to improve the properties of pulp and paper made from wheat straw [9]. Here we show the efficient expression of this endo-β-1,4-glucanase in *S. cerevisiae* as an active and stable enzyme secreted into the growth medium or retained on the cell wall. For this, we have used translational fusion to the Pir4 cell wall protein [37], a technique we have successfully used previously for the secretion of recombinant enzymes and antigens in *S. cerevisiae* [1–3, 36, 40, 41].

Materials and methods

Strains and media

Escherichia coli DH5α was used as the cloning host; it was cultivated in Luria–Bertani broth supplemented with 100 μg of Ampicillin per milliliter when necessary. The standard *Saccharomyces cerevisiae* strains BY4741 (MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1) and *mnn9* (MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, ypl050c::kanMX4), used in this study were obtained from the EUROSCARF collection (Heidelberg, Germany). Table 1 presents a summary of the yeast strains used in this study. Yeast strains were cultivated in YPD (1%yeast extract, 2% Bacto peptone, 2% glucose, pH 6.5), or synthetic minimal medium YNB: 0.7% yeast nitrogen base without amino acids, 2% glucose, pH 6.5 with amino acids added as required (uracil, 35 mg/l; methionine, 20 mg/l; histidine, 80 mg/l; leucine, 20 mg/l). Cultivation took place on an orbital shaker at 28°C and 180 revolutions per minute for 24–48 h.

Reagents

Agar, yeast extract, peptone, and yeast nitrogen base were purchased from Pronadisa (Madrid, Spain); DNA restriction and modification enzymes were from Roche, New England Biolabs, Inc. (Beverly, MA) and Amersham-Pharmacia (Amersham, UK). Phenylmethyl sulfonyl fluoride (PMSF), Tris base, HCl and other buffer reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and from Panreac (Barcelona, Spain). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA). Nitrocellulose membranes, the chemiluminescence ECL reagents for developing Western immunoblots and the goat anti-rabbit IgG-peroxidase were from Amersham-Pharmacia and Roche. Carboxymethyl cellulose (CMC), Congo Red and all reagents for activity and zymogram assays were purchased from Sigma.

Transformation of strains and DNA isolation

Basic DNA manipulation and transformation in *E. coli* was performed as described by Sambrook et al. [46]. Yeast transformation was carried out following the lithium acetate method [20, 25]. Plasmid DNA from *E. coli* was prepared using the Quantum Prep® Plasmid Miniprep Kit (Bio-Rad Laboratories) and DNA fragments were purified from agarose gels using the Agarose Gel DNA Extraction Kit (Roche).

Construction of the gene fusion between *PIR4* and *cel*A

Construct C1 consisted of the insertion of the coding sequence of *Paenibacillus barcinonensis cel*A (GenBank

Table 1 List of *S. cerevisiae* strains used in this study

Strains	Genotype	Source
BY4741	*MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1*	Euroscarf
mnn9	*MATα, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1,ypl050c::kanMX4*	Euroscarf
C1-BY4741	*MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, (YEplac195-PIR4/celA-BglII)*	This study
C2-BY4741	*MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1, (YEplac195-PIR4celA-BglII-SalI)*	This study
C1-mnn9	*MATα, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1,ypl050c::kanMX4, (YEplac195-PIR4/celA-BglII)*	This study
C2-mnn9	*MATα, ura3Δ0, leu2Δ0, met15Δ0, his 3Δ1,ypl050c::kanMX4, (YEplac195-PIR4/celA-BglII-SalI)*	This study

Access Number Y12512) gene [9], lacking the 5′region coding the leader peptide, in the *Bgl*II site of *PIR4* (ORF YJL158C; www.yeastgenome.org). The truncated 1,091-bp fragment of *cel*A was amplified from pCelA [9] using oligonucleotides CB5-CB3 (Table 2). The primers contained the restriction sites for the enzyme *Bgl*II and had been designed in such a way that the *cel*A fragment would be inserted in-frame in *PIR4* in construct pIA1, based on YEplac112 [2]. The PCR fragments, amplified using Expand High Fidelity DNA Polymerase (Roche), were initially subcloned in the *Hinc*II site of pUC18; digested out with *Bgl*II and inserted into the corresponding sites in pIA1. Orientation of the inserts was confirmed by performing colony PCR on transformants using primers PIR5 and CB3 (Table 2).

Construct C2 involved the substitution of a fragment of *PIR4* by the coding sequence of *cel*A. In this construct, the 1,091-bp fragment of *cel*A was amplified using primers CB5 and CS3 (Table 2) and plasmid pCelA as template. The amplified fragment was subcloned in the *Hinc*II site of pUC18, digested out with *Bgl*II and *Xho*I and subcloned in pIA1 previously digested with enzymes *Bgl*II and *Sal*I with the loss of 365 bp of the 5′region of the *PIR4* ORF.

Isolation of cell wall mannoproteins

Cell walls were purified and extracted with β-mercaptoethanol as follows: cells in the early logarithmic phase were harvested and washed twice in buffer A (Tris–HCl 10 mM, pH 7.4, 1 mM in PMSF). The harvested biomass

Table 2 Primers used to amplify the coding sequence of the *celA* gene minus the region coding the leader peptide (CB5-3, CS3) and for confirmation of the orientation of inserts (PIR5-3)

Primer	Sequence
PIR5	5′-TGCATTCCATACGATTTCCACGGG-3′
PIR3	5′-GTGTATATTAAAGGCTGCATGTGG-3′
CB5	5′-GGGGATATAGATCTTATCGTTAACGGCTGCTCAG-3′
CB3	5′-TGCAGAAGATCTTCTGCATGCCCTGCATGATG-3′
CS3	5′-ATGCAACTCGAGCTGCATGCCCTGCATGATG-3′

Restriction sites used in subcloning are *underlined*

was resuspended in buffer A in a proportion of 2 ml per gram wet weight. Glass beads (0.45 mm in diameter) were added up to 50% of the final volume, and the cells were broken by shaking four times for 30 s, with 1-min intervals, in a CO_2 refrigerated MSK homogenizer (Braun Melsungen, Germany). Breakage was confirmed by phase contrast microscopy and the walls were washed six to eight times in buffer A. Removal of non-covalently bond proteins was achieved by boiling the walls in buffer A containing 2% SDS (10 ml per gram of walls, wet weight) for 10 min, followed by six to eight washes in buffer A. The purified cell walls were finally resuspended in 10 mM ammonium acetate buffer, pH 6.3, containing 2% (v/v) β-mercaptoethanol (5 ml per gram of walls, wet weight) and incubated for 3 h at 30°C in an orbital incubator at 200 revolutions per minute. The β-mercaptoethanol cell wall extract was separated from the cell walls by centrifugation and concentrated by lyophilization.

SDS–polyacrylamide gels and Western-blot analysis

Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [27] in 10% polyacrylamide gels. The proteins separated by SDS–PAGE were transferred onto Hybond-C nitrocellulose membranes as described by Towbin et al. [58] and Burnette [11]. Membranes were blocked overnight in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% nonfat milk. The blocked membranes were washed three times in TBST and incubated for 1 h in TBST containing an antibody that reacts with Pir cell wall proteins [37] at a dilution of 1:5,000. After another three washes in TBST, membranes were incubated for 20 min in TBST containing goat anti-rabbit IgG-peroxidase at a dilution of 1:12,000 and washed again in TBST. Finally, antibody binding was visualized on X-ray film by using the ECL method (Amersham).

Determination of cellulase activity

Cellulase activity was detected on plates according to the method described by Teather and Wood [55], based on the reactivity of Congo Red dye with glucose polymers, and

modifying the composition of the media as described by Strauss et al. [53]. The different strains were plated on YPD plates containing carboxymethyl cellulose (CMC) at a concentration of 0.5% and cultivated at 28°C for 72 h. The plates were then flooded with a solution of Congo Red at 0.1% for 20 min and destained in 1 M NaCl until clear halos could be seen around colonies as a consequence of the degradation of the CMC substrate. Zymogram assays were performed on SDS–PAGE gels containing CMC at a final concentration of 0.2% [9, 44]. Samples were boiled for 2 min in SDS–PAGE sample buffer before loading. Once the electrophoresis was completed, gels were washed in 2.5% Triton X-100 for 30 min to eliminate SDS and allow the renaturalization of the separated proteins. The gels were then rinsed for another 30 min in 50 mM acetate buffer at pH 4, followed by an incubation of 2 h in the same buffer at 45°C. The gels were stained in 0.1% Congo Red solution for 15 min and destained in NaCl 1 M until clear bands indicative of cellulase activity became visible. The gels were then immersed in a 5% solution of acetic acid, which causes the background to turn blue, and the gels were then photographed.

Cellulase activity quantification was performed on samples of growth medium supernatant concentrated 20-fold as previously described [9], based on the determination of reducing sugars released by the action of cellulase from CMC. Samples were incubated in a final volume of 1 ml 50 mM acetate buffer (pH 4.4), containing 1.5% CMC as substrate, for 15 min at 45°C and the amount of reducing sugars released was determined by the Somogyi Nelson technique [52]. Test tubes were centrifuged at 16,000 × *g* for 5 min before determining absorbance at 520 nm [9]. One international unit of activity was defined as the amount of enzyme necessary to release 1 μmol of reducing sugars, measured as μmol of glucose, per minute under the assay conditions described. Values of activity are expressed as U/ml of non-concentrated supernatant.

Results

PIR4/celA gene-fusion strategies and expression in *Saccharomyces cerevisiae*

Two different gene fusion strategies were used to achieve the targeting of the endoglucanase A from *Paenibacillus barcinonensis* either to the cell wall or to the growth medium. Pir4 belongs to the family of PIR cell wall proteins of *Saccharomyces cerevisiae* (PIR-CWPs), all of which share the presence of a signal peptide and that of a pro-peptide (subunit I) that is processed at the Golgi by the Kex2p protease. The mature protein (subunit II) includes a 19-amino-acid repetitive domain and a conserved carboxy-terminus that contains four cysteine residues at fixed positions, which, considering the extractability of some PIR-CWPs by reducing agents [37, 38], should be responsible for cell wall retention. The first fusion strategy consisted of inserting the coding sequence of the *celA* gene, lacking the 5′ fragment coding the leader peptide, in the naturally occurring *Bgl*II site close to the amino-terminus of subunit II of *PIR4*, to achieve cell wall retention (Fig. 1). In the second strategy, the *Bgl*II-*Sal*I region of *PIR4* was substituted by the coding sequence of the *celA* gene, lacking the leader peptide, to achieve secretion of the cellulase to the growth medium (Fig. 1). For this, the *celA* sequence was amplified using plasmid pCelA [9] as template and primers which included in their 5′ends restriction sites compatible with the *Bgl*II or *Bgl*II-*Sal*I sites in *PIR4*, and which had been designed to fit in frame in the corresponding sites in the ORF of *PIR4*.

The two constructs, named C1 (*Bgl*II, cell wall targeting) and C2 (*Bgl*II-*Sal*I, secretion), based in YEplac112, were then transformed to the parental BY4741 and the *mnn9* [22] glycosylation-deficient strains of *S. cerevisiae*, and the resulting recombinant strains were tested for cellulase activity in carboxymethyl cellulose plates (Fig. 2). As can be deduced from the halos formed around the colonies, both constructs C1 and C2 confer cellulase activity to both the standard and the glycosylation-deficient strains harboring them. This activity is more noticeable in the strains harboring the C2 construction, designed to drive the secretion of the recombinant protein into the growth medium and it does not seem to be affected by expression in a standard yeast strain in which the bacterial enzyme may be hyperglycosylated after passing through the secretory pathway.

Study of the localization of the recombinant Pir4-CelA fusion proteins by Western-blot analysis and zymograms

To find out if the Pir4-CelA fusion proteins derived from constructs C1 and C2 were being correctly targeted to the

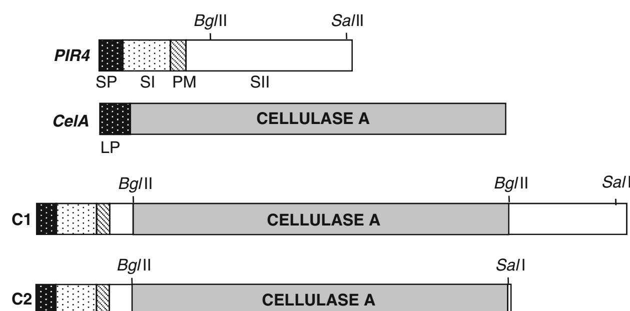

Fig. 1 Schematic representation of the *CelA* and *PIR4* genes together with the C1 and C2 gene fusions. *SP* signal peptide; *SI* subunit I; *PM* PIR motive; *SII* subunit II; *LP* leader peptide. Mature Pir4 corresponds to subunit II after removal of subunit I by Kex2 protease at the Golgi

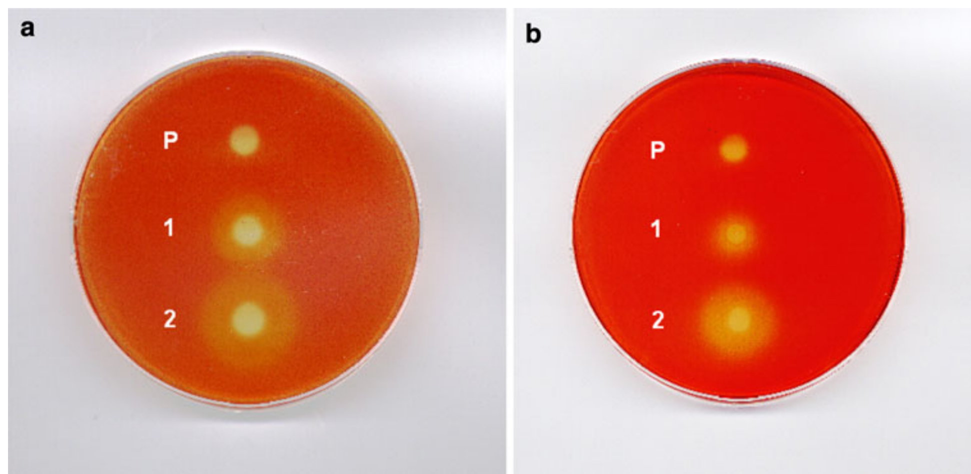

Fig. 2 Cellulase plate assay of the different strains harboring constructs C1 (1) and C2 (2) on YPD plates containing 0.5% CMC as substrate; halos around the colonies represent the degradation of the substrate as highlighted by Congo Red staining. Strains based on BY4741 (**a**), and *mnn9* (**b**). *P* corresponds to the parental strains BY4741 (**a**) or *mnn9* (**b**)

cell wall or the growth medium, β-mercaptoethanol extracts of purified cell walls of the different strains, and concentrated samples of growth medium, were probed by Western immunoblot using an antibody that reacts with Pir-CWPs of *S. cerevisiae* [37]. The results in the case of the standard *S. cerevisiae* BY4741 strain (Fig. 3a) show specific bands of over 100 kDa and around 75 kDa in the β-mercaptoethanol extracts from purified cell walls of the C1 strain. These bands are absent in the extracts of either the parental or the C2-transformed strain, indicating the correct targeting of the Pir4-CelA fusions derived from construction C1. The higher than the 55 kDa expected size together with the lack of definition of the bands suggest that the fusion protein is profusely glycosylated, the lower molecular weight band possibly corresponding to a degradation product or to a less glycosylated form of the protein. Identical analysis in the C1-transformed glycosylation-deficient *mnn9* strain (Fig. 3c) revealed the presence of bands of lower and better-defined apparent size, 70 and 30 kDa, the second one possibly a degradation product. This indicates that in both strains, the Pir4-CelA fusions derived from construct C1 are correctly targeted to the cell wall and suggest that the degree of glycosylation, as expected, is lower in the *mnn9* strain. The presence of Pir4-CelA fusions was also probed in the growth medium of the strains harboring the different constructs. In the case of the concentrated growth medium from the BY4741 strain transformed with construction C2 (Fig. 3b), it was possible to detect the presence of two polypeptides of around 100 and 70 kDa that were not present in the parental strain nor in the strain transformed with construction C1. A low-molecular-band (18 kDa) was also detected in the growth medium of the C1-transformed BY4741 strain, however, this band may represent either a degradation product of the partially leaked C1-derived protein fusion or a degradation product that is no longer retained by the cell wall. Specific bands were also found in the concentrated growth medium from the C2-transformed *mnn9* strain (Fig. 3d), although in this case, with a size of around 70 kDa. This band, however, was also apparent in the concentrated growth medium of the C1-transformed *mnn9* strain, a fact that would indicate a partial leakage of the cell wall-targeted polypeptide to the growth medium. As with the cell wall-targeted fusions, the results from the concentrated growth medium indicate both that construction C2 drives the secretion of the Pir4-CelA fusion, and that the size of the fusion protein is lower when expressed in the *mnn9* strain, probably as a consequence of the limited glycosylation in this strain.

To confirm that the polypeptides detected were indeed responsible for the cellulase activity observed in plate assays, zymograms were performed on the concentrated growth medium from the different strains. The results show the presence of activity bands of around 70–75 kDa and over 100 kDa both in the growth medium of the C2-transformed BY4741 strain and, to a lesser extent, in the growth medium of the C1-transformed strain, but not in the growth medium of the parental strain (Fig. 4a). Similarly, the assay performed on the *mnn9*-based strains revealed the presence of activity bands of around 70 kDa, both in the growth medium of the C1- and C2-transformed *mnn9* strains that were not present in the *mnn9* parental strain (Fig. 4b). This result confirms complete secretion, as expected, in the case of the C2-derived fusion protein, and leakage or partial secretion of the C1-derived fusion in both the standard and glycosylation-deficient *mnn9* strain, as detected in Western blot.

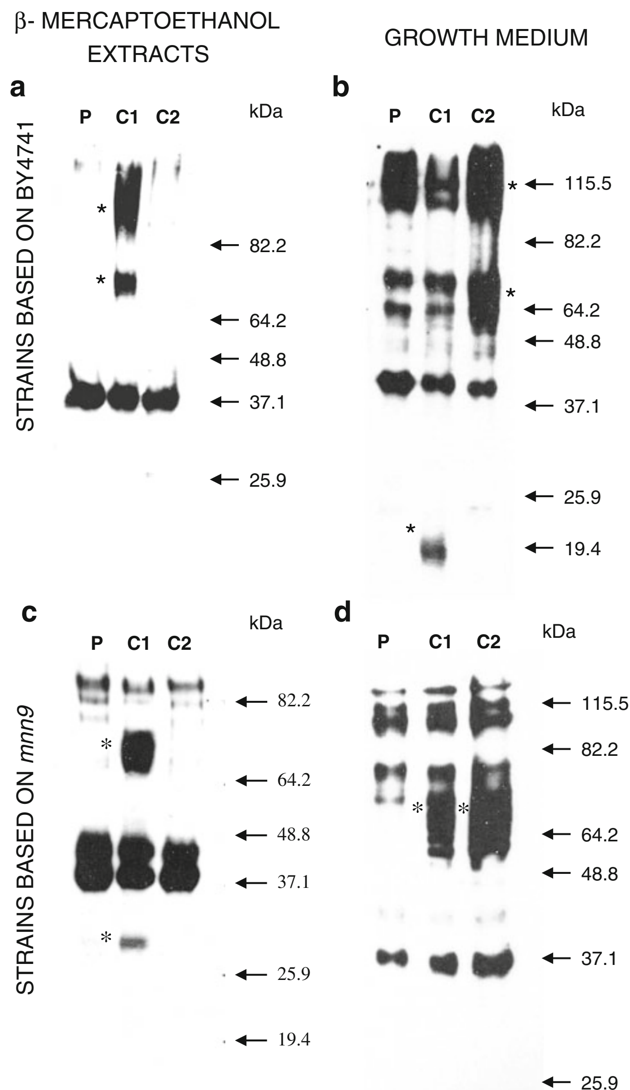

Fig. 3 Immunoblot localization of the different recombinant fusion-proteins derived from constructs C1 (*lane 2*) and C2 (*lane 3*) in strain BY4741 (**a**, **b**) and *mnn9* (**c**, **d**), using an antibody that reacts with the Pir4 protein. **a**, **c** *β*-Mercaptoethanol extracts from purified cell walls. **b**, **d** Growth medium. *Lane 1* (P) corresponds to the untransformed BY4741 or *mnn9* strains. 10 μg of total protein was loaded in each lane. *Stars* indicate recombinant proteins

Quantification of cellulase activity secreted into the growth medium

The activity of the cellulase that was secreted into the growth medium was quantified by determining the amount of reducing sugars released from CMC [9] after incubation with concentrated growth medium from the different strains constructed. The assay was performed in triplicate, in three successive experiments on each strain, after 24 h (BY4741-based strains) or 48 h (*mnn9*-based strains) cultivation in YNB selective medium, reaching cellular densities of around 3 O.D units for the BY4741-based strains and 1 O.D units for the *mnn9*-based strains. The values of CMCase activity detected in the growth medium are consistent with the results obtained in both plate assays and zymograms and, to a lesser extent, with those from the Western-blot analysis using specific antibodies (Table 3). Maximum activity values were around 1.2 units/ml after 24-h growth (C2-transformed BY4741) or 1.3 units/ml after 48-h growth (C2-transformed *mnn9*). Taken together, these results confirm correct targeting of the C2-derived fusion proteins to the growth medium and suggest the existence of at least partial leakage of the C1-derived fusion proteins targeted to the cell wall, into the growth medium. No reliable determination of cellulase activity could be performed on whole cells or cell wall extracts, possibly due to problems of accessibility of the substrate or because of loss of activity in the extraction process.

Discussion

Cellulase-expressing recombinant *Saccharomyces cerevisiae* strains have potential applications in bioethanol production from cellulosic substrates, as part of a consolidated bio-processing strategy [16, 33–35, 62, 64], in wine production, as a way to improve the efficiency of the maceration process [31, 61], and in beer brewing, by reducing the content of barley *β*-glucans in fermenting wort and enhancing filterability as a result [4, 21, 23, 32].

The aim of the work we present was to evaluate the possibility of expressing the *celA* (GenBank Access No. Y12512)-coded endo-1,4-*β*-glucanase A from *Paenibacillus barcinonensis*, an enzyme showing a high polysaccharide degrading potential [8–10, 13, 43, 47, 48], in *S. cerevisiae*. For this, we have used translational fusion with the naturally secreted Pir4 cell wall protein of *S. cerevisiae*, to create hybrid proteins Pir4-celA that, depending on the way the fusion was made, could be targeted either to the cell wall or the growth medium.

Two fusions were made; C1 involved insertion of the *celA* gene in the coding sequence of *PIR4*, which should lead to the cell wall targeting of the hybrid protein, while C2 involved substituting the cell wall retention domain of Pir4 by celA, leading to the secretion of the C2-derived fusion protein. These two constructs were transformed to both a BY4741 *S. cerevisiae* strain and, because of the presence of 5 potential *N*-glycosylation sites in the sequence of celA, in a glycosylation-deficient *mnn9* strain of *S. cerevisiae*. The two fusions tested, C1 and C2, did exhibit cellulase activity both on plate assays and by colorimetric determinations when expressed either in the BY4741 strain or in the *mnn9* glycosylation-deficient strain of *S. cerevisiae*. Western-blot analysis and zymograms of the BY4741 strain showed the existence of two bands; one

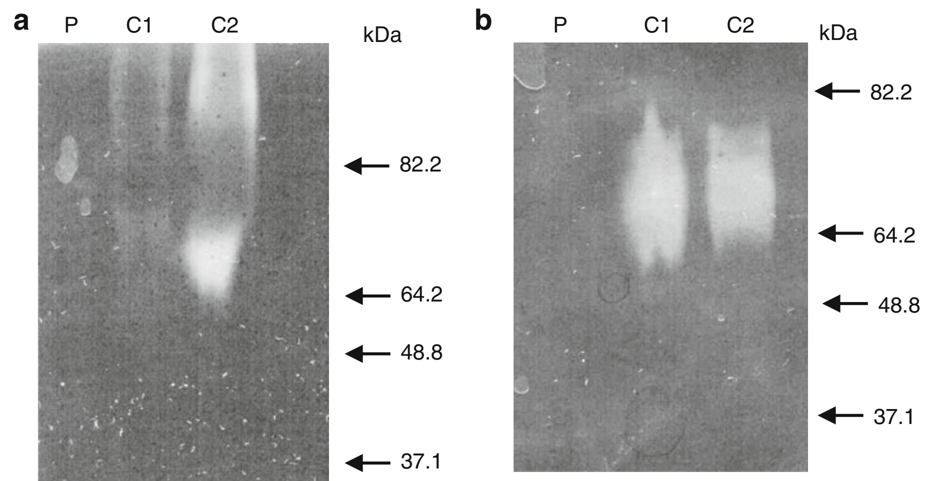

Fig. 4 Zymogram of samples of growth medium from the BY4741 (**a**), and the *mnn9* (**b**) strains of *S. cerevisiae* harboring constructions C1 (*lane 2*) and C2 (*lane 3*) after separation on SDS–polyacrylamide gels containing 0.2% CMC. *Lane 1* corresponds to the untransformed parental strains. 10 μg of total protein was loaded in each lane

Table 3 Cellulase activity determined in growth medium of strains BY4741 and *mnn9* of *S. cerevisiae*, untransformed or harboring constructions C1 or C2

Strain	CM case activity (u/ml)	±
A		
BY4741	0.02	0.003
C1-BY4741	1	0.070
C2-BY474	1.2	0.170
B		
mnn9	0.01	0.001
C1-mnn9	0.6	0.013
C2-mnn9	1.3	0.020

Cultures were grown 24 h (BY4741 strains) or 48 h (*mnn9* strains) in YNB selective medium. Cell density of the cultures reached 3 O.D units in the strains based on BY4741 and 1 O.D units in the *mnn9*-based strains. The results presented correspond to the mean of three experiments, in which the activity of each sample was determined by triplicate

of around 70–75 kDa, and another of between 80 and 100 kDa. Qin et al. [45] found a similar result when expressing Cel5A (endoglucanase II) from *Trichoderma reesei* in *S. cerevisiae*, detecting two bands that differed in the degree of glycosylation, the larger hyperglycosylated one corresponding to a smear with a size of between 85 and 200 kDa that was reduced to the approximate size of the lower molecular weight band by treatment with Endoglycosidase H. In our case, expression in the *N*-glycosylation-deficient *mnn9* strain led to the detection of a single band with a size similar to the lower-molecular-weight band detected in the BY4741 strain, both in Western blot and zymogram. This result suggests that as in the case of the endoglucanase II of *T. reesei*, the two bands detected in the BY4741 strain could correspond to different degrees of glycosylation of the same protein. If this is the case, it can be concluded from the zymograms that hyperglycosylation does not seem to affect the activity on CMC of the endoglucanase A from *P. barcinonensis* expressed in a BY4741 strain of *S. cerevisiae*. This result is significant since, contrary to endoglucanases from fungi, endoglucanases from bacteria are not naturally glycosylated, and confirms the results of previous attempts to express *Bacillus* [14, 23] or other bacterial endoglucanases [7, 24, 29, 51, 59, 60] in *S. cerevisiae*, where glycosylation did not seem to interfere with activity.

Correct targeting of the C1-coded fusion proteins to the cell wall was confirmed by cell wall purification and extraction of the fusion protein by reducing agents. Immobilization to the cell wall of recombinant enzymes by fusing them to the cell wall-retaining domains of *S. cerevisiae* cell wall proteins, apart from facilitating the recovery of the enzyme and its reusability, offers enzymes a physical support that improves their stability [19, 21, 39, 42, 50, 54]. However, in our case, Western-blot experiments also showed the presence of fusion proteins or their degradation product in the growth medium of the C1-transformed strains, a result that was confirmed by zymograms and cellulase activity determinations, suggesting the existence of at least partial leakage to the growth medium of the C1-derived fusion proteins targeted to the cell wall.

Cellulase activity in the growth medium, tested using CMC as substrate, reached values of around 1.2 units/ml for the C2-transformed BY4741 strain after 24-h growth and 1.3 units/ml for the C2-transformed glycosylation-deficient *mnn9* strain after 48 h of growth, in both cases in selective medium. These values compare quite favorably with the activity data reported for other endoglucanases expressed in *S. cerevisiae*, specifically those of fungal origin [28, 57, 65]. The results we present in this paper show the efficient secretion of *celA*-coded endoglucanase from the newly characterized species *P. barcinonensis* in *S. cerevisiae*. Further to this, we have shown that despite the five potential *N*-glycosylation sites present in the sequence

of CelA, hyperglycosylation does not seem to affect activity when this endoglucanase is expressed in *S. cerevisiae*. These results, together with the reasonable values of activity obtained, confirm the potential usefulness of the *celA*-coded endoglucanase of *P. barcinonensis* expressed in *S. cerevisiae*.

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