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ORIGINAL PAPER

Production of heterologous polygalacturonase I from *Aspergillus kawachii* in *Saccharomyces cerevisiae* in batch and fed-batch cultures

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Abstract The *pg1* gene from the filamentous fungus *Aspergillus kawachii,* which codifies for an acid polygalacturonase, was cloned into the pYES2 expression vector, giving rise to the pYES2:*pg1ΔI* construct. Engineered *Saccharomyces cerevisiae*, transformed with pYES2:*pg1ΔI* construct, both expressed and exported an active polygalacturonase with a MW of ~60 kDa and an isoelectric point of 3.7, similar to those reported for the wild-type enzyme. The recombinant enzyme has the ability to hydrolyze polygalacturonic acid at pH 2.5. Heterologous PG1 production was studied under controlled conditions in batch and fed-batch systems. A simultaneous addition of glucose and galactose was found to be the most suitable feeding strategy assayed, resulting in a final PG1 production of 50 U/ml. The production process proposed in this study could be applied for the industrial production of a novel and useful polygalacturonase.

Keywords *Aspergillus kawachii* · Acid polygalacturonase · *Saccharomyces cerevisiae* · Heterologous expression · Fed-batch culture

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Introduction

Pectins are important components of plant cell walls and facilitate many of the functions that the latter perform in plant tissues. The main structural feature of the pectin molecule is a linear chain of β-(1-4)-linked D-galactosyluronic-acid residues in which varying proportions of the acid groups are present as methyl esters. Pectins are generally associated with neutral polysaccharides (arabinans, arabinogalactans, and galactans). Pectins are used in the food and pharmaceutical industries as natural ingredients because of their ability to form gels at low concentrations and increase the viscosity of liquid foods [24]. Pectinolytic enzymes are widely used in the food and beverage industries to clarify fruit juices and wines, to improve cloud stability in fruit and vegetable nectars, and to increase the protein content of fruit juices. The ability to degrade pectins could be used as an alternative method of pectin extraction from by-products of the fruit industry and also to macerate vegetable tissues in order to produce single-cell foods [18].

Some strains of the genus *Aspergillus* are known to be capable of growth in extremely acidic environments (pH 2) and to produce acid-stable enzymes that are peculiar to them and with appropriate properties for a potential use on an industrial scale. *Aspergillus kawachii* (IFO 4308), a white koji mold used in the Japanese shochu process, produces several acidic depolymerases such as α-amylases and glucoamylases [16], xylanases [14], and acidophilic proteinases [25]. An acidophilic polygalacturonase (PGase) from *A. kawachii* has been reported by Contreras Esquivel et al. and was referred to by the authors as PG1. This enzyme has been purified and some properties, including the N-terminal amino-acid sequence, determined [6]. Moreover, Mikami and Fukuda have reported the nucleotide sequence of an

acidic pectinase from *A. kawachii* called PgaA (GenBank accession no. **AB080269**). Since the N-terminus of PgaA and PG1 were found to be identical, the two enzymes are probably one and the same.

A. kawachii, like many other fungi, produces small quantities of PG1. Even when culture conditions have been optimized for enzyme expression, the activity obtained has not been sufficient for industrial purposes. For this reason, the cloning of the *pg1* gene and the appropriate genetic engineering for its overexpression would be a desirable approach in order to increase enzyme production. Since the PG1 protein is a eukaryotic gene product, the expression of the *pg1* gene would most reasonably be engineered within a eukaryotic system. The brewer's yeast *Saccharomyces cerevisiae* has been extensively used for the production of ethanol, single-cell proteins, and as bakery yeast. *S. cerevisiae* has certain properties that have been shown to enable the expression of heterologous proteins of biotechnological interest. Gene expression systems in yeast offer a broader range of potential industrial applications than do the corresponding bacterial systems. For example, yeasts are as easy to manipulate and grow as bacteria, but, unlike the latter, their subcellular organization is capable of the posttranslational processing of many heterologous proteins [20]. *S. cerevisiae* in particular has been used successfully as a vehicle for the heterologous expression of different enzymes from *A. kawachii* as well as the PGases from other fungi [9, 12, 13, 17]. Furthermore, *S. cerevisiae* has been universally recognized as having the status of GRAS (Generally Regarded As Safe) and moreover secretes a small number of native proteins into the culture medium at relatively low levels. This latter property would facilitate the purification of any recombinant protein.

When an expression system containing the galactose-inducible promoter is used, cells are usually first cultivated with glucose as the carbon and energy source (CES) to obtain the necessary biomass before the previously cloned gene is induced by the addition of galactose. The molar ratio of galactose to glucose and the absolute concentration of galactose are the key parameters in determining the level of gene expression [10].

Although many other genes, including those encoding pectinase, have been expressed in *S. cerevisiae*, most are active under neutral or only slightly acid conditions. Since the stability of PG1 under extreme acid conditions (pH 2.5) is desirable for this enzyme's utility within an industrial context, the cloning and overexpression of the *pg1* gene would be indispensable for obtaining sufficiently large amounts of this gene product for the appropriate industrial applications.

We report here a molecular cloning of the gene for an acid PGase from *A. kawachii*, its heterologous expression in *Saccharomyces cerevisiae* in batch and fed-batch cultures, and its biochemical characterization. To our knowledge, this is the first report on the expression in *S. cerevisiae* of a pectinase gene from *Aspergillus kawachii*.

Materials and methods

Chemicals

Polygalacturonic acid (PGA) and glass beads G-9268 (425-600 μm) were obtained from Sigma-Aldrich (St Louis, MO, USA); the restriction endonucleases (*Eco*RI, *Bam*HI), Taq DNA polymerase, calf intestinal alkaline phosphatase, T4 DNA ligase, Wizard SV Gel, and the PCR Clean-up System from Promega (Madison, WI); the DNA purification kit Illustra™ MicroSpin Columns from GE Healthcare; and the *Pfl*MI from New England Biolabs (Ipswich, MA, USA). All of these products were used as recommended by the manufacturers. All other chemicals were of analytical grade.

Buffer compositions

Yeast lysis buffer (YLB): 2% (v/v) Triton× 100, 1% (w/v) SDS, 100 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl pH 8. Citric phosphate buffer (CPB): 50 mM citric acid and 25 mM Na_2HPO_3 pH 2.5.

Strains, plasmids, and culture conditions

A. kawachii IFO 4308 was used as source of DNA. *Saccharomyces cerevisiae* INVSc1 (INVITROGEN®); (MATa *his3Δ1 leu2 trp1-289 ura3-52*/MATα *his3Δ1 leu2 trp1-289 ura3-52*) was used as the recipient for the expression of the cloned *pg1* gene. *Escherichia coli* strain TOP10F′ (F′ {*lac*Iq *Tn10* (TetR)} *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *end*A1, *rec*A1, *ara*D139, Δ(ara, leu) 7697, *gal*U, *gal*K, *nup*G, *rps*L (StrR)) was used for DNA manipulation and plasmid preparation.

Plasmid pYES2 (Invitrogen®) was used as the vector for cloning, DNA manipulation, and protein expression.

A. kawachii was grown according to Contreras et al. [6]. One-liter Erlenmeyer flasks with 200 ml of growth medium (1.0 g/l K_2HPO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 2.5 g/l tryptone, and 5.0 g/l glucose) was inoculated with 10^6 spores/ml and incubated for 48 h on an orbital shaker at 200 rpm and 30°C. After depletion of the glucose in the medium, the mycelium was harvested from the culture broth by filtration and used for DNA extraction.

E. coli TOP10F′ was cultured in LB medium according to the manufacturer's protocols.

S. cerevisiae INVSC1 was cultured in SC-URA medium according to the manufacturer's protocol: 10.0 g/l CES,

3.0 g/l $(NH_4)_2SO_4$ (or 1.25 g/l urea), 1.0 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 0.1 g/l NaCl, 0.1 g/l $CaCl_2$, 1.0 ml/l of vitamin stock solution, 10.0 ml/l of amino-acid stock solution, and 1.0 ml/l of trace element stock solution. The vitamin stock solution contains: 2.0 mg/l biotin, 400 mg/l calcium pantothenate, 2.0 g/l folic acid, 2.0 g/l *myo*inositol, 400 mg/l nicotinic acid, 200 mg/l *p*-aminobenzoic acid, 400 mg/l pyridoxine, 200 mg/l riboflavin, and 400 mg/l thiamine. The amino-acid stock solution contains: 0.1 g/l of adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, and uracil plus 0.05 g/l of aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, and valine. The trace element stock solution contains: 0.5 g/l H_3BO_3, 40 mg/l $CuSO_4$, 100 mg/l KCl, 200 mg/l $FeCl_3$, 400 mg/l $MnSO_4$, and 150 mg/l $(NH_4)_2MoO_4$ pH 1.5.

DNA manipulation and transformation

Fungal DNA extraction: About 1 g of fungal mycelium was suspended in 2 ml of yeast lysis buffer in a centrifuge tube containing glass beads and mixed until no liquid phase was visible (about 30 s). The mixture was next vigorously agitated for 60 s by vortexing and immediately cooled in an ice bath for 30 s. These steps were repeated nine times. After addition of 2 ml of phenol/chloroform/isoamyl alcohol (25/24/1), the admixture was agitated for 1 min by vortexing and returned to the ice bath for 30 s. These steps were repeated four times before returning the sample once again to the ice bath for a final 20 min. The liquid mixture was separated from the glass beads, transferred to 1.5-ml Eppendorf tubes, and centrifuged for 5 min at 15,000×*g* and 4°C. Of the aqueous fraction, 500 µl were transferred to clean Eppendorf tubes containing 2 volumes of ice-cold 96% (v/v) aqueous ethanol and mixed by inversion. The sample was left on ice for 2 min to sediment the DNA and was then centrifuged again for 2 min at 15,000×*g*. The supernatant was discarded, and the pellet was washed twice with cold 70% (v/v) aqueous ethanol, before drying at room temperature and final resuspension in 50 µl of sterile miliQ water for storage at −20°C until use as PCR template.

Plasmid manipulations were performed by means of standard protocols as described by Sambrook et al. [21]. The *E. coli* and *S. cerevisiae* transformations were carried out by electroporation according to Sambrook et al. [21] and Becker and Guarente [2], respectively.

The open reading frame (ORF) that encodes PG1 was obtained from *A. kawachii* genomic DNA by PCR through the use of a set of primers based on the *pgaA* sequence. The primers were called PGfw (5′-*TTTATC*<u>*GGATCC*</u>AT GCCTTCTGCCAAGCC-3′) and PGrv (5′-*TTTATC*<u>*GAA TTC*</u>TT ACTGACTGCAGGAAGCGC-3′. Italicized letters correspond to noncoding residues. Based on the sequence of the polylinker region from pYES2, *Bam*HI, and *Eco*RI restriction sites (underlined) were added to the respective primers in order to insure the correct orientation. Amplification was performed in a DNA Amplifier Mastercycler® (Eppendorf AG, Hamburg, Germany) with Taq DNA polymerase (Promega) and standard concentrations of buffer, primers, and templates. The amplification profile was: (a) 95°C for 60 s; (b) 8 cycles of 94°C for 30 s, 54°C for 90 s, 72°C for 100 s; (c) 30 cycles of 94°C for 30 s, 64.5°C for 50 s, 72°C for 100 s; (d) 72°C for 5 min. The DNA fragment obtained, referred to as *pg1*, was recovered by ethanol precipitation from the PCR mixture [21]. Both *pg1* and pYES2 were digested with *Eco*RI and *Bam*HI. The plasmid was also dephosphorylated with calf intestinal alkaline phosphatase to avoid recircularization. The DNA was precipitated with ethanol [21] and resuspended in distilled water. The *pg1* gene was ligated to the pYES2-inducible vector by means of T4 DNA ligase, thereby generating the plasmid pYES2:*pg1*. The absence of mutations was confirmed by DNA sequencing (Macrogen Inc., Gasan-dong, Seoul, 153-023, Republic of Korea).

Intron excision from the *pg1* gene

The intron was excised from *pg1* as follows: First, the need for a restriction enzyme with a recognition site corresponding to a unique sequence located inside the second exon and near its 5′ end was fulfilled by *Pfl*MI. A primer, called PG1wI, was designed according to the intron-adjacent sequences and was hence composed of 16 bases at the 3′ end of the first exon and 31 bases at the 5′ end of the second exon, including the recognition site of *Pfl*MI (underlined): PG1wI: 5′ ACTC<u>CCAGGTGGTGG</u>TTCC CTCAAAGGTGACGGTGGTGCCATCGTTG 3′. In order to amplify the first exon, a PCR reaction was done with pYES2:*pg1* as template and PGfw and PG1wI as primers. The reaction was carried out in 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. The amplification product (228 bp) was purified from the PCR mixture through the use of the DNA purification kit Illustra™ MicroSpin Columns and digested with *Bam*HI and *Pfl*MI. Plasmid pYES:*pg1* was then digested with *Bam*HI and *Pfl*MI to release a cassette composed of the first exon, the intron, and the 5′ end of the second exon. The largest fragment (the vector ligated to the upstream portion of the second exon) was next purified on a 1% (w/v) agarose gel by means of the Wizard SV Gel and PCR Clean-up System (Promega). Finally, both the amplification product and the digested plasmid were ligated together to generate the pYES:*pg1ΔI* construct, whose DNA was sequenced in order to confirm the presence of the ORF and the excision of the intron. After amplifying this construct in *E. coli*

TOP10F′ as indicated above, competent *S. cerevisiae* INVSc1 cells were transformed with pYES:*pg1ΔI*, plated onto SC-URA medium, and screened for *pg1ΔI* by colony PCR with PGfw and PGrv as primers.

Expression studies

Batch cultures in flasks

The expression of *pg1* in submerged cultures in flasks was carried out through two different protocols. Preliminary studies were conducted according to the Invitrogen recommendations (Raffinose protocol). The transformed *S. cerevisiae* was cultured overnight in SC-URA medium with raffinose as the CES. An aliquot of the culture was centrifuged at 1,500×*g* for 15 min, and the pellet resuspended and inoculated into 100 ml of induction medium [SC-URA medium with 1% (w/v) raffinose as the CES containing 2% (w/v) galactose as the inducer] at an initial OD_{600} of 0.4. Samples (5 ml) were thereafter taken every 2 h for a total of 10 h. The OD_{600} and pH were measured and an aliquot centrifuged and frozen for further analytical assays and determination of enzyme activity.

A second strategy (the glucose protocol) was also used. Transformed *S. cerevisiae* cells were cultured overnight in SC-URA medium with glucose as the CES. An aliquot of culture was centrifuged at 1,500×*g* for 15 min. The pellet was resuspended and inoculated into 100 ml of SC-URA medium plus 1% (w/v) glucose as the CES. When the glucose became depleted and exponential growth ceased, the culture was subjected to starvation for 2 h to avoid glucose repression and the expression system then induced by the addition of galactose to a final concentration of 2% (w/v). The sample processing was similar to that of the raffinose protocol.

Bioreactor cultures

Batch and fed-batch cultures were carried out in a 1.5 l BioFlo 310 Benchtop Bioreactor (New Brunswick Scientific Corp., Edison, NJ, USA) with SC-URA medium following the glucose protocol, at 30°C, with aeration (1.0 vvm) and agitation (450 rpm). The culture pH was measured with a glass electrode (Mettler-Toledo) and automatically controlled at either 5.5 (growth phase) or 4.5 (induction phase) with 1 N H_2SO_4 or 1 N NaOH. The dissolved oxygen was measured with a polarographic-type electrode (Mettler Toledo). The outlet gas was analyzed with a paramagnetic O_2 detector (Series 1100, Servomex, USA) and an infrared CO_2 detector (Pir 2000, Horiba, Japan). The O_2 uptake and CO_2 production rates were calculated according to Cooney et al. [7].

In order to obtain the culture to be seeded for fermentation, cells from the glycerol stock kept at −70°C were first inoculated into SC-URA solid medium and grown overnight, then inoculated into 100 ml of liquid SC-URA medium, grown once again overnight, and then collected by centrifugation. After resuspension, these cells were inoculated into the fermentor containing SC-URA medium.

Batch fermentation was carried out in 1.1 l of SC-URA medium. After glucose depletion an induction similar to that of the glucose protocol was carried out. The kinetics and stoichiometric parameters of *S. cerevisiae* INVSc1:*pg1ΔI* growth were determined during the growth phase (with glucose as the CES).

Fed-batch fermentations were carried out through the use of three different feeding and induction strategies: (1) after a glucose growth phase with a single pulse of galactose [at a final concentration of 10% (w/v)]; (2) after a glucose growth phase, with a double pulse of galactose [10% (w/v)]; and (3) a simultaneous feeding of both glucose and galactose [10% (w/v)] as the CES and inducer, respectively. The same conditions of batch fermentation were maintained during feeding phase except that the shaking speed was regulated according to the dissolved O_2 concentration.

The fed-batch protocol (with respect to the glucose concentration and a constant feeding rate) was designed according to the equations derived from the mass balances for the substrate and biomass in carbon-limited cultures by means of the kinetic and stoichiometric parameters calculated in the batch cultures.

$$\mathrm{Sr} = \frac{X_f V_f - X_0 V_0}{Y_{X/S} \cdot (V_f - V_0)} \quad F = \frac{\mu_0 X_0 V_0}{Y_{X/S} \cdot \mathrm{Sr}}$$

where Sr is the concentration of limiting substrate (CES) in the feeding medium; X_0, X_f is the biomass concentration at the beginning and the end of the feeding phase (g/l), respectively; V_0 and V_f, the initial and final volume (l); F, the feeding rate (l/h), μ, the specific growth rate (h^{-1}); and $Y_{X/S}$, cellular yield coefficient based on carbon source consumption (g cell/g carbon source).

Enzyme assay

PG1 activity was determined as described by Contreras et al. [6] with minor modifications. The enzyme activity was measured at pH 2.5 with PGA [0.2% (w/v) in CPB] as the substrate. When low activity was expected, the reaction time was extended to up to 2 h. One unit of enzyme activity was defined as the production of 1 μmol of reduced sugar as galacturonic acid per min.

Enzyme characterization

The influence of pH on the enzyme activity was determined with 0.2% (w/v) PGA in CPB as substrate after varying the pH from 2.0 to 5.0. The thermal stability of the expressed

PG1 was evaluated by incubating the enzyme at pH 2.5 in CPB for 10 h at different temperatures. Samples were taken every 2 h and kept at 0°C until the time of assay.

The protein concentration was measured by the Bradford technique with Coomassie brilliant blue G-250 as the chromophore and bovine serum albumin as the standard. SDS-PAGE was carried out in a Mighty Small II Unit (Hoefer SE 260) according to Smith [23], while isoelectric focusing was performed with an LKB 2117 Multiphor II on a 7.5% (w/v) polyacrylamide gel containing 5% (v/v) Pharmalyte (pH 2.5–5.0) according to the manufacturer's instructions. PG1 was visualized after staining with Coomassie brilliant blue R-250.

Results and discussion

Cloning of the *pg1* gene

With an aim at designing specific primers to amplify the *pg1* ORF, a BlastP search was performed throughout the GenBank database http://www.ncbi.nlm.nih.gov/ [3] on the basis of the mature PG1 N-terminal amino-acid sequence [6]. A sequence of a PGase from *A. kawachii,* called PgaA, was found (GB: **AB080269**). The PgaA ORF consisted in 1,160 bp with a C + G content of 59.5%. An intron of 47 bp was located between bases 228 and 276. The intron/exon junction was consistent with the GT-AG rule, and a stretch within the intron of YGCTAAC, which sequence resembled the internal conserved sequence of filamentous fungi, was also present.

The high similarity between the known N-terminal amino-acid sequence of the mature PG1 and that of the fragment of PgaA near to the methionine residue of the deduced sequence suggested that both amino-acid sequences could either belong to the same protein family or have arisen from the same ORF. In order to investigate this hypothesis, an additional bioinformatic search was carried out. Upon analysis of the PgaA protein by the server SignalP (http://www.cbs.dtu.dk/services/SignalP/ [19]), a predicted 5′-leader sequence was indicated immediately followed by a sequence similar to the mature N-terminus of PG1. This analysis *prima facie* would indicate that the PgaA protein's first 32 amino-acid residues have a similar sequence to a processed peptide of the mating pheromones in *S. cerevisiae*. This sequence includes the dipeptide Lys31-Arg32, a natural target for the Kex2 proteinase [15]. This finding suggests that the enzyme is translated as a preproenzyme as other fungal PGases [4].

In addition, Contreras Esquivel et al. [6] have characterized PG1 biochemically in terms of its molecular weight, isoelectric point, and degree of glycosylation. Moreover, the theoretical biochemical properties of PgaA, obtained by *in silico* analysis through the servers http://www.expasy.ch/cgi-bin/protparam [1] and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), were similar to those previously reported for PG1.

Based on these results, the *pgaA* nucleotide sequence was used as a template for the design of primers called PGfw and PGrv. Next, genomic DNA from *Aspergillus kawachii* was extracted and used as a template for PCR amplification directed by the PGfw and PGrv primers. A single fragment of approximately 1,200 bp was observed in agarose gel electrophoresis (Fig. 1), as would have been expected on the basis of the sequence of *pgaA*.

The amplification product was removed from the PCR mixture by ethanol precipitation [21], digested with *Bam*HI and *Eco*RI, and ligated to pYES2. The plasmid thus generated, pYES2:*pg1*, was used to transform competent *E. coli* cells. In the several positive clones sequenced, the *pg1* and *pgaA* ORFs were found to be identical (Fig. 2).

Construction of the expression vector for yeast

S. cerevisiae INVSc1 was selected as the host for PG1 expression. In order to analyze the possibility of obtaining a functional splicing system, a bioinformatic analysis was performed to evaluate the presence of the requisite acceptor and donor sites for splicing into *Saccharomyces* sp. (Splice View, http://zeus2.itb.cnr.it/~webgene/wwwspliceview.html). The results indicated the capability of *Saccharomyces* sp. to recognize a donor, but not an acceptor, site. For this reason, it was decided to carry out the *pg1* 47-bp intron deletion in vitro through the following strategy. First, pYES2:*pg1* was used as a template for PCR with the PGfw and PG1wI primers. A fragment of ca. 228 bp was obtained, purified from the PCR product mixture with Wizard SV Gel and PCR Clean-up System, and then digested with *Bam*HI and *Pfl*MI (product A). Next, pYES2:*pg1* was digested with *Pfl*MI and *Bam*HI, and the larger fragment separated from the released cassette—this latter consisting of the first exon plus the intron (product B). Both products (A and B) were ligated. The plasmid so generated, pYES2:*pg1ΔI*, was finally used to transform competent *E. coli* TOP10′cells. Plasmids from several colonies (10) were obtained by miniprep and used as templates for PCR. The amplification products were digested with *Pfl*MI and analyzed by electrophoresis in 3% agarose gels. Five clones were analyzed and found to contain the correct construction (plasmid sequences along with the *pg1* gene lacking the intron).

A sequencing of these clones verified that the intron had been excised from all of them, and no alterations were present in their ORFs. Figure 2 shows the nucleotide sequences of *pgaA* and two clones encoding the recombinant *pg1* in alignment along with the deduced amino acid

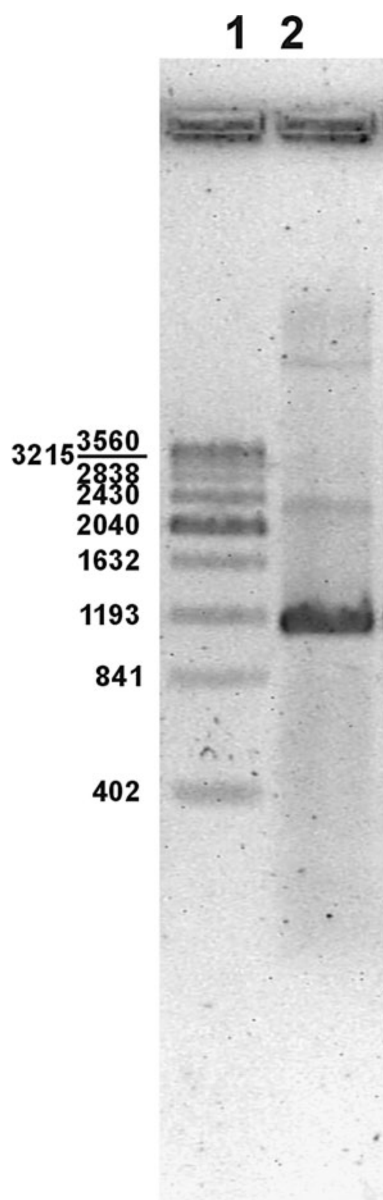

Fig. 1 Agarose gel electrophoresis (1%) showing DNA amplification from *A. kawachii* by PCR with primers PGfw and PGrv. *Lane 1* Marker 400 bp (PBL®). *Lane 2* Amplified *Pg1* ORF

sequence of the predicted PGase. Accordingly, the nucleotide sequences of the two *pg1* clones and of *pgA* gene are seen to be 100% coincident. This finding reinforces the contention that the PgaA and PG1 proteins are one and the same.

Expression of *pg1ΔI* gene in yeast

Batch cultures in flasks

Competent *S. cerevisiae* INVSc1 was transformed with pYES2:*pg1ΔI*, and the transformants that recovered Ura-synthesis capabilities (grown on SC-URA medium) were selected. Colonies were analyzed by PCR and two positive clones detected. In order to confirm the functionality of the constructs, both clones were screened upon cultivation in liquid SC-URA medium following the Invitrogen induction protocol. The detection of PGase activity confirmed that the engineered yeast INVSc1/*pg1ΔI* containing and expressing the *pg1* gene had been obtained. Moreover, the ability of *S. cerevisiae* to export PG1 indicates that the PG1 leader sequence is correctly recognized and processed by the yeast.

In order to study the ability of INVSc1:*pg1ΔI* to produce the heterologous PG1, two series of galactose-induction experiments were carried out in Erlenmeyer flasks. In the first attempt, the protocol suggested by Invitrogen was used without any modification. Both clones were cultured aerobically at 30°C in 100 ml of SC-URA medium with raffinose as the CES up to the CES depletion point. Raffinose was used as the CES because this trisaccharide does not repress the GAL10 promoter as glucose or other sugars do. The cultures were then supplemented with further raffinose and induced with galactose. A PGase activity of 2 U/ml was obtained after 24 h of induction under these conditions (Fig. 3). Though this value may seem low, the activity is 100 times higher than that of the PGase obtained in *A. kawachii* cultures [5]. As can be seen in Fig. 3, a culture of INVSc1 transformed with pYES2, not containing the *pg1* gene, and incubated under similar conditions as a negative control does not exhibit any polygalacturonase activity, showing that the yeast strain used for pg1 expression does not have polygalacturonase endogenous coding genes.

Since raffinose is too expensive to be used as the CES in an industrial fermentation process, we carried out a second strategy for induction. INVSc1/*pg1ΔI* was first grown in SC-URA medium with glucose as the CES, then subjected to 2 h of starvation before a final galactose induction in order to avoid glucose repression. Since no major differences in PGase induction between the two experimental approaches were observed (Fig. 4), it was decided to use the second protocol for further studies. Because of the potential industrial application of this enzyme, a low-cost medium would be critical, given the degree of scaling-up necessary for the industrial production process.

Batch cultures in a bioreactor

Figure 5a shows the time course of biomass growth and substrate consumption in a batch culture. From these data, a maximal specific growth rate (μ_m) of 0.28 h^{-1} was calculated; this value is lower than that reported for wild-type *Saccharomyces cerevisiae* in synthetic medium. The decline

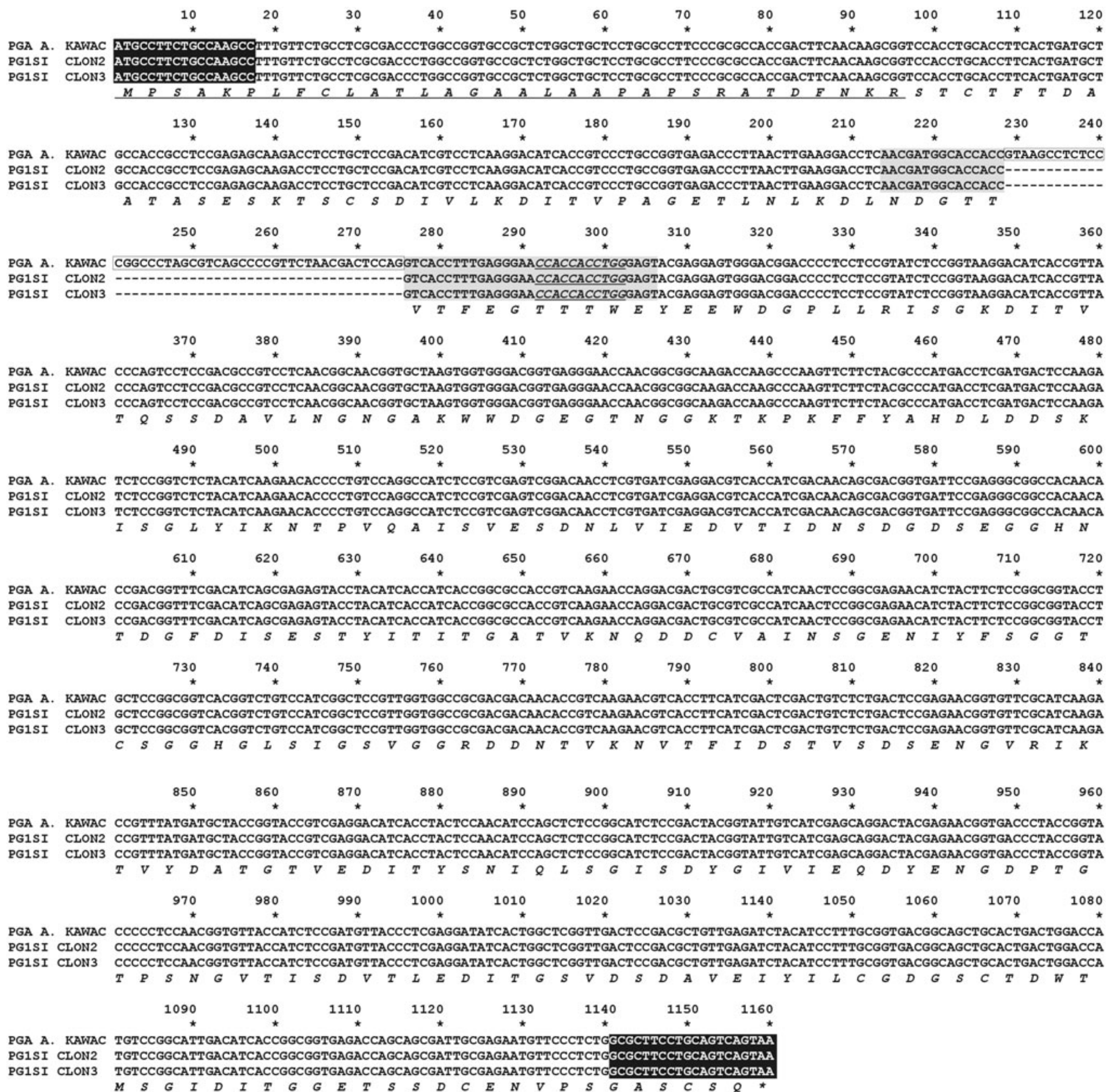

Fig. 2 Sequence comparison of *A. kawachii pgaA* gene and *pg1* recombinant clones. The *black-shaded* and *white symbols* indicate the sequence of the PCR primer PGfw and the sequence complementary to the PCR primer, PGrv, respectively. The *boxed letters* in the *pgaA* sequence correspond to the intron eliminated through PCR. The *grey-shaded* nucleotides indicate the sequence complementary to the PG1wI primer. Inside this stretch, the *italicized* and *underlined* characters show the *Pfl*MI target sequence. Below the nucleotide sequence alignment, the PGase amino-acid sequence is shown. The *underlined* amino acids correspond to the signal peptide

in the μ_m value is understandable in view of the metabolic burden caused by the foreign construct [11]. The dissolved oxygen concentration was always above 60% saturation, thus insuring oxygen levels were nonlimiting. Figure 5b depicts the time course for the rates of O_2 consumption and CO_2 production. The respiratory quotient was always above 1, typical of an overflow metabolism (i.e., from the Crabtree effect).

Table 1 shows the stoichiometric parameters of the culture. The carbon and energy balances were calculated according to Erickson et al. for product-generating cultures [8] and found to be close to unity, indicating that only biomass, ethanol, and CO_2 are produced during cultivation under these conditions.

Once the growth phase had ended, PGase expression was induced by a pulse of galactose as described above (for

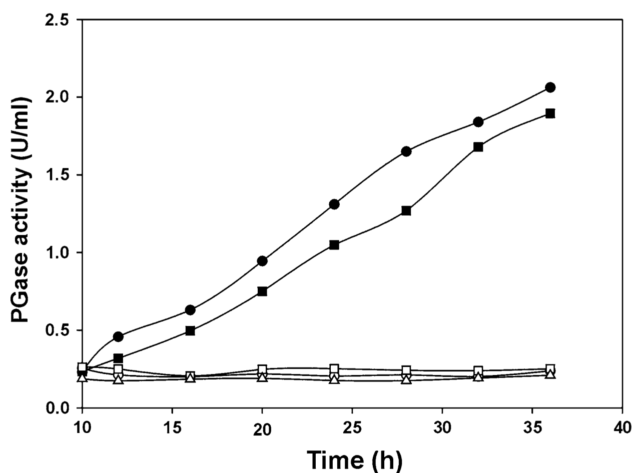

Fig. 3 PGase expression by recombinant *S. cerevisiae* following the raffinose protocol. Batch cultures in SC-URA medium were induced with galactose after 10 h of exponential growth in raffinose. *Filled circle* Clone 1 (induced), *filled square* clone 2 (induced), *open circle* clone 1 (noninduced), *open square* clone 2 (noninduced), *open triangle* negative control (without the *pg1* gene)

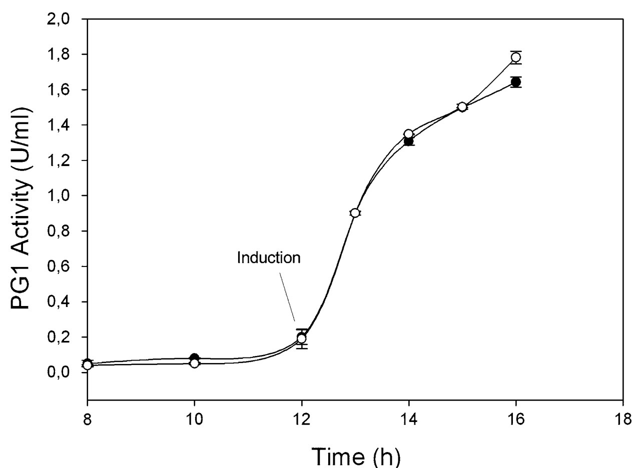

Fig. 4 Induction of *pg1* gene cloned in *S. cerevisiae* grown in raffinose or glucose as CES. Induction was carried out after 8 h of exponential growth. *Filled circle* Glucose; *open circle* raffinose

the glucose protocol). After the induction phase, the total biomass concentration, protein concentration, and enzyme activity were 2.2 g/l, 10 mg/l, and 3 U/ml, respectively, to give a productivity of 0.06 U/ml h. This enzyme concentration is higher than that obtained with batch cultures in flasks, probably as a result of the degree of environmental control (e.g., pH, dissolved O_2 levels) attained in the bioreactor. Contreras et al. have reported that PG1 is stable only within a pH range between 2.5 and 5.0. For this reason, the pH control was extremely critical in maintaining the enzyme stability during the expression phase. Furthermore, the biomass yield during this last phase was lower than that obtained during the growth phase. Considering that glucose and galactose are similar to CESs, the decrease in yx/s (cf. Table 1) could result from the metabolic burden caused by expression of the foreign gene. In the present situation the carbon contribution of the enzyme to the carbon balance is negligible because of the extremely small amount of mass that the former represents in the supernatant of the culture.

Fed-batch cultures in bioreactor

In an attempt to increase PG1 productivity, a number of fed-batch fermentations were conducted using different strategies for introducing galactose into the growing culture. In order to control the rate of galactose feeding in fed-batch cultures so as to avoid an accumulation of the CES and a consequent production of ethanol through the Crabtree effect, fundamental fermentation parameters were estimated from the batch culture data (Table 1). The final biomass concentration desired was 15 g/l. For this level of biomass the corresponding parameters were: $X_0 = 2.20$ g/l, $V_0 = 0.8$ l, $V_f = 1.12$ l, Sr = 320 g/l, F = 10 ml/h, $\mu_0 = 0.28\ h^{-1}$, $y_{x/s}$ (c-mol/c-mol) = 0.173.

The various strategies used for galactose feeding were: (1) after a glucose growth phase, the addition of a single pulse of galactose at a final concentration of 10% (w/v); (2)

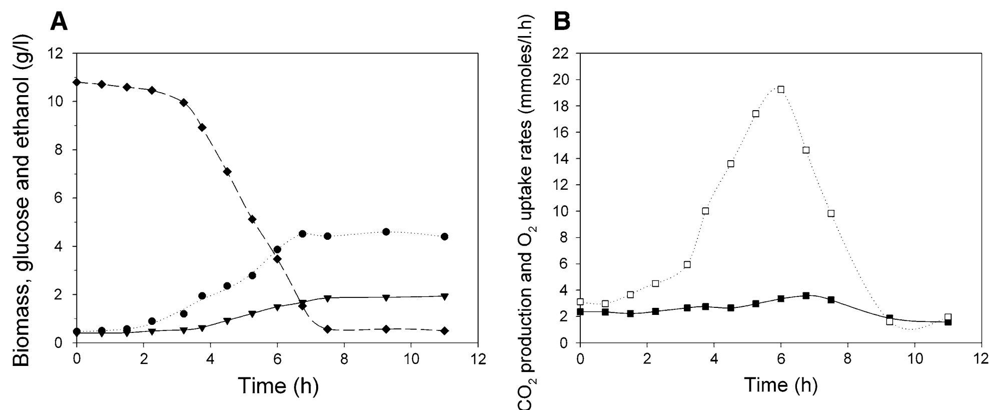

Fig. 5 Profiles of the rates of growth, glucose consumption, ethanol production, volumetric O_2 consumption, and CO_2 production during the batch cultivation of *S. cerevisiae* INVSC1 containing the pYES2:*pg1ΔI* construction. **a** *Filled diamond* Remaining glucose; *filled inverted triangle* biomass concentration; *filled circle* ethanol concentration, **b** *open square* CO_2 production rate; *filled sqare* O_2 consumption rate

Table 1 Stoichiometric parameters of *S. cerevisiae* batch culture

$Y_{X/S}$ (C-MOL)	$Y_{CO2/S}$ (C-MOL)	B (MOL)	$Y_{P/S}$ (C-MOL)	A (MOL)	CB	ΓB
0.173	0.291	0.082	0.523	0.0345	0.95	1.05

The carbon and energy balances were calculated according to Erickson et al. for product-generating cultures

CB Carbon balance, γB reduction degree (energy) balance

after a glucose growth phase, the introduction of a double pulse of galactose at the same final concentration; and (3) a simultaneous feeding of glucose and galactose, both at a final concentration of 10% (w/v), as the CES and the inducer, respectively.

In the first two strategies, the final biomass concentration obtained in this culture system before induction was 11.11 g/l. Once the glucose feeding was halted, the expression of the enzyme was induced by the addition of galactose. With strategy (1), after the glucose depletion from the medium and a single pulse of galactose, the final PG1 concentration and total protein content obtained were 12.2 U/ml and 67 mg/l, respectively, after 63 h of culture to give a productivity of 0.19 U/ml h. These yields are higher than those obtained in batch cultures. The reason for the improved PG1 productivity was probably the greater biomass concentration achieved in this culture system.

Strategy (2), with the addition of a double pulse of galactose, gave a final PG1 concentration and a total protein content of 16 U/ml and 73.6 mg/l, respectively, after 78 h of culture, corresponding to a productivity of 0.21 U/ml h. This strategy therefore resulted in only a marginal increase in PG1 productivity relative to strategy (1).

Strategy (3), involving simultaneous growth and induction phases, was based on the idea that during the growth phase, glucose is used as the CES and its concentration thus becomes almost negligible, so that the galactose promoter should not be repressed, and the *pg1* gene would therefore be expressed [22]. This feeding strategy yielded a final PG1 concentration and a total protein content of 50.0 U/ml and 54 mg/l, respectively, after 38 h of culture to give a productivity of 1.32 U/ml h, a value almost 500 times higher than the productivity of 0.0025 U/ml h reported by Contreras Esquivel et al. [5] for wild-type *Aspergillus kawachii*.

On comparison of the results from these three approaches to the level of PG1 production by the fed-batch mode, it was concluded that the simultaneous feeding of glucose and galactose was by far the most suitable strategy for the generation of this enzyme.

An industrial production process must be capable of yielding large amounts of products while still keeping the fermentation time as short as possible; moreover, the substrate should be completely utilized at the same time since the cost of raw material is the predominant manufacturing expense. In addition, an industrial production strategy must take into account the regulation of expression of the specific gene in question, a feature determined by its promoter. For these reasons, this last approach would appear to be the most promising one for the recombinant production of PG1 in large-scale experiments.

Partial characterization of the recombinant protein

In order to compare the recombinant protein with the wild-type PG1, some biochemical properties were determined. The recombinant protein had a molecular weight of 60.1 kDa (Fig. 6) and an isoelectric point of 3.7.

With PGA as substrate a maximum of activity occurred at around pH 4.0, but enzyme activity was still recorded at a pH of 2.0 (at 20% of the maximal activity) (Fig. 7a). Moreover, the recombinant PG1 retained no activity at pH 5.0, similar to the previously reported pH profile for the nonrecombinant enzyme [6]. The activity of PG1 at pH 2.5 would render it functional for the maceration of vegetable tissues and their subsequent pectin extraction since that low a pH would minimize any possible microbial contamination within the preparation.

The thermal stability of PG1 was studied at pH 2.5. PG1 activity proved to be highly stable at 50°C, with only 15% of the activity being lost after 10 h incubation at that temperature, even when no protective agent was added (Fig. 7b). Temperatures higher than 55°C, however, resulted in a rapid

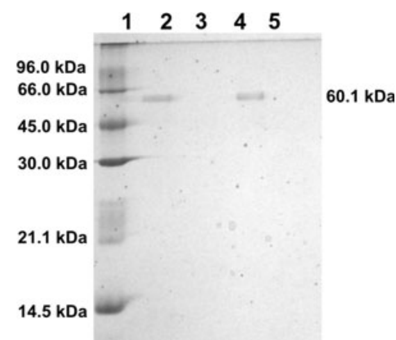

Fig. 6 SDS–PAGE of the recombinant PG1. *Lane 1* Low-molecular-weight protein standard (GE). *Lane 2* Recombinant PG1 from clone 1. *Lane 3* Negative control (noninduced clone 1). *Lane 4* Recombinant PG1 from clone 2. *Lane 5* Negative control (noninduced clone 2)

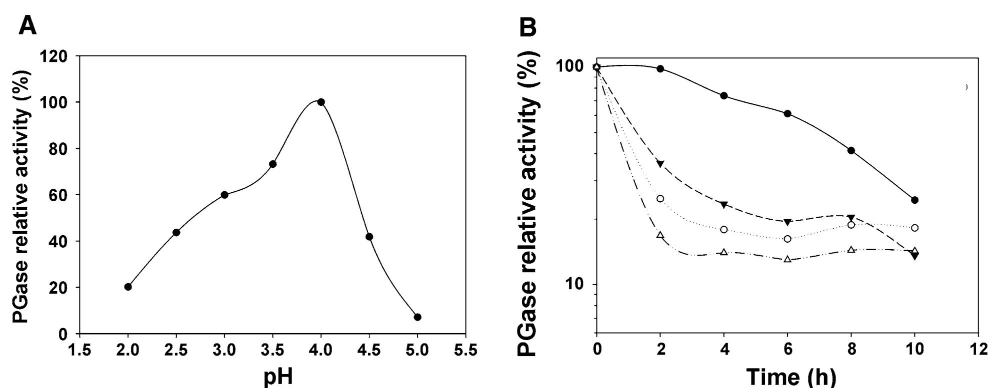

Fig. 7 Biochemical characterization of the recombinant PG1. **a** Effect of pH on the activity of *S. cerevisiae* PG1. CPB buffer adjusted to various pHs was used in the measurement of enzymatic activity under the standard conditions. **b** Effect of temperature on PG1 stability. The enzyme solution was incubated at the indicated temperatures, and at the selected time intervals samples were taken and chilled in an ice bath. The residual PG1 activity was then measured under standard conditions. *Filled circle* 50°C, *filled inverted triangle* 55°C, *open circle* 60°C, *open triangle* 70°C

loss of catalytic activity. Finally, the enzyme in CBP buffer, pH 2.5, without any protective agent, was not inactivated after two cycles of freezing and thawing.

Conclusions

Since both PgaA and PG1 showed similar properties (MW, IP, pH activity profile), it could be concluded that PG1 and PgaA represent one and the same protein.

Optimization of the recombinant PG1 production in bioreactors indicates that fed-batch fermentation with a simultaneous feeding of glucose and galactose is the most convenient strategy for the production of PG1. For this reason, and considering the productivity achieved with a low cost culture medium, the proposed process could be applied for industrial production of a novel and useful polygalacturonase.

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