Glucose-Tolerant Expression of *Trichoderma longibrachiatum* Endoglucanase I, an Enzyme Suitable for Use in Wine Production

Angélica Ganga, Luis González-Candelas, Daniel Ramón, and José A. Pérez-González*

Departamento de Biotecnología de los Alimentos, Instituto de Agroquímica y Tecnología de los Alimentos, CSIC, Poligono de la Coma S/N, 46980 Paterna, Valencia, Spain

A *Trichoderma longibrachiatum* transformant has been constructed constitutively expressing the homologous *egl1* gene under the control of the *Aspergillus nidulans* glyceraldehyde phosphate dehydrogenase gene (*gpdA*) promoter. Consequently, the *egl1* gene can be expressed in glucose-containing media. The *egl1* product (EGL1) has been purified from such a medium. The physicochemical and kinetic properties of EGL1, which has both endoglucanase and xylanase activities, have been determined. Analysis of the effects of certain enological factors such as temperature, pH, and glucose, ethanol, and SO_2 concentrations on the enzyme's activities indicates that it can be used in wine production.

Keywords: *Trichoderma longibrachiatum transformation; gpdA promoter; endoglucanase; purification; characterization; wine aroma*

INTRODUCTION

Aroma is an important characteristic of a quality wine, and the most important compounds contributing to it are monoterpenes (Bayonove and Cordonier, 1971). These compounds are present in must and wine in two fractions: one volatile that contributes to the aroma and one nonvolatile comprised of monoterpenyl diglycosides (Günata et al., 1986; Williams et al., 1982). The latter compounds constitute a potential source of additional aroma. The use of enzymes such as β-glucosidases, arabinofuranosidases, and rhamnosidases that can release monoterpenes from these precursors has been suggested previously (Günata et al., 1990a−c). In addition, the use of cell wall degrading enzymes such as endoglucanases and xylanases could contribute to wine aroma by increasing the amount of monoterpenyl-diglycoside precursors in the must. These compounds can be released from the walls of grape cells by the action of these enzymes.

Some *Trichoderma* species produce high levels of endoglucanase activities. Endoglucanase I from *Trichoderma reesei* has been purified (Shoemaker et al., 1983) and the corresponding gene cloned and sequenced (Arsdell et al., 1987; Penttilä et al., 1986). The *Trichoderma longibrachiatum* endoglucanase I encoding gene (*egl1*) has also been cloned and sequenced (González et al., 1992), and its expression in a wine yeast strain has been reported (Pérez-González et al., 1993). The commercial enzyme preparations used in wine production and other food processes are crude preparations that mainly consist of concentrated *Trichoderma* or *Aspergilli* culture filtrates and contain a large number of different activities. A way to increase their specificity is the use of fungal strains transformed with the gene encoding an enzyme of interest controlled by a glucose-tolerant promoter. In this manner the fungal culture filtrates will contain mainly the enzyme of interest as most of the other activities produced by the strain will not be induced in glucose-containing medium. This strategy has already been employed for the production of *T. reesei* cellulases (Nakari-Setälä and Penttilä, 1995) and xylanases (Kurzatkowski et al., 1996) using different *T. reesei* glucose-tolerant promoters. The glyceraldehyde phosphate dehydrogenase gene (*gpdA*) promoter of *A. nidulans* has also been used following this strategy (Punt et al., 1991). Here we present both the construction of a *T. longibrachiatum* transformant that can produce endoglucanase activity in the presence of glucose using the *gpdA* promoter and the purification and characterization of this enzyme.

MATERIALS AND METHODS

Chemicals. Azo-barley-β-glucan, azo-birchwood-xylan, wheat flour xylan, and xylooligosaccharides were purchased from Megazyme (Sydney, Australia). Carboxymethylcellulose (CMC), barley β-glucan, laminarin, birchwood xylan, oat spelt xylan, *p*-nitrophenyl glucoside, and all other monosaccharides and chemicals were from Sigma (St. Louis, MO).

Strains and Culture Conditions. *T. longibrachiatum* CECT 2606 was the host strain used for transformation. For enzyme purification, *T. longibrachiatum* C1 was grown on a Mandel's modified medium which contained 1% glucose, 0.1% peptone, 0.14% $(NH_4)_2SO_4$, 0.20% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.075% $CaCl_2$, 0.00125% $FeSO_4$, 0.0004% $MnSO_4$, 0.00035% $ZnSO_4 \cdot 7H_2O$, and 1.05% citric acid for 24 h at 30 °C with vigorous shaking.

Plasmids. Plasmid pAN7-1 (Punt et al., 1987) confers hygromycin B resistance that was the phenotype used for selection of transformants. Plasmid pPGPDEGL1 contained the *T. longibrachiatum egl1* cDNA under the control of the *A. nidulans gpdA* gene promoter and was constructed as follows. A DNA fragment containing the *gpdA* gene promoter was synthesized as previously described (Sánchez-Torres et al., 1994), digested with *Bam*HI and *Eco*RI, and cloned into pUC18 yielding plasmid pPGPD. A DNA fragment containing the *T. longibrachiatum egl1* cDNA was synthesized by the polymerase chain reaction (PCR) using pTLEGC1 (González et al., 1992) as template and the oligonucleotide EGL6 (5′-GTCAGC-CATGGCGCCCTCAGC-3′) and the universal primer of M13 as primers. The PCR fragment was restricted with *Nco*I (a synthetic *Nco*I site having been introduced into the EGL6 primer) and ligated to plasmid pPGPD previously digested

* Address correspondence to this author at the Departamento de Biotecnología de los Alimentos, Instituto de Agroquímica y Tecnología de los Alimentos, Apartado de Correos 73, 46100 Burjassot, Valencia, Spain (telephone 34-6-3900022; fax 34-6-3636301; e-mail jantonio @iata.csic.es).

S0021-8561(96)00904-1 CCC: $14.00

with the same enzyme. The resulting plasmid pPGPDΔEGL contains part of the *egl1* cDNA downstream of the *A. nidulans gpdA* gene promoter. To get the entire *egl1* cDNA under the control of the *gpdA* gene promoter, plasmid pTLEGC1 was restricted with *Eco*RV and *Hin*dIII and the fragment containing the 3′ end of *egl1* cDNA separated, purified, and ligated to the large fragment obtained by digestion of pPGPDΔEGL with *Eco*RV and *Hin*dIII, yielding plasmid pPGPDEGL1.

Transformation Experiments. Protoplasts from *T. longibrachiatum* CECT 2606 were obtained and cotransformed with 2.5 μg of plasmid pAN7-1 and 25 μg of plasmid pPGPDEGL1 according to the previously described poly(ethylene glycol) method (Sánchez-Torres et al., 1994). Transformants were selected on OSCM medium (Sánchez-Torres et al., 1994) containing 100 μg/mL hygromycin B. Forty randomly selected transformants were then picked onto complete medium (CM) (Pontecorvo et al., 1953), nonselective medium, and CM with 50 μg/mL hygromycin B. All media also contained 0.01% Triton X-100 to reduce colony size. After 3 days of incubation at 30 °C, the plates were overlaid with 5 mL of a 1% CMC/0.7% agar solution in 50 mM sodium citrate buffer (pH 5.0) and incubated at 50 °C for 6 h. The agar surface of the plates was flooded with a 0.1% Congo Red solution and left to stand for 15 min. The dye was then removed, and the plates were washed several times with a 1 M NaCl solution to visualize the clear halos surrounding CMCase-producing colonies.

Enzyme Assays. For endoglucanase activity determinations, CMC or azo-barley-β-glucan was used as substrate. In the first case, the assay samples (300 μL) contained 10 mg of CMC/mL and an appropriate enzyme solution volume in 50 mM sodium acetate buffer (pH 5.0). The reaction mixtures were incubated at 50 °C for 15 min, and reducing sugars liberated were determined according to the method of Wood and Bhat (1988). One unit of CMCase activity is defined as the amount of enzyme that released 1 μmol of glucose equiv/min. When dye-modified substrate was used, 250 μL of the commercial substrate solution was mixed with an appropriate volume of enzyme solution in a total reaction volume of 500 μL and incubated at 50 °C for 15 min. The reaction was stopped by adding 1 mL of precipitant solution (prepared as described by the substrate solution manufacturer), and the samples were centrifuged for 5 min in a microfuge. Absorbance of the supernatants was then measured at 590 nm. One unit of endoglucanase activity measured according to this method is defined as the amount of enzyme that gives 1 unit of absorbance at 590 nm in 1 h. In the case of xylanase activity, birchwood xylan or azo-birchwood-xylan was used as substrate. When the former was used, the assay samples (300 μL) contained 50 mg of xylan/mL and an appropriate enzyme solution volume in 50 mM sodium acetate buffer (pH 4.5). The reaction mixtures were incubated at 60 °C for 30 min and reducing sugars liberated determined as above. In the second case the determination of xylanase activity was as described for endoglucanase activity except that the incubation of samples was for 30 min at 60 °C. One unit of xylanase activity measured according to this method is defined as the amount of enzyme that gives 1 unit of absorbance at 590 nm in 1 h.

Protein Estimation. Protein concentration was measured according to the Bradford method (Bradford, 1976). Protein content in chromatography fractions was estimated by measuring absorbance at 280 nm.

Enzyme Purification. A 1 L culture was filtered through Nytal mesh. The filtrate was concentrated to 100 mL in a Minitan ultrafiltration system (Millipore, Bedford, MA) using a 10 000 molecular mass cutoff polysulfone filter and then dialyzed against 20 mM Bis-Tris buffer (pH 6.5). The concentrate was loaded onto a DEAE-Bio-Gel A column (2 × 18 cm) equilibrated with 20 mM Bis-Tris buffer (pH 6.5). Elution of adsorbed proteins was performed with a linear NaCl concentration gradient (0−0.2 M in the same buffer) at a flow rate of 0.5 mL/min. Endoglucanase samples were pooled and dialyzed/concentrated to 5 mL in a Omegacell unit (Filtron, Northborough, MA). This fraction was applied to a Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM Bis-Tris buffer (pH 6.5). Elution was carried out with a linear NaCl concentration gradient (0−0.2 M in the same buffer).

Electrophoresis and Isoelectric Focusing. SDS−polyacrylamide gel electrophoresis was performed according to the procedure of Smith (1984) using an acrylamide concentration of 10% (w/v). The gel was stained with silver as described previously (Merril et al., 1981). The low molecular mass calibration mixture from Pharmacia was used as standard. Analytical isoelectric focussing was carried out on precast gels with a pH gradient from 3.5 to 9.3 (Pharmacia-LKB) following the instructions of the manufacturer.

Measurement of Enzyme Properties. The effect of pH on reaction rates was determined by incubating the reaction mixtures in Teorell and Stenhagen universal buffer (Stauffer, 1989) at various pH values between 2.0 and 9.0 at 50 °C. For the corresponding study of temperature effects, reaction mixtures were incubated at different temperatures in the range 30−70 °C. Thermal stability was monitored by incubating the enzyme preparation at 37, 45, and 60 °C and assaying activities at various time points using the standard assays. The influence of various reagents on activity was also tested.

Kinetic parameters were determined on CMC for endoglucanase activity and on birchwood xylan for xylanase activity of the enzyme. Samples were incubated in sodium acetate buffer at pH 4.5 for 30 min at 50 °C (for endoglucanase activity) or at 60 °C (for xylanase activity). CMC and birchwood xylan were assayed in the concentration ranges of 0−30 and 0−100 mg/mL, respectively.

Polymer Hydrolysis Experiments. The analysis of products of hydrolysis of barley β-glucan and birchwood xylan was performed by HPLC using a SugarPack 1 column (Waters, Milford, MA) equilibrated and eluted with Milli-Q water at a flow rate of 0.5 mL/min. Peaks were detected by differential refractometry and identified by comparing elution times with those of appropriate standards. Substrates (at a final concentration of 12 mg/mL) were incubated with enzyme for 3, 10, or 24 h at 50 °C. The reaction mixtures were then centrifuged, and 20 μL of the supernatants was injected onto the column.

RESULTS AND DISCUSSION

Construction of a *T. longibrachiatum* Transformant Constitutively Expressing the *egl1* Gene. Nineteen of the 40 *T. longibrachiatum* transformants originally selected maintained the hygromycin resistance phenotype and showed CMC-degrading activity, indicating that they were cotransformants. The CMCase activity phenotype was conferred by the *egl1* cDNA copy contained in plasmid pPGDPEGL1 as the expression of the genomic *egl1* gene is repressed by the glucose present in CM medium. Six of the original 40 transformants were only transformed to hygromycin resistance as they grew on antibiotic containing plates but did not produce CMCase activity. Two transformants seemed to be transformed only to the CMCase-producing phenotype as they could not grow in the presence of antibiotic due to instability after initial selection but produced clear halos in CMC-containing CM medium. After additional passages on nonselective CM medium, these transformants lost their CMCase activity. The remaining transformants did not maintain the hygromycin resistance phenotype, nor did they show CMCase activity. Four of the 19 cotransformants showing the highest CMCase activity were selected and subjected to several passages on nonselective CM medium with the aim of obtaining a stable transformant sensitive to the antibiotic but showing constitutive CMCase activity. Only one showed the desired phenotype. Southern analysis of this transformant followed by image analysis using electronic autoradiography indicated the ectopic integration of a single copy of pPGDPEGL1 in the genome of the recipient strain (results not shown). This transformant was named C1.

Enzyme Purification. The *T. longibrachiatum* endoglucanase I (EGL1) has been purified to homogene-

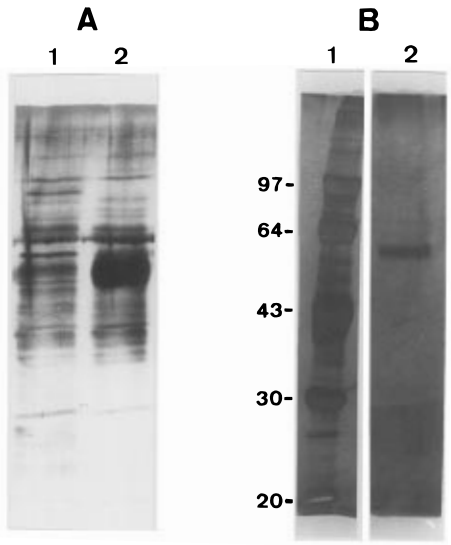

Figure 1. SDS−PAGE analysis: (A) *T. longibrachiatum* (lane 1) and *T. longibrachiatum* C1 transformant (lane 2) culture broth; (B) protein molecular mass standards (lane 1) and pure EGL1 protein (lane 2). The numbers to the left indicate the molecular mass (kDa) of the standards.

Table 1. Purification of Endoglucanase EGL1 from *T. longibrachiatum* C1

step	total activity (units)	total protein (mg)	spec activity (units/mg)	recovery (%)	purifn factor
culture fluid	268.13	13.900	19.29	100	1
DEAE-Bio-Gel	11.96	0.553	21.63	4.46	1.1
Mono Q	1.61	0.024	67.30	0.6	3.5

ity from *T. longibrachiatum* C1 transformant culture filtrate (see Figure 1 and Table 1), following the protocol described under Materials and Methods. The endoglucanase activity was purified by ion exchange chromatography in two steps using a DEAE-Bio-Gel A column and a Mono Q column, successively. Xylanase activity assays were done in parallel with endoglucanase assays during the purification procedure. A xylanase activity copurified with the endoglucanase activity in both chromatographic steps. Separation of the activities could not be achieved, thus suggesting that the single *T. longibrachiatum* enzyme has the same broad specificity as *T. reesei* endoglucanase I. This may be expected given the high level of similarity between the deduced amino acid sequences of the enzymes of both species.

Characterization of the Enzyme. The molecular mass of purified EGL1 enzyme was 65.5 kDa as calculated by SDS−PAGE migration (see Figure 1B). Glycosylation of the protein (five potential glycosylation sites have been detected in the amino acid sequence) may be the cause of an observed molecular mass higher than that (48.3 kDa) calculated from the deduced amino acid sequence (González et al., 1992). Isoelectric focusing of the enzyme yielded an isoelectric point of 5.0 (result not shown).

The enzyme showed activity in a narrow pH range, the optimum pH determined for the endoglucanase and xylanase activities being 4.5. At pH 4 and 5.5 enzyme retained 35% of the maximum endoglucanase activity. Enzyme retained 50% of the maximum xylanase activity. The optimum temperature for endoglucanase activity at pH 4.5 was 50 °C, and the activation energy calculated up to 50 °C from the slope of an Arrhenius plot was 16.6 kJ/mol. The optimum temperature for xylanase activity at the same pH was 60 °C and the activation energy 8.8 kJ/mol. The enzyme reached 30% of the maximum endoglucanase and xylanase activities

Table 2. Effects of Some Compounds on EGL1 Enzyme Activities

compound[a]	rel endoglucanase activity (%)	rel xylanase activity (%)
none	100.0	100.0
$ZnCl_2$	85.2	0.0
$CaCl_2$	73.8	5.3
DTT	100.0	73.6
$MgCl_2$	67.8	100.0
EDTA	123.5	100.0
EDTA (10 mM)	152.0	100.0
$CdCl_2$	84.1	115.0
$CoCl_2$	116.0	18.5
$CuCl_2$	0.0	20.1

[a] All compounds were used at a final concentration of 2 mM with the exception specified.

Table 3. Enzyme Substrate Specificity[a]

substrate	spec activity (units/mg)	substrate	spec activity (units/mg)
carboxymethylcellulose	98.9	birchwood xylan	59.8
barley β-glucan	189.4	oat spelt xylan	47.5
laminarin	19.6	wheat flour xylan	39.7
pNPG	0.0		

[a] All substrates were used at a final concentration of 12.5 mg/mL except pNPG, which was used at 2 mM.

at 40 °C. The enzyme retained 50% endoglucanase activity after a 4 h incubation period at 60 °C and lost it completely after 9 h of incubation at this temperature. Xylanase activity of the enzyme was more resistant at the same temperature, 50% remaining after an incubation period of 10 h and totally lost only after 30 h of incubation.

No positive influence of divalent cations on the enzyme activities at 2 mM concentration was observed (Table 2). Endoglucanase activity (but not xylanase activity) increased in the presence of EDTA. This may indicate that the chelating agent suppresses the inhibitory effect of one or more metal cations present in the reaction mixtures upon that activity.

The Michaelis kinetic parameters were determined for both endoglucanase and xylanase activities of the enzyme. K_m and V_{max} of the endoglucanase activity when CMC was used as substrate were found to be 13.77 mg/mL and 176.7 μmol min^{-1} (mg of protein)$^{-1}$, respectively. The corresponding values for the xylanase activity were found to be 8.88 mg/mL and 83.55 μmol min^{-1} (mg of protein)$^{-1}$, respectively, when birchwood xylan was used as substrate.

The specific activities of the enzyme on different polysaccharides and pNPG were determined (see Table 3). The results confirm the lack of specificity of EGL1 as it is able to hydrolyze not only CMC but also different substituted xylans. Similar characteristics have also been described for *T. reesei* EGL1 (Penttilä et al., 1989).

Analysis of the products of barley β-glucan hydrolysis by purified enzyme showed an increase in the amount of glucose, cellobiose, and cellotetraose released after 3, 10, and 24 h of incubation. Very low amounts of cellotriose and cellopentaose were produced even after 24 h of incubation. Xylooligosaccharides from 2 to 5 xylose units were produced in approximately equal amounts and also increased after 3, 10, and 24 h of incubation of xylan samples with the purified enzyme (results not shown).

As one of the potential uses of this enzyme is the treatment of must in wine production (Pérez-González et al., 1993), we have also tested the effects of some enological parameters on the enzyme activities. One of the parameters tested was ethanol concentration (see Figure 2). The enzyme retains 50% endoglucanase

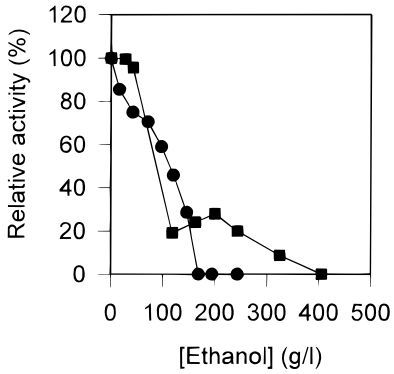

Figure 2. Effect of ethanol concentration on EGL1 endoglucanase (●) and xylanase (■) activities.

activity at an ethanol concentration of 115 g/L at pH 4.5 after an incubation period of 30 min. This activity was completely lost at ethanol concentrations >168.5 g/L. Loss of 50% of xylanase activity was achieved at an ethanol concentration of 100 g/L, but 20% activity was retained at concentrations as high as 150−250 g/L. Glucose concentration did not have an inhibitory effect on enzyme activity. In fact, activity increased up to 180% in the glucose concentration range of 0−168 g/L and returned to its original activity level in the range 168−250 g/L. SO_2 has an inhibitory effect on endoglucanase and xylanase activities only at concentrations higher than 35 and 30 ppm, respectively (results not shown).

Using a molecular genetics approach, we have constructed a *T. longibrachiatum* strain that is able to produce EGL1 constitutively in glucose-containing media. The high activity levels retained by the enzyme in the presence of ethanol, glucose, and SO_2 make this enzyme suitable for wine production. It can be very active at the beginning of the process, when the ethanol concentration is low, hydrolyzing cellulose- and xylan-containing material, and therefore increasing the amount of aroma precursors such as terpenyl diglycosides released into the must.

ACKNOWLEDGMENT

We thank Dr. A. P. MacCabe for critical reading of the manuscript.

LITERATURE CITED

Arsdell, J. N. van; Kwok, S.; Schweickart, V. L.; Ladner, M.; Gelfand, D. H.; Innis, M. A. Cloning, characterisation, and expression in *Saccharomyces cerevisiae* of endoglucanase I from *Trichoderma reesei*. *Bio/Technology* **1987**, *5*, 60−64.

Bayonove, C. L.; Cordonier, R. E. Recherches sur l'arôme du muscat. III. Etude de la fraction terpénique. *Ann. Technol. Agric.* **1971**, *20*, 347−355.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

González, R.; Ramón, D.; Pérez-González, J. A. Cloning, sequence analysis and yeast expression of the *egl1* gene from *Trichoderma longibrachiatum*. *Appl. Microbiol. Biotechnol.* **1992**, *38*, 370−375.

Günata, Y. Z.; Bayonove, C. L.; Baumes, R. L.; Cordonier, R. E. Stability of free and bound fractions of some aroma components of grapes cv. Muscat during the wine processing: preliminary results. *Am. J. Enol. Vitic.* **1986**, *37*, 112−114.

Günata, Y. Z.; Bayonove, C. L.; Cordonier, R. E.; Arnaud, A.; Galzy, P. Hydrolysis of grape monoterpenyl glycosides by *Candida molischiana* and *Candida wikerhamii* β-glucosidases. *J. Sci. Food Agric.* **1990a**, *50*, 499−506.

Günata, Y. Z.; Bayonove, C. L.; Tapiero, C.; Cordonier, R. E. Hydrolysis of grape monoterpenyl β-D-glycosides by various β-glucosidases. *J. Agric. Food Chem.* **1990b**, *38*, 1232−1236.

Günata, Y. Z.; Dugelay, I.; Sapis, J. C.; Baumes, R. L.; Bayonove, C. L. Action des glycosidases exogènes au cours de la vinification: liberation de l'arôme a partir de précurseurs glycosidiques. *J. Int. Sci. Vignes Vin* **1990c**, *24*, 133−144.

Kurzatkowski, W.; Törrönen, A.; Filipek, J.; Mach, R. L.; Herzog, P.; Sowka, S.; Kubicek, C. P. Glucose-induced secretion of *Trichoderma reesei* xylanases. *Appl. Environ. Microbiol.* **1996**, *62*, 2859−2865.

Merril, C. R.; Goldman, D.; Sedman, S. A.; Ebert, M. H. Ultrasensitive stain for proteins in polyacrylamide gel shows variation in cerebrospinal fluid proteins. *Science* **1981**, *211*, 304−307.

Nakari-Setälä, T.; Penttilä, M. Production of *Trichoderma reesei* cellulases on glucose-containing media. *Appl. Environ. Microbiol.* **1995**, *61*, 3650−3655.

Penttilä, M.; Lehtovaara, P.; Nevalainen, H.; Bhikhabhai, R.; Knowles, J. Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. *Gene* **1986**, *45*, 253−263.

Penttilä, M.; Lehtovaara, P.; Knowles, J. Cellulolytic yeast strains and their application. In *Yeast Genetic Engineering*; Barr, P., Brake, A., Valenzuela, P., Eds.; Butterworth: Boston, MA, 1989; pp 247−267.

Pérez-González, J. A.; González, R.; Querol, A.; Sendra, J.; Ramón, D. Construction of a recombinant wine yeast strain expressing β-(1,4)-endoglucanase and its use in microvinification processes. *Appl. Environ. Microbiol.* **1993**, *59*, 2801−2806.

Pontecorvo, G.; Roper, J. A.; Jemmons, L. J.; MacDonald, K. D.; Buft, A. W. J. The genetics of *Aspergillus nidulans*. *Adv. Genet.* **1953**, *5*, 141−238.

Punt, P. J.; Oliver, R. P.; Dingemanse, M. A.; Powels, P. H.; van den Hondel, C. A. M. J. J. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* **1987**, *56*, 117−124.

Punt, P. J.; Zegers, N. D.; Busscher, M.; Powels, P. H.; van den Hondel, C. A. M. J. J. Intracellular and extracellular production of proteins in *Aspergillus* under the control expression signals of the highly expressed *Aspergillus nidulans gpdA* gene. *J. Biotechnol.* **1991**, *17*, 16−34.

Sánchez-Torres, P.; González, R.; Pérez-González, J. A.; González-Candelas, L.; Ramón, D. Development of a transformation system for *Trichoderma longibrachiatum* and its use for constructing multicopy transformants for the *egl1* gene. *Appl. Microbiol. Biotechnol.* **1994**, *41*, 440−446.

Shoemaker, S.; Watt, K.; Tsitovsky, G.; Cox, R. Characterisation and properties of cellulases purified from *Trichoderma reesei* strain L27. *Bio/Technology* **1983**, *1*, 687−690.

Smith, B. J. SDS polyacrylamide gel electrophoresis of proteins. In *Methods in Molecular Biology. 1 Protein*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1984; pp 41−56.

Stauffer, C. E. In *Enzyme Assays for Food Scientists*; Van Nostrand Reinhold: New York, 1989; pp 77.

Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wines. *Phytochemistry* **1982**, *21*, 2013−2020.

Wood, T. M.; Bhat, K. M. Methods for measuring cellulase activities. *Methods Enzymol.* **1988**, *160*, 87−112.

Received for review December 2, 1996. Revised manuscript received March 6, 1997. Accepted April 2, 1997.[⊗] This work has been supported by Grants ALI93-0809 and ALI96-0431 from the Comisión Interministerial de Ciencia y Tecnologia of the Spanish Government. A.G. is the recipient of fellowships (C-52859) and Mutis of the Fundación Andes and Instituto de Cooperación Iberoamericana, respectively.

JF960904X

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.