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EVALUATION OF THE USE OF PHASE-SPECIFIC GENE PROMOTERS FOR THE EXPRESSION OF ENOLOGICAL ENZYMES IN AN INDUSTRIAL WINE YEAST STRAIN

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SUMMARY

Genes as *POT1*, *HSP104* and *SSA3*, which are late expressed in laboratory culture conditions are expressed only during the first few days in microvinifications in wine yeast cells. This effect is probably due to the different growth conditions and leads to useless levels of enzyme activity for a reporter gene. However the *ACT1* promoter, which is constitutively expressed in laboratory conditions, produces sufficient amounts of enzyme activity in late fermentation phases.

INTRODUCTION

During the last few years many winemakers have used pure yeast cultures isolated from their own wine region for inoculation into fresh must in order to perform controlled fermentations. Recently, the imposition of an inoculated wine yeast strain has been demonstrated using molecular markers. This microbiological simplification of the fermentation process opens the way to the use of genetic engineering techniques in the wine yeasts. As an example, one yeast strain expressing both the K1 and K2 killer toxins which may give it a broader competitive advantage has been constructed. Also *Saccharomyces cerevisiae* strains that express the L(+)-lactate dehydrogenase gene from *Lactobacillus casei* have been used to deacidify wines (reviewed in Querol and Ramón, 1996).

We are mainly interested in the study of wine aroma. For this purpose our group has constructed a recombinant wine yeast strain expressing a *Trichoderma longibrachiatum* β-(1,4)-endoglucanase that increases the fruity aroma of the wine produced (Pérez González *et al.*, 1993). In certain cases, temporary expression of these foreign genes could be of technological relevance. In *S. cerevisiae*, various gene promoters have temporal patterns of expression in laboratory conditions: some are induced when yeast cells reach stationary phase (reviewed in Werner-Washburne *et al.*, 1996) but most of them are mainly expressed during the logarithmic phase. We have studied here the expression pattern of several representative genes of both types in microvinification conditions.

MATERIALS AND METHODS

Microbial strains. The industrial *S. cerevisiae* strain T_{73} (CECT1894) (Querol *et al.*, 1992) commercialized by Lallemand Inc. (Montreal, Quebec, Canada) or its plasmid-transformed derivatives were used. *Escherichia coli* strain DH5α was used for the production of all plasmid constructions.

Culture conditions. For laboratory cultures, yeast cells were grown in 100 ml of YPD (2% bacteriological peptone, 2% glucose, 1% yeast extract) in 0.5 liter flasks with vigorous orbital agitation at 30°C. For microvinification experiments, synthetic must (20% glucose, 0.5% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.04% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract, pH 3.8) or red grape Bobal must were used. In the last two cases the medium was contained in 1 liter capped bottles filled with 1 liter and with gentle orbital agitation without aeration (Pérez-González *et al.*, 1993). In all the cases the yeast inoculum was of 2.5 10^5 cells/ml. *E. coli* cells were grown in LBA (1% tryptone, 1% NaCl and 0.5% yeast extract, supplemented with 50 μg/ml of ampicillin).

Plasmids and cloning procedures. YEpCR21 and pTLEGY3 plasmids have been described previously (Pérez-González *et al.*, 1993). The *egl1* gene from *Trichoderma longibrachiatum* which codes for a β-(1,4)-endoglucanase (González *et al.*, 1992) was fused to the *POT1* promoter (Igual *et* al., 1992) to yield the yeast plasmid pTLEGY4 by the method described in Fig. 2. A deletion mutant of the *POT1* promoter, from the -203 nucleotide (*POT1*-203), lacking the upstream repressing sequence (URS) was also constructed (see Fig. 2) and cloned into a yeast plasmid to yield pTLEGY5. The molecular biology techniques described by Sambrook *et al.*, (1989) were followed throughout this work.

Transformation protocols. *E. coli* was transformed following standard procedures (Sambrook *et al.*, 1989) and transformants selected on LBA plates (LBA plus 1.5% agar). Transformation of yeast cells was achieved using the procedure of Gietz *et al.* (1995). Selection and maintenance of transformants were done on YPD plates (YPD plus 2% agar) containing 1 μg/ml of cycloheximide. Restriction enzymes, *Taq* DNA polymerase, T4 DNA ligase and Klenow DNA polymerase were from Boehringer Mannheim.

Endoglucanase determinations. Enzyme activity was determined in culture and must filtrates using azobarley β-glucan as substrate in a commercial kit from Biocon, according to the instructions of the manufacturer. One unit of endoglucanase activity was defined as the amount of enzyme giving an increase of 1 U of A_{590} in one hour.

RNA isolation and analysis. Total RNA was isolated from yeast cells using low pH phenol and glass beads according to Sherman *et al.* (1986). 5 μg RNA samples were electrophoresed in 1% agarose formaldehyde-containing gels, blotted to Hybond-N (Amersham) and hybridized with random-primed labelled DNA probes according to standard procedures (Sambrook *et al.*, 1989). rRNA amounts were determined by video imaging analysis (UVP Imagestore GDS 5000) and specific mRNA amounts by direct radioactivity measurements with an Instant Imager apparatus (Packard Instruments).

RESULTS

Study of the expression patterns of several phase-specific genes in yeast strain T_{73}

To evaluate the potential use of several phase-specific yeast gene promoters we have measured the transcriptional rates of the *POT1*, *HSP104* and *SSA3* genes along the growth curve of the wine yeast strain T_{73} in microvinification conditions. We have used laboratory growth conditions as a control.

As can be seen in Fig. 1A, under laboratory growth conditions the pattern of expression of the *POT1*, *HSP104* and *SSA3* genes is in strain T_{73} similar to that previously found for laboratory strains under identical conditions (Igual *et al.*, 1992, Sánchez & Lindquist, 1990). The expression of *SSA3* is delayed with regard to the other two genes. On the other hand a control gene, *ACT1*, is expressed constitutively and decays after entry into stationary phase, similarly for the case of laboratory strains (Igual *et al.*, 1992). The amount of 28S rRNA was used as an internal loading control. The smaller amount of total RNA loaded in

samples from days 1 and 5 may explain their apparently irregular behavior. We have corrected this by making specific radioactivity measurements (not shown).

The expression pattern in microvinification conditions however, shows that all the genes are expressed only during the first few days and subsequently show a continuous decline (Fig. 1B). In this case there is only a slight delay in the expression of *SSA3*. The same results were obtained using synthetic must (not shown).

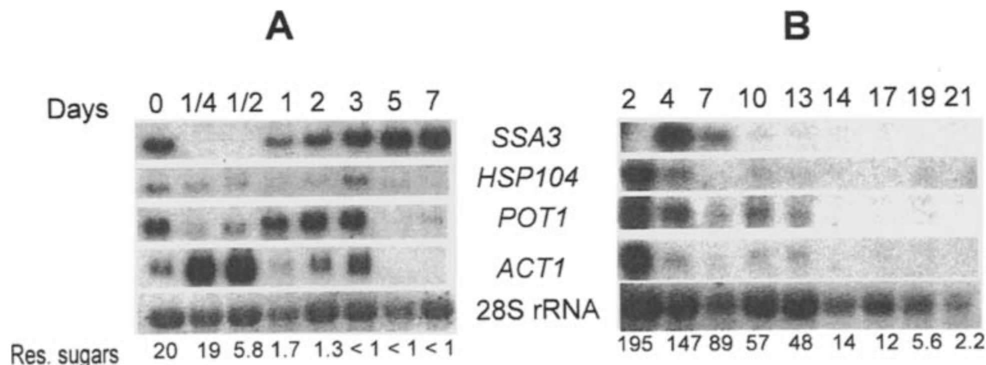

Figure 1. Northern blot analysis of RNAs from T_{73}. A) Laboratory culture. **B)** Microvinification in red grape Bobal must. The gel was stained with ethidium bromide for rRNA measurements, blotted and successively hybridized with different DNA probes (as indicated). Days of culture and residual sugar concentration (in g/l) are indicated. Day 0 in laboratory culture is RNA obtained from an aliquot sample of the inoculum.

Study of *POT1-egl1* gene fusion expression in T_{73} wine yeast cultures

To further analyze the utility of a late phase-specific promoter we have studied the case of *POT1*. We have made a *POT1-egl1* gene fusion in an autonomous-replicating yeast plasmid, pTLEGY4, (see Fig. 1) and used it to transform the well-known industrial wine yeast strain T_{73} (Querol *et al.*, 1992). The resulting transformant has been used to test β-(1,4)-endoglucanase activity under laboratory culture conditions. As can be seen in Figure 2A, the enzyme activity pattern indicates that *POT1*-directed expression was delayed two days with regard to the gene fusion constructed previously using the strong constitutive *ACT1* promoter in plasmid pTLEGY3 (Pérez-González *et al.*, 1993). The maximum enzyme activity levels obtained with *POT1* were six times lower than those obtained with *ACT1*, probably resulting from both the higher transcriptional activity of *ACT1* and the longer period of expression of this promoter during yeast growth.

We have tested both plasmid constructions in microvinification conditions. It was difficult to obtain clear results from natural must fermentations because of interference problems with the endoglucanase enzymatic activity assay hence we decided to use synthetic must (see Materials and Methods). We have found that, in this case, the expression patterns are different between the two plasmids as *POT1*-driven expression is almost negligible: compare enzyme profiles for pTLEGY3, pTLEGY4 - containing yeast strains in Fig. 2B. The *POT1* promoter is strongly repressed by glucose (Igual *et al.*, 1992) and this repression is dependent on an upstream repressing sequence (URS) located between -238 and -203 with regard to the translational start. For this reason we constructed a deleted copy of the *POT1* promoter

lacking this region (*POT1*-203) and transformed T_{73} with a plasmid, pTLEGY5, identical to pTLEGY4 except for the deletion in the *POT1* promoter (see Fig. 2). In this case the pattern of enzyme expression was similar to that of the *ACT1*-driven *egl1* gene but with slower kinetics and lower levels of expression both under laboratory conditions (Fig. 3A) and in microvinification (Fig. 3B).

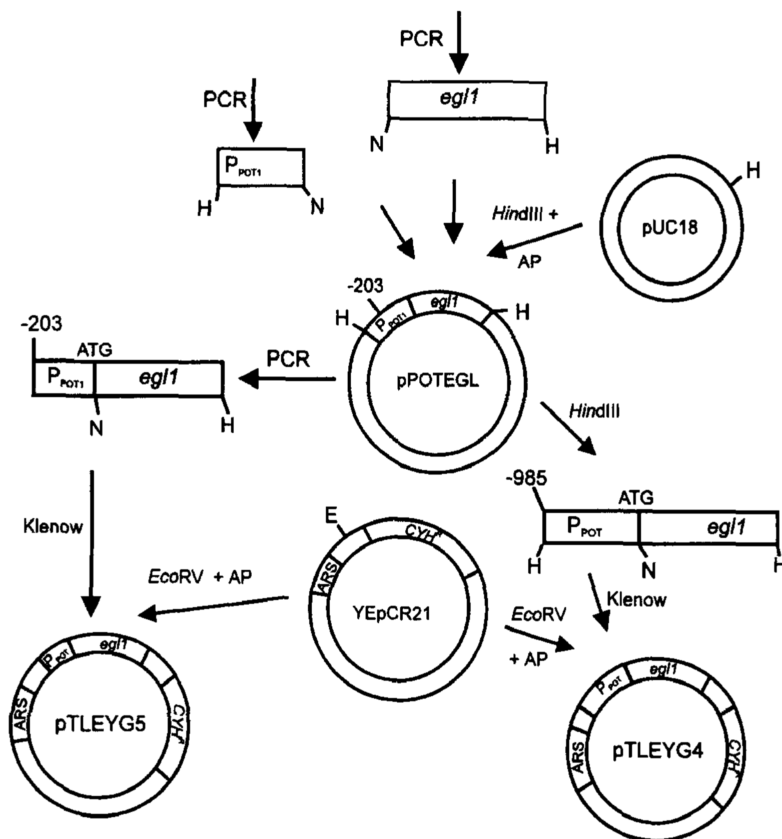

Fig. 2. Construction of pTLEGY4 & pTLEGY5 plasmids. 'PCR' indicates amplification with specific oligonucleotides. Treatments with alkaline phosphatase (AP) and with Klenow fragment of DNA pol I (Klenow) are indicated. Restriction digestions are indicated by the names of the enzymes. Names of plasmids and significant genetic elements are indicated. E: *Eco*RV, H: *Hin*dIII, N: *Nco*I. ATG is the translation origin.

DISCUSSION

The use of genetic engineering has opened the door to manipulate wine yeast strains in order to obtain better wine producers (reviewed in Querol and Ramón, 1996). One of the most direct potential benefits of this technology could be the introduction in wine yeast of exogenous genes coding for enzymes useful in vinification but not present in natural wine yeasts. In many cases it is not of technological relevance that the expression of those genes, and therefore of the enzymatic activities, be constitutive. For instance, flavor-producing enzymes could be more useful during the late phases of vinification in order to avoid flavor evaporation. On the contrary, several enzymes, such as pectinases, would be more useful if

they were to be expressed during the first stages of vinification when grape wastes, and therefore several potential substrates for the enzymes, are still present in the must.

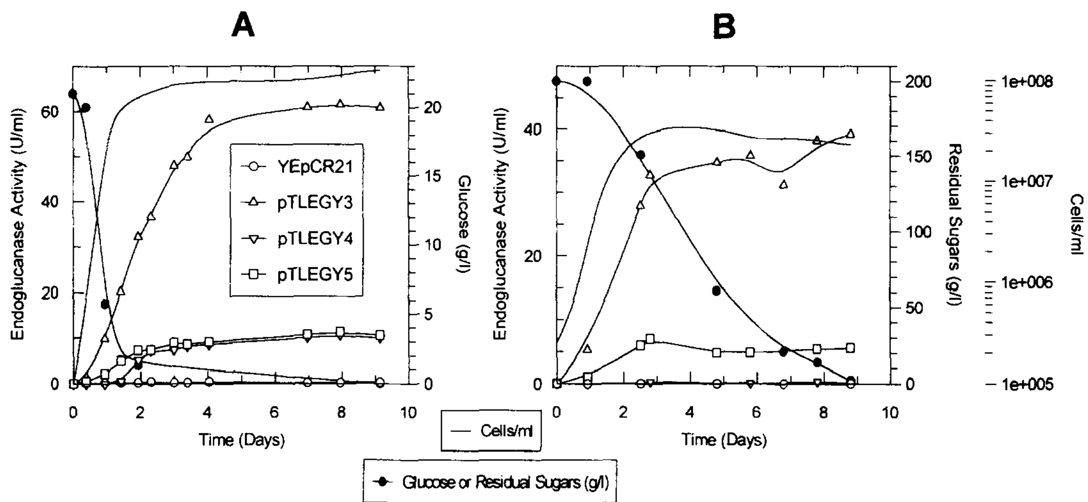

Fig. 3. Endoglucanase activity along the growth curve of several T_{73} derivatives. A) Laboratory cultures. **B)** Microvinification experiments using synthetic must. Enzyme activities are shown on the left. Cell densities and glucose concentrations are shown on the right. Plasmids contained in each strain are indicated. YEpCR21 was used as a negative control of endoglucanase activity.

The use of yeast gene promoters that are only, or mainly, expressed during the late phases of the growth curve has been the subject of much attention recently (Riou *et al.*, 1995; Polotnianka *et al.*, 1995). Here, we have evaluated the potential use of these promoters by comparing their expression patterns in laboratory culture and microvinification conditions. As can be deduced from the experiments with the *POT1* gene fusions and northern analyses with this and other late-expressed genes, there is no selective late expression of these genes under microvinification conditions. The reason for that could be the very different growth conditions used in microvinification (and therefore in real vinifications) with regard to those of laboratory cultures. Vinifications are characterized by very high contents of glucose and fructose compared with usual laboratory conditions (20-25% versus 1-3%), comparatively poor media (musts versus YPD or similar) and anaerobic conditions (non-aerated vinifications versus highly aerated laboratory cultures). These conditions greatly affect the expression of many genes. As glucose concentrations above 0.5% repress many yeast genes (Entian, 1986) they cannot be derepressed in vinifications where this sugar concentration is only reached at the end of the fermentation when most of the yeast cells are dead. Also the lack of any respiration could exert important effects on gene expression. This has been demonstrated for *POT1* expression. In this gene, full derepression requires glucose deprivation, stationary phase (Igual *et al.*, 1992) and respiratory conditions (Igual and Navarro, 1996). All these effects are mediated by means an URS element located between -203 and -238 with regard to the translational start (Navarro and Igual, 1994). Our results with the *POT-203* promoter, which lacks the URS element, show that *POT1* is expressed constitutively in its absence. Similar elements have been found in other late-expressed genes (Werner-Washburne *et al.*, 1996) and it is likely that the case of *POT1* promoter can be generalized.

On the contrary, we have shown that the *ACT1* promoter is more suitable for certain cases of late expression in which early expression of the gene in question does not result in undesirable consequences. If the gene product of the fusion is stable enough (like the β-(1,4)-endoglucanase), high and constant levels of activity during much of the vinification process are produced (see Fig. 3B).

We can also conclude that the search for late-expression promoters should be done in the future by means of direct analysis of gene expression in wine yeasts under vinification or microvinification conditions because the results from laboratory conditions cannot be directly extrapolated to wine fermentations.

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