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Engineering of an oenological *Saccharomyces cerevisiae* strain with pectinolytic activity and its effect on wine

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Abstract

A pectinolytic industrial yeast strain of *Saccharomyces cerevisiae* was generated containing the *S. cerevisiae* endopolygalacturonase gene (*PGU*1) constitutively expressed under the control of the 3-phosphoglycerate kinase gene (*PGK*1) promoter. The new strain contains DNA derived exclusively from yeast and expresses a high polygalacturonic acid hydrolyzing activity. Yeast transformation was carried out by an integrative process targeting a dispensable upstream region of the acetolactate synthase locus (*ILV*2), which determines sulfometuron methyl resistance. Microvinification assays were performed on white and red musts with the transformed UCLMS-1M strain and with the same strain untransformed. It was found that the changes in the pectic polysaccharide contents did not directly affect the taste or flavor of the wine. From the data reported, it is deduced that the chief advantage of using the modified strain is that it improves the yield of must/wine extraction, while it also positively affects some variables relating to appearance.

Keywords: *Saccharomyces cerevisiae*; Polygalacturonase activity; *PGU*1; Wine

1. Introduction

Pectinases are enzymes able to degrade pectic substances by hydrolysing the ester bond between galacturonic acid and methanol (pectin esterases) or by cleaving the glycosidic bonds of specific polymers (polygalacturonases, pectin, and pectate lyases), and are synthesized by plants (Ali and Brady, 1982) and microorganisms (Pascualli et al., 1991). Pectins may cause problems in the food industry by giving rise to turbidity and viscosity during the extraction, filtration, and clarification of fruit juices.

Most commercial pectinase preparations used in the food industry are derived from *Aspergillus niger*, which in addition to producing large quantities of these

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enzymes is a GRAS (Generally Recognized as Safe) microorganism. However, this mould secretes other enzymes that are less desirable for the production of wine or fruit juices for instance arabinofuranosidase, which can cause turbidity (Whitaker, 1984).

Given the role played by yeasts, especially of the genus *Saccharomyces*, in fermented products, further research into their pectinolytic enzymes would be useful for two purposes: one, so that yeast can be used to synthesize then purify the enzymes for addition to fruit juices as clarification and extraction enhancers; and two, in the case of fermented products, so that the enzyme can be produced by the yeast as part of the process rather than having to be added to the medium.

Fungal or bacterial genes encoding for these enzymes have been cloned and expressed in yeasts (Laing and Pretorius, 1993; Lang and Looman, 1995; González-Candelas et al., 1995; Iguchi et al., 1997; Blanco et al., 1998; Gognies et al., 1999; Vilanova et al., 2000; Sieiro et al., 2003).

Probably the chief drawback to the use of yeast-derived pectinolytic enzymes in industrial processing is the low yield of fermentation activity. An alternative would therefore be to clone and overexpress the structural genes responsible for these enzymatic activities so as to obtain a wine yeast strain that facilitates must and wine clarification during fermentation, thus producing cost savings. Such strains could further enhance the color and the aroma of wines (Van Rensburg and Pretorius, 2000).

Gainvors et al. (1994a) found that the strain SCPP, recently identified as *Saccharomyces bayanus* (Naumov et al., 2001) produced three types of pectinolytic enzyme: pectin methyl esterase (PME), pectin lyase (PL), and polygalacturonase (PG). They also demonstrated (Gainvors et al., 1994b) that when the enzymatic extract from that strain was added to a fresh must, the effects on turbidity were the same as when a commercial enzymatic preparation (Endozyme) was added.

Blanco et al. (1997) reported that when PG+ strains of *Saccharomyces cerevisiae* were used in vinification, in some cases the filtration time was reduced by half without any appreciable changes in viscosity.

The *PGU*1 gene encoding the endoPG of *S. cerevisiae* strains has been characterized, cloned, and overexpressed (Blanco et al., 1998; Gognies et al., 1999, 2001; Jia and Wheals, 2000; Vilanova et al., 2000; Gainvors et al., 2000; Blanco et al., 2002, and Fernández-González et al., 2004), and the protein sequences of some of them show different amino acids from the protein sequence of the database SGD (http://genome-www.stanford.edu/Saccharomyces).

Blanco et al. (1998), Naumov et al. (2001), and Veiga-Crespo et al. (2004) suggested that the coding region of the *PGU*1 gene (ORF YJR153w) was probably present and conserved in both PG positive and negative strains, but Fernández-González et al. (2004) evaluated a total of 61 *S. cerevisiae* strains, 60 of them isolated from wine ecosystems for the presence of this gene and for PG activity, founded that 9 strains lacked the gene and of the 52 strains possessing *PGU*1 gene, only 36 showed PG activity. These results showed that such inconsistent occurrence of the *PGU*1 gene in different strains of *Saccharomyces* suggests that this gene is not typical of *S. cerevisiae*, and hence may be used as an intraspecific marker for genetic and phenotypic characterisations.

In this work the wine strain UCLMS-1, which has good enological qualities, was transformed with *PGU*1 gene from the strain UCLMS-39 (Fernández-González et al., 2004), transcriptionally bonded to the promoter of *PGK*1 gene, in order to enhance its expression during vinification. The *PGK*1 gene promoter presents high constitutive expression in the presence of glucose and is repressed by other non-fermentable carbon sources like glycerol (Kingsman et al., 1990). The data in the literature indicate that this promoter can increase the level of expression of different genes in *S. cerevisiae*, such as those for immunoglobulin (Wood et al., 1985), β-1,4-endoxylanase (Crous et al., 1995), α-amylase (Yánez et al., 1998), and alcohol acetyl transferase (Lilly et al., 2000).

Microvinification was assayed in white and red musts with the transformed UCLMS-1M strain, and with the same strain untransformed, and the results were compared.

2. Material and methods

2.1. Strains and media

Strains and plasmids used in this study are summarised in Table 1. The host used for maintenance

Table 1
Microbial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	References
Bacteria		
E. coli DH5 α	*end*A1 *hsd*R17 (r_k^- m_k^+) *sup* E44 *thi*1λ$^-$ *rec*A1 *gyr* A, *re*l A1 Δ (*lac*ZY A-*arg*F) U169 (φ80*lac*ZΔ M15)	Gibco-BRL
Yeasts		
S. cerevisiae W3031b	*Matα*, *ade2-1*, *his3-11,15 leu2–3,112 ura3-1 trp1-1 can1–100*	CBMSO, UAM-CSIC
S. cerevisiae WGUa/b	W303 1b pYES2-*PGU*1	Fernández-González et al. (2004)
S. cerevisiae WKU	W303 1b YCp50+PGK1p-*PGU*1	This work
S. cerevisiae UCLMS-39	Wine yeast strain isolated in our laboratory, pectinolytic activity	Fernández-González et al. (2004)
S. cerevisiae UCLMS-1	wine yeast strain isolated in our laboratory, pectinolytic activity-deficient control strain	Fernández-González et al. (2004)
S. cerevisiae UCLMS-1M	UCLMS-1 *SMR*1-410 *PGK1*p-*PGU*1	This work
Plasmids		
YCp50	*Hind*III – *Bam*HI – *Sph*I – URA3 – CEN4 – ARS1 – pMB1 ori – bla.	CBMSO, UAM-CSIC
YKU	YCp50-PGK1p-*PGU*1	This work
PWX509	bla SMR1-410	Casey et al. (1988)
PKUSa/b	pWX509-PGK1p-*PGU*1	This work

and propagation of the plasmids was *Escherichia coli* DH5α strain, which was grown in Luria–Bertani (LB) medium (bactotryptone 1%, yeast extract 0.5%, and NaCl 0.5%), supplemented with ampicillin (Ap; 100 μg/ml) or tetracyciline (TC; 25 μg/ml) as required. *S. cerevisiae* was cultivated at 30 °C in both media: SC (0.67% amino acid-free yeast nitrogen base, supplemented with the required aminoacids, and 2% glucose) and YPD (containing 1% yeast extract, 2% peptone, and 2% glucose). The haploid *S. cerevisiae* transformants were selected on synthetic complete (SC) medium without uracyl, and the wine yeast transformants were selected on SC plates complemented with sulfometuron methyl (SMM) 15 mg/l. Solid media contained 2% agar. Polygalacturonase (PG) activity evaluation of the yeast transformants, was carried out on plates complemented with polygalacturonic acid (PGA) as sustrate (Charoenchai et al., 1997), and the quantification was done using the method described by Milner and Avignad (1967), using PGA as substrate, and the results were expressed as μmol galacturonic acid (GALA) $\cdot$ ml^{-1} of supernatant (Fernández-González et al., 2004).

2.2. Enzymes and reagents

The restriction enzymes *Bam*HI, *Eco*RI, *Sal*I, *Xba*I, *Xho*I, and *Sma*I were supplied by Roche and *Sau*3A by Amersham Life Science. Other enzymes used in the handling of nucleic acids were: DNA ligase of bacteriophage T4, alkaline phosphatase (Roche), Klenow fragment of DNA polymerase I (*E. coli*; Promega), P*wo* DNA polymerase (Roche), Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and RNAsa A (Sigma). The oligonucleotide primers were TIB molbiol (Roche). Other enzymes used were lysozyme and proteinase K, both from Sigma. All these enzymes were used in accordance with the protocols in the literature (Sambrook et al., 1989) or following the supplier's instructions.

The antibiotics ampicillin and tetracycline were supplied by Sigma and the herbicide sulfometuron methyl by Supergo.

The various organic and inorganic products were supplied by Merck, Panreac, Roche, Bio-Rad, and Sigma. The ingredients of the culture media were supplied by Difco, Pronadisa, Oxoid, and Panreac.

2.3. DNA manipulation, cloning, and transformation

The competent cells of *E. coli*, DH5α, were prepared according to the SEM (simple and efficient method; Inoue et al., 1990). The state of competence of cells was maintained for a prolonged period by freezing them with 7% DMSO at −70 °C. The

method described by Inoue et al. (1990) was used for *E. coli* transformations.

Small-scale DNA isolation from *E. coli* was obtained by alkaline lysis mini-preparations (Birnboim and Dolly, 1979). Alternatively, the plasmidic DNA of recombinant clones was purified with Promega's Wizard® Plus SV Minipreps (DNA Purification System) kit.

Yeast genomic DNA was obtained following the protocol "Isolation of genomic DNA for Southern Blot Analysis" (Rose et al., 1990), and recovery of plasmids for *E. coli* transformation was according to Hoffman and Winston (1987).

Yeasts were transformed following the protocol LiAc/SS-DNA/PEG described by Gietz and Shiestl (1995).

Standard procedures were used for restriction–ligation reactions (Sambrook et al., 1989).

2.4. Polymerase chain reaction (PCR)

The 583nt (−580 to +3) fragment containing the *S. cerevisiae PGK*1 gene promoter, was fused to the *PGU*1 gene coding region by PCR (Fig. 1) and the oligonucleotides used are in Table 2.

The oligonucleotide CT-*PGU*1 is reverse complementary (nt 1 to 33) to the nt sequence of ORF *PGU*1 (nt +1063 to +1086) and also contains the recognition sequence for the enzyme *Bam*HI (nt 5 to 10).

The oligonucleotide 5′-p*PGK*1 is homologous (nt 8 to 28) to the nt sequence in the region promoting the gene *PGK*1 (nt −580 to −560). This oligonucleotide further contains the recognition sequences for the restriction enzymes *Nco*I (nt 1 to 6) and *Bam*HI (nt 5 to 10) in its 5′ end (Yánez et al., 1998).

The oligonucleotide R-*PGU*1-p*PGK*1 is reverse complementary (nt 1 to 22) to the nt sequence of the ORF *PGU*1 (nt +1 to +22) and is also reverse complementary (nt 19 to 38) to the nt sequence of the chain promoting the gene *PGK*1 (nt −16 to +3).

The oligonucleotide F-p*PGK*1-*PGU*1 is homologous (nt 1 to 16) to the nt sequences of the region promoting the gene *PGK*1 (nt −13 to +3) and is also homologous (nt 13 to 30) to the nt sequence of the ORF *PGU*1 (nt +1 to +17).

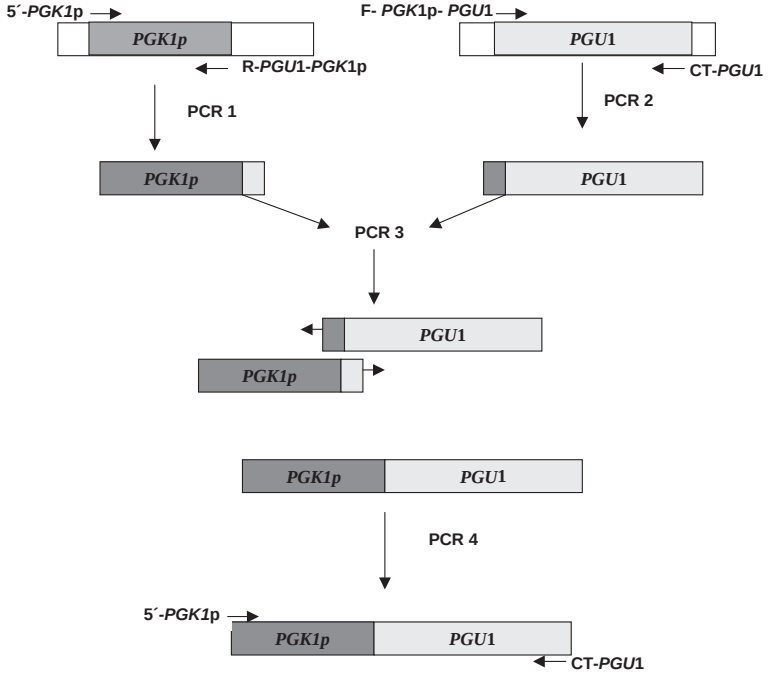

Fig. 1. Schematic representation of the fusion of the *S. cerevisiae* 3-phosphoglycerate kinase gene promoter (*PGK*1p) to the endopolygalacturonase gene (*PGU*1) coding region, using the PCR technique.

Table 2
Oligonucleotides used as primers to generate PCR products

Oligonucleotides	Sequences (5′ to 3′)	Nt
CT-*PGU*1	5′CTGCGGATCCTTAACAGCTTGCACCAGATCCAG 3′	33
5′-PGK1p	5′CCATGGAtcctccttcttgaattgatg 3′	28
R-*PGU1*-PGK1p	5′GTAATGAATTAGCAGAAATCATtgttttatatttgttg 3′	38
F-PGK1p-*PGU*1	5′ caaatataaaacaATGATTTCTGCTAATTC 3′	30

*Bam*HI recognition site (indicated by underlined letters) and *Nco*I (italics letters), 3-phosfoglicerate kinase gene promoter (*PGK1p*) sequence (small letters) and endopolygalcturonase gene (*PGU*1) coding sequence (capital letters). nt, nucleotide.

All amplification reactions were done in a Perkin-Elmer model 2400 Thermocycler apparatus. The volume of each reaction (PCR1, PCR2, and PCR4) was 50 μl, with a final concentration of: PCR buffer 1X; 0.2 mM of each dNTP; 1 μM of each of the oligonucleotides (5′-p*PGK*1 and R-*PGU*1-p*PGK*1 for PCR1; CT-*PGU*1 and F-p*PGK*1-*PGU*1 for PCR2; and 5′-p*PGK*1 and CT-*PGU*1 for PCR4); 1.25 U of P*wo* DNA polymerase; and 5–15 ng DNA. Amplification conditions were 94 °C/4 min followed by 30 cycles of 94 °C/30 s, 55 °C/30 s, and 72 °C/30 s (PCR1), 1 min (PCR2) and 2 min (PCR4), and finally 72 °C/5 min.

In the PCR3 reaction, the concentrations and components for a final volume of 50 μl were: PCR buffer 1X; 0.2 mM of each dNTP; 1.25 U of P*wo* DNA polymerase; and 100 ng of each of the reaction products of PCR1 and PCR2. Amplification conditions (hybridation) were 94 °C/4 min, followed by 30 cycles of 94 °C/30 s, 52 °C/1 min, and 72 °C/1.5 min, and finally 72 °C/10 min.

The fragment obtained by PCR3 was treated with *Bam*HI. T4 DNA ligase was used to bind it to plasmid YCp50, previously linearized with *Bam*HI, then *E. coli* DH5α was transformed with the plasmid, which formed (YKU). The constructions were checked by restriction and PCR4.

The plasmidic DNA extracted from the DH5α transformants with the target construction was used to transform *S. cerevisiae* W3031b, selecting by the Ura^+ phenotype and then by PG+.

2.5. Construction of a S. cerevisiae UCLMS-1M wine strain with pectinolytic activity

In order to obtain a wine yeast strain capable of hydrolysing pectins during vinification, the construction described in the previous section was used to transform the UCLMS-1 *S. cerevisiae* strain. The UCLMS-1. *S. cerevisiae* wine strain was isolated in wines from the Valdepeñas region (Spain) and selected as being of clear oenological interest to the wine-making of this region (Briones et al., 1995). Integration of this construction in the *ILV*2 locus (*SMR*1, *THI1*), ORF YMR108W, of chromosome XIII, which codifies for biosynthesis of acetolactate synthase (ALS), confering resistance to the herbicide sulphometuron methyl was the strategy followed (Falco et al., 1985) being used in the transformation of some industrial yeasts (Gasent-Ramirez et al., 1995; Marín et al., 2001). This was achieved with the integrative vector pWX509 (Casey et al., 1988) by insertion of the construction p*PGK1-PGU*1, obtained from the YUK plasmid using the *Bam*HI site. This fragment was treated with Klenow polymerase to obtain non-cohesive ends; then, after sub-cloning in the *Sna*BI site of the vector pWX509, *E. coli* was transformed by selecting Ap^R clones.

The plasmidic DNA (pKUSa/b) was treated with *Sal*I and the wine yeast was transformed with the resulting linear fragment, containing the *SMR*1 region, the p*PGK*1-*PGU*1 cassette and the 5′ and 3′ flanking sequences of locus *SMR*1, selecting for resistance to sulfometuron methyl (SMM^R).

Following transformation, it was found that the yeast (UCLMS-1M) expressed polygalacturonase activity in solid and liquid media (Fernández-González et al., 2004) and that it contained the target insert confirmed by PCR4.

2.6. Microvinification assays

Microvinification assays were performed in quintuplicate in white and red musts, following the traditional wine-making methods. The starter cultures

were *S. cerevisiae* UCLMS-1M and UCLMS-1 used as control.

White must from "Airen" grape variety and red must from "Cencibel" were stored at −20 °C until use. The musts with 30 ppm SO_2 added were used in the preparation of the inoculum and for microvinification trials. These assays were performed in 1-l Erlenmeyer flasks fitted with Müller valves. Samples were inoculated with 10^7 cells ml^{-1}. White musts were fermented at a controlled temperature of 18–20 °C until the sugars were exhausted. Red musts were fermented at 24–25 °C in contact with the skin for 72 h, and as much semi-fermented matter as possible was extracted by manual crushing. The yield of wine-must extraction was calculated by comparing the initial weight of the whole grapes and the final skin residue. Semi-fermentation after removal of the skin continued at the same temperature until the sugars were exhausted.

After fermentation, the wines were centrifuged to remove lies (2260×*g*, 4 °C/5 min) and then stored under refrigeration until analysis.

2.6.1. Viability of starter cultures

Samples were taken periodically to estimate cellular viability; these were plated on Wallenstein agar (Oxoid). The resulting colonies were counted after 48 h of incubation at 30 °C.

To ascertain the dominance of the inoculated strain, 10 isolates were selected at random and subjected to mitochondrial DNA restriction analysis using *Hin*fI (Querol et al., 1992). To confirm that the UCLMS-1M strain retained pectinolytic activity, these isolates were grown on PGA medium at pH 3.5 (Charoenchai et al., 1997).

2.6.2. Physicochemical parameters

The wines were analyzed for conventional parameters according to OIV recommendations. In addition, absorbances at 420 nm, 520 nm, and 620 nm were measured in red wines to determine the coloring intensity (IC=A420+A520+A620; Glories, 1984) and the tonality (*T*=A420/A520; Sudraud, 1958).

2.6.3. Filtration rate

Polygalacturonase activity of the *S. cerevisiae* UCLMS-1M strain on wine filtration behavior was determined by measuring the time required for 100 ml of final product to pass through a 0.45 μm filter using a tangential filtration system (Minitan, Millipore).

2.6.4. Measurement of viscosity

Viscosity was measured at 40 °C in 10 ml of filtered wine, using a Cannon-Fenske 5354/2 viscosimeter for transparent liquids (Comecta S.A). Kinematic viscosity (KV), expressed in centistockes (csk), was calculated by the formula KV=*Kt*, where $K_{40\ ^\circ C}$=0.0016709 and *t* is the time in seconds.

2.6.5. Major volatiles

Acetaldehyde, methanol, 1-propanol, 2-butanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and ethyl acetate were analyzed using 3-pentanol as internal standard. A Perkin-Elmer gas chromatograph equipped with a VINICOL packed column was used (González and González Lara, 1994). The conditions were the following: detector temp.: 225 °C; Injector temp.: 200 °C; Carrier gas: N_2; Flow: 15 ml/min; injection volume: 1 μl. Oven program: initial temp. 40 °C/3 min; 6 °C/min up to 60 °C, isotherm 16 min; and 6 °C/min up to 126 °C, isotherm 12 min.

2.6.6. Sensory analysis

Sensory evaluations were performed to determine whether there were significant differences between wines made with the modified strain and with the control strain, in both white and red wines. All evaluations were carried out in a standardized tasting room (Spanish standard UNE 87004:1979; AENOR, 1997) using standard wine-tasting glasses (Spanish standard 87022: 1992 and ISO standard 3591:1977) (AENOR, 1997).

A triangular test (Spanish standard UNE 87006:1992 and ISO standard 4120:1983; AENOR, 1997) was designed to ascertain the effect of modified yeast strain on wine. The taste panel was composed of 25 tasters who were familiar with the product.

2.6.7. Statistical analysis

To determine whether there were significant differences between wines made with UCLMS-1 and UCLMS-1M strains, the Student's *t*-test was applied to data for related samples, using the SPSS statistical program (version 11).

3. Results and discussion

3.1. Modification of gene PGU1 expression

Once it was known that the PG+ activity of the strain UCLMS-39 was due to the gene *PGU*1 and that this was functional under its own promoter and the *GAL*1 promoter (Fernández-González et al., 2004), the *PGU*1 gene promoter was replaced by the strongly-expressing *S. cerevisiae PGK*1 gene promoter in order to raise the level of expression (Fig. 1).

In the first PCR (PCR1), a region containing the *PGK*1 gene promoter, nt −580 to +3, with *Nco*I and *Bam*HI recognition sequences at the 5′ end, and a short region "upstream" *PGU*1 gene, nt +1 to +22, at the 3′ end was amplified.

In another reaction mixture (PCR2), a region including a short portion "downstream" *PGK*1 gene promoter, nt –13 to +3, the 5′ end *PGU*1 gene, nt +1 to +1086, and a *Bam*HI recognition sequence at the 3′ end was amplified.

A third reaction (PCR3) produced a DNA fragment of 1665 bp containing the *PGK*1 gene promoter, nt −580 to +3, bonded to the *PGU*1 gene, nt +1 to +1086. This fragment was purified and bonded to the plasmid YCp50 before transformation of *E. coli* DH5α. The plasmidic DNA with the target construction (YKU-2, -15, and -16) was used to transform *S. cerevisiae* W3031b strain by selection in the appropriate media (see Material and methods).

3.2. Construction of a S. cerevisiae UCLMS-1M wine strain with pectinolytic activity

The *S. cerevisiae* UCLMS-1 strain lacking the *PGU*1 gene, as confirmed by PCR and Southern Blot (Fernández-González et al., 2004), was transformed with the p*PGK*1-*PGU*1 linear fragment. The pKUSa/b plasmids were treated with SalI, producing 30 transformed clones with the KUSa construction and 35 with the KUSb, SMM^R construction. More than half of these exhibited stable PG+ activity in a solid medium. *S. cerevisiae* UCLMS-1M strain is a genetically modified but not a transgenic organism since it contains no phage or bacterial sequences.

One transformed strain was randomly selected (UCLMS-1M), and the presence of the 1665 pb fragment corresponding to the construction p*PGK*1-*PGU*1 was confirmed by PCR4. This strain was also used for the quantification assays.

Table 3 shows the μmol GALA released by the transformed, the donor and the untransformed strains when grown without and with PGA.

The UCLMS-1M strain was much more active than the donor strain UCLMS-39 in all cases, and in only 1 h of contact with PGA it released large quantities of galacturonates, increasing progressively with time. Moreover, during growth it hydrolyzed PGA, and to a far lesser extent pectin, at both pH 3.5 and 5.5.

3.3. Microvinification assays

The kinetic behavior of all microvinification types was similar irrespective of the yeast strain used (UCLMS-1/-1M), and cell viability was adequate; counts were normal for this type of process.

As to implantation of the cultures, all the isolates analyzed presented the same mitochondrial DNA restriction profile as the starter cultures (patterns shown in Fig. 2). As can be observed in the plate with PGA, the isolates from microvinifications with the UCLMS-1M strain exhibited polygalacturonase activity.

Vilanova et al. (2000), used the recombinant strain M-20T with the pBJ16-*PGU*1 plasmid (Blanco et al., 1998), leaving its fermentation ability essentially unchanged. The results revealed that cells were gradually losing the plasmid. After fermentations, 70% of the cells still retained the plasmid, suggesting

Table 3
Polygalacturonase activity in supernatant and in broth-culture of donor (UCLMS-39), receptor (UCLMS-1), and modified (UCLMS-1M) yeast strains, expressed as μmol of galacturonic acid (GALA) released for mL of supernatant (μmol GALA mL S^{-1}), using polygalacturonic acid (PGA) as substrate

	(1) μmol GALA mL S^{-1}			(2) μmol GALA mL S^{-1}			
	1 h	5 h	24 h	PGA3.5	PGA5.5	PT3.5	PT5.5
UCLMS-39	1.01	2.05	3.26	3.06	2.94	0.38	0.30
UCLMS-1	0.00	0.01	0.02	0.01	0.05	0.00	0.02
UCLMS-1M	3.82	5.06	6.26	9.12	7.96	1.47	1.50

(1) GALA released from PGA, using the supernatant from 3 days of culture in YNB-glucose medium, incubated in PGA medium at 37 °C pH 5,5 at different times (1 h, 5 h, 24 h).
(2) Direct quantification of GALA released from PGA or pectin (PT) into the 3 days' culture medium at pH 3.5 or 5.5.

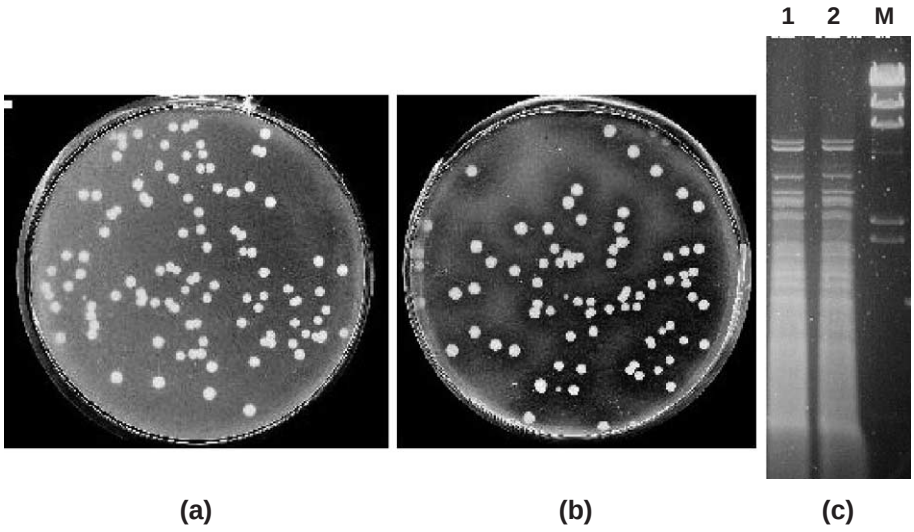

Fig. 2. Polygalacturonase activity on polygalacturonic acid medium at pH 3.5 after incubation at 30 °C/24 h. (a) UCLMS-1; (b) UCLMS-1M. (c) mtDNA patterns obtained using the restriction enzyme *Hin*fI, corresponding to UCLMS-1 and -1M strains (lines 1, 2). Line M. Marker II (Roche).

a high level of mitotic stability, but in this work, 100% of cells analyzed have PG activity because of chromosomal integration of the construction of interest (p*PGK*1-*PGU*1), and transformants were stable during vinification process.

The physicochemical parameters recorded for white and red wines are shown in Table 4. The Student's *t*-test for related samples showed no significant ($p \leq 0.05$) differences in any of the target physicochemical parameters in white wines. However, in red wines made with UCLMS-1M (RWM), the amount of reducing sugars was significantly ($p \leq 0.05$) higher than in the control (RWC). These findings were to be expected given that maceration with pectinolytic enzymes produces slow hydrolysis of the skins cell walls and hence a greater presence of enzyme-resistant

Table 4
Physicochemical parameters

	WWC		WWM		RWC		RWM	
	X	S.D.	*X*	S.D.	*X*	S.D.	*X*	S.D.
PH	3.84	0.02	3.84	0.03	4.15	0.05	4.14	0.04
Volatile acidity (g/L acetic acid)	0.10	0.01	0.11	0.00	0.10	0.02	0.10	0.01
Total acidity (g/L tartaric acid)	5.10	0.09	5.00	0.09	4.34	0.16	4.45	0.18
Residual sugars (g/L)	0.53	0.25	0.57	0.06	2.85[a]	0.31	4.67[b]	0.21
Alcohol degree (%v/v)	12.3	0.0	12.3	0.0	15.2	0.1	15.3	0.1
A420	0.118	0.001	0.131	0.007	0.384	0.039	0.455	0.021
A520					0.558[a]	0.062	0.703[b]	0.033
A620					0.154	0.022	0.189	0.007
IC=A420+A520+A620					10.96[a]	1.22	13.46[b]	0.61
T=A420/A520					0.69[a]	0.01	0.65[b]	0.01
Filtration time (s)	68.7	3.8	81.2	13.1	550.2	36.2	545.6	34.6
Kinematic viscosity (csK)	1.10	0.03	1.11	0.03	1.24	0.01	1.25	0.01
Yield of extraction (%)					75.3[a]	1.6	82.3[b]	0.5

WWC, white wine fermented with UCLMS-1; WWM, white wine fermented with UCLMS-1M; RWC, red wine fermented with UCLMS-1; RWM, red wine fermented with UCLMS-1M. *X*=mean value; S.D.=standard deviation. [a,b] Significant differences ($p \leq 0.05$).

pectic polysaccharides such as Rhamnogalacturonan II, which can increase the reducing power (Pellerin, 2001). We would note, however, that there were no significant differences in final alcohol concentration.

Also, the RWM sample exhibited more intense coloring accompanied by red tones (A520 nm), a feature very much appreciated in red wine fermentations, although the tone of the control was higher. This is consistent with the findings of González San José et al. (1998) in a study of the effect of treatment with commercial pectinolytic enzymes.

As regards the parameters identified with improvement of the process, the major technological improvement was an increased yield of extraction of semi-fermented from red grapes in wines inoculated with the UCLMS-1M strain, where the yield was 7% higher than in the control. This was because the secreted enzyme facilitated cleavage of the grape cell walls favoring also the extraction of anthocyans from anthocyanoplasts (Rogerson et al., 2000), which are responsible for increased colorant intensity. There were no significant differences in filtration time or kinematic viscosity between wines made with either strain.

Fig. 3 shows the GC analysis values of the major volatiles. The analyzed compounds did not differ significantly in the white wines; however, the methanol concentration increased in the red wines made with the UCLMS-1M strain, which is the logical outcome of degradation of the grape pectins. Nevertheless, these values were well below the maximum permitted by European regulations (500 mg/l).

3.3.1. Sensory analysis

A triangular test was conducted with white and red wines elaborated with UCLMS-1 and -1M strains. The statistical data revealed there were no significant differences (at the 95% level) between white wines elaborated with UCLMS-1 (WWC) and UCLMS-1 (WWM), and the same occurs for RWC and RWM; therefore, a more exhaustive sensory analysis was not possible because tasters were not able to difference the wines elaborated with UCLMS-1M and UCLM-1.

The results of the sensory analysis demonstrated that wines elaborated with the UCLMS-1M strain are of the same quality as those obtained with the UCLMS-1 strain and, moreover, they preserved the typical characteristic of wines elaborated with the Cencibel and Airen grape variety. Alterations to the polysaccharide content therefore have no direct effect on the taste or flavor of the wine.

Vilanova et al. (2002) obtained the same results with Albariño grape variety using the recombinant strain M-20T with the pBJ16-*PGU*1 plasmid (Blanco et al., 1998), leaving its fermentation ability essentially unchanged. Wines obtained with the recombinant strain and the untransformed counterpart did not differ in their physicochemical parameters or major sensory characteristics. With respect to wines obtained from must supplemented with commercial pectic enzymes, these wines display aromas that are less typical or not typical at all.

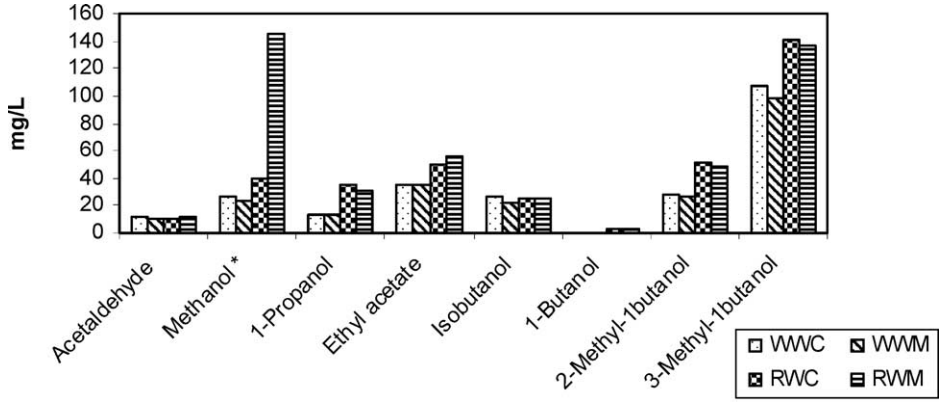

Fig. 3. Major volatile compounds in red and white wines, fermented with UCLMS-1 and UCLMS-1M. WWC, white wine fermented with UCLMS-1; WWM, white wine fermented with UCLMS-1M; RWC, red wine fermented with UCLMS-1; RWM, red wine fermented with UCLMS-1M. *Significant differences ($p<0.05$) in red wines.

4. Conclusions

The work reported is directed to the construction of a *S. cerevisiae* wine yeast strain, selected as being of clear oenological interest to the wine-making, containing *PGU*1 endopolygalacturonase gene constitutively expressed under the control of *PGK*1 gene promoter, used in trial fermentations in the search for an alternative to the use of commercial pectic enzymes of fungal origin.

Transformants obtained in this way are stable during vinification process, with solely nucleotide sequences of interest. The absence of bacterial DNA integrant structure, agree with the current GMO regulations in USA and Europe for the commercial application of recombinant DNA technology in food and beverage industry.

The major technological improvement was an increased yield of extraction of semi-fermented from red grapes in wines inoculated with the UCLMS-1M strain, where the yield was 7% higher than in the control, and increased colorant intensity.

When fermentation tanks are in short supply, the adventage of enzyme treatment is obvious, since the faster colour extraction will allow pomace to be pressed up earlier.

In white wines, any modification was observed, probably because of maceration techniques were not used, and Airen variety is a non-aromatic one.

However, additional work is required to determine the effectiveness of it in large-scale winemaking trials, in order to obtain information on the effect of the enzyme on the clarification and filtration of the wine.

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