

## Construction of a Genetically Modified Wine Yeast Strain Expressing the *Aspergillus aculeatus rhaA* Gene, Encoding an $\alpha$ -L-Rhamnosidase of Enological Interest

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**The *Aspergillus aculeatus rhaA* gene encoding an  $\alpha$ -L-rhamnosidase has been expressed in both laboratory and industrial wine yeast strains. Wines produced in microvinifications, conducted using a combination of the genetically modified industrial strain expressing *rhaA* and another strain expressing a  $\beta$ -glucosidase, show increased content mainly of the aromatic compound linalool.**

Monoterpenes such as geraniol, linalool, and  $\alpha$ -terpineol present in grapes determine the varietal flavor properties of young quality wines made from Muscat varieties (for reviews, see references 19 and 21). Geraniol and linalool are considered to be the most important of the monoterpene alcohols, as they are present in greater concentrations and have lower flavor thresholds than other major wine monoterpenes. In particular, linalool is thought to be responsible for the grapelike aroma of wines produced from the Muscat variety. A large proportion of these compounds are found as odorless diglycoside conjugates, such as 6-*O*- $\alpha$ -L-arabinofuranosyl- $\beta$ -D-glucopyranosides, 6-*O*- $\alpha$ -L-rhamnopyranosyl- $\beta$ -D-glucopyranosides, and 6-*O*- $\beta$ -D-apiofuranosyl- $\beta$ -D-glucopyranosides. This fraction constitutes a potential pool of aroma precursors, and its enzymatic hydrolysis has been studied as a method to release free monoterpenes and enhance grape juice and wine flavor (10, 34, 35). It is now well established that this enzymatic hydrolysis occurs in two steps (11). During the first step, and depending on the conjugate, the glycosidic linkage is cleaved by either a  $\beta$ -D-apiosidase, an  $\alpha$ -L-arabinofuranosidase, or an  $\alpha$ -L-rhamnosidase, and the corresponding monoterpenyl- $\beta$ -D-glucosides are released. In the second step, monoterpenes are liberated by the action of a  $\beta$ -D-glucosidase. Consequently, research interest has been focused on those glycosidases able to release flavor compounds from glycosides. Several glycosidases, such as  $\alpha$ -L-arabinofuranosidases (14, 30, 31, 36),  $\beta$ -D-apiosidases (13),  $\alpha$ -L-rhamnosidases (2, 3, 15, 17, 18, 20, 22, 32, 33), and  $\beta$ -D-glucosidases (1, 12, 20, 26, 30, 31), from different microbial sources have been characterized for their potential application in increasing the aroma of wine.

An alternative way to achieve increases in the content of volatile compounds is the inoculation of must with genetically

modified wine yeasts expressing genes coding for these enzymes (25). Previous work in our laboratory has reported the use of several transgenic wine yeasts expressing a *Candida molischiana*  $\beta$ -D-glucosidase and an *Aspergillus niger*  $\alpha$ -L-arabinofuranosidase as tools to increase the free monoterpene content in wine (27, 28). We have also expressed genes encoding a *Trichoderma longibrachiatum* endoglucanase (23) and different *Aspergillus nidulans* xylanases (4, 5) in industrial wine yeast strains, which resulted in increased levels of some volatile compounds.

Recently, we cloned two genes from *Aspergillus aculeatus* encoding the  $\alpha$ -L-rhamnosidases RhaA and RhaB (18). In the present work, the expression of the gene encoding the *A. aculeatus* RhaA in a commercial wine yeast strain was investigated. For this purpose, a 2.2-kb *Bam*HI fragment carrying the *rhaA* cDNA (18) (which includes a 19-amino-acid signal sequence) was ligated between the *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase gene promoter (i.e., *GPD<sub>p</sub>*) and the phosphoglycerokinase (i.e., *PGK<sub>t</sub>*) terminator and polyadenylation signals in plasmid pG-1 (29), thereby generating plasmid pR31. Plasmids pR31 and pG-1 (a control) were used independently to transform the laboratory yeast strain W303-1A (*MATa ade2-1 his3-11,15 leu2-3,112 can1-100 trp1-1 ura3-1*) using the LiAc-ss-DNA-polyethylene glycol protocol described by Gietz and Woods (7). Tryptophan prototrophic transformants were selected on synthetic dextrose minimal supplemented medium (0.67% yeast nitrogen base without amino acids, 20 g of glucose per liter, 20 mg of adenine per liter, 20 mg of uracil per liter, 20 mg of histidine per liter, and 30 mg of leucine per liter) and analyzed for  $\alpha$ -L-rhamnosidase production by plating on synthetic dropout minimal-supplemented media containing 4-methylumbelliferyl  $\alpha$ -L-rhamnopyranoside (MUR) as the substrate (16). The hydrolysis of MUR by the action of  $\alpha$ -L-rhamnosidase resulted in the release of 4-methylumbelliferone, which can be visualized under UV illumination as fluorescent halos surrounding yeast colonies. Only yeast cells transformed with pR31 showed the corre-

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sponding activity. Eight randomly selected pR31 transformants and a control strain transformed with pG-1 were grown in liquid selective medium for 18 h, and  $\alpha$ -L-rhamnosidase activity was determined in culture filtrates by using *p*-nitrophenyl- $\alpha$ -L-rhamnopyranoside (pNPR) as the substrate (18). As expected, activity was detected only in pR31 transformants. These data confirm that the *A. aculeatus rhaA* gene encodes an  $\alpha$ -L-rhamnosidase and demonstrate that it can be efficiently expressed in *S. cerevisiae*. From those transformants exhibiting the highest levels of  $\alpha$ -L-rhamnosidase activity, one of them, designated YR6, was isolated for further studies. This transformant produces 130 mU of  $\alpha$ -L-rhamnosidase activity per ml (specific activity of 2.6 U/mg) after 72 h of growth in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% bacteriological peptone, 2% glucose).

Following the protocol previously developed except for the use of 10 mM sodium citrate buffer (pH 3.8) (18), the *A. aculeatus* RhaA enzyme was purified to apparent homogeneity from transformant YR6 by using cation-exchange chromatography. The level of recovery was 12% of that of the original  $\alpha$ -L-rhamnosidase activity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of RhaA revealed a single protein band after N deglycosylation of an apparent molecular mass of 70 kDa, a finding which was in agreement with the molecular mass of RhaA purified from *A. aculeatus* (18). Optimum pH and stability as well as kinetic constants using pNPR as the substrate were similar to those described for wild-type RhaA (18). Certain factors that may affect enzyme activity under enological conditions were also tested (28). Ethanol at a concentration of 15% (vol/vol) reduced RhaA activity by 55%, whereas at 20% (vol/vol) ethanol, the enzyme retained 35% of its original activity. The presence of SO<sub>2</sub> at concentrations of up to 150 mg/liter, a level that can be found at different stages during wine fermentation, did not significantly affect enzyme activity. Glucose concentration did however affect RhaA activity, and inhibition levels of 59, 84, and 93% were observed for glucose concentrations of 0.15, 0.50, and 1 M, respectively. Finally, to test the ability of RhaA to hydrolyze Muscat must glycosides, the release of L-rhamnose was analyzed by high-performance liquid chromatography using a Waters Sugar-Pak I column (dimensions, 6.5 by 300 mm) at 80°C and a refraction index detector. From our data, it is possible to conclude that the heterologously expressed RhaA at a final concentration of 48  $\mu$ g/ml in the incubation mixture is able to release 46 nmol of L-rhamnose after 24 h of incubation at 40°C. These experiments indicate that the *A. aculeatus* enzyme has enologically relevant properties.

In order to express the *A. aculeatus rhaA* gene in a commercial wine yeast strain, we isolated the *GPD<sub>p</sub>::rhaA::PGK<sub>t</sub>* expression cassette from plasmid pR31 as a 4.2-kb *Hind*III fragment and ligated it into the *Hind*III site of plasmid YEpCR21 (23), yielding plasmids pR37 and pR38 (corresponding to each of the two possible orientations with respect to the vector). The industrial wine yeast strain T<sub>73</sub> (24) was transformed with plasmids YEpCR21 (control), pR37, and pR38, and positive colonies were tested for  $\alpha$ -L-rhamnosidase activity on YPD plates supplemented with 1  $\mu$ g of cycloheximide per ml and containing MUR as the substrate. No activity was detected in T<sub>73</sub> or in a T<sub>73</sub> strain (YR13) transformed with the vector YEpCR21. However, transformants generated with pR37 and pR38 gave

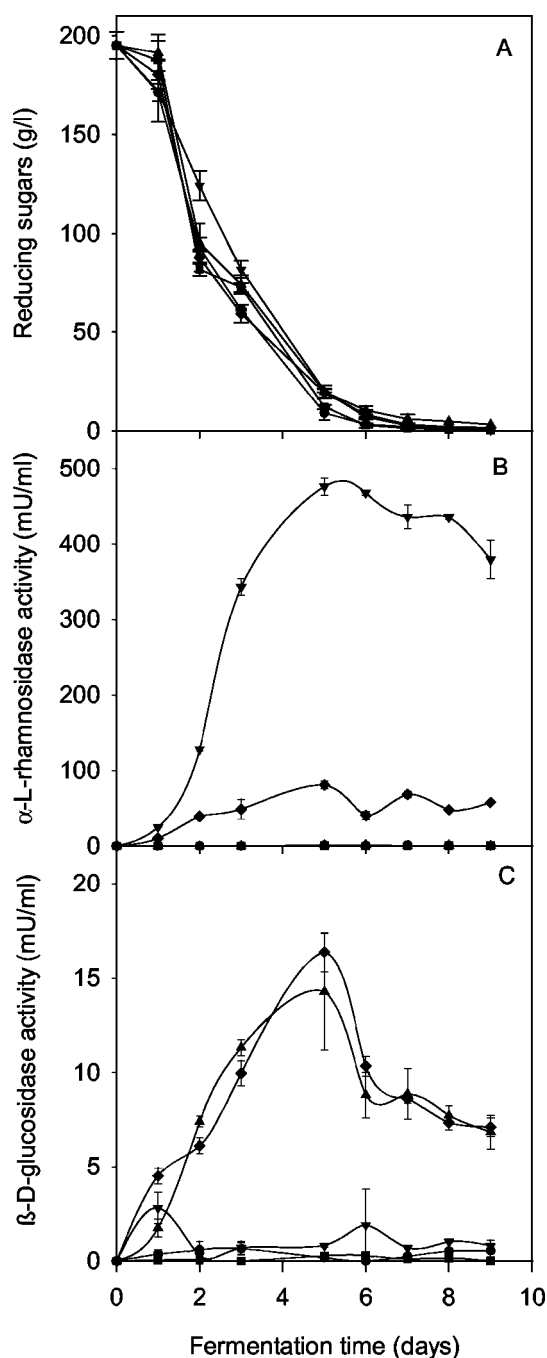


FIG. 1. Analysis of microvinifications carried out with the commercial wine yeast strain T<sub>73</sub> (●), the YR13 control strain transformed with the vector YEpCR21 (■), the YCB<sub>35</sub> transformant strain expressing the *C. molischiana*  $\beta$ -D-glucosidase (▲), the YR8 transformant strain expressing the *A. aculeatus*  $\alpha$ -L-rhamnosidase (▼), and a combination of the YCB<sub>35</sub> and the YR8 strains (◆). Shown are the kinetics of reducing sugar consumption (A) and  $\alpha$ -L-rhamnosidase (B) and  $\beta$ -D-glucosidase (C) activities, respectively, during the course of fermentation.

similar levels of  $\alpha$ -L-rhamnosidase activity, demonstrating that all the signals necessary for expression (i.e., promoter and terminator) are present on the 4.2-kb *Hind*III fragment and are independent of orientation within the vector. From the

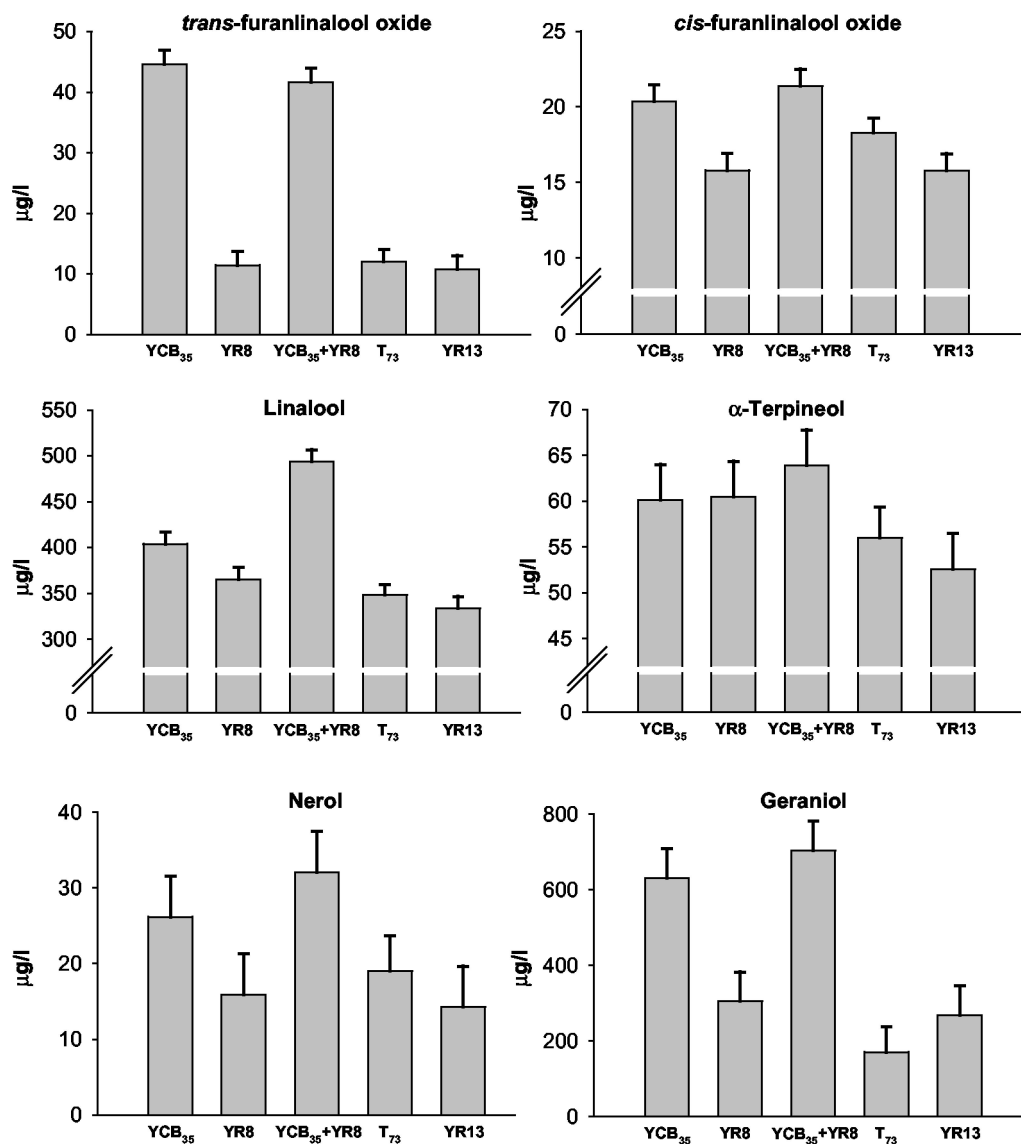


FIG. 2. Concentrations expressed as mean values with Tukey's honestly significant difference intervals at 95% confidence level of various terpenols in the final wines produced in the microvinifications (aroma threshold values in micrograms per liter: *trans*-furanlinalool oxide and *cis*-furanlinalool oxide, >6,000; linalool, 100;  $\alpha$ -terpineol and nerol, 400 to 500; geraniol, 130) (19).

latter, transformant YR8 was selected and used for microvinification experiments. This transformant secretes 600 mU of  $\alpha$ -L-rhamnosidase per ml after 72 h of growth in YPD medium, a sixfold increase in production with respect to strain YR6. Following previously described protocols (9), we detected no differences in RhaA cellular location between the strains YR6 and YR8 (30% intracellular, 10% cell wall bound, and 60% extracellular).

Due to the fact that the enzymatic hydrolysis of grape rhamnoglucosides occurs in two steps and that the actions of an  $\alpha$ -L-rhamnosidase and a  $\beta$ -D-glucosidase are needed, microvinification experiments were carried out both singly and with a combination of transformant YR8 expressing the *A. aculeatus rhaA* gene and a T<sub>73</sub> strain (YCB<sub>35</sub>) expressing the *C. molischiana* gene *bglh*, which codes for a  $\beta$ -D-glucosidase (28). Controls were done with the untransformed T<sub>73</sub> strain and the

transformant YR13. Microvinifications were performed in triplicate and carried out at 18°C in 375-ml glass bottles containing 300 ml of Muscat grape must inoculated with 10<sup>5</sup> cells/ml. The progress of the fermentation was similar in all the microvinifications (Fig. 1A). After 9 days of fermentation, the final wine products contained approximately the same ethanol concentration: 11.8% (vol/vol) in wines produced by T<sub>73</sub> and 12.0% (vol/vol) in those produced by the YR13 transformant and the combination of the transformants YR8 and YCB<sub>35</sub>. Figure 1B and C show that  $\alpha$ -L-rhamnosidase activity is only found in the presence of transformant YR8 and that although  $\beta$ -D-glucosidase activity was mainly detected in the presence of YCB<sub>35</sub> strain, as previously reported (28), very slight levels of activity could be detected in all fermentations. The highest levels of both enzyme activities occurred during the first 5 days of fermentation. High levels of  $\alpha$ -L-rhamnosidase activity (470 mU/

ml) were reached in the fermentation carried out by strain YR8, whereas in the presence of the YCB<sub>35</sub> transformant, around 90 mU of  $\alpha$ -L-rhamnosidase activity per ml was found.  $\beta$ -D-Glucosidase activity reached levels of approximately 15 mU/ml in the YCB<sub>35</sub> fermentation and also in the presence of the YR8 transformant. Further studies will be done to find out if the differences in  $\alpha$ -L-rhamnosidase activity between single and double fermentations are a consequence of differences in growth or enzyme production rates. Regarding possible effects on aroma compounds, volatiles were extracted by using C<sub>18</sub> cartridges and analyzed by gas chromatography-mass spectrometry (8) (Fig. 2). Fermentations carried out by YCB<sub>35</sub> alone showed marked increases in *trans*-furanlinalool oxide and geraniol contents. These data suggest the possible existence of monoglucosides of these terpenes or the capability of the *C. molischiana*  $\beta$ -D-glucosidase to hydrolyze diglucosides with different specificities, depending on the aglycon. Similar results have been reported for purified *C. molischiana*  $\beta$ -D-glucosidase added directly to Muscat wine (6). Although a slight increase in linalool content was also detected, greater levels of linalool were observed for wines cofermented by YCB<sub>35</sub> and the novel genetically modified yeast strain YR8 producing the *A. aculeatus*  $\alpha$ -L-rhamnosidase A. Moreover, significant increases in  $\alpha$ -terpineol and nerol were detected only in wines produced by both yeast strains in combination compared to individual T<sub>73</sub> and YR13 fermentations. These data indicate the biotechnological feasibility of the combination of wine yeast strains producing  $\alpha$ -L-rhamnosidase and  $\beta$ -D-glucosidase to increase free monoterpenol content. To our knowledge, this is the first report on the use of a genetically modified wine yeast strain producing an  $\alpha$ -L-rhamnosidase of enological relevance. Problems related to the social acceptance of genetically modified foods, mainly in the European Union, make the use of such products an alternative for the future but not for the present.

P.M. and M.O. contributed equally to this report.

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