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PROOF

Screening for yeasts able to hydrolyse arbutin in the presence of glucose or ethanol

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Abstract - The study was carried out using 212 glucose-fermenting yeasts isolated from Calabrian and Sicilian samples of must and wine. They were screened for β-glucosidase activity in Petri plates containing arbutin agar medium. Eleven strains (three apiculate yeasts and eight elliptic yeasts) were able to perform arbutin hydrolysis, but only strain C5, identified as *Pichia anomala*, maintained the ability to hydrolyse arbutin even in the presence of remarkable amounts of glucose or ethanol. This strain did not show the capacity to excrete the β-glucosidase into the medium; effectively, the enzymatic activity in the culture supernatant was near to zero. However, its intracellular activity was high.

Key words*:* wine yeast, *Pichia anomala*, arbutin hydrolysis, β-glucosidase activity.

INTRODUCTION

β-Glucosidase-producing yeasts are attractive for their possible utilisation in the food industry, such as the production of aromatic juices, wines, or vinegars, to increase product flavour properties.

Several authors have studied β-glucosidase activity in wine yeasts in order to use them or the enzyme produced by them for the hydrolysis of grape monoterpenyl glycosides. This ability has been found in genera, such as *Candida* (Günata *et al*., 1990; Freer and Skory, 1996; Saha and Bothast, 1996), *Debaryomyces* (Yanai and Sato, 1999), *Dekkera* (Blondin *et al*., 1983), *Hanseniaspora* (Vasserot *et al*., 1989), *Hansenula* (Grossmann *et al*., 1987), *Kloeckera* (Vasserot *et al*., 1990), and *Kluyveromyces* (Raynal and Guerineau, 1984). In *Saccharomyces cerevisiae* β-glucosidase activity is rather rare and poor (Delcroix *et al*., 1994; Restuccia *et al*., 2002). Thus, several attempts to perform heterologous expression of the β-glucosidase genes in *Saccharomyces cerevisiae* have been carried out (Leclerc *et al*., 1987; Adam *et al*., 1995; Skory *et al*., 1996; Sánchez-Torres *et al*., 1998).

In the presence of glucose, Table activity is often reduced and, more frequently, it is inhibited in the presence of ethanol. Therefore, its technological use seems to be restricted to the first stages of the winemaking process (Mateo and Di Stefano, 1997).

The aim of the present study was to select yeasts able to maintain Table activity even in the presence of glucose or ethanol. This could be very useful, because glucose and ethanol are natural constituents of food products where Table activity is required, but in normal conditions they can inhibit the activity of many yeasts.

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MATERIALS AND METHODS

Yeasts strains. The study was carried out using 212 glucose-fermenting yeasts, isolated from Calabrian and Sicilian samples of must and wine: 174 elliptic yeasts - the great majority *Saccharomyces*, 29 apiculate yeasts - all *Hanseniaspora*, and nine *Schizosaccharomyces*. Consequently, a large number of wine yeasts were screened to determine Table activity using arbutin.

Arbutin test. The yeast strains were screened in Petri plates for Table activity according to Kersters and De Ley (1971). Arbutin agar medium, sterilised by autoclaving for 15 min at 121 °C, with the following composition (w/v) was utilised: 10% yeast extract, 2% agar, 0.5% arbutin (sterilised by filtration), 40 drops/100 mL of a 1% ferric ammonium citrate solution (sterilised by filtration). Each plate of arbutin agar was inoculated in duplicate with one strain and incubated at 25 °C for 10 d. A control strain of *Saccharomyces cerevisiae* ATCC4098 was employed as a negative control.

Arbutin test in the presence of glucose or ethanol. The strains able to hydrolyse arbutin were tested in duplicate for their activity in the presence of increasing amounts of glucose or ethanol. For several tests, glucose, sterilised by filtration, was added to the arbutin agar medium plates, giving a final concentration of 2.5%, 5%, 7.5%, 10%, 12.5%, and 15% (w/v). For other tests, ethanol was added to the arbutin agar medium plates, giving a final concentration of

3%, 6%, 9%, 12%, and 15% (v/v). Inoculated plates were evaluated for the intensity of colour change and given a score from 0 (no change) to 3 (dark brown).

Enzyme activity assay. The yeast strain with the highest Table activity in the presence of glucose or ethanol was checked in duplicate in liquid culture conditions to determine, according to Rosi *et al*., 1994, the enzymatic activity values. Cells were grown in liquid medium (6.7 g Yeast Nitrogen Base, 20 g dextrose, and 1 L distilled water at pH 5.0) on a shaker (150 rev/min) at 25 °C for 24 h. Cell growth was evaluated by measuring absorbance at 600 nm (OD_{600}), using un-inoculated medium as a control. After 24 h of growth, the culture was centrifuged (4000 rev/min, 10 min, 4 °C) and both the pellet and the supernatant were used for the enzyme assay. The pellet from 5 mL of centrifuged liquid was washed twice in distilled water and resuspended in 1 mL of citrate-phosphate buffer (100 mmol/L, pH 5). b-Glucosidase activity was tested measuring the quantity of p-nitrophenol (pNP) liberated from p-nitrophenyl-b-D-glucopyranoside (pNPG) hydrolysis. Enzyme activity was expressed as nmol of pNP/mg of protein (nmol/mg). Enzymatic activity was measured in duplicate and the reported data are the average values.

Evaluation of protein content. Protein content was carried using the Bio-Rad protein assay (Bradford, 1976). To measure protein content of whole cells, the pellet obtained after centrifugation (4000 g; 10 min; 4 °C) was resuspended in 1 mL of citrate-phosphate buffer (100 mmol/L; pH 5). To 0.8 mL of this suspension, 0.2 mL of Bradford reactive was added for spectrophotometric reading at 595 nm.

Molecular identification. Molecular identification was carried out by partial sequencing and analysis of the 26S of rDNA. DNA for sequencing was amplified using primers NL-1 and NL-4, as described by Kurtzmann and Robnett (1998). Sequences were analysed with the Staden package (Dear and Staden) and the GCG Wisconsin package (Genetic Computer Group, Madison, USA) and compared to sequences in GenBank. The identification was confirmed by PCR/RFLP of the ITS regions (Esteve Zarzoso *et al*., 1999; Pulvirenti *et al*., 2001).

RESULTS AND DISCUSSION

The results of the screening for Table activity showed that, among the 212 tested yeasts, only 11 strains (three apiculate yeasts and eight elliptic yeasts) were able to perform arbutin hydrolysis. Their enzymatic activity was determined in the presence of glucose or ethanol.

As reported in Table 1, strain C5 maintained strong Table activity, almost unchanged, up to 10% of glucose. Higher concentrations inhibited this enzymatic activity. Strain L1 maintained good Table activity in the presence of 2.5% of glucose. At 5% of glucose, the strain exhibited poor Table activity that disappeared when higher concentrations of glucose were tested. Strains C2, C6, C14, L3, S3, S6 and K2249 maintained Table activity in the presence of 2.5% of glucose. In the plates with higher concentrations of glucose, the brown colour showing that arbutin was hydrolysed, was not produced.

TABLE 1 – Behaviour of the eleven yeasts able to perform arbutin hydrolysis compared to the control strain ATCC4098 in the presence of increasing concentrations of glucose

	C2	C5	C6	C14	L1	L3	S3	S6	H1141	H2577	K2249	ATCC4098
Control arbutin 0.5% (w/v)	2	3	1	3	3	1	2	2	1	1	2	0
Arbutin + glucose 2.5% (w/v)	1	3	1	2	3	1	2	2	0	0	1	0
Arbutin + glucose 5% (w/v)	0	3	0	0	1	0	0	0	0	0	0	0
Arbutin + glucose 7.5% (w/v)	0	3	0	0	0	0	0	0	0	0	0	0
Arbutin + glucose 10% (w/v)	0	3	0	0	0	0	0	0	0	0	0	0
Arbutin + glucose 12.5% (w/v)	0	0	0	0	0	0	0	0	0	0	0	0
Arbutin + glucose 15% (w/v)	0	0	0	0	0	0	0	0	0	0	0	0
Control glucose 2.5% (w/v)	0	0	0	0	0	0	0	0	0	0	0	0

0, no colour change; 1, light brown; 2, brown; 3, dark brown.

TABLE 2 – Behaviour of the eleven yeasts able to perform arbutin hydrolysis compared to the control strain ATCC4098 in the presence of increasing concentrations of ethanol

	C2	C5	C6	C14	L1	L3	S3	S6	H1141	H2577	K2249	ATCC4098
Control arbutin 0.5% (w/v)	2	3	1	3	3	1	2	2	1	1	2	0
Arbutin + ethanol 3% (v/v)	0	3	0	3	3	0	0	0	0	0	0	0
Arbutin + ethanol 6% (v/v)	0	3	0	3	1	0	0	0	0	0	0	0
Arbutin + ethanol 9% (v/v)	0	3	0	1	1	0	0	0	0	0	0	0
Arbutin + ethanol 12% (v/v)	0	3	0	0	0	0	0	0	0	0	0	0
Arbutin + ethanol 15% (v/v)	0	3	0	0	0	0	0	0	0	0	0	0
Control ethanol 3% (v/v)	0	0	0	0	0	0	0	0	0	0	0	0

0, no colour change; 1, light brown; 2, brown; 3, dark brown.

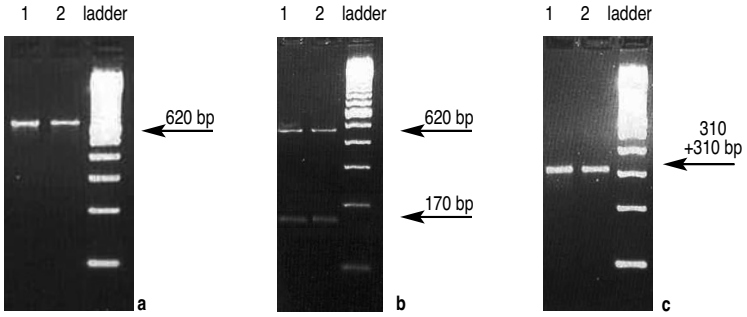

FIG. 1 – Restriction analysis of ITS regions with the endonuclease *Hae* III (a), *Rsa* I (b), and *Hinf* I (c). From left to right: 1, CBS 5759; 2, C5 strain; ladder, 100 bp.

As reported in Table 2, strain C5 maintained strong Table activity, unchanged at all the tested concentrations of ethanol. In particular, Table activity was remarkable in the plate containing 15% of ethanol, even if the produced biomass was decreased. Strain C14 maintained good Table activity up to 6% of ethanol. At 9% of ethanol, it was still possible to observe the Table activity, but the produced biomass was clearly decreased. Strain L1 maintained the Table activity unchanged in the presence of 3% of ethanol. At 6% and 9% of ethanol this activity was reduced. With higher concentrations, the ability to hydrolyse arbutin was completely inhibited. None of the other strains showed the Table activity in the presence of ethanol.

Thus, only strain C5 maintained the ability to hydrolyse arbutin even in the presence of remarkable amounts of glucose or ethanol. The C5 yeast strain was preliminarily identified on the basis of the D1/D2 domain as *Pichia anomala*, according to Kurtzmann and Robnett (1998). Correct identification was confirmed by PCR/RFLP of the ITS regions, with three restriction enzymes, by comparing restriction profiles with the type strain of *Pichia anomala* CBS 5759. The strains examined had an amplicon of 650 bp and a restriction profile identical to that of the *P. anomala* type strain (Caggia *et al*., 2001). The restriction enzyme *Hae* III (Fig. 1a) showed only one fragment of 620 bp, the same as the *P. anomala* type strain. The restriction profile obtained with the enzyme *Rsa* I displayed two bands, one at 450 bp and the other at 170 bp, highlighting a single restriction site for the strains (Fig. 1b). The enzyme *Hinf* I produced a single cut site in the defined region as well. In this digestion by *Hinf* I the two fragments had the same size 310 bp and the restriction pattern of the examined strain showed a single intense band (Fig 1c), characteristic of the *P. anomala* type strain.

Strain C5 showed a reduced capacity to excrete Table into the medium; enzymatic activity in the culture supernatant was negligible, 0.5 nmol mL^{-1} h^{-1}. However, enzymatic activity of the whole cell was high: the pellet activity was 71.78 nmol mL^{-1} h^{-1}, or 10.35 nmol mg^{-1} of protein.

The presence of Table activity in 11 wine yeasts indicated their possible use to hydrolyse glycoside precursors of the terpenes in grape juice processing. In particular, strain C5 showed a Table activity that was barely inhibited by glucose and totally unaffected by ethanol until 15% (v/v).

A previous study on the properties of Table of a *Pichia anomala* strain showed that this endogenous enzyme is active at the ethanol concentrations typically found in wine (12-14%); moreover, glucose, a not-competitive inhibitor, despite lowering activity, actually protects the enzyme from factors that could damage it (Spagna *et al*., 2002).

Our preliminary study was performed in synthetic media. Further research may confirm the ability of this strain to hydrolyse monoterpenyl glycosides in food products. For winemaking this strain could be used together with *Saccharomyces cerevisiae* selected strains, during or after alcoholic fermentation.

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