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Short Communication

Isolation and selection of yeasts from wine grape ecosystem secreting cold-active pectinolytic activity

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ABSTRACT

The present study was undertaken with the purpose of selecting yeasts from wine grapes that are able to produce extracellular cold-active pectinases. After two consecutive selections yeast isolates were identified by pheno- and genotyping, and pectinolytic activity was preliminarily characterised at proximate winemaking conditions. Out of 1023 indigenous microorganisms isolated from grape skins of D.O. San Rafael (Mendoza, Argentina) viticulture region, 565 (55%) showed pectinolytic activity on plates and, among them, 96 (17%) were chosen in a primary selection. Ten isolates were finally selected for exhibiting the greatest activity at low temperature (12 °C) and identified as *Aureobasidium pullulans*. GM-R-22 strain demonstrated the highest pectinolytic activity (0.751 U/mL) at pH 3.5 and 12 °C. Yeast pectinases were constitutively produced. This study is the first report about strains of *A. pullulans* producing pectinases which are able to show good activity at low temperature. These pectinolytic strains could be of interest in wine production.

1. Introduction

Pectinolytic enzymes are widely used in the beverage industry to clarify fruit juices and wine. They can degrade pectic substances, which are mainly composed of partially methyl-esterified galacturonic acid subunits joined by α(1–4)-glycosidic bonds. These enzymes play an important role in winemaking (Kashyap et al., 2001). They can help to improve liquefaction, clarification and filterability, releasing more colour and flavor compounds entrapped in the grape skins, thereby making a positive contribution to the wine bouquet, and facilitating the liberation of phenolic compounds (Van Rensburg and Pretorius, 2000). In a previous work carried out in our laboratory, an autochthonous-microbial pectinolytic preparation was applied to red-skin maceration with very good results in pigment and polyphenols extraction (Cabeza et al., 2009).

Most strains of *Saccharomyces cerevisiae*, usually used in wine-making, do not show the capacity to degrade pectic substrates. A few wild strains have been reported to have the ability to degrade pectin in wine fermentation (Blanco et al., 1994; Fernández-González et al., 2005). Recently, there has been interest in the application of non-*Saccharomyces* wine yeasts, but the ability of these yeasts to secrete pectinases has been scarcely studied (Charoenchai et al., 1997; Fernández et al., 2000; Strauss et al., 2001).

Fermentation conducted at low temperatures (15–20 °C) can increase the production and retention of volatile compounds, improving the aromatic profile of wines (Molina et al., 2007). However, it is well known that colour extraction in red wines is reduced within this temperature range. Consequently, pectinolytic enzymes able to be active at low temperature, both for extraction and clarification, are needed. Few attempts have been made to isolate and characterise cold-active pectinases from yeasts (Birgisson et al., 2003; Nakagawa et al., 2004).

"San Rafael" Designation of Origin (D.O.), a Western Argentinean wine-making area, stands at the foot of Los Andes principal mountain range. It presents a special microclimate that contributes to distinguish its vineyards and wines. Grapes from this region may have a unique biota of wine yeasts with potential to produce pectinases that act at low temperatures.

This work was aimed at the selection and identification of yeasts isolated from wine grapes, which are capable of secreting cold-active pectinases. It reports the preliminary characterisation of pectinolytic activity at some vinification conditions such as low pH (3.5), low temperature (12 °C) as well as at 28 °C.

2. Materials and methods

2.1. Study area, sampling and yeast isolation

The samples were collected from different districts of "San Rafael" D.O., in Southern Mendoza (34.5°–36° South latitude and 70°–66.5° Western longitude). All samples were taken in 2007 vintage, at

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optimum ripeness and in good sanitary conditions. Clusters of 11 grape varieties were randomly harvested in vineyards, transported to the laboratory aseptically, and kept cold until their study.

Ten berries of each grape variety were placed in a flask containing 10 mL of sterile peptone water (0.1%). The system was shaken at 165 rpm during 1 h at room temperature. An aliquot was spread in surface on three different media: WL nutrient agar (Merck); YGC (Merck); and grape must-agar medium containing per litre: concentrated grape must (868 g reducing sugar/L), 2.3 mL; yeast extract, 1.0 g; agar, 2.0 g; pH 4.5. The plates were incubated at 28 °C for 24–48 h. Approximately 30 colonies were isolated from each plate according to their macroscopic features and frequencies.

2.2. Screening on plates for pectinolytic yeast selection

2.2.1. Primary selection

The isolates were point-inoculated onto a mineral medium containing citric pectin as a sole carbon source: (g/L) citric pectin, 2.0; yeast extract, 1.0; agar, 15.0; KH_2PO_4, 0.2; $CaCl_2$, 0.05; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.8; $MnSO_4$, 0.05; pH 4.5, reported by Moyo et al. (2003) with slight modifications. The production of pectinolytic enzymes was detected on plates incubated at 28 °C for 48 h. Enzyme activity was indicated by the formation of a clear halo around the colonies against a purple-brown background on pectin plate after lugol's solution addition (Fernandes-Salomão et al., 1996).

2.2.2. Secondary selection

A second selection was carried out according to the capability of pre-selected yeasts to grow and produce pectinases that are active at low temperature (12 °C). Two criteria were taken into account, the first one was to select the yeasts producing the widest halo diameter/colony diameter relation (Dh/Dc) and the second criterion was to choose those that produced the largest colony size (Dc) and, at the same time, secreting important amounts of enzyme, although this originated a medium value of Dh/Dc relation.

Moreover, the pre-selected isolates were tested to detect pectinolytic activity at 28 °C with the same criteria, in order to choose the best pectinase-secreting yeasts at this temperature.

2.3. Enzyme assays under proximate oenological conditions

2.3.1. Production of extracellular enzymatic extracts

For enzyme production, the 21 selected yeast strains were inoculated in a basal medium (g/100 mL citric-citrate buffer 50 mM, pH 3.5: D-(+)-glucose, 2.0; soy peptone, 1.0; meat peptone, 1.0; yeast extract, 1.0) at 5% of pre-cultures grown in the same medium. Pectinolytic strains were incubated with agitation (130 rpm) at 12 °C during 6 days or 28 °C during 3 days (according to previous assays, data not shown). After removing cells by centrifugation (5000×*g*, 15 min at 4 °C), the supernatants were filtered through a 0.22 μm pore size membrane. These cell-free supernatants were used to assay the enzymatic activity.

2.3.2. Evaluation of pectinolytic activity by releasing of reducing sugars

Pectinolytic activity was assayed by quantification of reducing sugars released from a pectin dispersion (0.25% pectin in 50 mM citric-citrate buffer, pH 3.5) using 3,5-dinitrosalicylic acid reagent (DNS) (Miller, 1959). Galacturonic acid was used as standard (Sigma Chem. Co.). The reaction mixtures containing 0.45 mL of substrate and 0.05 mL of enzymatic extract were incubated at 12 °C or 28 °C for 30 min. The reaction was stopped by adding 0.5 mL DNS reagent. After cooling, 1.5 mL of distilled water was added and the absorbance measured at 530 nm. One unit of pectinase activity (U) was defined as the amount of enzyme required to release 1 μmol of reducing sugar per min under assay conditions. All tests were carried out in triplicate.

Total protein determination was performed using bovine serum albumin as standard (Bradford, 1976).

2.4. Pheno- and genotypic identification of cold-active-pectinase-secreting yeasts

Based on the purpose of this work only the strains selected at 12 °C as the best pectinolytic yeasts were identified at species level following the taxonomic criteria described by Kurtzman and Fell (2000) based on their morphological and physiological characteristics as well as by PCR-RFLP analysis of the ITS1-5.8S–ITS2 region from the nuclear rDNA gene complex.

PCR reaction was carried out according to protocols described by Esteve-Zarzoso et al. (1999) with some modifications using universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) already described by White et al. (1990). PCR products were digested with CfoI, HinfI and HaeIII restriction enzymes following the supplier's instructions. Amplified products and their restriction fragments were electrophoresed on 1.4 and 2.2% agarose gels, respectively, in 1×TAE (Tris-acetic acid-EDTA) buffer. Gels were stained with ethidium bromide, visualized, and photographed under UV light. Fragment sizes were estimated by comparison against a DNA standard (100-bp ladder). All PCR-RFLP reagents were from Promega Co. (USA).

2.5. Statistical analysis

ANOVA and Fisher LSD test with $\alpha = 0.05$ were performed by mean comparison, using STATGRAPHICS Plus 5.1 (Manugistics, Rockville, MD). The data normality and variance homogeneity in the residuals were verified by modified Shapiro–Wilks and Levene's test, respectively.

3. Results and discussion

3.1. Screening of pectinolytic activity for yeast selection

One thousand and twenty three microorganisms isolated from wine grape skins were firstly evaluated for their potential to secrete extracellular pectinases on plates. Among them, 565 (55%) showed pectinolytic activity. Finally, 96 pectinolytic yeast isolates producing the greatest clarification halos at 28 °C were pre-selected. These isolates were subjected to a secondary screening according to their capacity to grow and produce pectinases active at low temperature (12 °C) and also at 28 °C.

In the secondary screening the Dh/Dc relations at 12 °C varied between 1.5 and 6.0 units while at 28 °C this parameter differed between 1.8 and 11.2 units. In Table 1 are summarised the selected strains at 12 and 28 °C. Among the best isolates at both temperatures half of them were chosen for their largest Dh/Dc relations, and the rest of the strains were picked for their growth capacity apart from the large halo size.

Out of 96 isolates chosen in the primary selection only GM-Se-320 strain was picked either at 12 or 28 °C, the remaining isolates selected at 12 °C were different from those chosen at 28 °C, indicating that a direct relation was not maintained at both temperatures respect to the best pectinolytic yeasts.

3.2. Evaluation of pectinolytic activities by selected yeasts under proximate oenological conditions

In view of the potential application of these pectinolytic strains to winemaking process, the pectinase production and enzyme activity were assayed at pH and temperatures related to vinification. All selected yeasts showed good pectinolytic activity at pH 3.5 and 12 °C or 28 °C. As shown Fig. 1, the pectinase activity varied slightly among the isolates, although statistically significant differences were observed. According to activity level, five groups were constituted among the strains selected at 12 °C (Fig. 1A), while the yeasts chosen at 28 °C showed four clusters (Fig. 1B). Particularly, among yeasts tested at 12 °C, strains R-22 and Ch-24 exhibited the highest enzymatic activities under the test conditions