Fructans hydrolysis in extracts obtained from *Saccharomyces cerevisiae* isolates

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Abstract

Fructans are the main carbohydrates of reserve in the Agave genus, comprising between the 65 to 97 % of total carbohydrates. Cooked musts obtained from some Agave plants are used to produce fermented spirits such as tequila or mezcal. Main microorganisms involved in these fermentations belong to the Saccharomyces complex, and some strains of this species show fructanase activity, which is characterized by the hydrolysis of β-2,1 and β-2,6 linkages of the fructans to release fructose. In this study we analyzed the fructanase potential of four Saccharomyces cerevisiae strains isolated from Agave spp. must. Specifically, the enzymatic extracts of these yeasts were obtained under different induction conditions by using inulin, sucrose or levan and quantifying their fructanase activity over sucrose, inulin and levan. The enzymatic extracts did not hydrolyze sucrose, but had an activity between 2 to 8 % over inulin and levan. Best hydrolysis percentage on levan substrate were obtained for strains S. cerevisiae 3y8 (6 %) and Fermichamp (8 %) when these were induced with levan. Finally best incubation time for levan and inulin hydrolysis was 40 min.

Keywords: *Fructans, Agave, enzymatic hydrolysis, Saccharomyces cerevisiae.*

1 Introduction

Fructans are the main carbohydrates of reserve in the *Agave* genus comprising between the 69 % to 97 % of total carbohydrates, in mature plants of 2 to 6.5 years-old, respectively [1],[2]. The heat-induced fructans hydrolysis (cooking for hours) allows the production of important spirits such as tequila and mezcal [3], but enzymatic hydrolysis is a much cleaner and selective way to utilize such important carbohydrates. At biochemical level, this process includes the enzymatic breakdown of β-2,1 or β-2,6 linkage of the carbohydrate backbone to release fructose; these reactions are mediated by a plethora of enzymes named together: fructanases [4],[5]. To industrial level, this process mainly included the use of high temperatures, acid-thermal treatment and enzymatic hydrolysis [6]-[8]. However, due to structural complexity of Agave fructans [9],[10], sometimes it is necessary use the combination of several processes to increase yields of fructose [8],[9]. Nevertheless, the use of thermal acid hydrolysis is associated with the production of unwanted by-products (hydroxymethyl furfural and fructose dianhydride) that can affect further biological steps or the purity of final products [11],[12]. Also, the exclusive application of acid hydrolysis, are being strongly questioned because of the negative impact they pose to the environment. The specific use of the enzymatic hydrolysis is a friendly-environmental alternative for producing fructose avoiding the risk of chemical degradation.

In the natural fermentation of Agave must participate a high amount of microorganisms, mainly *Saccharomyces cerevisiae* strains [13]. These yeasts show different phenotypes according to environmental substratum where these inhabited [14]. Therefore, the main aim of this study was to characterize the fructanase hydrolysis of extract obtained from *S. cerevisiae* strains isolated from Agave must. Enzymatic hydrolysis in these yeasts was induced by using different common fructans present in Agave and other genera of plants. Finally, the enzymatic

breakdown of β-2,1 or β-2,6 linkages on sucrose, inulin and levan was used as an indicator of this fructanase potential.

2 Materials and methods

2.1 Microorganisms

Three *S. cerevisiae* yeast strains belong to the Mezcal LBI-CBG yeast collection were selected and the commercial wine strain *S. cerevisiae* Fermichamp (DSM Food Specialties B.V., The Netherlands) used as a control for its fructophilic character. LBI-CBG yeasts were the 3y8, 3y5 and 3y4 isolates. Fermentative profiles of these yeasts were reported [15].

2.2 Fructanase hydrolysis induction

Yeasts were grown overnight in solid YPD medium (yeast extract, 10 g L^{-1}; peptone, 20 g L^{-1}; glucose, 20 g L^{-1} and agar, 2 %) to 29 °C. Subsequently the yeasts were grown for 24 h in YPD broth (yeast extract, 10 g L^{-1}, peptone 20 g L^{-1} and glucose 20 g L^{-1}) at 29 °C under agitation at 250 rpm. Then, 2×10^{6} yeast cells L^{-1} were inoculated into the induction medium containing yeast extract 10 g L^{-1}, peptone 20 g L^{-1} and supplemented with sucrose 20 g L^{-1}, or inulin 20 g L^{-1} or levan 20 g L^{-1}. Medium YP without sugar or carbohydrate supplementation was used as control of the basal hydrolytic potential in all *S. cerevisiae* yeasts of this study.

2.3 Crude extract characterization

After grow for 48 h under induction conditions, yeast cultures were harvested by centrifugation at 6797 X g for 15 min, and immediately were used for hydrolysis experiments. Protein concentration in each crude enzymatic extract was determined according to Bradford method [16]. Protein curves were built by using different concentrations of BSA [0.0025, 0.005, 0.0075 and 0.01 g L^{-1}] and its resultant regressions were used to interpolate protein contents in the enzymatic extracts (Figure 1).

2.4 Sucrose enzymatic hydrolysis

Fructanase potential was determined by the enzymatic breakdown of β-2,1 and β-2,6 linkage of the substrates such as sucrose, inulin and levan. An aliquot of 500 µl of the enzymatic extract was mixed with 500 µL of substrate solution (2 % w/v in 100 mM of acetate buffer, pH 4.5) and incubated for 0, 40, 80 and 90 min at 50 °C. Subsequently quantifying reducing sugars, it was performed by adding 666 µL of sample (from different incubation times) and 334 µL of DNS

reagent, the reaction was maintained at 80 °C for 7 minutes. After, the reaction is stopped by incubating at 100 °C for 10 min. The blank was obtained in the same conditions described above, with difference that the extracts not were incubated. Absorbance was measured in spectrophotometer Optizen at 580 nm. Reducing sugars were determined by interpolate absorbance values, at 580 nm, of according to linear regressions obtained from DNS curves built using different concentrations of glucose (0.1 to 0.6 g L^{-1}). Fructanase hydrolytic potential was defined as the percentage of reducing sugar release from 1 g de sucrose in each reaction time (0, 40, 60 and 90 min). The time zero represent the enzymatic reactions without incubation time, corresponding to residual sugar present in the extract, hence the statistical analysis was carried out only at higher time values.
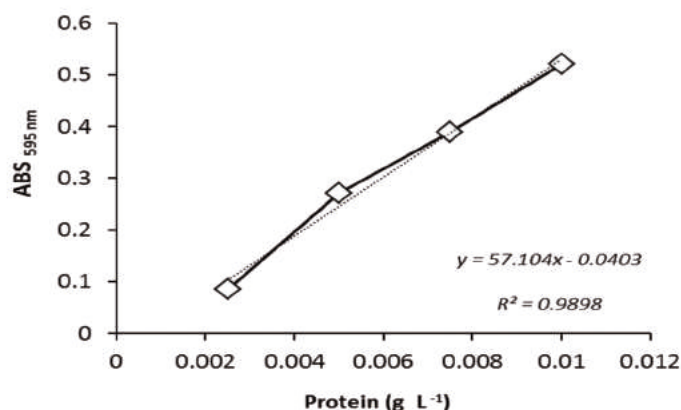

Figure 1 Regression curve used to interpolate the protein values in the crude enzymatic extracts.

2.5 Statistical analysis

The data were analyzed and plotted with SAS/STAT® (Statistical Analysis Software) and Origin 9.1 Software (Data Analysis and Graphing Software). An analysis of variance (ANOVA) was done and the mean differences were determined by using Tukey test at $P \leq 0.05$.

3 Results and discussion

Fructanase hydrolysis potential was determinate by sucrose hydrolysis, as it constitutes the basic component in all fructans, since from this are polymerized the fructose chains [3],[17]. Two experiments were initially conducted, first one, the sucrose hydrolysis potential of the different enzymatic extracts without induction, or basal enzymatic activity,

which was conducted by using only YP medium. Secondly the sucrose hydrolysis performance induced by the same sucrose (YPS medium). Figure 2 shows the relation between both experiments. Results obtained showed not significant differences between the hydrolysis percentages obtained for different times incubation in comparison to time zero, indicating that these *Saccharomyces* strains did not hydrolyze sucrose, as it has been reported in studies carried out in the same study group [18].
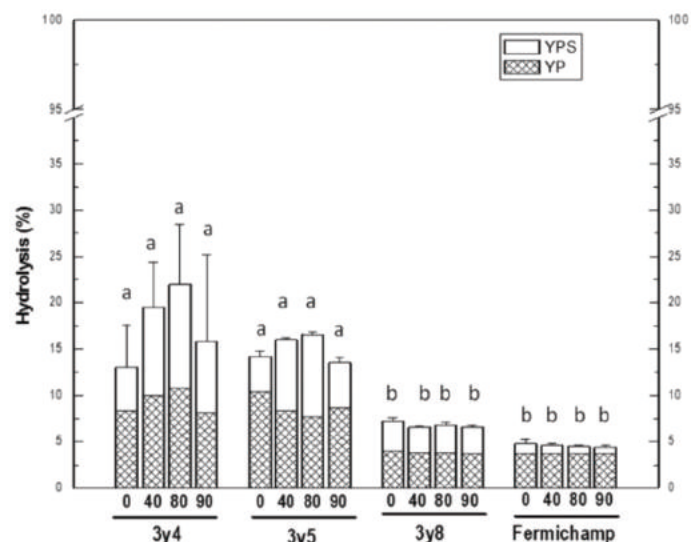
Hydrolytic behavior induced by inulin does not discriminate by its origin (mezcal or wine) to these strains, although here it is important to mention that both strains have demonstrated a high industrial application by its fermentative performance. In fact, *S. cerevisiae* Fermichamp (DSM Food Specialties B.V., The Netherlands) strain is widely used as a control for its fructophilic character, which is used to reactivate stuck fermentations [19], and *S. cerevisiae* 3y8 which also exhibits a fructophilic performance [15].
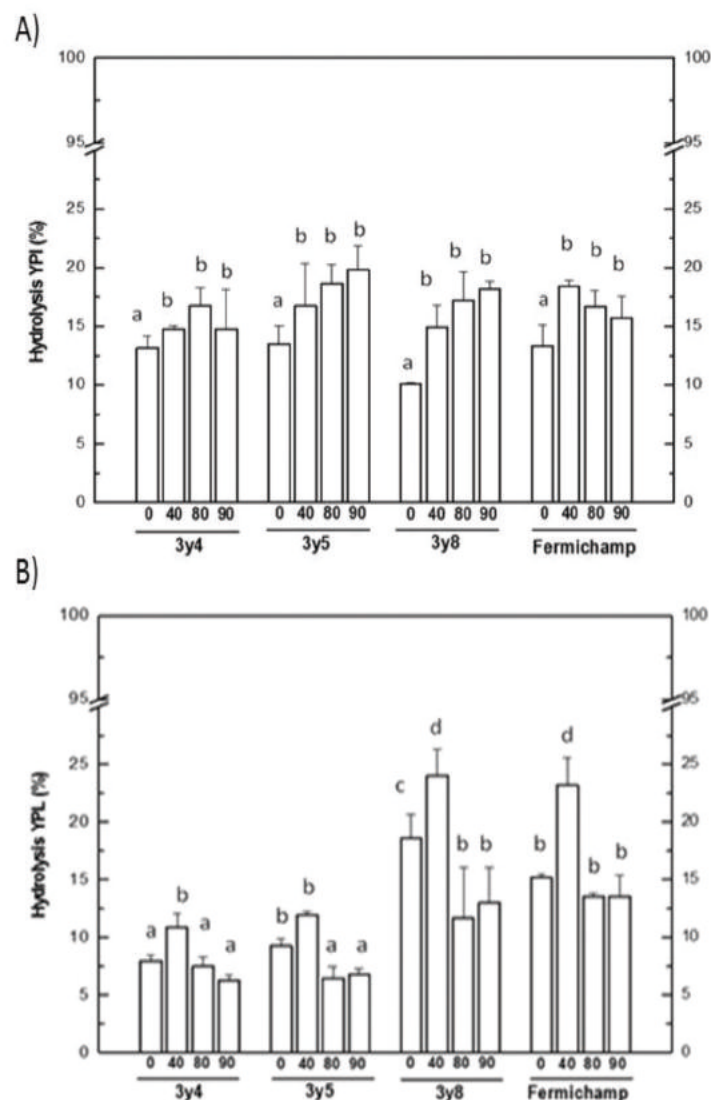

Figure 2 Sucrose hydrolysis in *S. cerevisiae* strains isolated from mezcal (3y4, 3y5 and 3y8) and control (Ferm) in different times (min) of incubation. White bars correspond to hydrolysis percentage obtained in enzymatic extracts induced with sucrose (YPS). Dark bars corresponded to basal sucrose hydrolysis without induction (YP). Error bars correspond to standard error (SE) of three measurements.

Fructanase potential was also analyzed by using as inductors common agave fructans as inulin and levan. The inulin represents up the 43 % of fructans in *Agave tequilana* [17] and the content of levan is still unknown. Figure 3 shows the results of both experiments without regarding to basal values obtained in medium YP, because the values of hydrolysis of inulin and levan were zero.

Fructan induction with inulin allows observe a preserved hydrolytic performance of each *S. cerevisiae* strains with regarding to each inductor. In general, inulin was a better inductor for this inulin hydrolysis activity in all the *S. cerevisiae* strains (Fig. 3A). Inulin hydrolytic percentages ranged among 2 and 8 %, respect to time zero. The times incubation analyzed for inulin hydrolysis showed not differences significant.

Figure 3 Inulin and levan hydrolysis in *S. cerevisiae* strains isolated from mezcal (3y4, 3y5 and 3y8) and control (Ferm) at different times (min) of incubation. **A)** Inulin induction and **B)** Levan induction. Columns correspond to hydrolysis percentage obtained in enzymatic extracts induced with inulin (YPI) and levan. Error bars correspond to standard error (SE) of three measurements.

These differences in the hydrolytic behavior of the *S. cerevisiae* strains of study also were observed when used levan as inductor (Fig. 3B). However, with this inductor shows a more remarkable hydrolytic activation in the 3y8 and Fermichamp strains in comparison to 3y4 and 3y5. The values percentage of levan hydrolysis in Fermichamp and 3y8 were 4 to 8%, respect to time zero. For this part the 3y4 and 3y8 strain only present 2 % of levan hydrolysis. The best incubation time for levan hydrolysis was 40 min.

Yeast non-*Saccharomyces*, isolated from musts "Mezcal Oaxaca", have been show exhibit values of fructanase activities of between 0.02 and 0.27 U mL^{-1}, using as substrate sucrose, inulin, levan and fructans of *Agave tequilana* (ATF) [20]. In addition, *S. cerevisiae* isolated from tequila must show high fructanase activity (31.1 U mL^{-1}) using as substrate ATF [21] and 3y5 strain have been show fructanase activity on ATF (5 U mL^{-1}) and on Inulin (1 U mL^{-1}) substrate [18]. In ATF are present the substrates such as inulin, levan, and sucrose, because it is not surprising that the yeasts analized in this research display hydrolytic potential on inulin and levan substrates. More experiments remain to be done to understand the high regulation of the fructanase activity and the substratum adaptation of these *S. cerevisiae* strains. The differences in fructanase potential displayed by these strains could be a result of its previous adaptation to substrates rich in inulin and levan.

4 Conclusions

Fructanase hydrolysis indirectly calculated by determine the breakdown of the sucrose β-2,1 linkage was calculated in *S. cerevisiae* strains from Mezcal LBI-CBG collection and in the commercial wine strain Fermichamp. These *Saccharomyces* extracts were able to hydrolyze between 5 to 25 % of sucrose. Hydrolysis performace vary of according to the incubation time or to the induction condition. In general, the best incubation time was of 40 min and the best inductor was inulin. However, the best sucrose hydrolytic results (~25 %) were obtained for 3y8 and Fermichamp strains induced with levan. The differences in fructanase potential displayed by these strains could be a result of its previous adaptation to substrates rich in inulin.

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