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Fructanase and fructosyltransferase activity of non-*Saccharomyces* yeasts isolated from fermenting musts of Mezcal

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ABSTRACT

Fructanase and fructosyltransferase are interesting for the tequila process and prebiotics production (functional food industry). In this study, one hundred thirty non-*Saccharomyces* yeasts isolated from "Mezcal de Oaxaca" were screened for fructanase and fructosyltransferase activity. On solid medium, fifty isolates grew on *Agave tequilana* fructans (ATF), inulin or levan. In liquid media, inulin and ATF induced fructanase activities of between 0.02 and 0.27 U/ml depending of yeast isolate. High fructanase activity on sucrose was observed for *Kluyveromyces marxianus* and *Torulaspora delbrueckii*, while the highest fructanase activity on inulin and ATF was observed for *Issatchenkia orientalis, Cryptococcus albidus*, and *Candida apicola*. *Zygosaccharomyces bisporus* and *Candida boidinii* had a high hydrolytic activity on levan. Sixteen yeasts belonging to *K. marxianus*, *T. delbrueckii* and *C. apicola* species were positive for fructosyltransferase activity. Mezcal microbiota proved to showed to be a source for new fructanase and fructosyltransferases with potential application in the tequila and food industry.

1. Introduction

Agave tequilana fructans (ATF) are polymers of fructose with one internal or external glucosyl residue. The complex branched structures combine fructose moieties linked by β (2 → 1) and β (2 → 6) bonds. The degree of polymerization ranges from 3 to 29 fructose units (Lopez et al., 2003). The proportion of free and polymerized fructose as well as the branching degree of ATF changes as a function of plant age (Arrizon et al., 2010). ATF are very important for Mexican agro-industry as they constitute the principal raw material for tequila production (Arrizon et al., 2010). In the traditional tequila process, ATF are hydrolyzed by cooking, generating a fructose-rich syrup and a cooking honey (containing non hydrolyzed fructans). In some industries, an extra acid-thermal treatment is applied to the honey to complete hydrolysis of the ATF (Waleckx et al., 2008). Alternative enzymatic processes have been evaluated to reduce energy consumption and to increase sugar recovery from ATF (Muñoz-Gutierrez et al., 2009; Ávila-Fernández et al., 2009; Waleckx et al., 2011); however, the identification of enzymes capable of efficiently degrading the complex structure of ATF remains an important problem (Muñoz-Gutierrez et al., 2009). The structural complexity of ATF (Lopez et al., 2003; Arrizon et al., 2010), may require the use of multiple enzymes with both exo- and endo-hydrolase capabilities. Non distilled *Agave* beverages are a well-known source of fructanase (β-fructofuranosidase)-hyperproducing yeast strains (Cruz-Guerrero et al., 2006; García-Aguirre et al., 2009) and recently a new fructanase from *Kluyveromyces marxianus* isolated from the fermentation of Mezcal was purified and biochemically characterized and showed a high substrate affinity for ATF and exo-hydrolase activity (Arrizon et al., 2011). The fermentation process of "Mezcal de Oaxaca" has naturally yeast strains well adapted to *Agave* fructan degradation (Lappe-Oliveras et al., 2008). Therefore, the purpose of this study was to evaluate yeast strains isolated from fermenting musts of "Mezcal de Oaxaca" for fructan hydrolytic activity using different fructose-containing substrates. Yeasts were previously isolated and characterized through PCR-restriction fragment length polymorphism (PCR-RFLP) of internal transcribed spacer (ITS) region of the 5.8 rRNA gene (ITS 5.8) and sequencing of the D1/D2 region of the 26S rRNA gene (Segura, 2010).

As ATF have prebiotic properties (Gómez et al., 2010), it is possible to envisage the production of short-chain fructooligosaccharides (FOS) by controlling ATF enzymatic hydrolysis. In addition, since FOS can be obtained directly from sucrose (Buchholz and Seibel, 2008) via reverse β-fructofuranosidase activity (Velázquez-Hernández et al., 2009), fructosyltransferase capacity will also be evaluated. This is the first report of Mezcal microbiota

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screening for both fructanase and fructosyltransferase activity utilizing yeast species. This work is useful for the discovery of new fructanase and fructosyltransferase producers for the development of industrial enzymatic processes.

2. Methods

2.1. Yeast strains and chemicals

The yeast strains, representing twenty distinct species, belong to the culture collection of the "Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco" (Guadalajara, México). They were previously isolated and characterized by PCR-RFLP of the ITS-5.8 regions and D1/D2 sequencing region of the 26S rRNA gene (Segura, 2010). Glucose, fructose, sucrose and levan from *Erwinia herbicola* were purchased from Sigma–Aldrich (St. Louis, USA), and inulin from Dalhia tubers was from Fluka (Steinheim, Germany). ATF was provided by BUSTAR Alimentos (Guadalajara, Mexico).

2.2. Solid medium

An aqueous solution containing urea (4 g L^{-1}), K_2HPO_4 (5 g L^{-1}), $MgSO_4$ (1 g L^{-1}) was adjusted to pH 4.5 with HCl (0.25 N), then agar was added (2%, w/v) and the medium was sterilized (15 min, 121 °C). After the medium was cooled to 50 °C, the oligosaccharide solution at 50 °C was added by filtration (0.45 μm sterile filters) to reach saturation (15, 5 and 1% w/v for ATF, inulin and levan, respectively).

2.3. Evaluation of growth on solid media

Each yeast strain colony was deposited by a sterile wood stick on the solid medium containing either ATF or inulin and growth measurements (colony diameter) were conducted at 0, 24, 48 and 72 h. Colonies growing on ATF or inulin-containing medium were tested on levan medium. Experiments were performed in duplicate (two colonies for each isolate).

2.4. Preparation of an induction medium for evaluation of fructanase activity

An induction medium composed of urea (8 g L^{-1}), K_2HPO_4 (10 g L^{-1}), $MgSO_4$ (2 g L^{-1}) dissolved in water was sterilized (121 °C) for 15 min. When the media had cooled to 50 °C, ATF or inulin was added by filtration (0.45 μm sterile filters) to obtain a final concentration of 20 g L^{-1}.

2.5. Preparation of enzymatic extracts

Yeasts selected from solid medium screening were grown overnight (250 rpm, 30 °C) in aqueous YPD medium (yeast extract 10 g L^{-1}, peptone 20 g L^{-1}, glucose 20 g L^{-1}). Then, 2×10^6 yeast cells were inoculated into the aqueous induction medium. Depending on the yeast, after 24–48 h of cultivation (30 °C, 250 rpm) cells were removed by centrifugation (15 min, 6797 g) and the supernatant (enzymatic extract) was screened for fructanase and fructosyltransferase activity. All experiments were performed in duplicate.

2.6. Evaluation of fructanase activity

Fructanase activity was determined with different substrates (sucrose, inulin, ATF and levan) as previously reported (Arrizon et al., 2011). Fifty microliter of the enzymatic extract was mixed with 50 μL of substrate solution (1% w/v in 100 mM of acetate buffer, pH 4.5) and incubated for 15 min at 50 °C. The reaction was stopped by addition of 100 μL of dinitrosalicylic acid (DNS) and boiling for 5 min at 100 °C, then the mixture was placed on ice. The blank was obtained by inactivation of 50 μL of enzymatic extract with 100 μL of dinitrosalicylic acid (DNS), adding 50 μL of substrate solution (1% w/v), incubation for 15 min followed by boiling for 5 min and placing on ice. The absorbance was measured with a microplate reader at 540 nm. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol of reducing sugars per minute. All experiments were performed in duplicate.

2.7. Characterization of fructanase production for ATF degradation with selected yeasts

Six yeast strains selected through fructanase activity screening were grown in YPD medium and inoculated into aqueous induction medium with ATF as inducer as described in Section 2.4. Samples were removed at 0, 12, 24, 48 and 72 h of culture. Biomass production was evaluated by dry weight determination. Protein was determined according to the Bradford method (Bradford, 1976). Fructanase activity with sucrose, inulin, ATF and levan was evaluated as described before (Section 2.6). Experiments were performed in duplicate.

2.8. Fructosyltransferase activity screening

Two sucrose solutions (300 and 600 g L^{-1} in 100 mM acetate buffer, pH 5.0) were used for fructosyltransferase activity determinations. Each enzymatic extract (100 μL) was added to sucrose solution (900 μL) and the reaction mixture was incubated at 50 °C. Samples were removed at 0, 24 and 48 h and the concentration of fructose, glucose and sucrose was determined by HPLC with a Biorad HPLC Carbohydrate Analysis column (Aminex HPX-87 C column, 300 × 7.8 mm; Biorad, Hercules, CA, USA) at 80 °C and elution with degassed ultrapure water at a flow rate of 0.6 mL min^{-1}. Glucose, fructose and sucrose were used as standards. Reaction samples were diluted in distilled water to approximately 20 g L^{-1} and filtered (0.45 μL membrane) before analysis. For each reaction, the moles of sucrose, glucose and fructose were calculated. The fructosyltransferase activity was calculated considering the difference between moles of glucose and fructose (equivalent to the moles of fructose transferred, moles of sucrose transfructosylated). One unit of enzyme activity was defined as the amount of enzyme transferring 1 μmol of sucrose per minute. All experiments were performed in duplicate.

3. Results and discussion

3.1. Screening of growth capacity of yeasts in solid medium with different fructan substrates

Differences in the growth capacity between yeasts as a function of the fructans were observed, even within the same species (Table 1). A growth with a >5 mm colony diameter was observed on ATF with 39 yeasts strains (Table 1). On inulin-containing medium, a growth of 2–5 mm colony diameter was observed with 24 yeast strains (Table 1). Yeasts positive on ATF and inulin were tested on solid medium containing levan (Table 1), a growth of <3 mm diameter was observed with 17 yeasts. ATF was the substrate with the highest number of growing yeasts. *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Rhodotorula mucilaginosa* and *Zygosaccharomyces bisporus* were the principal growing yeast species in solid medium, with differences in growth capacity depending on the substrate. The differences observed on the different substrates tested showed the metabolic diversity of yeasts,