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Bioconversion of *Agave tequilana* fructans by exo-inulinases from indigenous *Aspergillus niger* CH-A-2010 enhances ethanol production from raw *Agave tequilana* juice

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Abstract *Agave tequilana* fructans are the source of fermentable sugars for the production of tequila. Fructans are processed by acid hydrolysis or by cooking in ovens at high temperature. Enzymatic hydrolysis is considered an alternative for the bioconversion of fructans. We previously described the isolation of *Aspergillus niger* CH-A-2010, an indigenous strain that produces extracellular inulinases. Here we evaluated the potential application of *A. niger* CH-A-2010 inulinases for the bioconversion of *A. tequilana* fructans, and its impact on the production of ethanol. Inulinases were analyzed by Western blotting and thin layer chromatography. Optimal pH and temperature conditions for inulinase activity were determined. The efficiency of *A. niger* CH-A-2010 inulinases was compared with commercial enzymes and with acid hydrolysis. The hydrolysates obtained were subsequently fermented by *Saccharomyces cerevisiae* to determine the efficiency of ethanol production. Results indicate that *A. niger* CH-A-2010 predominantly produces an exo-inulinase activity. Optimal inulinase activity occurred at pH 5.0 and 50 °C. Hydrolysis of raw agave juice by CH-A-2010 inulinases yielded 33.5 g/l reducing sugars, compared with 27.3 g/l by Fructozyme® (Novozymes Corp, Bagsværd, Denmark) and 29.4 g/l by acid hydrolysis. After fermentation of hydrolysates, we observed that the conversion efficiency of sugars into ethanol was 97.5 % of the theoretical ethanol yield for enzymatically degraded agave juice, compared to 83.8 % for acid-hydrolyzed juice. These observations indicate that fructans from raw *Agave tequilana* juice can be efficiently hydrolyzed by using *A. niger* CH-A-2010 inulinases, and that this procedure impacts positively on the production of ethanol.

Keywords Inulinases · Enzymatic hydrolysis · *Agave tequilana* fructans · Ethanol production · Tequila

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Introduction

Fructans are the primary storage carbohydrate in plants of the family *Agavaceae*. They are fructose polymers with β-fructofuranosyl residues that according to the type of β-fructofuranosyl linkage are classified into five groups: 1. Inulin, with β-(2-1)-fructofuranosyl linkages; 2. Levan, with β-(2-6) linkages; 3. Graminans, which are branched fructans containing types 1 and 2 linkages; 4. Inulin neoseries, containing a glucose molecule between two β-(2-1)-linked units; 5. Levan neoseries, containing a central sucrose moiety flanked by β-(2-1)- and β-(2-6)-linked fructofuranosyl units on either end. *Agave tequilana* Weber

var. azul (*A. tequilana*), commonly known as blue agave [26] is rich in highly branched fructans, with both β-(2-1) and β-(2-6) linkages presenting internal and external glucose moieties [14]. Blue agave fructans are considered an inexpensive source of fructose for various industrial applications [24] including the production of tequila. Fructose may be obtained by acid hydrolysis of fructans at 80–100 °C. Nevertheless, fructose degrades under these conditions, resulting in the formation of difructose anhydrides [5], which are colored products with no sweetening capacity. In addition, physicochemical methods, such as acid hydrolysis, are being strongly questioned because of the negative impact they pose to the environment.

Tequila is a traditional Mexican liquor produced by distillation and rectification of a previously fermented juice obtained from fructan-rich stems of *A. tequilana*. Fructans contained in *A. tequilana* stems are the main source of fructose for the production of tequila. In modern distilleries, fructose is produced by acid hydrolysis. This proceeding generates residual wastewater with high concentrations of organic matter, suspended solids, acidic pH values (3.4–3.8), and temperatures ranging from 43 to 55 °C. Wastewater from the tequila industry flows into surrounding rivers, producing a highly negative environmental impact.

Enzymatic hydrolysis is an alternative strategy for producing fructose avoiding the risk of chemical degradation, as well as reducing hazards to the environment. Hydrolysis of fructans may be achieved by the action of inulinases. Inulinases are enzymes that catalyze the hydrolysis of inulin-like fructans to produce fructose and fructooligosaccharides. Endoinulinases are 2,1-β-D-fructan fructanohydrolases (EC 3.2.1.7) that hydrolyze internal β-2,1 fructofuranosidic linkages to yield inulotriose, inulotetraose, and inulopentaose. In contrast, exo-inulinases are β-D-fructan fructohydrolases (EC 3.2.1.80) that hydrolyze end-terminal, non-reducing 2,1-linked β-D-fructofuranose residues, with the concomitant release of β-D-fructose. Inulinases having β-fructosidase activity can be found in plants and microorganisms, including fungi, yeast, and bacteria. In fact, microbial inulinases can yield up to 95 % pure fructose by a single-step enzymatic reaction [25].

The potential use of microbial enzymes for the hydrolysis of *A. tequilana* fructans to produce fermentable sugars has not been fully explored. We have recently described the isolation of *Aspergillus niger* CH-A-2010 (*A. niger* CH-A-2010), an indigenous strain that produces extracellular inulinases by using agro-industrial waste from the tequila industry as the sole carbon source [12]. Thus, the aim of the present work was to investigate the potential application of *A. niger* CH-A-2010 inulinases for the saccharification of fructans from raw *A. tequilana* juice. *A. niger* CH-A-2010 inulinases were characterized. The efficiency of *A. niger* CH-A-2010 inulinases for the bioconversion of fructans was compared to that of commercially available enzymes, and to that produced by acid hydrolysis. The hydrolysates obtained were subsequently fermented by *Saccharomyces cerevisiae* to determine the efficiency of ethanol production.

Materials and methods

Microorganisms

Aspergillus niger CH-A-2010 was isolated from the soil of *A. tequilana* fields at Jalisco, Mexico, and purified as described previously [12]. The strain of *S. cerevisiae* used for fermentations was isolated from samples of agave juice obtained in a traditional tequila distillery, and it was identified by standard methods using yeast taxonomy. The cells were immobilized on the surface of glass beads as described elsewhere [10], and stored at −20 °C until used.

Production of microbial enzymes

All reagents used to prepare culture medium were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). *A. niger* CH-A-2010 was grown in 500-ml Erlenmeyer flasks containing 200 ml of liquid medium containing 4 g/l $(NH_4)_2SO_4$, 500 mg/l KH_2PO_4 and 500 mg/l K_2HPO_4, adjusted to pH 4.5. *A. tequilana* leaves were used as a substrate to produce extracellular inulinases. Fresh *A. tequilana* leaves were thoroughly washed with water, cut into 1-cm^3 pieces, and added to the liquid medium at a concentration of 1 % w/v. The medium was autoclaved at 20 lbs for 20 min. The flasks containing medium were inoculated with one mycelial disc (8 cm in diameter) obtained from a 5-day-old culture on a Petri dish. Flasks were incubated at 37 °C on a rotary shaker at 200 rpm for 120 h. Medium samples (15 ml) were collected every 24 h to monitor production of inulinases. Samples were centrifuged for 20 min at 3,000 × *g* at room temperature to remove biomass. Supernatants were filtered through a Whatman no. 41 filter, and kept frozen at −20 °C until used. After 120-h incubation, enzyme-containing medium was centrifuged as described and exhaustively dialyzed for 6 h, at 4 °C, against distilled water. The enzyme-containing retentate was then lyophilized and stored at −20 °C. To investigate the effect of different carbon sources on the production of inulinases, agave leaves were substituted for 1 % w/v solutions of glucose, purified chicory root inulin (Sigma-Aldrich Inc. St. Louis, MO, USA), or nanofiltered agave fructans. To obtain fructans, agave juice was filtered through a 10-μm ceramic filter, and nanofiltered using a

conventional Spray Dryer-SD (GEA Process Engineering, Gladsaxevej, Denmark) with a 10,000-Da membrane.

Production of enzymes was also carried out in a 14-l fermentor. For inoculum preparation, one mycelial disc of *A. niger* CH-A-2010, obtained from a 5-day-old culture on Petri dish, was grown in a 2,800-ml Fernbach flask containing 1 l of sterile liquid medium supplemented with *A. tequilana* leaves at a concentration of 1 % w/v, at 37 °C on a rotary shaker at 200 rpm for 24 h. The mycelium produced was used as inoculum for a 10-l culture contained in a 14-l fermentor (New Brunswick Scientific, Enfield, CT, USA) Connecticut). The reaction was carried out at 37 °C, 200 rpm, and 1 vvm, for 120 h. Sterile samples were obtained from the fermentor every 24 h for inulinase activity analysis. The fermented broth was clarified by centrifugation at 5,000 × *g* for 1 h at room temperature. Supernatant was collected and concentrated using a Pellicon 10 000 MWCO-ultrafiltration system (Millipore Co. Billerica, MA, USA). The final concentrated volume (500 ml) was lyophilized and stored at −20 °C until use.

Determination of inulinase activity and biomass quantification

Inulinase activity was determined by hydrolyzing a 2.5 % solution of chicory root inulin (Sigma-Aldrich Inc. St. Louis, MO, USA) in 0.1 M sodium acetate buffer (pH 5.5), at 50 °C for 30 min. Enzymatic activity was determined by measuring the released reducing sugars by the 3,5-dinitrosalicylic acid (DNS) reagent method [16] using fructose as standard. Inulinase activity was expressed as Units (U). One enzyme unit is defined as the amount of enzyme that releases 1 µmol/l of reducing sugars per minute under assay conditions.

To measure biomass, the fungal mycelia were removed from the flasks by passing through a dried and preweighed Whatman no. 1 filter paper and washed twice with 50 ml of sterile water. The biomass-containing filter was dried at 90 °C until a constant weight was obtained.

Effect of pH and temperature on inulinase activity

The effect of pH on the activity of *A. niger* CH-A-2010 inulinases was determined using 0.5 ml of enzyme-containing filtrates, diluted in 1 ml McIlvaine's buffer containing the appropriate amounts of 0.1 M citric acid and 0.2 M Na_2HPO_4 to give pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, and supplemented with 1 % *A. tequilana* leaves. The mixture was incubated at 50 °C for 60 min. To measure the effect of temperature on the inulinases activity, the reaction mixture was prepared using McIlvaine's buffer at the pH in which the maximum activity of inulinases was demonstrated. Incubation was performed at 20, 30, 40, 50, 60, 70, and 80 °C for 60 min. Inulinases activity was determined as described above.

Amplification of *InuB* and *InuE* genes from *A. niger* CH-A-2010 by PCR

Genomic DNA was extracted as described previously [28]. Endoinulinase gene (*InuB*) was amplified using the InuBf (5′ GAA TTC ATG TTG AAT CCG 3′) and InuBr (5′ GCG GCC GCA ATG CTG TCT CA 3′) primers, which amplify a 1,640-bp fragment from *A. niger InuB* gene. Exo-inulinase gene (*InuE*) was amplified using the InuEf (5′ TAC GTA ATG GCT CGT CTT TTG AAG 3′) and InuEr (5′ GCG GCC GCT TAA TTC CAC GTC GAA GT 3′) primers that amplify a 1,675-bp fragment from *A. niger InuE* gene. The PCR protocol was: 94 °C for 5 min, 30 cycles of 95 °C for 30 s, 29 °C (for *InuB*) or 43 °C (for *InuE*) for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min. PCR products were analyzed by using agarose gel electrophoresis.

Production of antibodies to *A. niger* inulinases

Polyclonal anti-inulinases antibodies were prepared in mice using a standard protocol. Animals were used in accordance with the Research Animals Use and Care Guidelines of the Institute of Biomedical Research, Mexico. Groups of 6 to 8-week-old female BalbC mice were immunized intradermally with 10 µg of commercial inulinases from *A. niger* (BioChemika from Sigma-Aldrich Inc. St. Louis, MO, USA), diluted in 40 µl Hank's medium and 50 µl Freund′s adjuvant on day 0, and boosted with 1 µg of inulinases diluted as before on days 15, 30, and 45. Mice were bled by retro-orbital puncture before immunization and 1 week after the last boosting. Sera was separated by centrifugation and stored at −20 °C until used. The presence of antibodies was determined by ELISA using commercial inulinases as target antigen. Plates were coated overnight at 4 °C with 500 ng of inulinases diluted in 100 µl PBS per well. The plates were washed four times with washing solution (Tris-buffered saline containing 0.5 % Tween 20). Non-specific binding sites were blocked by adding 200 µl blocking solution (2 % bovine serum albumin in washing solution) to each well for 2 h at 37 °C. Mice serum samples were diluted 1:10 in blocking solution. All samples were serially diluted twofold down the microtiter plate. Bound antibodies were detected with anti-mouse IgG-alkaline phosphatase-conjugated secondary antibody (Dako Co. Carpinteria, CA, USA). Alkaline phosphatase substrate Sigma 104 was diluted in a 10 % diethanolamine (Sigma-Aldrich Inc. St. Louis, MO, USA) solution and added to the plates. Reaction was read at 405 nm in an ELISA plate reader (EL × 800, Bio-Tek). All samples were tested in triplicate.

Endpoint titers were defined as the reciprocal of the highest dilution with an absorbance value greater than three times the mean absorbance value for the control pre-immunization serum.

Analysis of proteins by SDS-PAGE and Western blotting

Total protein content was determined using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules CA, USA). Proteins were washed three times with lysis buffer, resolved by 10 % SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Co. Billerica MA, USA). Membranes were incubated overnight with mouse anti-inulinases serum (diluted 1:100) at 4 °C. The next day, membranes were washed and incubated with a horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Invitrogen Co. Carlsbad CA, USA), diluted 1:500. Proteins were detected by chemiluminescence using the Amersham ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway NJ, USA).

Thin layer chromatography (TLC)

Thin layer chromatography was used for qualitative analysis of the products of inulin hydrolysis with inulinases of *A. niger* CH-A-2010. The samples were spotted onto pre-coated TLC Silica gel 60 plates (Merck KGaA, Darmstadt, Germany), and developed with the solvent system ethyl acetate:acetic acid: 2-propanol:formic acid:water (25:10:5:1:15 v/v). Fructose, glucose, sucrose, 1-kestose, nystose, and inulin (all from Sigma-Aldrich) were used as standards. The TLC plates were sprayed with freshly made detection reagent containing 2 % (w/v) diphenylamine, 2 % (v/v) aniline, 100 ml acetone, and 15 ml of 85 % (v/v) ortho-phosphoric acid. Sugars were detected by heating the plates at 110 °C for 20 min.

Acid hydrolysis

Agave juice was obtained from 6 to 7-year-old *A. tequilana* plants. The juice was produced from shredded, raw *A. tequilana* stems using a juice extractor (Extractor International EX-S, Mexico). The juice was clarified by centrifugation at 5,000 × *g*, collected, and immediately stored at −20 °C. For acid hydrolysis, 100 ml of *A. tequilana* juice was mixed thoroughly with 0.01 ml of concentrated sulfuric acid and then autoclaved at 121 °C at 1 atm for 3 h. After cooling, the concentration of reducing sugars was determined.

Enzymatic hydrolysis

Erlenmeyer flasks (250 ml) containing 50 ml 1 M acetate buffer (pH4.8), 1,000 U of lyophilized *A. niger* CH-A-2010 inulinase, or Fructozyme® (Novozymes Corp. Bagsværd, Denmark), and the following substrates: 10 ml *A. tequilana* juice, or 2.5 g nanofiltered agave fructans, or 2.5 g inulin from chicory (Sigma-Aldrich Inc. St. Louis, MO, USA), were incubated for 120 h at 50 °C with agitation at 200 rpm. Samples of 1 ml samples were taken at 3-h intervals and analyzed for reducing sugars production. Samples were stored at −20 °C, and thawed prior to analysis. To produce hydrolysates for alcoholic fermentation, 1-l Erlenmeyer flasks containing 40 ml 1 M acetate buffer (pH 4.8), 400 ml *A. tequilana* juice diluted to 8.6° Brix, 30 μg/ml chloramphenicol, and inulinase (2 U/ml of juice), in a total reaction volume of 440 ml were incubated for 24 h at 50 °C with agitation at 200 rpm. The concentration of reducing sugars produced was determined before fermentation was carried out.

Fermentation

Frozen *S. cerevisiae* were activated by incubation of two glass beads in a broth containing 50 ml of hydrolyzed agave juice, diluted with distilled water to reach 6° Brix, supplemented with 0.9 g/l $(NH_4)_2HPO_4$, 0.8 g/l K_2HPO_4, and 0.7 g/l yeast extract, at 30 °C, 200 rpm, for 24 h. *S. cerevisiae* were propagated by incubating 2 ml of activated yeast in 100 ml of broth at 30 °C, with agitation at 200 rpm, for 18 h. These cells were used as inoculum for the fermentation tests. Fermentations were carried out in 1-l Erlenmeyer flasks using agave juice previously treated by either enzymatic hydrolysis or acid hydrolysis. Fermentation broth containing 0.45 g/l $(NH_4)_2HPO_4$, 0.4 g/l K_2HPO_4, 0.35 g/l yeast extract, 2 % *S. cerevisiae* inoculum, and 500 ml hydrolyzed agave juice, was incubated for 28 h at 35 °C. The ethanol concentration was determined spectrophotometrically by using the Enzymatic BioAnalysis for Ethanol/Food kit (Boehringer Mannheim GmbH., Mannheim, Germany) according to the instructions of the manufacturer.

Statistics

All assays were conducted in triplicate. To evaluate the differences in enzymatic activity and production of reducing sugars, a two-tailed *t* test was used. A p value < 0.05 was considered significant.

Results

Expression of extracellular inulinases by *A. niger* CH-A-2010

Aspergillus niger CH-A-2010 produces extracellular inulinases using *A. tequilana* leaves as the sole carbon source

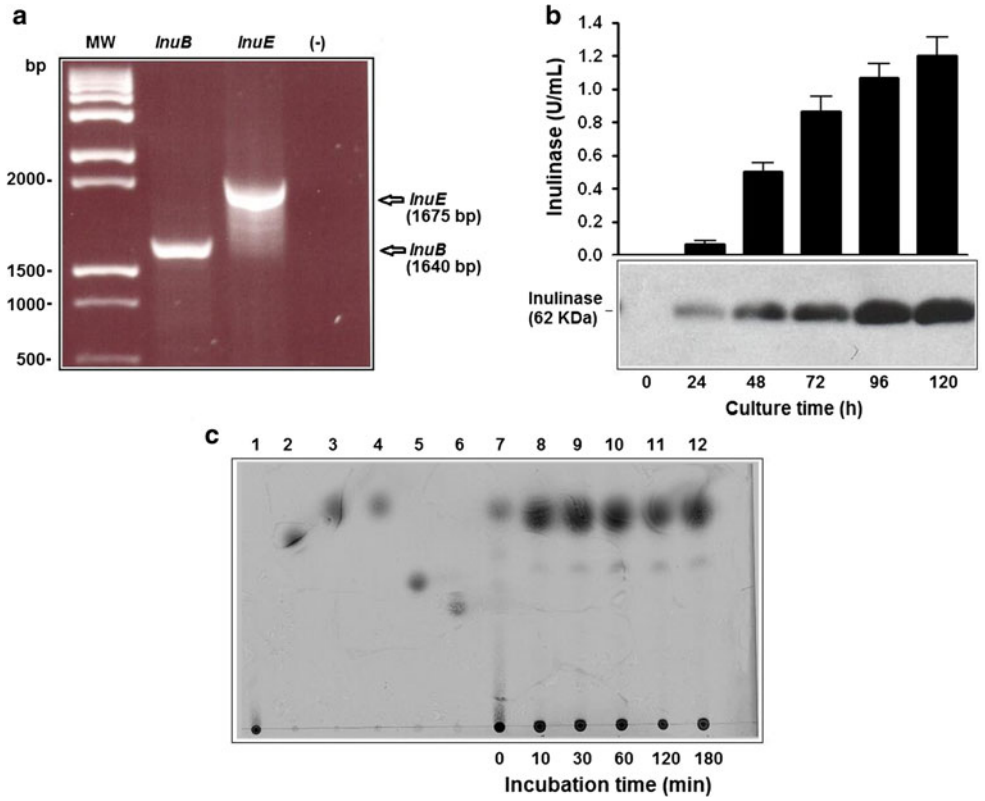

Fig. 1 Characterization of inulinases produced by *A. niger* CH-A-2010. **a** Amplification of endoinulinase and exo-inulinase genes; genomic DNA from *A. niger* CH-A-2010 was isolated and analyzed by PCR using specific primers for endoinulinase (*InuB*), and exo-inulinase (*InuE*) genes. Negative control, to assess the presence of contaminants, was set using purified water instead of template DNA (−). *MW* Molecular weight marker. **b** Time course of inulinases production. *A. niger* CH-A-2010 inulinases were produced using *Agave tequilana* leaves as a substrate. Extracellular inulinases were analyzed in cell-free medium collected at the indicated time points by the 3,5-dinitrosalicylic acid (*DNS*) reagent method. Values represent the mean of three independent experiments. *Error bars* indicate the standard error of the mean. Total extracellular protein was analyzed by Western blotting using a polyclonal anti-*A. niger* inulinases antibody. A predominant 62-kDa band was detected by chemiluminescence. **c** Thin layer chromatography analysis of the hydrolysis products of inulin by inulinases from *A. niger* CH-A-2010. The following standards were included: (*1*) inulin, (*2*) sucrose, (*3*) glucose, (*4*) fructose, (*5*) 1-kestose, and (*6*) nystose. The enzyme reaction was performed as described in the Materials and methods section. Hydrolysis products were analyzed at the indicated time points (*7–12*)

[12]. It is known that *A. niger* degrades inulin using both endo-inulinases (EC 3.2.1.7), encoded by the *InuA* and *InuB* genes [1, 23], and exo-inulinases (EC 3.2.1.80), encoded by the *InuE* gene [18]. Thus, we first investigated whether the inulinase activity produced by *A. niger* CH-A-2010 is mediated by endo- or exo-inulinases. Analysis of genomic DNA from *A. niger* CH-A-2010 by specific PCR amplification showed the presence of both *inuB* and *inuE* sequences (Fig. 1a). However, analysis of extracellular proteins by Western blotting revealed the presence of a dominant band of 62 kDa that showed an increasing level of expression associated with an increment in inulinase activity (Fig. 1b). Thin layer chromatography analysis of the hydrolysis products of inulin showed that monosaccharides were the predominant end product (Fig. 1c). A rapid conversion of inulin into monosaccharides was observed after 10-min incubation, suggesting the presence of an exo-inulinase in the crude inulinase-containing culture filtrate. This observation suggests that the dominant inulinolytic activity is mediated by an exo-inulinase enzyme. However, a trace amount of oligosaccharides was detected after 30 min incubation also pointing to the presence of low endo-inulinase activity.

Production of *A. niger* CH-A-2010 inulinases and effect of pH and temperature on inulinase activity

Culture conditions for the production of *A. niger* CH-A-2010 inulinases had been previously determined [12]. In the present work, the production of inulinases was scaled up from 0.5-l scale to 10-l scale. As shown in Fig. 2a, despite the difference in scale, the time course of inulinases production was similar. At 120 h, inulinases yield was 0.99 U/ml for the 0.5-l flask cultures, and 1.20 U/ml for the 10-l cultures, the difference was not significant ($p = 0.1$). It is known that different carbon sources may influence the

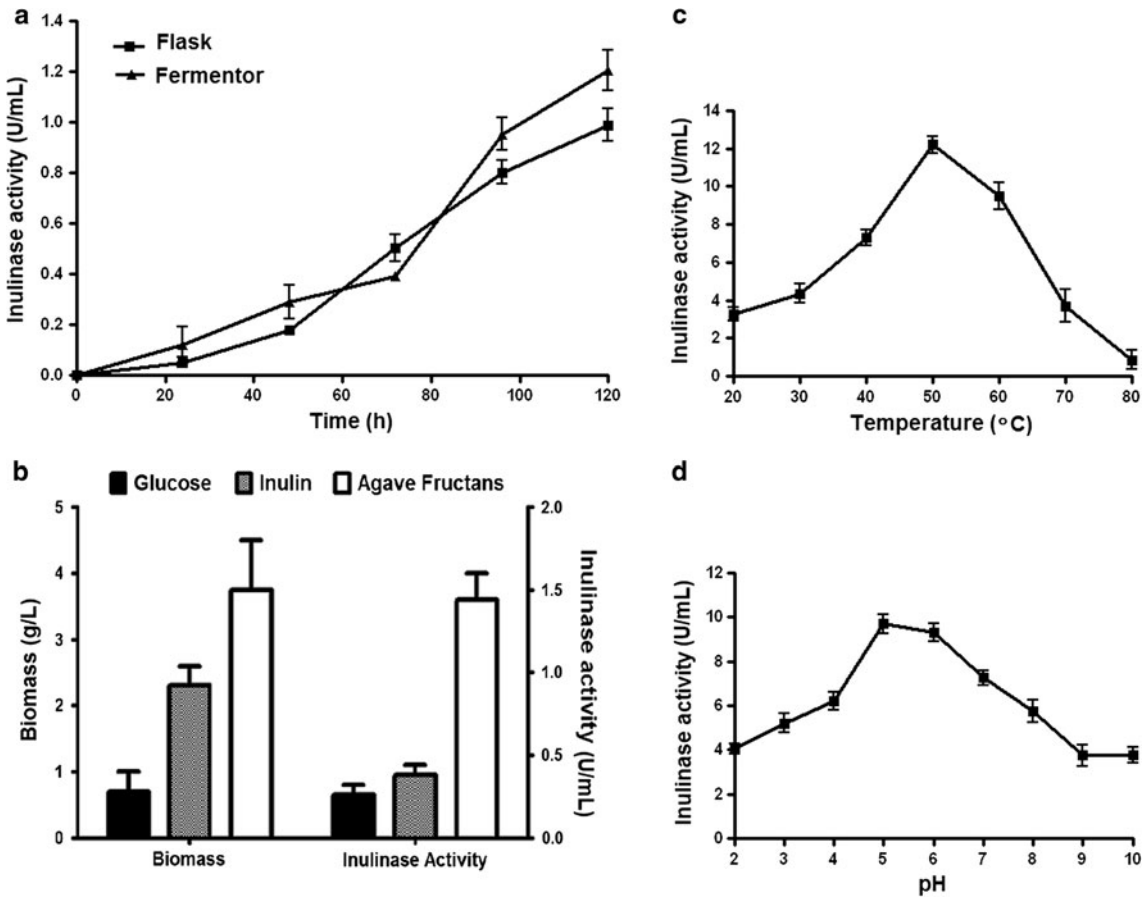

Fig. 2 Production of inulinases in 0.5-l flask and 10-l fermentor, effect of different carbon sources on inulinase production, and effect of pH and temperature on enzymatic activity. **a** Production of inulinases was scaled up from 0.5-l to 10-l scale. Extracellular inulinase activity was measured at 24-h intervals during 120 h. **b** Effect of different carbon sources on inulinase and biomass production was evaluated by incubating *A. niger* CH-A-2010 for 120 h in culture medium containing glucose, purified chicory root inulin (inulin), or agave fructans. Optimal conditions of temperature (**c**) and pH (**d**) for the enzymatic degradation of inulin were determined. The highest inulinase activity occurred at pH 5.0 and 50 °C. *Error bars* indicate the standard error of the mean of three independent experiments

production of inulinases. Thus, the effect of glucose, purified chicory root inulin, and agave fructans on inulinase production was tested. As seen in Fig. 2b, agave fructans were the best carbon source for the production of both inulinase and biomass. Significantly lower levels of inulinase and biomass were induced by purified inulin. The lowest inulinase and biomass were produced in medium containing glucose. To establish the optimal conditions for the enzymatic degradation of inulin, the effect of various pH and temperature conditions on the activity of *A. niger* CH-A-2010 inulinases was evaluated. The highest level of inulinase activity was observed at 50 °C, and a significant decrease of activity was evident at lower temperatures, whereas inulinase activity was abolished at 80 °C (Fig. 2c). As shown in Fig. 2d, the optimum inulinase activity occurred at pH 5.0.

Comparison of hydrolysis efficiency between *A. niger* CH-A-2010 inulinases, Fructozyme®, and acid hydrolysis on raw *A. tequilana* juice.

The use of commercially available inulinases for the bioconversion of *A. tequilana* fructans for the production of tequila has been recently suggested [4]. Thus, we performed a comparative study of the capacity of *A. niger* CH-A-2010 inulinases and commercial inulinases for the saccharification of untreated agave juice, nanofiltered agave fructans, and commercial chicory inulin. All substrates were incubated with 1,000 U/ml of either *A. niger* CH-A-2010 inulinases or Fructozyme® for 48 h. As seen in Fig. 3, the yield of reducing sugars obtained by degradation of the three substrates with *A. niger* CH-A-2010 inulinases was significantly higher than the yield of free sugars produced by using Fructozyme® ($p < 0.05$).

Acid hydrolysis is considered an efficient means for the production of monosaccharides from inulin and fructans. In fact, modern tequila distilleries use this methodology to obtain fructose-rich juices for alcoholic fermentation.

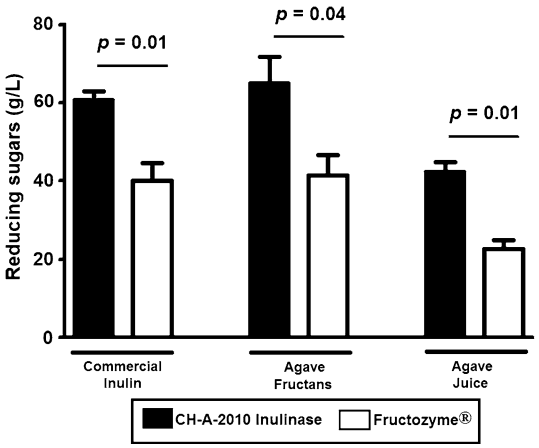

Fig. 3 Hydrolysis of commercial inulin from chicory root (commercial inulin), nanofiltered *A. tequilana* fructans (agave fructans), or *A. tequilana* raw juice (agave juice) by *A. niger* CH-A-2010 inulinases and Fructozyme®. Substrates were incubated with 1,000 U of either *A. niger* CH-A-2010 inulinases or Fructozyme®. The yield of reducing sugars was evaluated after 48-h incubation. Values represent the mean of three independent experiments. *Error bars* indicate the standard error of the mean. Statistical differences between hydrolysis by *A. niger* CH-A-2010 inulinases and Fructozyme® are indicated for each substrate, the *p* values were calculated using a two-tailed *t* test

Table 1 Production of reducing sugars by enzymatic or acid hydrolysis of raw agave juice and production of ethanol after 28-h fermentation of the hydrolysates

Hydrolysis technique	Reducing sugars (g/l)	Alcohol yield (g/g reducing sugar)	Efficiency of conversion into alcohol (%)
Enzymatic hydrolysis	33.5	0.48	97.5
Acid hydrolysis	29.4	0.41	83.8

Thus, we compared the efficiency of *A. niger* CH-A-2010 and Fructozyme® inulinases to that of acid hydrolysis in degrading *A. tequilana* fructans. As indicated in Table 1, the yield of reducing sugars produced by acid hydrolysis was 29.4 g/l. Meanwhile, after 48 h incubation with 1,000 U/ml of *A. niger* CH-A-2010 and Fructozyme®, yields of 33.5 g/l and 27.3 g/l reducing sugars were obtained, respectively (Table 1). There was no difference between the final concentration of sugars from the acid hydrolysis process and the yield obtained by using Fructozyme®. Although production of reducing sugars mediated by *A. niger* CH-A-2010 inulinase seemed to be higher than the yield obtained by acid hydrolysis, the difference was not significant ($p > 0.5$). These observations suggest that hydrolysis of agave fructans with *A. niger* CH-A-2010 inulinases is as efficient as acid hydrolysis.

Ethanol fermentation of hydrolyzed fructans

Ethanol fermentation by *S. cerevisiae* was assessed using agave juice pre-treated with either *A. niger* CH-A-2010 inulinases or acid hydrolysis. Equal volumes of agave juice were pre-treated as described, and then used directly for a 28-h fermentation process. As shown in Table 1, at the end of the fermentation, the ethanol yield produced from enzymatically treated fructans was 0.48 g compared with 0.41 g from acid-hydrolyzed fructans. The conversion efficiency of fructan-derived sugars into ethanol was 97.5 % of the theoretical ethanol yield for enzymatically degraded agave juice, compared to 83.8 % for acid-hydrolyzed juice. These observations indicate that ethanol productivity can be improved by the enzymatic saccharification of agave fructans.

Discussion

It has been demonstrated that inulinolytic enzymes of *A. niger* are simultaneously induced in the presence of inulin [32] by the activation of InuR, a positive-acting transcription factor that co-regulates the expression of the endo- and exo-inulinase genes [33]. Accordingly, in the present study, we showed that inulin-rich agave waste induced both the endo-inulinolytic and the exo-inulinolytic activities of *A. niger* CH-A-2010. The inulinolytic activity was predominantly mediated by an exo-inulinase enzyme. Extracellular exo-inulinases have been identified in a number of *Aspergillus* species, including *A. awamori* [2], *A. fumigatus* [11], *A. ficuum* [19], and *A. terreus* [7]. In general, *Aspergilli* exo-inulinases show molecular weights ranging from 57 to 69 kDa, acidic optimal pH (4.0–5.5), and optimum temperatures ranging from 50 to 60 °C. Accordingly, the molecular weight of the *A. niger* CH-A-2010 exo-inulinase was estimated to be 62 kDa, and it exhibited optimum pH and temperature values of 5.0 and 50 °C, respectively.

Inulinases are important enzymes currently used for the industrial production of high-fructose syrups and fructooligosaccharides. *A. niger* strains are commonly employed for the production of inulinases. Here, we found that *A. niger* CH-A-2010 yielded a maximum of 1.2 U/ml by using agave juice as carbon source. This is consistent with the reported yields of extracellular inulinases for wild-type *A. niger* strains, which ranges from 0.06 [24] to 23.98 U/ml [27]. Interestingly, increased productivities of inulinases have been obtained with UV light-mutagenized *A. niger* (377 U/ml) [31], as well as biochemical mutants of *A. niger* (35.18 U/ml) [27]. Since inulinases are inducible enzymes, their production is frequently based on the use of inulin-rich or even purified inulin-containing

substrate medium. However, we observed that agave fructans induced higher levels of inulinase than those produced by purified inulin. Thus, our results suggest that an alternative to produce inulinases at low cost might be the use of industrial medium containing fructans-rich agroindustrial residues. The use of industrial waste for the production of commercially important enzymes has been explored [6, 8, 13] because it is economically attractive. However, recent reports have shown that some agroindustrial residues contain inhibitory compounds that affect the recovery of inulinases [15, 29]. In the present work, we used agave waste as a substrate for the production of inulinases, obtaining modest yields of the enzyme. Therefore, further investigation will be needed to make the production process viable.

Tequila production relies on the efficient hydrolysis of *A. tequilana* fructans. Thus, for the optimization of fructan hydrolysis, the use of commercial inulinases has been proposed [4]. Fructozyme® is a commercial preparation of endo- and exo-inulinases produced by a strain of *A. niger*. In the present work, we found that inulinases produced by *A. niger* CH-A-2010 exhibited a higher hydrolysis efficiency than Fructozyme® on *A. tequilana* fructans, agave juice, and purified chicory inulin. *A. niger* CH-A-2010 was isolated from soil containing decomposing *A. tequilana* plants, therefore it is well adapted to degrade this type of fructans. In accordance with our findings, Arrizon and colleagues [3] demonstrated that a fructanase produced by a *Kluyveromyces marxianus* strain, isolated from the fermentation process of *Agave angustifolia*, exhibited higher enzymatic activity and specificity than Fructozyme® for the hydrolysis of *A. tequilana* fructans. In combination, these observations suggest that inulinases produced by naturally occurring microorganisms are specific and may have important industrial applications.

In modern distilleries, fructans are transformed into fermentable fructose by acid thermal hydrolysis. Here, we demonstrated that the yield of reducing sugars obtained by acid hydrolysis of *A. tequilana* fructans was similar to that produced by enzymatic degradation. This observation may be relevant because acid hydrolysis has many drawbacks. For instance, it is known that fructose undergoes significant degradation when treated at high-temperature (121 °C)/low-pH conditions [21]. Besides, this procedure may produce undesirable by-products such as 5-hydroxymethylfurfural, difructose dianhydrides, and color-forming compounds that have a negative impact on fermentable fructose output. Thus, strategies based on the use of enzymes have been proposed as an alternative for the production of fructans-derived sugars for alcoholic fermentation. The use of commercial inulinases from *Aspergillus ficuum* have proven useful for the production of fructose-rich Jerusalem artichoke juice, which can be further converted into ethanol by fermentation with *Zymomonas mobilis* [9]. Similarly, recombinant inulinases have been used to hydrolyze Jerusalem artichoke for the production of ethanol [34]. Interestingly, systems for the simultaneous saccharification and fermentation of inulin have been developed either by using *S. cerevisiae* inoculated into an inulinases-containing medium [22] or by using *Kluyveromyces marxianus*, an inulinolytic yeast that is able to directly convert inulin into ethanol [20]. In the present work, we observed that both enzymatic hydrolysis and acid hydrolysis of *A. tequilana* fructans produce a similar amount of reducing sugars. However, the enzymatic hydrolysis yields a higher concentration of ethanol after fermentation by *S. cerevisiae*. In fact, the conversion efficiency obtained in our system (97.5 %) was higher than that reported for other inulinases-based procedures, for instance the use of commercial inulinases and fermentative *Z. mobilis* (92 %) [9], and the simultaneous saccharification and fermentation of inulin using *A. niger* and *S. cerevisiae* (83–84 %) [22].

Fermentation of *A. tequilana* juice is a crucial step in tequila production. Here, we used a locally isolated strain of *S. cerevisiae* for the fermentation of *A. tequilana* juice, obtaining high yields of ethanol (43.1 g/l). Our observations are in line with a recent report showing that a strain of *S. cerevisiae* isolated from a tequila distillery was able to produce 40.08 g/l ethanol when the culture medium was supplemented with ammonium phosphate [17]. Additionally, it was reported that an indigenous strain of *Kloeckera africana* yielded 47.8 g/l ethanol by adding asparagine to the fermentation medium [30]. These observations may be interesting for the tequila industry, as they point out the convenience of introducing locally adapted yeast to improve ethanol productivity.

Our results indicate that the use of inulinases produced by *A. niger* CH-A-2010 may be included as a downstream step for the conversion of fructans into ethanol. In fact, there is evidence showing that tequila produced by enzymatic hydrolysis using Fructozyme® followed by a short thermal treatment is not significantly different in chemical composition and bouquet than the tequila prepared using standard methods [4]. In conclusion, fructans from raw *A. tequilana* juice can be hydrolyzed efficiently by *A. niger* CH-A-2010 inulinases, and this procedure has a positive impact on the production of ethanol. Thus, we propose that replacing the acid hydrolysis of agave fructans by the use of *A. niger* CH-A-2010 inulinases may be feasible and potentially attractive for the tequila industry.

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Conflict of interest The authors declare that they have no conflicts of interest.

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