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Agricultural waste from the tequila industry as substrate for the production of commercially important enzymes

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Abstract: *Approximately 1 million tons of Agave tequilana plants are processed annually by the Mexican Tequila industry, generating vast amounts of agricultural waste. The aim of this study was to investigate the potential use of Agave tequilana waste as substrate for the production of commercially important enzymes. Two strains of Aspergillus niger (CH-A-2010 and CH-A-2016), isolated from agave fields, were found to grow and propagate in submerged cultures using Agave tequilana waste as substrate. Isolates showed simultaneous extracellular inulinase, xylanase, pectinase, and cellulase activities. Aspergillus CH-A-2010 showed the highest production of inulinase activity (1.48 U/ml), whereas Aspergillus niger CH-A-2016 produced the highest xylanase (1.52 U/ml) and endo-pectinase (2.7 U/ml) activities. In both cases production of enzyme activities was significantly higher on Agave tequilana waste than that observed on lemon peel and specific polymeric carbohydrates. Enzymatic hydrolysis of raw A. tequilana stems and leaves, by enzymes secreted by the isolates yielded maximum concentrations of reducing sugars of 28.2 g/l, and 9.9 g/l respectively. In conclusion, Agave tequilana waste can be utilized as substrate for the production of important biotechnological enzymes.*

Key words: *Agave tequilana, Microbial enzymes, Agave waste, Aspergillus niger*
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Introduction

Tequila is the most consumed Mexican liquor worldwide. Tequila is obtained exclusively from *Agave tequilana* Weber var. Azul. *A. tequilana* belongs to the Agavaceae family. It is a succulent plant with leaves spirally arranged around a thick stem, forming a rosette. During the tequila production process, the leaves are removed to harvest the stems. Stems are used to produce tequila, whereas leaves are left alone on the fields and are considered by-products of the tequila industry. Approximately 1 million tons of *A. tequilana* are produced and processed annually in restricted areas in five counties (Anonymous, 2006), generating large amounts of agricultural waste.

Recently, agro-industrial wastes have been used as carbon, hydrogen, and oxygen sources to produce ethanol, proteins, and microbial enzymes (Oliveira *et al*., 2006). Applications of microbial enzymes extend from food and beverage manufacturing to biomass conversion, and waste treatment. Recently, the use of microbial enzymes for plant biomass hydrolysis has been encouraged by the increased demand for biofuels (Lyons, 2003). The bioconversion of plant biomass to fermentable sugars utilizes a wide range of microbial enzymes (Mtui and Nakamura, 2005; Kulkarni *et al.,* 2007), such as cellulases, xylanases, pectinases, and inulinases. Thus, naturally occurring microbial strains capable of secreting a variety of enzymes with the potential to hydrolyze plant biomass would be attractive for such applications.

Filamentous fungi of the genus *Aspergillus* are major agents of decomposition and decay. Therefore, they are capable of producing a broad range of enzymes, and using a wide variety of substrates. Different *Aspergillus* species have been found to produce extracellular enzymes (Gawande and Kamat, 1999; Zhang *et al*., 2004; Ragunathan and Swaminathan, 2005; Villena and Gutierrez-Correa, 2006).

The objective of the current study was to investigate the potential use of *A. tequilana* waste as substrate for the production of extracellular enzymes by *Aspergillus niger* strains isolated from agave fields. Inulinase, xylanase, cellulase, endo and exo-pectinase activities were assessed in submerged cultures containing *A. tequilana* waste as sole carbon source. The use of these enzymes for the saccharification of raw agave plants to produce fermentable sugars was also investigated.

Materials and Methods

***Agave tequilana* samples:** Fresh leaves and stems of *Agave tequilana* Weber var. Azul were obtained from the tequila-producing area of Jalisco, Mexico.

Collection and purification of microorganisms: The microorganisms used in this study were isolated from soil, decomposing *A. tequilana* leaves and dried leaves of the fields at Jalisco, Mexico. Microorganisms were selected by serial dilution methods. Briefly, fresh *A. tequilana* leaves (1% w/v) were thoroughly

washed with water, cut into 1 cm^3 pieces, and added to 500 ml Erlenmeyer flasks containing 200 ml of liquid medium [0.4% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% K_2HPO_4, pH 4.5]. The medium was autoclaved at 20 lbs for 20 min. The flasks containing medium were inoculated with individual colonies of the collected microorganisms. Flasks were incubated at 37°C, on a rotary shaker at 200 rpm. After 120 hr incubation 0.5 ml samples were collected and used to inoculate 500 ml Erlenmeyer flasks, containing liquid medium supplemented with *A. tequilana* leaves, and incubated as previously described. After seven rounds of selection, 0.1 ml samples were collected and inoculated on petri-dishes containing solid medium [0.4% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 3.0% agar, pH 4.5] supplemented with *A. tequilana* waste (0.5%). Single colonies were isolated and further purified by seven rounds of serial dilutions. Purified microorganisms were preserved in potato-dextrose-agar (PDA) slants at 4°C, or frozen in liquid medium containing sterile glycerol (15%) at -70°C for long-term storage.

Identification of microorganisms: Identification of isolated strains was achieved by cultural, morphological and molecular methods. Isolates were grown on czapek-dox agar and malt extract agar to confirm their purity and species identity based on macro- and micro-morphological criteria (Raper and Fennell, 1977). Identification was subsequently confirmed by sequencing the D1/D2 domains of the large subunit (26S) ribosomal DNA region. DNA was extracted as previously reported (Tapia-Tussell *et al*., 2006). The NL1 (5' GCA TAT CAA TAA GCG GAG GAA AAG 3') and NL4 (5'GGT CCG TGT TTC AAG ACG G 3') primer pairs were used for the amplification of the D1/D2 region. Amplification was performed as described elsewhere (Hinrikson *et al*., 2005) with minor differences in the thermal cycling conditions employed. Initial denaturation at 95°C for 12 min, followed by 40 cycles of 94°C for 1 min, 52°C for 55 sec, and 72°C for 2 min. A final extension step at 72°C for 8 min was then conducted. All PCR products were purified and sequenced on both strands using the primers described above on an automated capillary DNA sequencer ABI Prism 310 genetic analyzer (Perkin Elmer Applied Biosystems).

Production of extracellular enzymes: *A. tequilana* leaves were utilized as substrate to produce extracellular enzymes. The isolated fungi were grown in 500 ml Erlenmeyer flasks containing 200 ml of liquid medium supplemented with *A. tequilana* leaves 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 diameter) obtained from a 5 days old culture on PDA. Flasks were incubated at 37°C, on a rotary shaker at 200 rpm, for 120 hr. Fifteen ml medium samples were collected at regular time intervals. Samples were centrifuged for 20 min at 2500 rpm at room temperature to remove spores. Supernatants were filtered through Whatman No. 41, and kept frozen at -20°C until used. These preparations were used as enzyme-containing filtrates during the course of the study. To analyze the level of enzyme production on different carbon sources, 1% (w/v) microcrystalline cellulose, citrus pectin, Birchwood xylan, chicory root inulin, glucose, or dried lemon peel were added to the liquid medium before autoclaving.

Determination of protein concentration: The protein concentrations of enzyme-containing filtrates were determined by the Lowry method (Lowry *et al*., 1951). Bovine serum albumin was used as standard.

Enzymatic activity assays: Enzymatic activities were analyzed using 0.5 ml of enzyme-containing filtrates. Substrates to test specific enzyme activities were diluted in 0.1M sodium acetate buffer. Cellulase activity was determined by hydrolyzing a 50 mg strip of filter paper (Whatman No. 1) as substrate in 1 ml of sodium acetate buffer (pH 5.5), at 50°C for 30 min. Release of glucose caused by enzymatic activity was measured by the 3,5-dinitro salicylic acid (DNS) method (Miller *et al*., 1959), using D-glucose as standard. Exo-pectinolytic activity was evaluated using 1.0% citrus pectin as substrate in sodium acetate buffer (pH 5.5), at 45°C for 60 min. Soluble reducing sugars released were estimated by the DNS method using galacturonic acid as standard. Xylanase activity was determined by hydrolyzing 0.75% Birchwood xylan as substrate in sodium acetate buffer (pH 5.0), at 50°C for 10 min. Glucose release was measured by the DNS method, using D-xylose as standard. Inulinase activity was determined using a 2.5% solution of chicory root inulin as substrate in acetate buffer (pH 5.5), at 50°C for 30 min. Enzymatic activity was determined by measuring the released reducing sugars by the DNS method, using fructose as standard. In all cases enzyme activity was expressed as Units (U). One enzyme unit is defined as the amount of enzyme that releases 1μM of reducing sugars per minute under assay conditions. Endo-pectinolytic activity was determined by measuring the reduction of viscosity of a 1% citrus pectin solution in sodium acetate buffer (pH 4.2), at 30°C for 60 min. Enzyme activity is expressed as relative fluidity units (RFU). One RFU is equivalent to the amount of enzyme required to produce a decrement of 1.0 of the relative fluidity per second.

Enzymatic hydrolysis of agave biomass: Fresh *A. tequilana* leaves and stems were thoroughly washed with water and cut into 1 cm^3 pieces. 4 g of leaves or stems were incubated with 5.5 ml of enzyme-containing filtrates, 1 ml acetate buffer (pH 4.8) and 11ml distilled water, in 250 ml Erlenmeyer flasks, in a rotary incubator at 50°C, for 24 hr. Released soluble reducing sugars were estimated by the DNS method.

Statistical analysis: 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 basic significance value of $p<0.05$ was considered.

Results and Discussion

Two strains of filamentous fungi isolated from decomposing *Agave tequilana* leaves (CH-A-2010) and soil (CH-A-2016) were selected based on their capacity to grow and propagate in submerged cultures containing *Agave tequilana* as sole carbon source. The isolated strains were analyzed by cultural, morphological and molecular methods to determine their species identity. Both strains were identified as *Aspergillus niger* van Tieghem. To address the question of whether *Agave tequilana* waste could be used as substrate

Table - 1: Extracellular enzymatic activities produced by isolated fungi using *Agave tequilana* waste as substrate

Aspergillus niger strain	Inulinase (U/ml)	Xylanase (U/ml)	Cellulase (U/ml)	Exo-pectinase (U/ml)	Endo-pectinase (RFU)
CH-A-2010	1.48	1.07	0.03	0.1	0.8
CH-A-2016	0.40	1.52	0.18	0.38	2.7

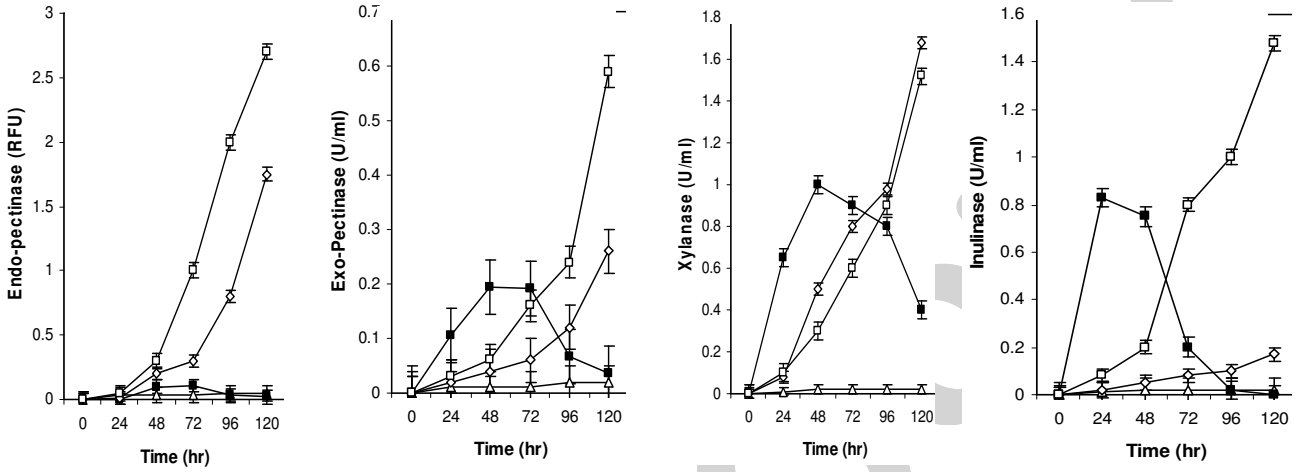

Fig. 1: Induction of enzyme production by *Agave tequilana* waste. Endo-pectinase, exo-pectinase and xylanase production by *Aspergillus niger* strain CH-A-2016, and inulinase production by *A. niger* strain CH-A-2010 were evaluated in submerged cultures containing *Agave tequilana* waste (□), lemon peel (◊), glucose (△), or the following polymeric carbohydrates (■): pectin, to determine endo- and exo-pectinase activities; xylan, to determine xylanase activity; and inulin, to determine inulinase activity. Fungi were cultivated as described in the Materials and Methods section. The experiments were conducted in triplicate

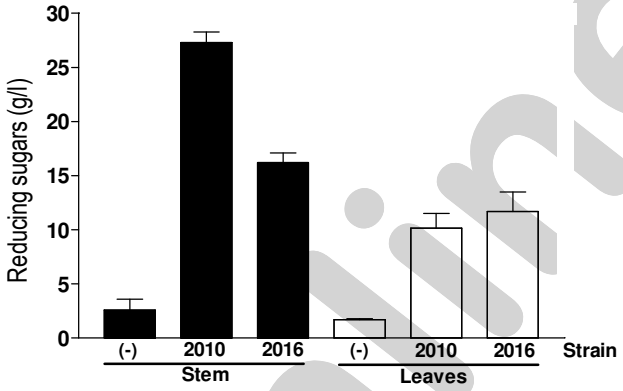

Fig. 2: Saccharification of *A. tequilana* stem and leaves by extracellular enzymes produced by *Aspergillus niger* CH-A-2010 (Strain 2010), and CH-A-2016 (Strain 2016). Raw stem and leaves were incubated with the enzymes at 50^{0}C for 24 hr. As a negative control (-) enzymes were replaced by sterile water. The experiments were conducted in triplicate

for the production of extracellular enzymes, we evaluated the production of cellulase, endo-pectinase, exo-pectinase, xylanase and inulinase activities by the isolates. As shown in Table 1, both strains were able to simultaneously secrete endo-pectinases, exo-pectinases, xylanases, and inulinases. This indicates the suitability of *A. tequilana* waste as a substrate for the secretion of these enzymes. However, both strains showed a low cellulase activity. *Aspergillus* sp are normally regarded as poor producers of cellulases. However, cellulose-degrading enzymes from *A. niger* have been reported previously (Seidle *et al*., 2004) and new methods to improve cellulase production by *A. niger* are in progress (Villena and Gutierrez-Correa, 2006). Utilization of other agricultural wastes as substrate for enzyme production by *Aspergillus niger* (Ong *et al*., 2004), *Aspergillus terreus* (Gawande and Kamat, 1999), and *Aspergillus oryzae* (Ragunathan and Swaminathan, 2005) has been previously described. However, simultaneous secretion of pectinases, xylanases, and inulinases by *Aspergillus niger* growing of agave waste had not yet been reported.

To study the production of enzymes by the isolates on different carbon sources, the levels of enzyme activities produced on agave waste, lemon peel, pectin, xylan and inulin in submerged cultures were compared. In all experiments glucose was included as a control. Strain CH-A-2016 was used to evaluate the production of endo-pectinase, exo-pectinase and xylanase activities. Strain CH-A-2010 was used to evaluate the production of inulinase. Endo-pectinase, exo-pectinase and inulinase activities on agave waste were significantly higher than those observed in cultures containing lemon peel ($p<0.01$), or specific polymeric carbohydrates ($p < 0.001$) (Fig. 1). Pectinases are widely used in the food industry, *Agave tequilana* waste was demonstrated to be a better inducer of pectinases

production by *A. niger* CH-A-2016 than lemon peel. However, to establish the conditions for a maximum production of pectinases, an optimization of the process will be needed (Patil and Dayanand, 2006). Xylanase activity by *A. niger* CH-A-2016 was also induced by *A. tequilana* waste. However, much higher levels of xylanase production by *A. niger* have been reported by using optimized processes (Dobrev *et al*., 2007). *A. tequilana* waste induced the production of inulinase by *A. niger* CH-A-2010. Production of inulinases by *A. niger* has been well characterized. *A. niger* inulinases have been cloned, expressed in yeasts (Zhang *et al*., 2004), and the genes coding for inulin-modifying enzymes have been transcriptionally analyzed (Yuan *et al*., 2006).

In all cases, enzyme production in cultures containing specific polymeric carbohydrates (cellulose, inulin, and pectin) was observed to reach maximum levels after 48-72 hr. In contrast, enzyme production peaks were observed after 120 hr in cultures containing agave waste. Interestingly, xylanolytic activity produced on agave waste was significantly higher than on xylan ($p < 0.001$), but similar to that observed on lemon peel ($p = 0.1$) (Fig. 1C). No enzymatic activity was detected in cultures containing glucose. These results suggest that agave waste can be used as substrate to increase the production of extracellular enzymes by *Aspergillus niger* CH-A-2010 and CH-A-2016.

Our results indicate that considerable levels of pectinase, inulinase, and xylanase activities are produced by the isolates when using agave waste as carbon source. To further investigate the potential use of these enzymes for the hydrolysis of plant biomass, we treated raw *A. tequilana* stems and leaves with the enzymes produced by the isolates. Results are shown in Figure 2. Enzymatic saccharification of stems by *A. niger* CH-A-2010 and CH-A-2016, yielded 28.2 g/l and 17.1 g/l of reducing sugars, respectively. These values were both significantly higher than that observed in the absence of fungal enzymes ($p < 0.005$). The yield of sugars produced by enzymes from strain CH-A-2010 was significantly higher than that produced by strain CH-A-2016. The superior performance of strain CH-A-2010, may be due to the fact that it produces three times more inulinase than strain CH-A-1016. As a consequence, the former is more efficient hydrolyzing inulin, which is the predominant polysaccharide in the stem of *A. tequilana* (Sanchez-Marroquin and Hope, 1953). On the other hand, comparable levels of reducing sugars were obtained by saccharification of agave leaves with enzymes from strain CH-A-2010 (8.8 g/l) and CH-A-2016 (9.9 g/l) ($p = 0.06$)

In the tequila industry, fermentable sugars for the production of ethanol are normally obtained by thermal and physical hydrolysis of *A. tequilana* stems (Pena-Alvarez *et al*., 2004). Our results indicate that considerable amounts of fermentable sugars can be obtained not only from untreated stems, but also from raw leaves by enzymatic saccharification using the isolated strains. This may be of practical importance for the tequila industry, and deserves further investigation.

Recently, a number of research projects have been focused on the use of agricultural wastes as substrate for the production of commercially important enzymes (Baig *et al*., 2003; Kahraman and Yesilada, 2001; Kansoh *et al*., 2001). However, attempts to use *A. tequilana* waste had been focused on the extraction of fibers for paper manufacturing (Idarraga *et al*., 1999; Iniguez-Covarrubias *et al*., 2001). Therefore, this is the first time that agricultural waste from the tequila industry, is used for the production of extracellular enzymes by filamentous fungi. Agro-industrial wastes normally have an extremely negative impact on the environment. In the case of the tequila industry, the problem concentrates in a very limited territory, affecting poor communities in low-technology areas, where waste management is highly inefficient. The present study strongly suggests that *A. tequilana* waste could be considered for prospective application in the production of important biotechnological enzymes. In order to improve the production of such enzymes using *A. tequilana* waste as sole carbon source, research on the optimization of the process is actually in progress.

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References

Anonymous.: Tequila Industry Statistical Information. Regulator Council of Tequila, Jalisco, Mexico (2006).

Baig, M.M.V., V.P. Mane, D.R. More, L.P. Shinde and M.I.A. Baig: Utilization of banana agricultural waste: Production of cellulases by soil fungi. *J. Environ. Biol*., **24**, 173-176 (2003).

Dobrev, G.T., I.G. Pishtiyski, V.S. Stanchev and R. Mircheva: Optimization of nutrient medium containing agricultural wastes for xylanase production by *Aspergillus niger* B03 using optimal composite experimental design. *Bioresour. Technol*., **98**, 2671-2678 (2007).

Gawande, P.V. and M.Y. Kamat: Production of *Aspergillus* xylanase by lignocellulosic waste fermentation and its application. *J. Appl. Microbiol.*, **87**, 511-519 (1999).

Hinrikson, H.P., S.F. Hurst, T.J. Lott, D.W. Warnock and C.J. Morrison: Assessment of ribosomal large-subunit D1-D2, internal transcribed spacer 1, and internal transcribed spacer 2 regions as targets for molecular identification of medically important Aspergillus species. *J. Clin. Microbiol*., **43**, 2092-2103 (2005).

Idarraga, G., J. Ramos, V. Zuniga, T. Sahin and R.A. Young: Pulp and paper from blue agave waste from tequila production. *J. Agric. Food Chem.*, **47**, 4450-4455 (1999).

Iniguez-Covarrubias, G., R. Diaz-Teres, R. Sanjuan-Duenas, J. Anzaldo-Hernandez and R.M. Rowell: Utilization of by-products from the tequila industry. Part 2: Potential value of *Agave tequilana* Weber *azul* leaves. *Bioresour. Technol*., **77**, 101-108 (2001).

Kahraman, S. and O. Yesilada: Industrial and agricultural wastes as substrates for laccase production by white-rot fungi. *Folia Microbiol.*, **46**, 133-136 (2001).

Kansoh, A.L., A. M-Ali and A. A-El-Gammal: Xylanolytic activities of Streptomyces sp 1-Taxonomy, production, partial purification and utilization of agricultural wastes. *Acta Microbiol. Immunol. Hung.*, **48**, 39-52 (2001).

Kulkarni, N.S., J.V. Jaiswal and M.G. Bodhankar: Influence of agro-waste amendment on soil microbial population in relation to plant growth response. *J. Environ. Biol.,* **28**, 623-626 (2007).

Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall: Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., **193**, 265-275 (1951).

Lyons, T.R.: Ethanol around the world: rapid growth in policies, technology and production. *In*: The alcohol textbook (*Eds.*: K.A. Jacques, T.P. Lyons and D.R. Kelsall). Nottingham University Press, Nottingham. pp. 1-8 (2003).

Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem*., **31**, 426-428 (1959).

Mtui, G. and Y. Nakamura: Bioconversion of lignocellulosic waste from selected dumping sites in Dar es Salaam, Tanzania. *Biodegradation*, **16**, 493-499 (2005).

Oliveira, L.A., A.L.F. Porto and E.B. Tambourgi: Production of xylanase and protease by *Penicillium janthinellum* CRC 87M-115 from different agricultural wastes. *Bioresour. Technol*., **97**, 862-867 (2006).

Ong, L.G., S. Abd-Aziz, S. Noraini, M.I. Karim and M.A. Hassan: Enzyme production and profile by *Aspergillus niger* during solid substrate fermentation using palm kernel cake as substrate. *Appl. Biochem. Biotechnol*., **118**, 73-79 (2004).

Patil, S.R. and A. Dayanand: Optimization of process for the production of fungal pectinases from deseeded sunflower head in submerged and solid-state conditions. *Bioresour. Technol*., **97**, 2340-2344 (2006).

Pena-Alvarez, A., L. Diaz, A. Medina, C. Labastida, S. Capella and L.E. Vera: Characterization of three *Agave* species by gas chromatography and solid-phase microextraction-gas chromatography–mass spectrometry. *J. Chromatogr. A*., **1027**, 131-136 (2004).

Ragunathan, R. and K. Swaminathan: Growth and amylase production by *Aspergillus oryzae* during solid state fermentation using banana waste as substrate. *J. Environ. Biol*., **26**, 653-656 (2005).

Raper, K.B. and D.I. Fennell: The Genus *Aspergillus*. Robert E. Krieger Publishing Co., New York (1977).

Sanchez-Marroquin, A. and H.P. Hope: Agave juices: Fermentation and chemical composition studies of some species. *J. Agric. Food Chem.*, **1**, 246-249 (1953).

Seidle, H.F., I. Marten, O. Shoseyov and R.E. Huber: Physical and kinetic properties of the family 3 beta-glucosidase from *Aspergillus niger* which is important for cellulose breakdown. *Protein J*., **23**, 11-23 (2004).

Tapia-Tussell, R., P. Lappe, M. Ulloa, A. Quijano-Ramayo, M. Caceres-Farfan, A. Larque-Saavedra and D. Perez-Brito: A rapid and simple method for DNA extraction from yeasts and fungi isolated from *Agave fourcroydes*. *Mol. Biotechnol.*, **33**, 67-70 (2006).

Villena, G.K. and M. Gutierrez-Correa: Production of cellulase by *Aspergillus niger* biofilms developed on polyester cloth. *Lett. Appl. Microbiol*., **43**, 262-268 (2006).

Yuan, X.L., C. Goosen, H. Kools, M.J. van der Maarel, C.A. van den Hondel, L. Dijkhuizen and A.F. Ram: Database mining and transcriptional analysis of genes encoding inulin-modifying enzymes of *Aspergillus niger*. *Microbiology*, **152**, 3061-3073 (2006).

Zhang, L., C. Zhao, D. Zhu, Y. Ohta and Y. Wang: Purification and characterization of inulinase from *Aspergillus niger* AF10 expressed in *Pichia pastoris*. *Protein Expr. Purif*., **35**, 272-275 (2004).