

Research Paper

## Utilization of molasses and sugar cane bagasse for production of fungal invertase in solid state fermentation using *Aspergillus niger* GH1

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### Abstract

Agro-industrial wastes have been used as substrate-support in solid state fermentation for enzyme production. Molasses and sugarcane bagasse are by-products of sugar industry and can be employed as substrates for invertase production. Invertase is an important enzyme for sweeteners development. In this study, a xerophilic fungus *Aspergillus niger* GH1 isolated of the Mexican semi-desert, previously reported as an invertase over-producer strain was used. Molasses from Mexico and Cuba were chemically analyzed (total and reducer sugars, nitrogen and phosphorous contents); the last one was selected based on chemical composition. Fermentations were performed using virgin and hydrolyzate bagasse (treatment with concentrated sulfuric acid). Results indicated that, the enzymatic yield (5231 U/L) is higher than those reported by other *A. niger* strains under solid state fermentation, using hydrolyzate bagasse. The acid hydrolysis promotes availability of fermentable sugars. In addition, maximum invertase activity was detected at 24 h using low substrate concentration, which may reduce production costs. This study presents an alternative method for invertase production using a xerophilic fungus isolated from Mexican semi-desert and inexpensive substrates (molasses and sugarcane bagasse).

**Key words:** by-products, virgin and hydrolyzated bagasse,  $\beta$ -fructofuranosidase, xerophilic.

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### Introduction

Invertase or  $\beta$ -fructofuranosidase (EC 3.2.1.26) catalyzes the hydrolysis of sucrose into glucose and fructose, acting on non-reducing fructofuranoside terminal residues of  $\beta$ -fructofuranosides. This enzyme is very important in food industry especially as a catalytic agent to obtain an artificial sweetener (Ashokkumar *et al.*, 2001). In addition, this enzyme has fructosyltransferase activity, which is important for synthesis of short-chain fructo-oligosaccharide compounds. This fact improves intestinal microflora and may prevent cardiovascular disease, colon cancer and osteoporosis (Linde *et al.*, 2009).

For years, production and kinetics of invertase enzyme have been studied using solid state fermentation (SSF) (Aguilar *et al.*, 2008; Aranda *et al.*, 2006), and/or

submerged fermentation (SmF) (Ashokkumar *et al.*, 2001, Robledo-Olivo *et al.*, 2009), these studies have revealed higher enzyme production in SSF (Aguilar *et al.*, 2008). Since 1917, *Aspergillus niger* has become a model organism for solid-state biotechnology. This microorganism is capable of producing enzymes with industrial application, including invertase (Hölker *et al.*, 2004).

Actually, great amounts of agroindustrial wastes are used for enzymes production with industrial interest using SSF (Aguilar *et al.*, 2008). Molasses and sugarcane bagasse are some by-products generated from sugar industry and these can be harnessed and converted with high valorization. Molasses is a viscous and dark liquid, final effluent obtained during the preparation of sugar by repeated crystallization (Leeson and Summers, 2000; Olbrich, 2006). Molasses chemical composition is very varied; where, su-

crose (60–63%), reducing sugars (3–5%) and trace minerals are the principal compounds (Tellez, 2004; Yopez, 1995). On the other hand, sugarcane bagasse is a residue that remains after sugarcane milling, which is realized for juice extraction (Basanta *et al.*, 2007). These by-products have been employed for animal feed, baking, fuel, paper and cardboard procurement and ethanol, lactic acid, citric acid, sorbitol productions, among others (Atiyeh and Duvnjak, 2003; Basanta *et al.*, 2007). In addition, sugarcane bagasse has been employed as good enzyme inducer, *i.e.*,  $\beta$ -glucosidase, cellulose, xylanase, tannase, inulinase and invertase (Echegaray *et al.*, 2000; Fouad *et al.*, 2005; Linde *et al.*, 2009; Mazzuti *et al.*, 2006; Olbrich, 2006; Tellez, 2004). However, to our knowledge, there are few reports about use of molasses and sugarcane bagasse for invertase production in SSF.

Based on these antecedents, the present study was carried out with the objective to utilize molasses and sugarcane bagasse for fungal invertase production, employing the invertase over-producer *A. niger* GH1 strain. The strain was isolated from rich-tannin plants from the Mexican semi-desert (Cruz-Hernández *et al.*, 2005) and has been reported as a good invertase producer (Veana *et al.*, 2011).

## Materials and Methods

### Microorganism and propagation conditions

*A. niger* GH1 (DIA/UAdC collection) was grown on potato dextrose agar at 30 °C for 4 days. The fungal spores were harvested with 0.1% of sterile Tween 80. The inoculum amount ( $1 \times 10^7$  spores/g bagasse) was determined using a Neubauer chamber.

### By-products preparation

Two samples of sugarcane molasses were evaluated: one commercially purchased molasses in México (unknown origin) and molasses proportionated for ICIDCA, La Habana, Cuba (Sugar Enterprise Uruguay, zafra 2009–2010). Sugarcane molasses were characterized through total and reducer sugar contents using the Lane-Eynon technique, nitrogen content was determined using Kjendhal technique and phosphorous content employing the ammonium metavanadate technique according to ICIDCA procedures for molasses (ICIDCA, 2009). Once, molasses composition was determined, the best molasses was selected and a nutrients balance was realized according to modified Czpek-Dox culture medium, with the following composition (g/L): NaNO<sub>3</sub> (7.65); KH<sub>2</sub>PO<sub>4</sub> (3.04); MgSO<sub>4</sub>·7H<sub>2</sub>O (1.52); KCl (1.52); and sucrose at 25 g/L, supplied by molasses sugarcane.

Prior to fermentation, the sugarcane bagasse was subjected to acid hydrolysis with concentrated sulfuric acid for 1 h boiling, in order to have greater availability of fiber and act as support in SSF. The bagasse (5 g) was placed in Erlenmeyer flasks (1 L), which were employed as reactors.

Therefore, two fermentation systems were development, one with virgin bagasse (VB) and other with hydrolyzated bagasse (HB) which was autoclaved at 121 °C for 15 min. Finally, molasses was added to both systems according nutrients balance realized previously.

### Solid State Fermentation

The flasks were inoculated with  $1 \times 10^7$  spores/g bagasse and humidity at 70%. The reactors were incubated at 30 °C. The kinetic was monitored every 24 h for 72 h. The enzymatic extracts were recuperated: 50 mL of water were added to flask and were filtered through filter paper. The enzymatic extracts were centrifuged at 1000 rpm for 15 min and filtered. The filtered was employed for enzymatic activity, sugars consumption and indirect real protein using the methodology described above.

### Enzymatic extract characterization

The invertase activity was determined by measuring the amount of reducing sugars released during the sucrose hydrolysis, using dinitrosalicylic acid reagent (Ashokkumar *et al.*, 2001). One unit of invertase (U) was defined as the amount of enzyme required to liberate 1  $\mu$ mol equivalent of reducing sugars per minute.

Sugars were determined by Lane-Eynon (ICIDCA, 2009) technique with the aim of observing substrate consumption by *A. niger* GH1 in the SSF. The substrate consumption was expressed in percentage; 25 g/L of sucrose was considered 100% of substrate.

The indirect real protein was determinate by Kjendhal technique (ICIDCA, 2009) with a previous treatment of bagasse; 1 g of bagasse was boiled in 70 mL of water and copper sulfate (25 mL) and sodium hydroxide (25 mL). The solution was cooled and filtered; bagasse washes with water were done in order to eliminate copper through the potassium ferrocyanide addition with a color change of filtered from purple to yellow-transparent. We performed a calibration curve with a standard biomass of *A. niger* Aa20 (0–60  $\mu$ g) for the determination of protein biomass of *A. niger* GH1 and absorbance at 595 nm was read; equation of the line was employed for protein obtention. The indirect protein was expressed in percentage (g of protein/ 100 g of fermented bagasse). In addition, invertase specific activity was estimated.

### Statistical analysis

A completely randomized design was proposed for molasses analysis. All results were analyzed using the general lineal procedure and comparison of means by Tukey test using SAS software. The kinetic graphics of invertase activity were plotted in Excel program of Microsoft 2010; standard deviations were added.

## Results and Discussion

Although the chemical composition of molasses was variable; according to Table 1 not statistical differences for total sugars and phosphorous contents were observed. However, Cuban molasses (Sugar Enterprise Uruguay, 2009-2010 harvest) was selected for SSF because free reducing sugars and phosphorous contents are within reference value (10-15 and 0.1-0.15%, respectively) (ICIDCA, 2009). In addition, slightly higher nitrogen content is presented.

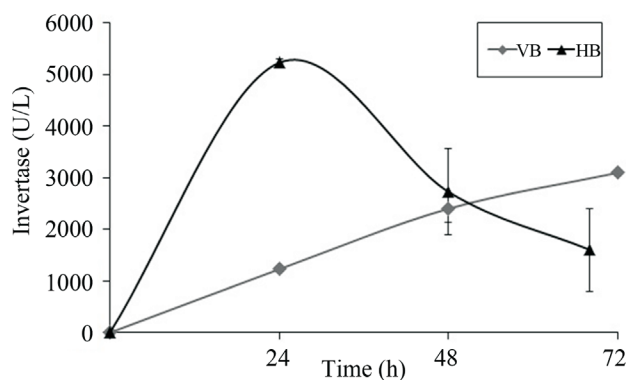
The maximum invertase activity (5232 U/L) was observed using HB (Figure 1). This can be attributed to acid hydrolysis, besides; high temperatures promote availability of fermentable sugars by lignocellulosic compounds hydrolysis (Fouad *et al.*, 2005), so bagasse becomes easily susceptible to the microorganisms attack and ultimately the enzymatic production rate increases (Javed *et al.*, 2007). Results in the present study are attributing to all these events.

Other authors have studied invertase production by *Aspergillus niger* strains. Romero-Gómez *et al.* (2000) evaluated the invertase production in SmF and SSF (employing polyurethane foam as support) with high sucrose concentration (100 g/L) using *A. niger* C28B25, *A. niger* N-402 and *A. niger* Aa20 strains. They demonstrated that using SSF system higher yield enzymatic rate in minor time are obtained, in comparison to SmF. In addition, the best enzyme producing strain was *A. niger* C28B25 (4488 U/L), followed by *A. niger* Aa20 (3411U/L) and *A. niger* N-402 (3089 U/L) in 36 and 48 h, respectively.

Asshokumar *et al.* (2001) employed sugarcane bagasse as support for invertase production by *A. niger* NRRL 330 with 50 g/L of sucrose, using SSF. The authors obtained 5890 U/L at 72 h of fermentation; this value is similar to that obtained in the present study (5232 U/L), however we detected this value at 24 h. This fact is important in industry in order to saving production cost. These also apply for lower substrate concentration (total sugars molasses concentration: 25 g/L).

Similarly, Rubio and Navarro (2006) obtained minor enzymatic yield (3000 and 4000 U/l) comparing with our results, they used sucrose (10 g/L) and glucose (5 and 10 g/L) in 48 h culture, not catabolic repression by glucose was observed.

The results obtained in this study are consistent with previous reports, the fact that invertase activity produced



**Figure 1** - Invertase activity detected during SSF using *A. niger* GH1. Sample of HB was monitored only for 68 h in SSF.

under SmF is lower than that produced in SSF (Aguilar *et al.*, 2008). Aranda *et al.* (2006) obtained invertase using *A. niger* Aa20 in SSF. These authors employed sucrose (25 g/L) and different glucose concentrations. The maximum activity of 212 U/L was obtained in glucose 100 g/L and in medium only with the inducer, 140 U/L were detected. In terms of productivity, in the present study we increased 30 times productivity than other authors using different *A. niger* strains in SmF and SSF (Aranda *et al.*, 2006; Ashokkumar *et al.*, 2001; Paranthaman *et al.*, 2008; Robledo-Olivo *et al.*, 2009; Rubio and Maldonado, 1995).

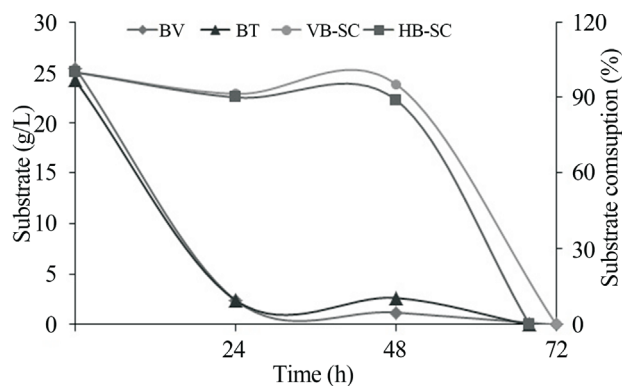
The substrate was consumed for *A. niger* GH1, according different needs and adaptation to SSF (Figure 2). At 24 h, 90% of substrate was consumed, time where the maximum invertase activity was detected in SSF with VB; at 48 h substrate was exhausted in two fermentation systems. While in SSF employing HB, a decrease in activity in the next hours was observed, which is due to insufficient substrate availability. Probably, *A. niger* GH1 begin the proteases secretion to culture medium, which degraded protein and reduced the culture yield.

Robledo-Olivo *et al.* (2009) reported that the maximum invertase activity of 3873 U/L was obtained with 12.5 g/L of sucrose followed by 25 g/L of sucrose, which recorded 2585U/L but there was no significant difference between 12.5 and 25 g/L of substrate. So, in the present study is correct the employment of 25 g/L of total sugars of molasses and comparing the invertase activity, the results are higher than those reported previously by different authors using other *A. niger* strains (Ashokkumar *et al.*, 2001; Romero-Gómez *et al.*, 2000). The maximum indirect pro-

**Table 1** - Chemical characterization of two molasses from different origin.

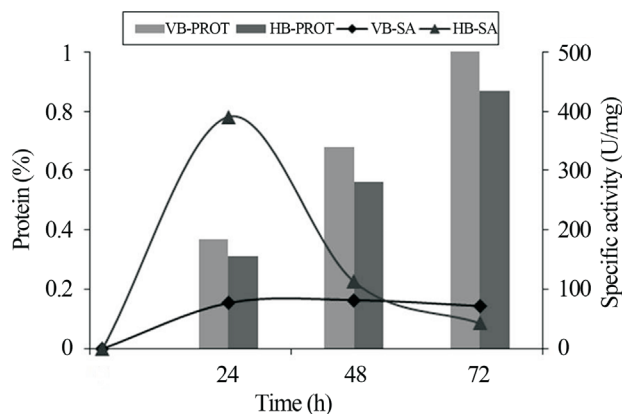
| Molasses | Total sugars (%)          | Free reducing sugars (%)  | Nitrogen (%)             | Phosphorous (%)          |
|----------|---------------------------|---------------------------|--------------------------|--------------------------|
| Mexico   | 51.63 ± 1.56 <sup>a</sup> | 18.15 ± 0.30 <sup>b</sup> | 0.28 ± 0.05 <sup>a</sup> | 0.17 ± 0.01 <sup>a</sup> |
| Cuba     | 50.58 ± 0.86 <sup>a</sup> | 12.31 ± 1.11 <sup>a</sup> | 0.43 ± 0.01 <sup>b</sup> | 0.14 ± 0.02 <sup>a</sup> |

Note: Means with same letter, in the same column, are not significantly different according Tukey test ( $\alpha = 0.05$ , 95% probability).



**Figure 2** - Percentage of substrate consumption by *A. niger* GH1 during SSF with initial concentration of sucrose (molasses) at 25 g/L. Sample of HB was monitored only for 68 h in SSF.

tein (1%) detected during SSF was present at 72 h (Figure 3) with VB and 0.87% at 68 h with HB, however this behavior is very different in specific activity, because there is not relationship between protein-invertase production. Specific activity is a parameter that indicates enzyme purity, if this value is high, indicates that of total protein content the main amount is invertase. 390 U/mg protein in crude extract during SSF at 24 h from *A. niger* GH1 was detected (Figure 3). Activity specific has been reported from crude extracts and with purification steps in previous studies and the obtained values has been lower than those reported in the present investigation. Boddy *et al.* (1993) obtained a specific activity of 143 U/mg protein in crude extract from *A. niger* B60. Different values were found by Rubio and Maldonado (1995), with a specific activity of 11.11 U/mg of protein in crude extract and 96 U/mg of protein after purification steps. Similarly, Nguyen *et al.* (2005) obtained 1.04 U/mg of protein in crude extract and 51.67 U/mg of protein in purified extract from *A. niger*



**Figure 3** - Indirect proteins (g of protein/ 100 g of fermented bagasse) and specific activity detected during SSF using *A. niger* GH1. Sample of HB was monitored only for 68 h in SSF. VB-PROT and HB-PROT: virgin bagasse and hydrolysate bagasse proteins, respectively; VB-SA and HB-SA: specific activities of virgin and hydrolysate bagasse, respectively.

IMI303386. Then, Vargas *et al.* (2004) obtained 5.18 and 5.38 U/mg of protein of *A. niger*

CCT7415 cultivated in molasses medium with 8 min of sonication and amplitude of 20 and 40, respectively. Later, Novaki *et al.* (2010) obtained 342 U/mg of protein by SSF of soybean bran using *Aspergillus caseiellus*. Comparing our results with those obtained by other authors, we observed that *A. niger* GH1 is a good alternative for invertase production in SSF using bagasse and sugarcane molasses. Specific activity detected in crude extract is high, so probably when this enzyme will be purified, purification steps will be less, therefore, the process will be cheaper.

## Conclusions

In the present study, the ability of the *A. niger* GH1 fungal strain for invertase production in less time under SSF, employing as substrates and support in SSF by-products (molasses and sugar cane bagasse) of sugar industry demonstrated. The enzyme industry can be beneficiate employing this by-products and *A. niger* GH1 fungal strain for invertase production, due to low enzyme production costs.

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