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Influence of Some Sugars on Xylanase Production by *Aspergillus awamori* in Solid State Fermentation.

Judith Liliana Solórzano Lemos[1*] **and Nei Pereira Junior**[2]

[1] Coordenação de Metalurgia Extrativa; Centro de Tecnologia Mineral; Avenida Ipê, 900; 21941-590; Ilha da Cidade Universitária - Rio de Janeiro - Brazil. [2] Departamento de Engenharia Bioquímica; Escola de Química; Universidade Federal do Rio de Janeiro; 21949-900; Ilha da Cidade Universitária - Rio de Janeiro - Brazil

ABSTRACT

Aspergillus awamori *showed high extracellular endoxylanase (100 U/ml) and β-xylosidase activities (3.5 U/ml) when grown on milled sugar cane bagasse as the principal carbon source without treatment. Partial characterization of xylanases showed that the apparent values of K_m were 3.12 ± 0.05 mg/ml for endoxylanase (in birchwood xylan) and 0.45 ± 0.05 mM for β-xylosidase (in p -nitrophenyl β-D-xylanopiranoside). Corresponding values of V_{max} were 6.63 ± 0.02 and 0.078 ± 0.02 μmol/min. Gradual repression of endoxylanase activity was observed when increasing concentrations of glucose and xylose (1, 2, 4, 6 and 8 g of carbohydrate / 4 g of sugar cane bagasse) were added to production media. In contrast, β-xylosidase activity was stimulated using low levels of carbohydrates (1 g xylose or glucose/ 4 g of sugar cane bagasse).*

Key words: *Aspergillus awamori*; endoxylanases; β-xylosidase; catabolic repression

INTRODUCTION

Amongst xylanolytic microorganisms, filamentous fungi have been more extensively studied, and the genus *Aspergillus* has been shown to be an efficient producer of xylanases on an industrial scale (Haltrich et al., 1996). Xylanases have been considered for clarifying fruit juices and wines, for food processing in combination with cellulases and for the improvement of the nutritional properties of agricultural silage and grain feed. Its main potential application though, relates to cellulosic pulp treatment where the biocatalyst has already been incorporated into commercial bleach sequences (Carmona et al., 1997).

The bioconversion of lignocellulosic materials to fermentable sugars for fuel alcohol production has been hindered by economical and technical aspects, and by the existence of more competitive sources of carbohydrates such as starch and sucrose. Nevertheless, there has been much effort in the sense of developing efficient bioprocesses using such raw materials, particularly those deriving from agricultural residues, which have no production cost attached to them, although costs for collection and transportation of these residues to centralized processing location may be incurred.

Genetic manipulation by classical mutation techniques, and more recently by use of recombinant DNA technology, have been used to increase the expression levels of a large number of microbial proteins (Walsh and Headon, 1994). Nonetheless, the use of modern techniques to improve the production of metabolites neither invalidate the search for wild microorganisms nor

* Author for correspondence

the effort to evaluate the potential of those already known to produce useful metabolites. Several enzymes are synthesized always, regardless of the conditions of the medium; they are constitutive enzymes. In contrast to these, there are inducible and repressible enzymes. The enzymes involved in substrate degradation are generally inducible. They are formed only when the corresponding substrate is present in the nutrient solution (Schlegel, 1989). Fungal, bacterial and actinomycete xylanolytic enzymes are generally induced by xylan, xylobiose, xylose and by lignocellulosic residues that contain xylan (Flores *et al*,. 1996; Archana and Satyanarayana, 1997; Kermnický and Biely, 1998).

The extension of induction is dependent upon the nature and the concentration of these carbon sources. An increase in substrate concentration is usually favorable to product synthesis, however, many fermentation products are subjected to carbon catabolite repression, that is, repression of the enzyme synthesis by easily metabolizable sugar (Piñaga et al., 1994; Flores *et al*,. 1996; Kadowaki et al., 1997; Archana and Satyanarayana, 1997; Kermnický and Biely, 1998). This phenomenon has been evidenced in many microorganisms, and widely studied in Saccharomyces cerevisiae. Nevertheless, the mechanism by which CREA (mediating factor for carbon catabolite repression) mediate glucose repression in filamentous fungi is not wholly understood (Ruijter and Visser, 1997). It is possible that the mechanism in filamentous fungi has some similarity to that in *S. cerevisiae*, where glucose repression is mediated by a protein that involves nucleosome positioning (Archer and Peberdy, 1997).

Considering the industrial importance of xylanases, the present study evaluated the use of milled sugar cane bagasse as carbon source, and the influence of xylose and glucose on the production of these enzymes by *Aspergillus awamori.*

MATERIALS AND METHODS

Organism and growth conditions

Aspergillus awamori was maintained on sterile sand for conservation. Conidia were generated by growing the fungus on agar tubes with Czapeck media. After 5-6 day cultivation at 30 ºC, conidia were scrapped off and transferred to sterile distilled water. This conidia suspension was used for medium inoculation in a final concentration of $5x10^6$ conidia/g bagasse.

Growth media and culture conditions

Fermentations for enzyme production were carried out in 500 ml conical flasks containing 4 g of roughly 1 mm-sized dry sugar cane bagasse, ground in a disc mill (Perten, Laboratory mill 3600). The sugar cane bagasse was moistened with 50 ml of an aqueous solution composed of (g/4g of bagasse): $NaNO_3$, 0.39; yeast extract, 0.5; NaCl, 0.2; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.04. The flasks were incubated in na incubator (FANEM, Retilinea, Brazil) at 30 ºC for 2.5 to 6 days when their whole content was extracted by adding 100 mL of distilled water. After a period of 30 minutes under agitation at 150 rpm and room temperature, the supernatant was separated by filtration and used for determination of enzymatic activities.

Enzymatic assays

Endoxylanase was determined by measuring the reducing sugars from hydrolysis of birchwood xylan (Sigma, St Louis, MO). 0.9 ml solution of 1% xylan was pre-incubated in 50 mM citrate phosphate buffer, pH 5, for 2 min at 60 ºC. To this solution, 0.1 ml of the diluted crude enzyme was added and the reaction stopped after 2 min by the addition of 1 ml DNS solution. One unit of activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar (xylose) per minute. **β-xylosidase** activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl β-D-xylanopiranoside (PNPX) from Sigma (St Louis, MO). The enzyme solution (0.1 ml) was incubated in a test tube with 0.5 ml of 1.25 mM PNPX (pre-incubated for 2 min) in 50 mM phosphate citrate buffer, pH 5 at 55 ºC. The reaction was stopped after 10 min by addition of 4 ml Na_2CO_3 0.25 M. One unit of activity was defined as the amount of enzyme required to release 1 μmol of p-nitrophenol per minute.

Kinetic parameters

In order to evaluate the kinetic parameters for endoxylanase and β-xylosidase, a microcomputer program (Oestreicher and Pinto, 1987) was used to

determine the degradation of birchwood xylan and PNPX using the following concentrations: 0.12, 0.20, 0.60, 0.75, 1.0, 1.50, and 3.0 % w/v for birchwood xylan, and 0.31, 0.41, 0.63 and 1.25 mM for PNPX. Both substrates were incubated with an appropriate diluted crude extract for up to 8 min for endoxylanase and for up to 130 min for β-xylosidase at 60 and 55 ºC, respectively. The computer program, written in BASIC can be run on any microcomputer with the CP/M operating system. Weighting of observed initial velocities is decided by the user by assessing constant variance, proportional variance or by incorporation of the variances calculated by a subroutine. The program also uses robust regression by bisquare weighting. All questions concerning data input, type of rate function, type of weight and the use of bisquare regression appear on the video display unit.

The program can be run on a microcomputer with less than 48 K of random access memory and is based upon a nonlinear least-square regression method, which uses a re-iterative Gauss-Newton procedure to refine initial estimates of the parameters generated by the same program.

RESULTS

Xylanases have been widely studied using the most different carbon sources. However, the activity of these enzymes have not been so expressive when the microorganisms were cultivated in sugar cane bagasse without any treatment. Table 1 showed the xylanase production by some of the organisms that used bagasse as carbon substrate. Figure 1 showed the estimation of kinetic parameters using Hanes Wolff plot. An analysis of the apparent kinetic parameters (Table 2) using both the microcomputer program and the Hanes Wolff plot revels that the results do not differ significantly from each other. The role of glucose and xylose as components of the media for xylanase production was also analyzed. Figure 2 showed that the higher the xylose and glucose concentrations were in the culture medium, the higher was the catabolic repression on endoxylanase activity.

Table 1 - Production of endoxylanase and β-xylosidase induced by sugar cane bagasse without treatment.

Strains	Endoxylanase ($UL^{-1}h^{-1}$)	β-xylosidase	Fermentation (time)	Reference
Aspegillus ochraceus	8.5 Uml^{-1} (51) 18.6 Uml^{-1} (48)	2.4 Uml^{-1} 3.8 Uml^{-1}	S [b] (7 days) SS [c] (16 days)	Biswas et al. 1988
Penicillium janthinellum	38.2 Uml^{-1} (796)	-	S [b] (48 h)	Milagres et al. 1993
Aspergillus sydowii	11.3 Umg^{-1}	2.2 Umg^{-1}	S [b] (4 days)	Gosh et al. 1993
Aspergillus sp	1.9 Uml^{-1} (16)	-	S [b] (5 days)	Wang et al. 1994
Thermomyceslanuginosus	824.5 Ug^{-1} (446)	-	SS [c] (7 days)	Alam et al. 1994
Thermoascus aurantiacus	292.6 Ug^{-1} (158)	-	SS [c] (7 days)	
Aspergillus fischeri Fxn1	10.7 Uml^{-1} (111)	-	S [b] (4 days)	Raj and Chandra 1995
Cellulomomas flavigena	18 Umg^{-1}	60.0 Umg^{-1}	S [b] (40 h)	Pérez-Avalos et al. 1996
Aspergillus tamarii	16.7 Uml^{-1} (139)	0.28 Uml^{-1}	S [b] (5 days)	Kadowaki et al. 1997
Trichoderma reesei	1900 Ug^{-1} [a] (1510)	-	SS [c] (72 h)	Gutierrez-Correa and Tengerdy 1998
Aspergillus niger	1750 Ug^{-1} [a] (1392)	-	SS [c] (72 h)	
Aspergillus phoenicis	0 Ug^{-1} [a] (0)	-	SS [c] (72 h)	
Aspergillus awamori	100 Uml^{-1} (1660) 60.5 Umg^{-1} 3750 Ug^{-1}	3.5 Uml^{-1} 2.1 Umg^{-1}	SS [c] (60 h)	Present work

[a] Alkali treated bagasse; [b] submerged fermentation; [c] solid state fermentation.

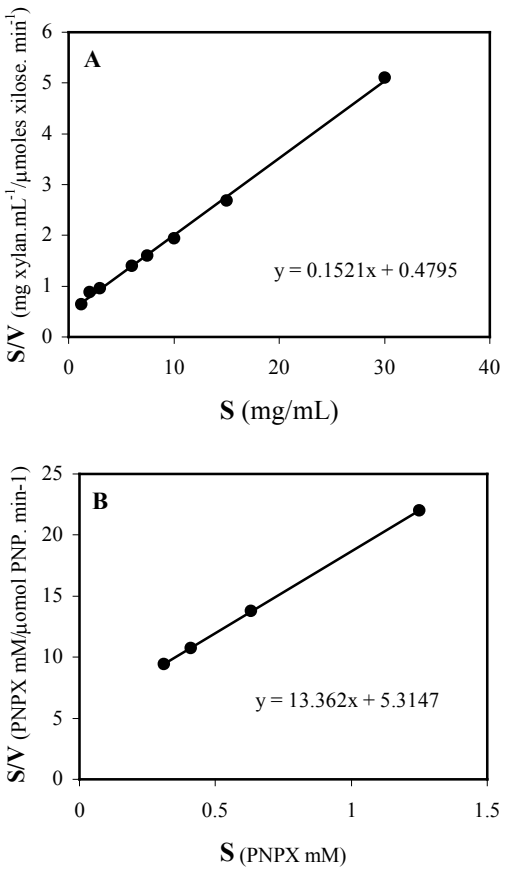

Figure 1 - Hanes Wolff plot for the determination of kinetic parameters for endoxylanase (**A**) and β-xylosidase (**B**).

Table 2 - Comparison of the kinetic parameters obtained from a computer program and Hanes Wolff plot

Endoxylanase		
Methods	K_m	V_m
Comp. Program	3.12 ± 0.05 mg/ml	6.63 ± 0.02 µmol/min
Hanes Wolff	3.15 ± 0.01 mg/ml	6.57 ± 0.01 µmol/min
β-xylosidase		
Methods	K_m	V_m
Comp. Program	0.45 ± 0.05 mM	0.078 ± 0.002 µmol/min
Hanes Wolff	0.4 ± 0.01 mg/ml	0.075 ± 0.001 µmol/min

Addition of glucose and xylose to the culture media containing another carbon source is known to affect negatively the enzymatic synthesis of many microorganisms (Flores *et al*,. 1996; Archana and Satyanarayana, 1997). Nevertheless, the influence of low concentrations of these carbohydrates on β-xylosidase production (Figures 2C and 2D) displayed different behavior. Apparently, they stimulated the enzyme activity. The time course of β-xylosidase production by *A. awamori* was studied in experiments containing 1 g of xylose or glucose / 4 g of bagasse (Figure 3). Results were compared with those obtained in a control experiment without any other carbon source, and they corroborated that low concentrations of easily metabolizable sugars improved the level of β-xylosidase production.

DISCUSSION

Table 1 showed extracellular xylanase production by several microorganisms. Our results showed that *A. awamori* grown on an inexpensive and abundant natural substrate in Brazil was able to produce high levels of hydrolytic enzymes, specially endoxylanase (100 U/ml) to a greater extent than other microorganisms also inoculated on untreated sugar cane bagasse. It could be important to point out that the superiority of xylanase production by *A. awamori* might be also evidenced by the volumetric productivity, 1660 $UL^{-1}h^{-1}$ for endoxylanase (Table 1). According to Gosh et al. (1993), agricultural residues efficiently induced the production of xylanases. In agreement with this, it could be noted that the results obtained with *A. awamori* reflected the high potential of sugar cane bagasse as an external stimulus on the production of xylanase.

The data presented in Figure 2A and 2B showed that the biosynthesis of endoxylanase decreased gradually when increasing concentrations of glucose or xylose were added. This inhibition of endoxylanase synthesis could be interpreted in terms of catabolite repression likewise described for other enzymes (Beguin and Aubert, 1994; Pereira-Meirelles et al. 1997; Siqueira et al. 1997; Kermnický and Biely, 1998). Catabolite repression refers to the repression of enzyme synthesis by glucose or other easily metabolized sugars.

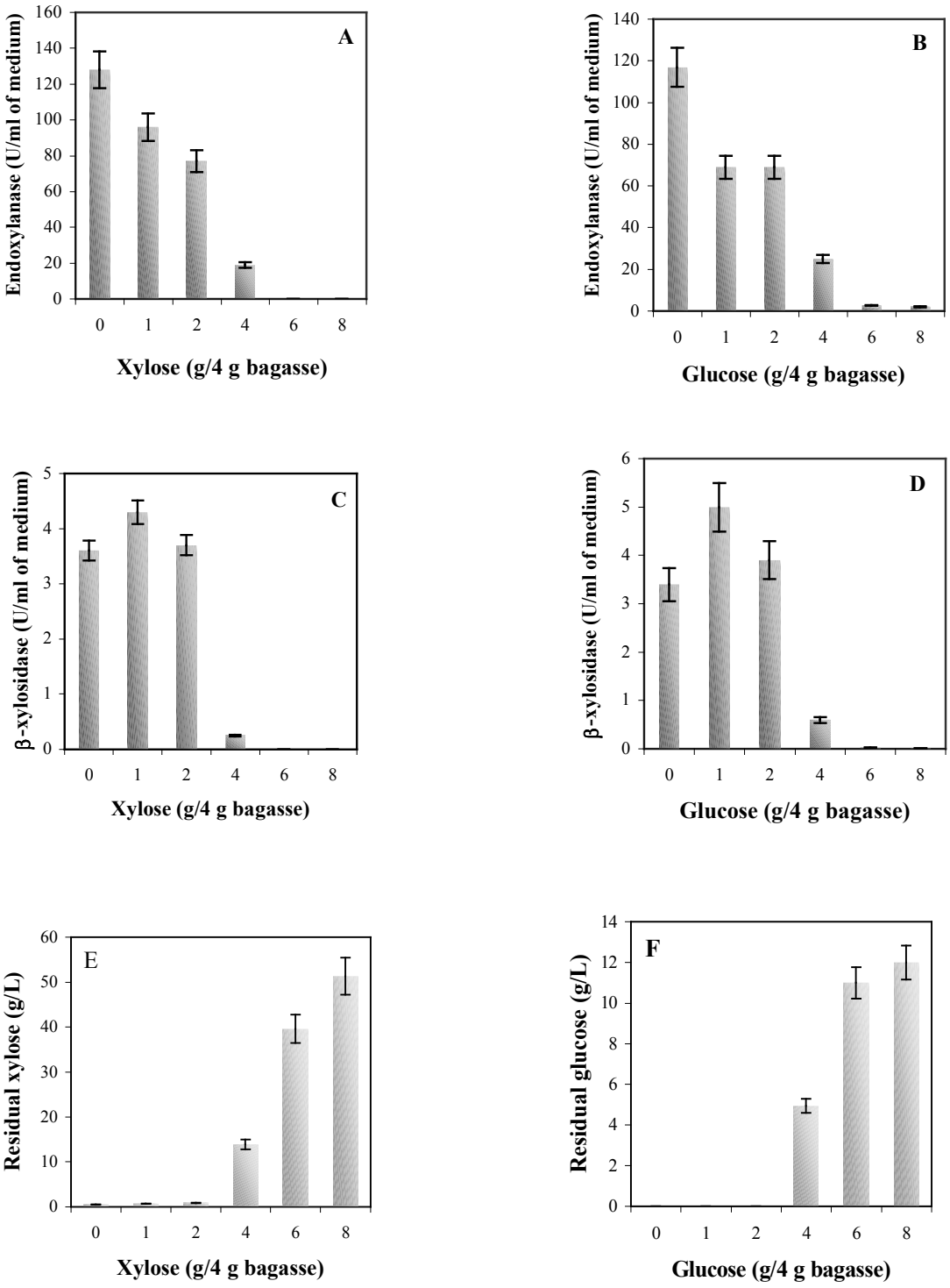

Figure 2 - Effect of xylose and glucose concentrations on endoxylanase (A and B) and β-xylosidase (C and D) production by *A. awamori* growing on sugar cane bagasse (4g/flask), on batch fermentation, during 2.5 days at 30 ºC, and pH 5.5. Residual xylose (E) and glucose (F) were also measured.

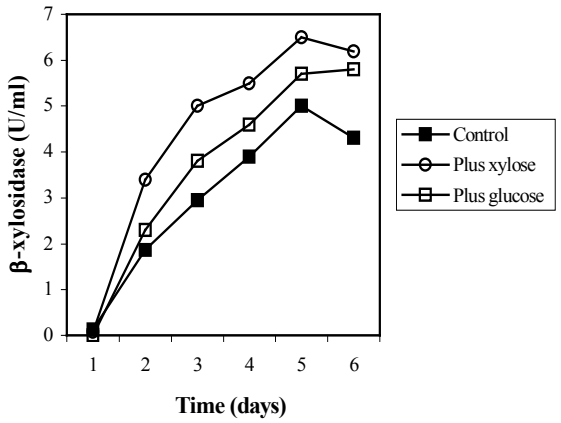

Figure 3 - Time course of β-xylosidase during incubation of *A. awamori* in media containing bagasse (■); bagasse plus 1 g xylose/4 g bagasse (O), and bagasse plus 1 g glucose/4 g bagasse ( ).

It is believed that some product of glucose metabolism, rather than glucose itself, is involved, because of the analogous effects of other easily metabolized sugars.

In contrast to the result obtained with endoxylanases, addition of 1.0 g xylose or glucose / 4 g of sugar cane bagasse caused an unusual increase in β-xylosidase expression, and 2.0 g xylose or glucose/4 g of sugar cane bagasse did not cause any effect on the activity of the enzyme when compared to the control (Figures 2C and 2D). However, 4.0, 6.0 and 8.0 g xylose or glucose/4 g of sugar cane bagasse strongly repressed β-xylosidase production.

These results suggested that a low supply of any easily metabolizable sugar triggered β-xylosidase induction. According to Flores et al. (1996), xylose has two possible effects. If xylose is added as sole carbon source it induces β-xylosidase biosynthesis, if xylose is added to a medium containing birchwood xylan it affects the enzymatic synthesis negatively, probably acting as end product repressor. Concerning to endoxylanase, probably, the repression by glucose or xylose is not solely related to the presence of sugar in the media (Figures 2E and 2F), considering that at low concentrations, carbohydrates were almost completely consumed. It was noted that although the consumption of glucose was higher than the consumption of xylose, the repressive effect on the activity of endoxylanase was similar for both monosaccharides.

Unfortunately, it is not yet possible to set down a final version of the repression mechanism complete in all respects. It is possible, however, to present, in outline, features of the process which are probably quite valid. In the first place, it is believed that all regulation of enzyme synthesis takes place by means of repression. Furthermore, repression seems to take place at the level of transcription of information between DNA and mRNA, by interfering with the synthesis of mRNA at the DNA template. Therefore, repression leads to a failure to make mRNA as well as protein (Gadd, 1988).

The time course of β-xylosidase production was studied in a following experiment to corroborate those results (Figure 3). Once again it was demonstrated that β-xylosidase biosynthesis was positively affected by low concentrations of carbon sources, probably by supporting the fungus growth, and thus allowing a higher production. Sunna and Antranikia (1997) commented that most of the reported β-xylosidases are completely inhibited by xylose. Nevertheless, in contrast to that consideration, *A. awamori* was able to produce a considerable amount of this enzyme when grown in media containing low xylose or glucose concentrations.

From the results presented in this study it is evident that, xylanases can be produced by developing an inexpensive process in solid state fermentation on sugar cane bagasse. The endoxylanase activity reported in this paper is higher than those reported by other researches using the same raw material. In addition, xylose and glucose exerted catabolic repression on endoxylanase but not on β-xylosidase activity at low concentrations.

RESUMO

O cultivo de *Aspergillus awamori* em bagaço de cana, finamente dividido e sem tratamento prévio, favoreceu a produção de níveis elevados de endo-xilanase (100 U/ml) e β–xilosidase (3.5 U/ml) extracelulares. A caracterização parcial das enzimas xilanolíticas permitiu estabelecer os parâmetros cinéticos aparentes, sendo K_m igual a 3.12 ± 0.05 mg/ml para endox-xilanase (xilana birchwood) e 0.45 ± 0.05 mM para β-xylosidase (p-nitrofenil β-D-xilanopiranosideo). Os correspondentes valores de V_m foram

6.63 ± 0.02 e 0.078 ± 0.02 μmol/min. Observou-se uma repressão gradual da atividade endo-xilanásica quando concentrações crescentes de glicose e xilose (1, 2, 4, 6 e 8 g de carboidrato / 4 g de bagaço de cana) foram adicionadas aos meios de produção. Contrariamente, a atividade β-xilosidásica foi estimulada quando níveis baixos de açúcares simples foram utilizados (1 g de xilose ou glicose/ 4 g de bagaço de cana).

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