Appl Biochem Biotechnol (2010) 162:1612–1625
DOI 10.1007/s12010-010-8942-z

Use of Mesophilic Fungal Amylases Produced by Solid-state Fermentation in the Cold Hydrolysis of Raw Babassu Cake Starch

Aline Machado de Castro · Thiago Vetter de Andréa · Leda dos Reis Castilho · Denise Maria Guimarães Freire

Received: 26 October 2009 / Accepted: 23 February 2010 /
Published online: 20 March 2010

Abstract Amylases constitute one of the most important groups of industrial enzymes, presenting several applications, such as in the food, textile, and ethanol manufacturing. In this work, a starchy residue from the Brazilian agroindustry, namely babassu cake, was used for the production of amylases by solid-state fermentation and for obtaining sugar hydrolysates, which can be used as building blocks for future bioconversions. Eight filamentous fungi from the genera *Aspergillus* and *Penicillium* were screened. Regarding amylase production, *A. awamori* strains showed well-balanced endoamylase and exoamylase activities, while *A. wentii* produced an amylolytic complex much richer in the endo-acting enzymes. Simultaneous liquefaction and saccharification using the crude enzyme extracts from the four most promising fungal strains was then investigated applying DOE techniques. The highest total reducing sugar content (24.70 g L^{-1}) was obtained by the crude extract from *A. awamori* IOC-3914, corresponding to a hydrolysis yield of 52% of total starch in the cake, while *A. awamori* IOC-3915 produced the most appropriate extract in terms of glucose release (maximum of 5.52 g L^{-1}). Multivariate analysis of the DOE studies indicated that these extracts showed their best performance at 50–57 °C under acid conditions (pH 3.6–4.5), but were also able to act satisfactorily under milder conditions (36 °C and pH 5.0), when TRS and glucose released were about 65% of the maximum values obtained. These data confirm the high potential of the enzyme extracts under study for cold hydrolysis of starch.

A. M. de Castro (✉)
Research and Development Center, Renewable Energy Division, PETROBRAS, Av. Horácio Macedo, 950. Ilha do Fundão, Rio de Janeiro, Brazil 21941-915
e-mail: alinebio@petrobras.com.br

A. M. de Castro · T. V. de Andréa · L. dos Reis Castilho
COPPE, Chemical Engineering Program, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

D. M. G. Freire
Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Keywords Mesophilic amylases · Babassu cake · *Aspergillus* · Filamentous fungi · Raw starch hydrolysis · Desirability function · Cold hydrolysis · Simultaneous liquefaction and saccharification

Introduction

Babassu (*Orbygnia* sp.) is a palm, which is cultivated in Brazil due to its potential industrial applications, such as in the food, detergent, and cosmetic sectors [1, 2] and more recently, as a source of oil for biodiesel production [3, 4]. In 2007, 115 million tons of babassu nut were harvested in Brazil, mostly in the Northeastern region [5].

According to Gombert et al. [6], the process for oil extraction generates a cake, composed mainly of protein (23%), carbohydrates (62%), and residual lipids (4.5%). It is estimated that for each ton of pressed babassu nut, 0.34 ton of cake is obtained [2].

The babassu cake, due to its composition, can be used for the production of several enzymes. It is reported that the starch content in the fermentation system is directly related to amylase synthesis, and that the yields for its production increased with further starch supplementation [6]. These enzymes act synergistically to break down the starch polysaccharides (linear-chain amylose and branched-chain amylopectin) to glucose. The complex is composed of endoamylases, exoamylases, and debranching enzymes. Endoamylases are liquefying enzymes, composed mainly of α-amylases (EC 3.2.1.1), which attack randomly the internal α-1,4 linkages, liberating oligossacharides of various lengths. Exoamylases are saccharifying enzymes composed mostly of glucoamylases (EC 3.2.1.3), which cleave primarily α-1,4 bonds at the chain terminals, liberating glucose as the main product, but can also break down α-1,6 linkages; and debranching enzymes, which act predominantly on α-1,6 linkages, such as pullulanase (EC 3.2.1.41) [7, 8].

Enzymes from the amylolytic complex present several industrial applications, such as in the food, textile, paper, detergent, and ethanol industries [9]. However, the use of amylases for ethanol production from corn starch processing, for example, usually requires high temperatures, for both starch gelatinization (up to 165 °C), liquefaction (80–90 °C), and saccharification (55–60 °C). Furthermore, the optimal pH values for the action of endoamylases and exoamylases are different, so that acidic solutions have to be added to adjust for suitable conditions between the first and second steps of hydrolysis [10–13]. These facts contribute to a higher energy demand and to chemicals consumption, which, added to the relevant costs of the enzyme and the main raw material, result in high production costs [14]. Regarding the energy requirement, Robertson et al. [13] has introduced the concept of fermentation-excess enthalpy, demonstrating that for traditional processes, which use high-temperature stages, the energy demand, apart from that necessary for the fermentation (commonly at 30 °C), corresponds to up to 20% of the energy contained in the produced ethanol.

Therefore, the objective of this work was to investigate the potential of an agroindustrial residue generated in Brazil, babassu cake, for the production of amylases by solid-state fermentation (SSF) and as a raw material to obtain fermentable sugars by cold hydrolysis process promoted by the enzyme extracts obtained by SSF. Focus was given on obtaining enzyme complexes active at temperatures below 63–73 °C, which do not cause gelatinization of babassu cake starch [15], so that the enzymes can be used for cost-effective simultaneous liquefaction

and saccharification processes, characterized by lower energy demands and lower capital investments than the traditional processes.

Materials and Methods

Raw Material

Babassu cake was kindly provided by TOBASA Bioindustrial de Babaçu S.A. (Tocantinópolis, Brazil). The cake was received with a mean particle size of (923±7) μm, which was estimated using a vibratory shaker (Viatest model 76773, Kuhardt, Germany) coupled with sieves from 8 to 150 mesh Tyler. Its C/N ratio (13.8) was determined using an elemental analyzer (model 2400 CNH from Perkin Elmer, Waltham, USA). A starch content of 50±2% was considered according to Baruque Filho et al. [15].

For both the solid-state fermentation and hydrolysis studies, the cake was dried, grounded, and sieved to obtain particles in the range of 210 to 297 μm (65 and 48 mesh Tyler, respectively).

Microorganism Maintenance

The strains evaluated in this work were isolated by Freire [16] from Brazilian soils (*Penicillium verrucosum* 8AF and *Aspergillus parasiticus*) or obtained from the Instituto Oswaldo Cruz (IOC) culture collection (*A. wentii* IOC-185, *A. sulphureus* IOC-4047, *A. niger* IOC-4003, *A. awamori* IOC-230, *A. awamori* IOC-3914, and *A. awamori* IOC-3915). Before inoculation, all fungi were propagated for 7 days at 30 °C in starch agar medium (in g L^{-1}, anhydrous mass: starch,10; sodium nitrate, 3; monopotassium phosphate, 1; magnesium sulfate, 0.5; iron sulfate, 0.001; and agar, 20).

Solid-state Fermentation (SSF) Experiments

Fungal spores (1.0×10^7/g of raw material) were inoculated in lab scale tray bioreactors containing 2.5 g of babassu cake. The initial moisture content was adjusted to 70%. The trays were incubated in humidified incubators for 96 h at 30 °C. Regularly, whole trays were taken as samples and submitted to enzyme extraction for 30 min at 37 °C and 200 rpm, using distilled water or Universal buffer [17] at pH values ranging from 3.6 to 6.4 (according to the hydrolysis experiments), followed by centrifugation for 20 min, at 25 °C and 11,000 *g*. Supernatants were aliquoted and frozen for further assays or for the hydrolysis experiments. All experiments were done in duplicate.

Simultaneous Liquefaction and Saccharification Experiments for Babassu Cake Cold Hydrolysis

Samples of 250 mg of babassu cake were incubated at 200 rpm in conic flasks at different temperature and pH values, using the design of experiments (DOE) techniques. A central composite design (CCD), as described in Table 1, was adopted. Enzyme extracts from different fungi containing 0.2 g L^{-1} of sodium azide (to prevent contamination) were added at a liquid:solid ratio of 12:1 to the samples. After 24 h, the contents of the flasks were centrifuged at 11,000 *g* for 3 min, the supernatants were boiled for enzyme inactivation and

Table 1 Central composite design for babassu cake hydrolysis.

Run	pH	Temperature (°C)
1	4.0	40
2	4.0	60
3	6.0	40
4	6.0	60
5	3.6	50
6	6.4	50
7	5.0	36
8	5.0	64
9 (CP)	5.0	50
10 (CP)	5.0	50
11 (CP)	5.0	50

CP, Central points

stored at −20 °C for further analysis. The results were analyzed using the software Statistica 7.0 (StatSoft Inc., Tulsa, USA).

Assays

All enzymatic analyses were based on previously reported methodologies, but in this work they were carried out at 40 °C, in order to determine the real potential of the enzymes to act at relatively mild conditions. Before measuring activity of samples, kinetic profiles were constructed for all assays to guarantee that reactions were carried out under initial rate conditions. All analyses were done in triplicate. Data are expressed as mean±1 standard deviation (SD).

Endoamylases were quantified according to Figueira and Hirooka [18] and Fernandes et al. [19]. The enzymatic extract (10 μL) was incubated for 3 min with 90 μL of 0.5% (m/v) soluble starch (Vetec, Rio de Janeiro, Brazil) solution in universal buffer (pH 5.0, containing 25.8 mL of 20-mM sodium hydroxide solution for each 74.2 mL of 40 mM of phosphoric, acetic, and boric acid solutions), followed by the addition of 90 μL of an iodometric reagent (Laborlab, São Paulo, Brazil) to quantify the residual starch. Finally, the absorbance was measured at 640 nm. One endoamylase activity was defined as the amount of enzyme that liquefies 1 mg of starch per minute, under the assay conditions.

Exoamylase activity was determined based on Riaz et al. [20], incubating 10 μL of enzyme extract with 90 μL of 1% (m/v) soluble starch solution in universal buffer (pH 5.0) for 10 min. Enzymes were inactivated by incubation for 5 min in a boiling water bath. The glucose released was quantified using a glucose measuring kit (Laborlab, São Paulo, Brazil), and the final absorbance was measured at 505 nm. One enzyme unit was defined as the amount that liberates 1 μmol of glucose per minute, under the assay conditions.

Protease activity was determined according to Bendicho et al. [21] and Charney and Tomarelli [22]. Enzyme extract (50 μL) was incubated with 500 μL of a 0.5% azocasein (Sigma Aldrich, St Louis, USA) solution in universal buffer at pH 5.0, for 5 min. Reaction was stopped by adding 1 mL of a 1 M HCl solution. One enzyme unit was defined as the amount of protease that promotes an increase of one absorbance unit (at 345 nm) per minute, under the assay conditions.

Total extracellular protein content was measured using the Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, USA), according to a previously described methodology [23]. BSA (Sigma Aldrich, St Louis, USA) was used as standard.

Total reducing sugars (TRS) concentration was determined using 3,5 dinitrosalicylic acid, according to the method described by Miller [24], using glucose as standard. Finally, glucose liberated during hydrolysis experiments was quantified using the same kit described for exoamylase activity determination.

Results and Discussion

Babassu Cake Fermentation for Enzyme Production

The profiles for endoamylases and exoamylases production are presented in Fig. 1. The yields are expressed as enzyme units per gram of initial dry babassu cake. Although many studies quantify amylolytic enzymes at higher temperatures, such as 50 [19, 25], 55 [26], 60 [27, 28], and 65 °C [29], in this work the enzyme activities were measured at 40 °C in order to evaluate the potential of filamentous fungi to produce enzymes that could act at relatively low temperatures.

It can be observed in Fig. 1 that the highest levels of exoamylases were achieved by the fungus *A. niger* and the three strains of *A. awamori*. Concerning the endoamylases, *A. wentii* and *A. awamori* IOC-3914 showed very similar activity profiles, but the latter presented a steadily increasing profile of up to 96 h of fermentation, whereas in the case of *A. wentii*, activity started decreasing at 72 h.

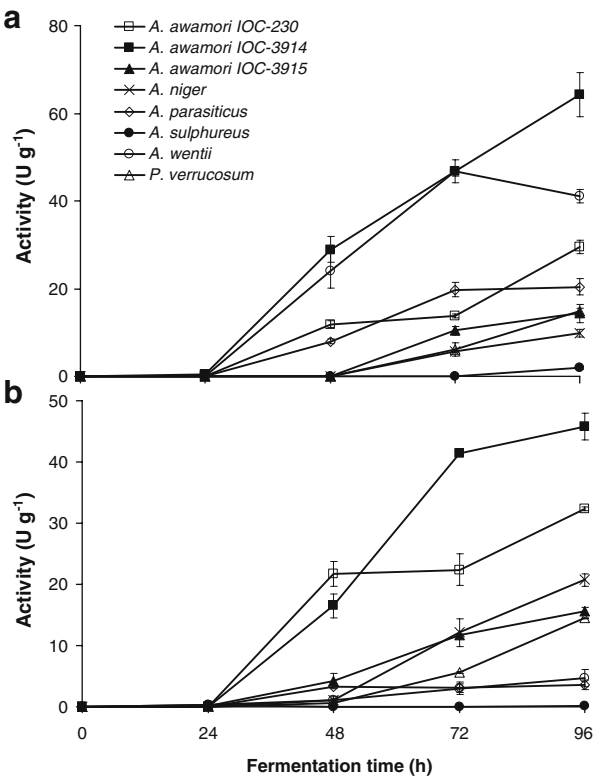

Fig. 1 Profiles for endoamylases (**a**) and exoamylases (**b**) production by filamentous fungi from babassu cake

According to these results, the babassu cake proved to be a promising raw material for the production of mesophilic amylolytic enzymes. This may be due to its adequate C/N ratio (13.8). Gombert et al. [6] compared the production of glucoamylase and other enzymes using a babassu cake with a C/N ratio of 12.4, and supplemented it to obtain different C/N ratios. The authors observed the best results when the carbon content was 14.1 times higher than the nitrogen content, a value very close to that observed in the present study.

The potential of agroindustrial materials for the production of amylases has been described by several authors in the literature. Ertan et al. [30] reported the potential of *Penicillium griseofulvum* to produce a mesophilic α-amylase (optimally active at 40 °C) from rice bran. Ramachandran et al. [31] compared the production of α-amylase by *A. oryzae* IFO-30103 using several agroindustrial residues: coconut oil cake, sesame oil cake, groundnut oil cake, palm kernel cake, olive oil cake, and wheat bran. The last one promoted the highest amylase production, which was increased to about 8% when the wheat bran was combined with groundnut oil cake.

In order to better characterize the behaviour of the eight evaluated strains in babassu cake, the protease profiles along fermentation time were determined and are shown in Fig. 2. The babassu cake has a low protein content (2.3%) that could induce the production of such enzymes [6]. The highest proteolytic activities were observed when *P. verrucosum* and *A. parasiticus* were used. The three strains of *A. awamori* and *A. niger*, which produced higher levels of amylases, did not promote high levels of proteases. *A. sulphureus*, besides being a poor amylase producer, showed also low levels of proteases. It was visually observed that this strain rarely grew in the raw material, probably due to its poor ability to metabolize the babassu cake components and/or to adapt to the limited water-content environment.

Table 2 presents the maximum specific activities (ratio of activity to total protein) and the time of incubation when they were observed. The values indicate that *A. awamori* IOC-230 and *A. awamori* IOC-3914 are the fungi with the highest potential for the production of amylolytic complex. Considering a 95% confidence interval (α= 0.05), the exoamylase-specific productions are equivalent for both strains, but the endoamylase are not. Based on this and on the fact that for both endo- and exoamylases, the highest activities were produced by *A. awamori* IOC-3914 (Fig. 3), this fungal strain was considered the most promising amylase producer from babassu cake, among the eight strains tested in this work.

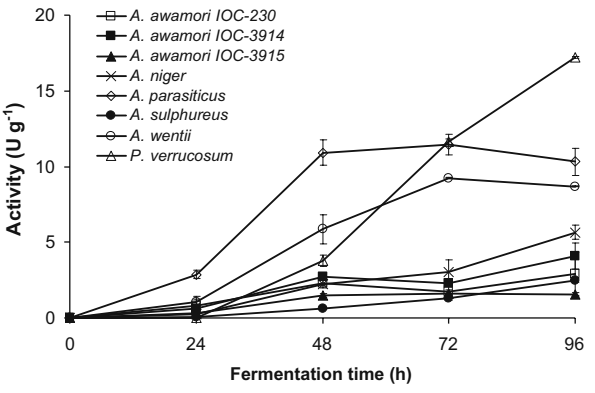

Fig. 2 Protease production by the eight filamentous fungi from babassu cake

Table 2 Maximum specific activities for enzymes produced by five filamentous fungi in babassu cake. Values in parentheses correspond to time of fermentation (h) when the values were observed.

Strain	Maximum specific activity (U mg^{-1}protein)		
	Endoamylase	Exoamylase	Protease
A. awamori IOC-230	5.97±0.41 (48)	10.90±1.03 (48)	2.87±0.83 (24)
A. awamori IOC-3914	14.86±1.51 (48)	8.49±1.01 (48)	1.41±0.12 (48)
A. awamori IOC-3915	5.15±1.16 (72)	6.42±0.05 (72)	1.75±0.12 (48)
A. niger	0.66±0.06 (96)	1.40±0.07 (96)	0.38±0.03 (96)
A. wentii	6.15±0.14 (72)	0.49±0.14 (96)	1.22±0.01 (72)

Although the production of amylases by some strains continuously increased up to 96 h of fermentation, it was observed that the specific endoamylase and exoamylase activities reached their maximum values commonly in shorter times. This fact is important for decision-making, since concomitant production of proteases is undesirable. For example, at 48 h of fermentation, for *A. awamori* IOC-3914, the protease specific activity was between six and ten times lower than the specific activities for amylases.

Variations in pH along fermentation are also important to evaluate, due to their possible effects on enzyme activity and stability. Figure 3 shows the pH profiles for the eight fungi. The initial pH was 5.80±0.05, in all cases. Concerning the gradual pH decrease observed for the three *A. awamori* strains until 48 h of fermentation, it may be due to the production of organic acids. Kumari et al. [32] studied rice straw degradation by a strain of *A. awamori* and detected the synthesis of citric, oxalic, formic, and maleic acids. The profiles for the other five fungi showed a continuous pH increase during the whole period, which is probably related to proteases production, resulting in hydrolysis of peptides and consequent liberation of ammonia [6].

Babassu Cake Hydrolysis

The crude extracts produced by the three *A. awamori* strains, which presented the best yields for amylase production, were used for the evaluation of their potential for babassu cake hydrolysis. In addition, due to the low protease level and to results from preliminary

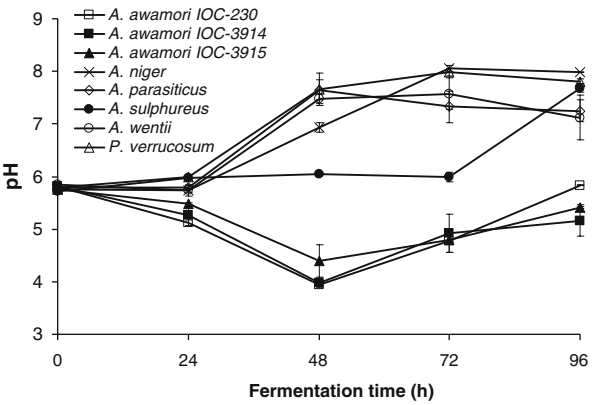

Fig. 3 pH behavior in solid-state fermentation of babassu cake, for the eight fungal strains evaluated

hydrolysis studies (data not shown), the enzyme extract from *A. niger* was also evaluated for its potential for babassu cake hydrolysis. The standard fermentation time for production of the extracts was set at 96 h, because most profiles for amylases production increased up to this time, and because for the four strains evaluated at this stage the highest amylolytic activities were obtained at 96 h.

The experiments followed a central composite statistical design (Table 1) under different pH and temperature conditions and aimed at determining the ability of enzyme complexes to hydrolyze raw starch under these conditions. The concentrations of glucose and total reducing sugars (TRS) obtained after a hydrolysis period of 24 h are presented for each run in Tables 3 and 4, respectively. Data shown are already net values due solely to the hydrolysis process, i.e., contributions from the enzyme extracts and from autoamylolysis (due to endogenous vegetable amylases present in the cake) have been subtracted from the total glucose/TRS concentrations measured after the hydrolysis step. The central point was carried out in triplicate, as well as the quantifications for all assays.

Based on the results presented in Tables 3 and 4, it can be observed that the glucose percentage in the TRS content varied between 16 and 32%. On a mass basis, the reducing power decreases as the number of glucose units in the oligosaccharide chain increases, thus it is expected that the higher the exoamylase contribution to the total amylolytic activity, the higher the glucose-to-TRS ratio. The results observed in this work indicate that even under mild-temperature conditions (36 °C, run 7) the enzymatic extracts produced by SSF were able to release considerable contents of fermentable sugars, when compared to the maximum release achieved for each extract. At such mild conditions, TRS concentration was between 62 (*A. awamori* IOC-3914 extract) and 76% (*A. niger* extract) of the maximum concentration observed in other runs at higher temperatures. Regarding glucose release, the relative concentration of this monosaccharide at 36 °C achieved even higher levels than for TRS, ranging from 71 (*A. awamori* IOC-3914 extract) to 84% (*A. awamori* IOC-230 extract) of the maximum glucose concentration obtained for each extract. Such process configuration (named cold hydrolysis) was pointed by Robertson et al. [13] as a future trend that could improve energy efficiency in grains-processing mills.

Besides the quantification of reducing sugars released during hydrolysis, the glucose and TRS contents in the enzymatic extracts (which were released during the fermentation stage) and also those liberated by autoamylolytic degradation of babassu cake were determined.

Table 3 Responses (glucose concentration, g L^{-1}) obtained for CCD experiments using enzyme extracts from four strains for babassu cake cold hydrolysis.

Run	*A. awamori* IOC-3914	*A. awamori* IOC-3915	*A. awamori* IOC-230	*A. niger*
1	4.56±0.16	5.39±0.14	3.45±0.09	4.31±0.05
2	4.70±0.09	3.89±0.06	4.23±0.09	4.96±0.15
3	3.82±0.10	3.82±0.12	3.89±0.04	3.31±0.03
4	2.68±0.02	2.52±0.02	3.13±0.04	4.08±0.08
5	4.56±0.11	5.37±0.04	4.98±0.12	4.75±0.09
6	3.04±0.05	3.63±0.17	3.47±0.10	4.11±0.05
7	3.51±0.05	4.61±0.14	3.88±0.04	4.33±0.04
8	2.92±0.07	4.63±0.04	3.13±0.04	5.46±0.11
9	4.92±0.10	5.42±0.13	4.18±0.08	5.26±0.16
10	4.19±0.06	5.52±0.15	4.22±0.14	5.19±0.03
11	4.30±0.06	5.27±0.23	4.38±0.15	5.26±0.07

Table 4 Responses (TRS concentration, g L^{-1}) obtained for CCD experiments using enzyme extracts from four strains for babassu cake cold hydrolysis.

Run	*A. awamori* IOC-3914	*A. awamori* IOC-3915	*A. awamori* IOC-230	*A. niger*
1	23.54±0.98	19.56±0.58	16.24±0.29	16.28±0.49
2	24.70±0.04	14.56±0.30	20.76±0.53	21.67±0.39
3	17.92±0.54	13.03±0.10	16.61±0.06	17.27±0.26
4	15.61±0.44	15.29±0.43	15.47±0.24	15.74±0.36
5	22.74±0.53	21.37±0.42	24.45±0.36	18.35±0.45
6	16.07±0.37	11.26±0.54	13.55±0.21	13.37±0.23
7	15.36±0.27	14.32±0.14	16.99±0.23	16.63±0.54
8	17.47±0.07	15.83±0.06	14.45±0.15	20.14±0.21
9	21.09±0.43	18.98±0.77	21.34±0.30	20.44±0.24
10	20.03±0.74	19.58±0.19	22.92±0.48	20.03±0.81
11	20.05±0.62	18.09±0.42	23.78±0.66	21.19±0.45

Extracts from *A. awamori* IOC-3914 and *A. awamori* IOC-230 contributed with low amounts of glucose (less than 5 and 10%, respectively) and TRS (less than 4 and 7%, respectively) of the total concentrations detected in the hydrolyzates after 24 h of enzyme action. On the other hand, the extracts produced by *A. awamori* IOC-3915 and *A. niger* corresponded to an input in glucose and TRS content of 30 and 13%, respectively, for the former and 20 and 13%, respectively, for the latter strain. Regarding the starch hydrolysis possibly promoted by endogenous enzymes from the babassu cake (autoamylolysis), a blank experiment was done, and the contribution for the total glucose and reducing sugar contents was approximately 1 and 2%, respectively. According to Roehr [33], cereals such as wheat and rye present autoamylolytical hydrolysis yields often higher than 95%, indicating that for those raw materials the addition of external enzymes is not crucial for hydrolysis performance. In the present work, instead of grains, a residual cake was used, and the expectation for a lower autoamylolytical yield in the babassu cake was confirmed.

The net glucose and TRS concentrations after hydrolysis by the enzyme complexes produced by SSF (having already subtracted the contents due to extracts and autoamylolysis) were submitted to analysis of variance (ANOVA) and validated according to their residues (difference between the observed and predicted values) and normality. Data were fitted to second-order models, and only the regression coefficients for the significant terms (considering a 95% confidence interval) and for the non-significant terms, which affected positively the adjusted regression coefficient (R_{adj}^2) were considered in the final models (Eqs. 1–8). Equations 1–4 present the models that describe glucose release due to extracts from *A. awamori* IOC-3914, *A. awamori* IOC-3915, *A. awamori* IOC-230, and *A. niger*, respectively, while Eqs. 5–8 represent the models for TRS release using extracts from the same strains as described above. The models are expressed in terms of the real, non-normalized values of the variables, which mean that temperature (T) values range from 36 to 64 °C and pH values from 3.6 to 6.4.

$$\text{Glucose}\left(\text{g L}^{-1}\right) = -9.12 + 1.00\text{*pH} + 0.50\text{*}T - 0.004\text{*}T^2 - 0.032\text{*pH*}T \quad (1)$$

$$\text{Glucose}\left(\text{g L}^{-1}\right) = -16.93 + 4.30\text{*pH} - 0.49\text{*pH}^2 + 0.49\text{*}T - 0.005\text{*}T^2 \quad (2)$$

$$\text{Glucose}\left(\text{g L}^{-1}\right) = -13.95 + 1.60\text{*pH} + 0.59\text{*}T - 0.0041\text{*}T^2 - 0.039\text{*pH*}T \quad (3)$$

$$\text{Glucose}\left(\text{g L}^{-1}\right) = -16.42 + 4.90\text{*pH} - 0.53\text{*pH}^2 + 0.33\text{*}T - 0.003\text{*}T^2 \quad (4)$$

$$\text{TRS}\left(\text{g L}^{-1}\right) = -1.85 - 2.91\text{*pH} + 1.25\text{*}T - 0.012\text{*}T^2 \quad (5)$$

$$\text{TRS}\left(\text{g L}^{-1}\right) = 2.18 - 1.44\text{*pH} - 0.99\text{*pH}^2 + 0.95\text{*}T - 0.019\text{*}T^2 + 0.181\text{*pH*}T \quad (6)$$

$$\text{TRS}\left(\text{g L}^{-1}\right) = -135.00 + 22.99\text{*pH} - 1.84\text{*pH}^2 + 4.20\text{*}T - 0.035\text{*}T^2 - 0.142\text{*pH*}T \quad (7)$$

$$\text{TRS}\left(\text{g L}^{-1}\right) = -96.56 + 25.66\text{*pH} - 1.84\text{*pH}^2 + 2.03\text{*}T - 0.011\text{*}T^2 - 0.173\text{*pH*}T \quad (8)$$

Fungal glucoamylases have been reported in the literature for their ability to hydrolyze raw starch, which is due to the presence of a starch-binding domain (SBD) in the structure of the enzymes. The SBD has a primary function of supporting enzyme adsorption onto the starchy chain, but it is also reported that this domain can be attached to the cell wall, keeping the catalytic domain very close to the cells, thus improving the glucose uptake by the microorganisms [7, 34]. In a lesser extent, α-amylase produced by the filamentous fungus *Aspergillus fumigatus* is also able to attack starch granules [35]. In the present work, the amylases produced by the eight evaluated strains were able to hydrolyze the starch contained in the babassu cake. The endoamylases present in the enzymatic complex play an important role in the hydrolysis, since the babassu starch is composed primarily of amylose rather than amylopectin, so the action of endo-active enzymes is required [15]. In a previous report on babassu cake hydrolysis by traditional processes, the cooking step was described as critical [15]. However, in the present work, using the enzyme extracts obtained by SSF of babassu cake, hydrolysis of this raw material did not need any cooking step.

In order to estimate the optimal conditions for babassu cake hydrolysis using the four fungal enzyme extracts, a multivariate analysis was performed, adopting a global desirability function (D) [36]. This global function is calculated according to Eq. 9, as the geometric mean of the individual desirability functions (d_i), which range from 0 to 1 ($0 \leq d_i \leq 1$). In the present work, glucose and TRS concentrations were converted to individual desirability values by normalizing them to the highest concentration obtained among the 11 experiments, and then using them to calculate D as a quantitative indicator of the global hydrolysis performance of the enzyme extracts.

$$D = (d_1\text{*}d_2)^{1/2} \quad (9)$$

The response surfaces for the desirability functions that describe the hydrolysis performance of the extracts from the four strains are shown in Fig. 4. It can be observed that enzymes produced by *A. awamori* IOC-3914 are more influenced by pH variations than by temperature

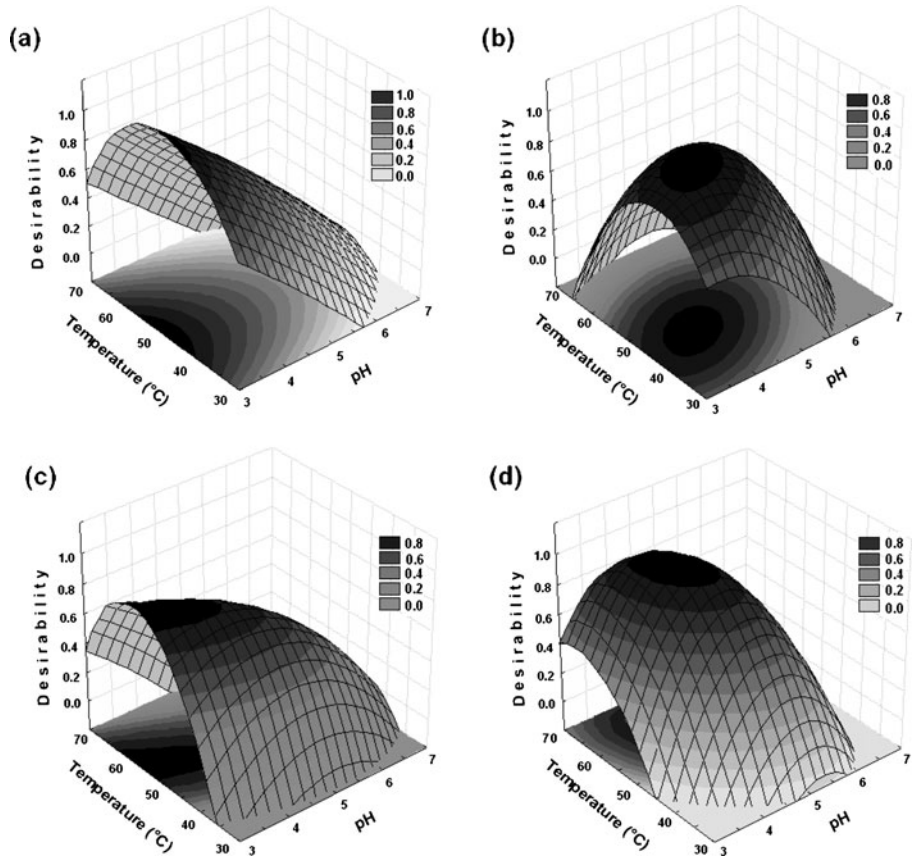

Fig. 4 Response surfaces of the desirability function used to represent the global hydrolysis performance of enzyme extracts from: **a** *A. awamori* IOC-3914; **b** *A. awamori* IOC-3915; **c** *A. awamori* IOC-230; and **d** *A. niger*

changes, and that they tend to act best at low pH values (under 5.0). Amylases from *A. awamori* IOC-230 and *A. niger* showed tendencies to act better at higher temperatures (above 40 °C) and, at lower temperatures, proved to be more sensitive at pH values in both extremes (considering the range studied). Finally, *A. awamori* IOC-3915 enzymes showed better performance under conditions close to the central point of the design.

Table 5 shows the optimal conditions and the corresponding predicted glucose and TRS concentrations, as determined by the dual analysis of responses with the desirability function, which was set to search for the optimal values of both variables, dividing the ranges set experimentally in 100 steps for the fitting. The higher the *D* value, the better the function represents the overall performance of the process, considering the two responses evaluated. It can be observed that the *D* value obtained for the analysis of the performance of amylases produced by *A. awamori* IOC-3914, *A. awamori* IOC-3915, and *A. niger* were very close to 1, which means that for each extract, the optimal release of both glucose and TRS was achieved at almost the same conditions of pH and temperature. This means that under such conditions, enzymes, which release reducing sugars of various lengths

Table 5 Predicted glucose and TRS concentrations at the optimal hydrolysis conditions, as determined by the multivariate global desirability analysis.

Strain	Optimal conditions		Glucose (g L^{-1})	TRS (g L^{-1})	*D* value
	pH	Temperature (°C)			
A. awamori IOC-3914	3.6	51	4.72	23.43	0.99
A. awamori IOC-3915	3.9	45	4.04	18.19	0.95
A. awamori IOC-230	3.6	54	4.21	21.28	0.86
A. niger	4.5	58	4.30	19.34	0.98

(primarily endoamylases), and enzymes, which liberate predominantly glucose (exoamylases) are expected to be acting synergistically, yielding the highest balance of glucose and TRS in the hydrolyzate.

The desirability function was very effective for the prediction of the best conditions for hydrolysis of the raw starch present in babassu cake. For example, Table 5 shows that based on a dual analysis, the enzymatic complex from *A. awamori* IOC-3914 acted best at 51 °C and pH 3.6. These are conditions very close to those conducted experimentally (Table 1, run 5), which, considering a 95% interval (1.96*SD), resulted in statistically identical concentrations of glucose (Table 3, range 4.34–4.79) and TRS (Table 4, range 21.71–23.78), compared to those predicted concentrations (Table 5).

Considering, as before, just the net hydrolysis due to the fungal amylases produced by SSF, up to 52% of the raw starch present in the cake was converted into TRS and up to 12% into glucose, promoting simultaneous liquefaction and saccharification of the babassu cake. Since the enzyme extracts in the present experiments were used without any prior concentration, it is expected that, by introducing a concentration step and by further optimizing the solid:liquid ratio and the enzyme load, yields can be further increased. Also, Baruque Filho et al. [15] determined that, regardless of the enzyme load, after hydrolysis always 5–7% of the babassu cake starch remains unhydrolyzed, and this was attributed to part of the starch in the babassu cake being resistant to hydrolysis.

Nagasaka et al. [37] studied the hydrolysis of raw starch from several sources at 40 °C. A purified glucoamylase from *Corticium rolfsii* showed a good performance (between 65 and 90% hydrolysis) when acting for 24 h on waxy corn, glutinuous, and nonglutinous rice. However, its action on tapioca, sago, sweet potato, and potato was poor, resulting in less than 50% of hydrolysis even after 96 h of process. Therefore, the enzymes produced in the present work show potential for starch hydrolysis, even at mild conditions (36 °C, Tables 3 and 4, run 7), since the raw fungal extracts, produced by a low-cost SSF process, without any further concentration or purification, could efficiently release fermentable sugars from raw babassu cake. It is also important to notice that the starch hydrolysis by amylases presents high specificity, thus no inhibitors, such as 5-(hydroxymethyl)furfural, are produced, as observed during acid hydrolysis of starch [38]. Furthermore, Melo et al. [39] compared acid and enzymatic hydrolysis of castor bean cake starch, proving that the latter promoted hydrolysis yields almost three times higher than the former.

Conclusions

Enzyme complexes containing amylases were produced from the agroindustrial residue babassu cake by filamentous fungi using a low-cost, simple and environment-friendly solid-

state fermentation (SSF) process. Among the eight fungal strains evaluated in this work, *A. awamori* IOC-3914 showed the highest endoamylase and exoamylase production (64.3 and 45.7 U g^{-1}, respectively) and the lowest specific activity for proteases, relatively to those observed for amylases. The enzyme complexes produced by the four most promising strains were studied for babassu cake hydrolysis according to a central composite statistical design, aiming at a simultaneous liquefaction and saccharification process. The dual analysis of glucose and TRS release using the global desirability function indicated that the enzymes are optimally active at pH values between 3.6 and 4.5 and at temperatures from 45 to 58 °C. Based on the desirability analysis, the enzymes produced by *A. awamori* IOC-3914 from babassu cake by SSF can promote the release of up to 4.7 g L^{-1} of glucose and 23.4 g L^{-1} of TRS, thus indicating their potential for the cold hydrolysis of raw starch.

References

1. Imirante (2008). Available from: http://imirante.globo.com/noticias/pagina171858.shtml. Accessed September 10, 2009.
2. DESER (2007). Available from: http://www.deser.org.br/pub_read.asp?id=113. Accessed June 05, 2009.
3. Alves, A. C., Carvalho, L. S., Trindade, J. M., Prazeres, A. F., & Cárdias, H. T. C. (2007). Available from: http://www.biodiesel.gov.br/docs/congresso2007/caracterizacao/22.pdf. Accessed September 10, 2009.
4. Lima, J. R. O., Silva, R. B., Silva, C. C. M., Santos, L. S. S., Santos, J. R., Jr., Moura, E. M., et al. (2007). *Quimica Nova, 30*, 600–603.
5. IBGE (2008). Available from: http://www.sidra.ibge.gov.br/bda/tabela/listabl.asp?c=289&z=t&o=17. Accessed September 10, 2009.
6. Gombert, A. K., Pinto, A. L., Castilho, L. R., & Freire, D. M. G. (1999). *Process Biochemistry, 35*, 85–90.
7. Norouzian, D., Akbarzadeh, A., Scharer, J. M., & Moo Young, M. (2006). *Biotechnology Advances, 24*, 80–85.
8. Van der Maarel, M. J. E. C., van der Veen, B., Uitdehaag, J. C. M., Leemhuis, H., & Dijkhuizen, L. (2002). *Journal of Biotechnology, 94*, 137–155.
9. Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. (2003). *Process Biochemistry, 38*, 1599–1616.
10. Polaina, J., & MacCabe, A. P. (Eds.). (2007). *Industrial enzymes—Structure, functions and applications* (1st ed.). Dordrecht, The Netherlands: Springer.
11. Bothast, R. J., & Schlicher, M. A. (2005). *Applied Microbiology and Biotechnology, 67*, 19–25.
12. Quintero, J. A., Montoya, M. I., Sánchez, O. J., Giraldo, O. H., & Cardona, C. A. (2008). *Energy, 33*, 385–399.
13. Robertson, G. H., Wong, D. W. S., Lee, C. C., Wagschal, K., Smith, M. R., & Orts, W. J. (2006). *Journal of Agricultural and Food Chemistry, 54*, 353–365.
14. McAloon, A., Taylor, F., Yee, W., Ibsen, K., & Wooley, R. (2000). NREL Report. Available from: http://www.nrel.gov/docs/fy01osti/28893.pdf. Accessed June 15, 2009.
15. Baruque Filho, E. A., Baruque, M. G. A., & Sant'Anna, G. L., Jr. (2000). *Bioresource Technology, 75*, 49–55.
16. Freire, D. M. G. (1997). PhD Thesis, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.
17. Britton, H. T. S., & Robinson, R. E. (1931). *Journal of the Chemical Society*, 1456–1462.
18. Figueira, E. L. Z., & Hirooka, E. Y. (2000). *Brazilian Archives of Biology and Technology, 43*, 461–467.
19. Fernandes, L. P., Ulhoa, C. J., Asquieri, E. R., & Monteiro, V. N. (2007). *Revista Eletrônica de Farmácia, IV*, 43–51.
20. Riaz, M., Perveen, R., Javed, M. R., Nadeem, H., & Rashid, M. H. (2007). *Enzyme and Microbial Technology, 41*, 558–564.
21. Bendicho, S., Martí, G., Hernández, T., & Martín, O. (2002). *Food Chemistry, 79*, 245–249.
22. Charney, J., & Tomarelli, R. M. A. (1947). *Journal of Biological Chemistry, 171*, 501–505.
23. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
24. Miller, G. L. (1959). *Analytical Chemistry, 31*, 426–428.
25. Bhanja, T., Rout, S., & Banerjee, R. (2007). *Bioprocess and Biosystems Engineering, 30*, 369–376.

26. Marlida, Y., Saari, N., Hassan, Z., Radu, S., & Bakar, J. (2000). *Food Chemistry, 71*, 221–227.
27. Guimarães, L. H. S., Peixoto-Nogueira, S. C., Michelin, M., Rizzatti, A. C. S., Sandrim, V. C., Zanoelo, F. F., et al. (2006). *Brazilian Journal of Microbiology, 37*, 474–480.
28. Costa, J. A. V., Colla, E., Magagnin, G., Santos, L. O., Vendruscolo, M., & Bertolin, T. E. (2007). *Brazilian Archives of Biology and Technology, 50*, 759–766.
29. Nahas, E., & Waldemarin, M. M. (2002). *Revista Latinoamericana de Microbiologia, 44*, 5–10.
30. Ertan, F., Yagar, H., & Balkan, B. (2006). *Preparative Biochemistry and Biotechnology, 36*, 81–91.
31. Ramachandran, S., Patel, A. K., Nampoothiri, K. M., Chandran, S., Szakacs, G., Soccol, C. R., et al. (2004). *Brazilian Archives of Biology and Technology, 47*, 309–317.
32. Kumari, A., Kapoor, K. K., Kundu, B. S., & Kumari Mehta, R. (2008). *Plant, Soil and Environment, 54*, 72–77.
33. Roehr, M. (Ed.). (2001). *The biotechnology of ethanol: Classical and future applications* (1st ed.). Weinheim, Germany: Wiley VHC.
34. Southall, S. M., Simpson, P. J., Gilbert, H. J., Williamson, G., & Williamson, M. P. (1999). *FEBS Letters, 447*, 58–60.
35. Tester, R. F., Qi, X., & Karkalas, J. (2006). *Animal Feed Science and Technology, 130*, 39–54.
36. Calado, V., & Montgomery, D. (2003). *Planejamento de Experimentos Usando o Statistica* (1st ed.). Rio de Janeiro, Brazil: E-papers Serviços Editoriais.
37. Nagasaka, Y., Kurosawa, K., Yokota, A., & Tomita, F. (1998). *Applied Microbiology and Biotechnology, 50*, 323–330.
38. Tasic, M. B., Konstantinovic, B. V., Lazic, M. L., & Veljkovic, V. B. (2009). *Biochemical Engineering Journal, 43*, 208–211.
39. Melo, W. C., Santos, A. S., Santa Anna, L. M. M., & Pereira, N., Jr. (2008). *Journal of the Brazilian Chemical Society, 19*, 418–425.