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# Antioxidant Capacity Improvement of Cashew Apple Bagasse

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The current study aimed to compare the influence of hydroethanolic treatment and enzymatic hydrolysis on the antioxidant activity of cashew apple bagasse extracts. A three-level Box-Behnken experimental design was applied to optimize the operational parameters. Hydrolysis parameters, including enzyme concentration (10 %, 15 % and 20 % respect to dry matter), solvent/sample ratio (1:1, 2:1 and 3:1) and sample granulometry were investigated. Hydroalcoholic extraction parameters were pH (3.0; 4.5 and 6.0), solvent/sample ratio (1:1, 2:1 and 3:1) and ethanol concentration (30 %, 50 % and 70 %). Based in preliminary hydrolysis data, the commercial cellulase Celluclast® 1.5 L (Novozymes) was selected. The antioxidant activity of cashew apple bagasse extracts were evaluated by $ABTS^{\cdot+}$ radicals method. Total phenolic content (TPC) was also determined using a Folin-Ciocalteu assay. Statistical results showed that the solvent/sample ratio was the only factor which influenced the antioxidant activity improvement, for both treatments. However, even when $ABTS^{\cdot+}$ quantification was higher, the TPC did not increase so expressively. This suggests that the availability of phenolic compounds were low, but the ones which were released from the cashew bagasse cell walls presented a meaningful antioxidant capacity.

## 1. Introduction

During the processing of many tropical fruits, by-products such as skins, seeds and fibres are discarded (Lima et al., 2014). In the cashew apple industry, only 10 % to 20 % of the waste production is used, which represents low yields of processing and high quantities of rejected biomass. Despite their promising economic potential, cashew apples are still underutilized (Gordon et al., 2012).
The use of bioactive compounds in different commercial sectors such as pharmaceutical, food and chemical industries signifies the need of the most appropriate and standard method to extract these active components from plant materials. Among conventional methods such as solvent extractions, enzyme technology is an alternative to obtain bioactive compounds from agro-industrial byproducts (Azmir et al., 2013). Furthermore, ethanol is a selective and environmentally friendly procedure to bioactive compounds recovery from biomass (Cruz et al., 2013).
Many studies have reported that cashew apple presents an important source of bioactive compounds like flavonoids (Lopes et al., 2012) carotenoids (Moo-Huchin et al., 2014) and anthocyanins (Silva et al., 2014). Moreover, several reports emphasize the presence of polyphenols like quercetin, myricetin, pentosides, hexosides and rhamnosides (Michodjehoun-Mestres et al., 2009). The dietary intake of some of those compounds is linked to antiproliferative properties being regularly referred to as anticancer due to their antioxidant properties (Gao and Hu, 2010).
Enzymatic hydrolysis is one of the techniques to compounds extraction from biomass without any organic solvents and toxic chemicals that give positive results and advantages among other conventional procedures

(Radenkovs et al., 2014). Furthermore, ethanol is a selective and environmentally friendly procedure to bioactive compounds recovery from biomass (Cruz et al., 2013). The aim of this work was to evaluate the total phenolic contents and antioxidant activity of cashew apple bagasse subjected to enzymatic hydrolysis and hydroalcoholic extraction treatments using a Box-Behnken statistical design.

## 2. Materials and methods

### 2.1. Reagents

Analytical grade chemicals and reagents, including ethanol used in the hydroalcoholic extraction, were purchased from commercial sources (Sigma/Aldrich and Merck). The enzyme Celluclast® 1.5 L was obtained from Novozymes (Dittingen, Switzerland).

### 2.2. Raw material

Cashew apple bagasse was obtained from fresh cashew apple fruits which were purchased in a local market of Aracaju, Sergipe, Brazil and transported to the Food Technology Department of Federal University of Sergipe. The pseudo fruits were submitted to two different washes with tap water and chlorine solution (50 $mg.L^{-1}$) at room temperature for 15 minutes. Later, they were cut and processed in a domestic blender to bagasse homogenization. Remain juice presented in the bagasse was removed by pressing it through a 42 mesh sieve. The bagasse was packaged and stored in a freezer at -22 °C until enzymatic treatment and specific analysis.

### 2.3. Enzymatic hydrolysis

Enzymatic hydrolyses were carried out using 150 mL conical flasks at 55 °C and 150 rpm inside an incubator shaker (CIENTEC, Model CT-712T) for 120 minutes. No buffer solution was applied, considering that the pH of cashew bagasse was in accordance with the optimum conditions for activity of Celluclast® 1.5 L, which range is 4.5 to 6.0. The loadings of enzymes varied between 10 % and 20 % relative to dry matter content. Experiments were conducted in triplicates to evaluate experimental repeatability. Samples with different granulometry were obtained by crushing the cashew bagasse for specific periods of time (2 min for level 0 and 4 minutes for level +1). At level -1 of experimental design, crushing step was not applied. In the hydrolysis process, 15 g of each sample were diluted in an enzyme-distilled water solution. After incubation, samples were placed in boiling water (100 °C) for 1 minute to enzyme inactivation and immediately cooled on ice, according to Krenek et al. (2014). Both, control and enzyme-treated samples were filtered under suction using a Büchner funnel, fitted with filter paper, attached to a Büchner flask connected to a low-vacuum pump. The filtrates were transferred to amber flasks and stored in freezer (-20 °C) until analysis.

### 2.4. Hydroalcoholic extraction

Fresh cashew apple bagasse samples were diluted in hydroethanolic solutions in order to evaluate the following parameters of phenolics extraction process: (i) pH; (ii) solvent/sample ratio, and (iii) ethanol concentration. All the experiments were performed in triplicates. Aliquots of 15 g of samples were mixed with different hydroethanolic solutions into 150 mL conical flasks which were placed into the same incubator shaker used previously for the enzymatic treatment. The same temperature (55 °C), rotation (150 rpm) and time (120 minutes) were applied. After incubation, control and hydroalcoholic-treated samples were cooled on ice, filtered through Büchner funnel and stored in amber glass bottles at -20 °C until analysis.

### 2.5. Box-Behnken design

A $2^3$ factorial design with 3 replicates at the centre point with a total number of 15 experiments per treatment was employed. This design is suitable for exploration of quadratic response surfaces and for construction of second order polynomial models, thus helping to optimize the process by using a small number of experimental runs (Kincl et al., 2005). A non-linear regression method was used to fit the second order polynomial equation to data and to identify the relevant model terms (Prakash Maran et al., 2013). Tables 1 and 2 show the Box-Behnken design matrixes of the experiments for enzymatic hydrolysis and hydroalcoholic extraction, respectively.

*Table 1: Coded values of variables used in Box-Behnken design for enzymatic hydrolysis*

| Independent variables | Symbol | Coded variable levels | | |
|---|---|---|---|---|
| | | -1 | 0 | 1 |
| Sample granulometry | $X_1$ | No smash | 2 min | 4 min |
| Solvent/sample ratio (w/w) | $X_2$ | 1:1 | 2:1 | 3:1 |
| Enzyme concentration (%; w/w) | $X_3$ | 10% | 15% | 20% |

*Table 2: Coded values of variables used in Box-Behnken design for hydroalcoholic extraction*

| Independent variables | Symbol | Coded variable levels | | |
|---|---|---|---|---|
| | | -1 | 0 | 1 |
| pH | $X_1$ | 3.0 | 4.5 | 6.0 |
| Solvent/sample ratio (w/w) | $X_2$ | 1:1 | 2:1 | 3:1 |
| Ethanol concentration (%; w/w) | $X_3$ | 30 | 50 | 70 |

### 2.6. Total phenolic content (TPC)

Total phenolic content of control, enzyme-treated and hydroalcoholic-treated samples was analysed in triplicate according to the spectrophotometric method using Folin-Ciocalteau reagent (Singleton et al., 1999). Results were expressed as milligram of gallic acid equivalents per 100 g of sample fresh weight (mg GAE.100g$^{-1}$ FW).

### 2.7. $ABTS^{\cdot+}$ assay

The antioxidant activity (AA) was measured based on the $ABTS^{\cdot+}$ radical cations method (Rufino et al., 2007). ABTS stock (7 mM) was reacted with a solution of potassium persulfate (145 mM) and the mixture stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. After the addition of 30 µL of sample or trolox standard to 3 mL of diluted $ABTS^{\cdot+}$ solution, absorbances were recorded at 6 min after mixing. Ethanolic solutions of known trolox concentrations were used for calibration and the results were expressed as µM Trolox per g of sample fresh weight (µM Trolox.g$^{-1}$ FW).

### 2.8. Statistical data analysis

Response surface methodology (RSM) was applied to analyse statistically the data obtained for both enzymatic hydrolysis and hydroalcoholic extraction processes. STATISTICA software (Trial 12.0, StatSoft Inc., USA) was applied for regression and graphical analyses of the experimental design results.

## 3. Results and discussion

### 3.1. Response surface analysis

TPC and $ABTS^{\cdot+}$ data after enzymatic hydrolysis of cashew apple bagasse, as designed by Box-Behnken, are presented in Table 3 which contains the coded factors considered in this study, observed values, predicted values as well as the residual values obtained. Results obtained for hydroalcoholic extraction are showed in Table 4.

It was observed a very strong agreement between TPC predicted by the model and the experimental data, however there was a low accordance among $ABTS^{\cdot+}$ observed and predicted values. This suggests that total phenolics content could not be considered directly proportional to the antioxidant activity ($ABTS^{\cdot}$+), i.e., when TPC is high does not mean that antioxidant activity will be higher too. Moreover, some trials presented low TPC values and high AA results, which propose that in certain experimental conditions several phenolic compounds are released in small quantities however they could present antioxidant functionality.

Pareto chart (Figure 1a) represents the estimated effects of the parameters sample granulometry ($X_1$), solvent/sample ratio ($X_2$) and enzyme concentration ($X_3$), affecting the TPC response. The length of each bar is proportional to the standardized effect (Marcos et al., 2013). As observed in that graph, the variables solvent/sample ratio and enzyme concentration presented a statistical significance for the TPC response. In comparison with $ABTS^{\cdot+}$ results (Figure 1b), variable $X_2$ was the only significant interaction, suggesting that the solvent/sample ratio was the most important factor that affected enhancement on availability of bioactive compounds according to the effects studied.

*Table 3: Enzymatic hydrolysis data for the observed TPC and $ABTS^{\cdot+}$, predicted TPC and $ABTS^{\cdot+}$ and respective residual values*

| Run | $X_1$ | $X_2$ | $X_3$ | TPC (mg GAE.100g$^{-1}$ FW) | | | $ABTS^{\cdot+}$ (µM Trolox.g$^{-1}$ FW) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Observed | Predicted | Residual | Observed | Predicted | Residual |
| 1 | -1 | -1 | 0 | 28.29 | 34.66 | -6.36 | 255.65 | 238.70 | 16.95 |
| 2 | 1 | -1 | 0 | 27.12 | 27.10 | 0.02 | 251.93 | 339.61 | -87.68 |
| 3 | -1 | 1 | 0 | 67.66 | 53.76 | 13.90 | 458.30 | 400.34 | 57.96 |
| 4 | 1 | 1 | 0 | 38.66 | 46.21 | -7.55 | 514.03 | 501.25 | 12.77 |
| 5 | -1 | 0 | -1 | 49.78 | 46.18 | 3.60 | 238.97 | 290.17 | -51.20 |
| 6 | 1 | 0 | -1 | 48.30 | 38.62 | 9.68 | 487.68 | 391.08 | 96.60 |
| 7 | -1 | 0 | 1 | 21.34 | 32.47 | -11.13 | 244.36 | 268.07 | -23.71 |
| 8 | 1 | 0 | 1 | 22.77 | 24.92 | -2.15 | 347.29 | 368.98 | -21.69 |
| 9 | 0 | -1 | -1 | 23.58 | 27.16 | -3.58 | 307.38 | 236.06 | 71.32 |
| 10 | 0 | 1 | -1 | 36.56 | 46.26 | -9.70 | 280.98 | 397.70 | -116.72 |
| 11 | 0 | -1 | 1 | 23.38 | 13.46 | 9.92 | 213.37 | 213.95 | -0.59 |
| 12 | 0 | 1 | 1 | 35.92 | 32.56 | 3.36 | 421.59 | 375.60 | 45.99 |
| 13 | 0 | 0 | 0 | 51.51 | 51.18 | 0.33 | 292.46 | 301.58 | -9.12 |
| 14 | 0 | 0 | 0 | 52.55 | 51.18 | 1.37 | 307.38 | 301.58 | 5.80 |
| 15 | 0 | 0 | 0 | 49.48 | 51.18 | -1.70 | 304.91 | 301.58 | 3.33 |

$X_1$ – Sample granulometry; $X_2$ – Solvent/sample ratio (w/w); $X_3$ – Enzyme concentration (%; w/w).

*Table 4: Hydroethanolic extraction data for the observed TPC and $ABTS^{\cdot+}$, predicted TPC and $ABTS^{\cdot+}$ and respective residual values*

| Run | $X_1$ | $X_2$ | $X_3$ | TPC (mg GAE.100g$^{-1}$ FW) | | | $ABTS^{\cdot+}$ (µM Trolox.g$^{-1}$ FW) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Observed | Predicted | Residual | Observed | Predicted | Residual |
| 1 | -1 | -1 | 0 | 12.73 | 11.82 | 0.91 | 579.08 | 773.13 | -194.05 |
| 2 | 1 | -1 | 0 | 13.28 | 13.09 | 0.19 | 731.93 | 602.58 | 129.35 |
| 3 | -1 | 1 | 0 | 34.35 | 35.04 | -0.69 | 1431.97 | 1371.53 | 60.44 |
| 4 | 1 | 1 | 0 | 35.90 | 36.31 | -0.41 | 1205.23 | 1200.98 | 4.26 |
| 5 | -1 | 0 | -1 | 22.33 | 22.72 | -0.39 | 1230.62 | 1091.51 | 139.11 |
| 6 | 1 | 0 | -1 | 23.90 | 23.98 | -0.08 | 865.61 | 920.96 | -55.35 |
| 7 | -1 | 0 | 1 | 23.47 | 23.30 | 0.17 | 1200.15 | 1205.65 | -5.50 |
| 8 | 1 | 0 | 1 | 24.87 | 24.56 | 0.30 | 956.84 | 1035.09 | -78.26 |
| 9 | 0 | -1 | -1 | 12.15 | 12.65 | -0.51 | 593.23 | 610.35 | -17.12 |
| 10 | 0 | 1 | -1 | 36.85 | 35.87 | 0.98 | 1142.11 | 1208.75 | -66.63 |
| 11 | 0 | -1 | 1 | 12.64 | 13.24 | -0.59 | 806.31 | 724.48 | 81.82 |
| 12 | 0 | 1 | 1 | 36.57 | 36.45 | 0.12 | 1324.82 | 1322.88 | 1.93 |
| 13 | 0 | 0 | 0 | 24.23 | 24.11 | 0.12 | 1119.46 | 1138.47 | -19.01 |
| 14 | 0 | 0 | 0 | 23.63 | 24.11 | -0.49 | 1250.21 | 1138.47 | 111.73 |
| 15 | 0 | 0 | 0 | 24.48 | 24.11 | 0.36 | 1045.75 | 1138.47 | -92.72 |

$X_1$ – pH; $X_2$ – Solvent/sample ratio (w/w); $X_3$ – Ethanol concentration (%; w/w).

Figure 2 shows the contour plots of the interactions among solvent/sample ratio (w/w) and enzyme concentration (%; w/w) at the fixed granulometry (2 minutes crush), as well as sample granulometry and enzyme concentration (%; w/w) at the fixed solvent/sample ratio (2:1; w/w) related to the response of TPC. The contour plots reveal that the maximum total phenol content (> 50 mg GAE.100g$^{-1}$ FW) during the enzymatic hydrolysis occurs when the solvent/sample ratio ($X_2$) between 2:1 (level 0) and 3:1 (level +1). The contour plots for $ABTS^{\cdot+}$ results also present higher quantified amounts when the solvent/sample ratio is bigger.

A similar behaviour of the contour plots presented in Figure 2 is showed for the hydroalcoholic extraction (Figure 3), where the highest quantitative results for TPC (> 35 mg GAE.100g$^{-1}$ FW) and antioxidant activity (> 400 µM Trolox.g-1 FW) are observed when the solvent/sample ratio is close to 3:1.

The impact of enzymatic treatments on the composition of bioactive compounds in tropical fruits has been reported (Krenek et al., 2014; Thitiratsaku and Anprung, 2014; Laaksonen et al., 2012), however the effect of enzymes with mainly cellulase activity in cashew apple bagasse is not well known. In the study of enhancement of phenolic compounds and antioxidant activity of cashew apple bagasse by enzymatic hydrolysis and hydroethanolic extraction the main parameter studied was the solvent/sample ratio suggesting that values greater than 2:1 (w/w) presented positive results probably due to the enzyme mobility which is facilitate when dilutions are high. High phenolic content extracts must be useful for future applications in the development of functional ingredients for industrial applications. Although the enzyme concentration was high,

this preliminary study was helpful for later optimization experiments referring to extraction of bioactive compounds from cashew apple bagasse.

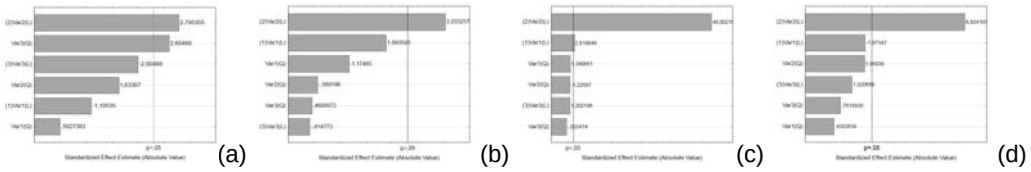
(a) (b) (c) (d)

*Figure 1. Pareto charts for enzymatic hydrolysis: (a) TPC and (b) $ABTS^{\cdot+}$ results; and hydroethanolic extraction: (c) TPC and (d) $ABTS^{\cdot+}$ responses.*

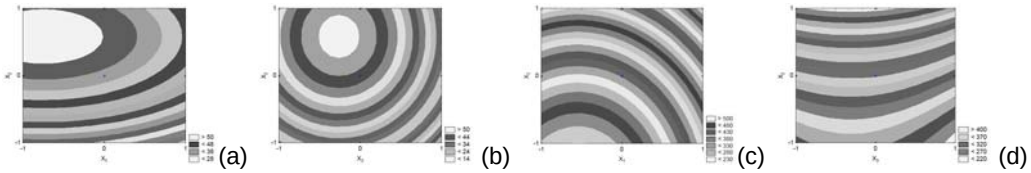
(a) (b) (c) (d)

*Figure 2. Enzymatic hydrolysis contour plots of TPC vs: (a) solvent/sample ratio ($X_2$; w/w); sample granulometry ($X_1$) and (b) solvent/sample ratio ($X_2$; w/w); enzyme concentration ($X_3$; % w/w). Contour plots of $ABTS^{\cdot+}$ vs: (c) solvent/sample ratio ($X_2$; w/w); sample granulometry ($X_1$) and (d) solvent/sample ratio ($X_2$; w/w); enzyme concentration ($X_3$; % w/w).*

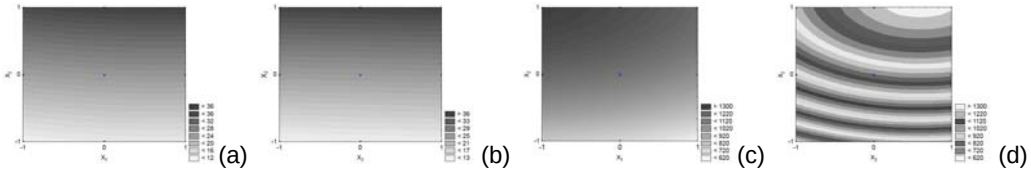
(a) (b) (c) (d)

*Figure 3. Hydroalcoholic extraction contour plots of TPC vs: (a) solvent/sample ratio ($X_2$; w/w); sample granulometry ($X_1$) and (b) solvent/sample ratio ($X_2$; w/w); enzyme concentration ($X_3$; % w/w). Contour plots of $ABTS^{\cdot+}$ vs: (c) solvent/sample ratio ($X_2$; w/w); sample granulometry ($X_1$) and (d) solvent/sample ratio ($X_2$; w/w); enzyme concentration ($X_3$; % w/w).*

## 4. Conclusion

The only parameter which affected enzymatic hydrolysis and hydroalcoholic extraction for enhancement of phenolic compounds and antioxidant activity of cashew apple bagasse was the solvent/sample ratio (w/w). The best proportion was two parts of solvent for one part of sample. Although in some experiments the amount of TPC was low, they presented a meaningful antioxidant capacity for both treatments (enzymatic hydrolysis and hydroalcoholic extraction), which suggests that TPC content may be not directly proportional when related to antioxidant activity. Extracts characterized in this report are usable for comprehension of cashew apple bagasse behaviour when submitted to enzymatic hydrolysis and hydroalcoholic extraction processes in reference to bioactive compounds.

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