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Solid-state fermentation as a tool for methylxanthine reduction and simultaneous xylanase production in cocoa meal

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ABSTRACT

Cocoa meal, the residue from cocoa processing, when used in animal feed can negatively affect health and performance because of the high concentrations of methylxanthines (theobromine and caffeine) and high fiber content. Solid-state fermentation (SSF) is one method to increase the use and value of the residue and to remove undesirable compounds. The fungus *Aspergillus awamori* IOC-3914 used in SSF was able to grow in cocoa meal, reduce methylxanthines and produce xylanase. Enzyme production reached about 66.5 U/g in 48 h before and 72 U/g after optimization. The xylanase showed good thermal stability, with residual activity between 97% and 90% after 4 h of incubation between 30 and 40 °C. The highest residual activity was obtained at pHs between 5.0 and 6.0. *Aspergillus awamori* reduced the theobromine and caffeine contents by about 69% and 63%, respectively, after the optimization process. Addition of feather meal significantly increased the reduction of methylxanthines. SSF in cocoa meal using *A. awamori* can expand the options for waste use, by producing enzymes and reducing the contents of undesirable caffeine and theobromine.

1. Introduction

The food, agricultural and forestry industries produce large volumes of waste, causing serious disposal issues. Because most agro-industrial waste has nutritional potential, its final destination is receiving increased attention (Orzua et al., 2009; Graminha et al., 2008). Examples of this waste include sugarcane bagasse (Song and Wei, 2010), coffee pulp (Hughes, 2014) and cocoa husks and bran (Adamafio, 2013).

The cocoa bean (*Theobroma cacao* L.) is processed by fermentation and drying for chocolate manufacture (Motamayor et al., 2008). The waste cocoa meal has a high fiber content (50%), composed of cellulose, hemicellulose and lignin, and (with the rind) contains about 16 protein and 8% lipid (Adamafio, 2013). Because of its composition, cocoa meal has been used as roughage in animal nutrition (Hamzat and Adeola, 2011; Bentil et al., 2015; Laconi and Jayanegara, 2015); however, the high levels of non-digestible fiber and the presence of methylxanthines such as caffeine and theobromine limit its use in animal feed. Methylxanthines are secondary metabolites derived from purine, which in high doses stimulate the central nervous system (CNS), causing nervousness, agitation, tremors and convulsions in animals (De Sena, 2011). Degradation of these compounds in cocoa meal is essential so that large amounts of it can be included in animal diets, thus reducing the cost of animal feed by replacing components such as soybean meal.

Solid-state fermentation (SSF) using agro-industrial residues as culture media to grow filamentous fungi has shown good results for degrading anti-nutritional or toxic compounds (Brand et al., 2000; Godoy et al., 2009; Belewu and Sam, 2010) as well as for producing exogenous enzymes such as xylanases (Chapla et al., 2010; Ang et al., 2013; Pirota et al., 2013; Kaushik et al., 2014; Castro et al., 2015).

Endo-β−1,4-xylanase enzymes (EC 3.2.1.8) are primarily responsible for hydrolyzing the β−1,4 bonds in xylan, the main component of hemicellulose (Dodd and Cann, 2009). Xylanases also have several applications in the food industry (fruit and vegetable processing, brewing, wine production, baking), as well as in other technical sectors (pulp and paper, textiles, bioremediation/bioconversion) (Collins et al., 2005). These enzymes can also be used as a nutritional supplement in animal feed, to increase the total digestibility and

Abbreviations: SSF, solid-state fermentation

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improve animal performance (Cowieson et al., 2003; Oso et al., 2015).

This study used SSF with the fungus *Aspergillus awamori* IOC-3914 as a method to reduce the content of anti-nutritional compounds (theobromine and caffeine) and to simultaneously produce xylanase in cocoa meal.

2. Materials and methods

2.1. Microorganism and inoculum propagation

Solid-state fermentations were carried out with *A. awamori* IOC-3914, obtained from the culture collection of the Instituto Oswaldo Cruz (IOC; Rio de Janeiro, Brazil). The culture was maintained at 4 °C in starch agar medium containing potato dextrose agar (PDA) (Vetec) and nutrient agar (NA) with monthly subculturing. To obtain *A. awamori* conidia, the fungal strain was grown for 7 days at 30 °C in an incubator (Fabbe-Primar).

2.2. Solid-state fermentation

The culture medium used for solid-state fermentation (SSF) was based on cocoa meal, kindly provided by the Executive Committee of the Cocoa Crop Plan – CEPLAC (Ilhéus, Brazil). The waste was ground in a laboratory knife mill and separated in a sieve shaker (screen mesh 20; Tyler). Final waste particle sizes ranged from 1.18 to 1.70 mm in diameter. The cocoa meal medium was supplemented with urea (1% and 2%, w/w) and feather meal (6% and 12%, w/w). Urea and feather meal were used as nitrogen sources to balance the carbon: nitrogen (C: N) ratio to 13 and 15. The total carbon content was determined in a Multi EA 2000 automatic analyzer, and the total nitrogen content was determined according to the Association of Official Analytical Chemists (AOAC International, 2002).

The fermentations were carried out in laboratory-scale tray-type bioreactors (cylindrical bioreactors, 10 cm in diameter and 15 cm in height), containing 15 g of waste and 60% (w/w) water. The resulting packed-bed formed a 1 cm-deep layer in the bioreactor. The medium was inoculated with 10^7 spores/g of dry solid (determined in a cell-counting chamber) and incubated in a chamber with conditions set to 30 °C and 90% water saturation. Fermentation samples (whole trays) were removed at 24-h intervals for up to 96 h.

2.3. Chemical analysis of samples

The samples were analyzed for lipid, ash and protein using standard methods of the Association of Official Analytical Chemists AOAC International (2002). The fiber fraction, neutral detergent fiber (NDF) and acid detergent fiber (ADF) were estimated by the method of Van Soest et al. (1991). Hemicellulose was calculated as the difference between NDF and ADF, while cellulose was calculated as the difference between ADF and lignin content.

2.4. Water activity and moisture determination

Water activity (Aw) and moisture content of the solid samples were determined using a water activity meter (Aqualab, USA) and an MX-50 moisture analyzer (A & D, USA), respectively.

2.5. Ergosterol determination

Biomass was quentified indirectly through the determination of ergosterol in the fungal biomass. The experimental procedure for determining ergosterol followed the method described by Barajas-Aceves et al. (2002). The biomass concentration was indirectly expressed as mg of ergosterol per gram of dry cocoa meal (mg/g).

The specific product yield ($Y_{P/X}$) was calculated as the ratio of xylanase activity (U/g) to ergosterol content (mg/g), and expressed as units per mg of ergosterol (U/mg).

2.6. Enzyme extraction

Acetate buffer (50 mM, pH 5.0, 5 ml/g) was added to each tray containing the fermented solids, and the enzyme was extracted in a rotary shaker at 35 °C and 200 rpm for 20 min. Next, the solid and liquid were separated by pressing followed by centrifugation at 2000 × *g* for 5 min (Gombert, 1995). The supernatant was stored at −20 °C and used to determine the xylanase activity.

2.7. Xylanase activity determination

The activity of xylanases was assayed based on the capacity of these hydrolytic enzymes to release xylose (measured as reducing sugars), by using a solution of Birchwood xylan (Sigma-Aldrich, USA) as substrate. Ten µl of enzyme extract was incubated with 90 µl of 1% (w/v) xylan solution for 5 min. Then, total reducing sugars (TRS) were calculated by the DNS method (Miller, 1959). Absorbance was measured at 540 nm. One enzyme unit was defined as the amount that produces 1 µmol of TRS per minute, under the assay conditions.

2.8. Xylanase stability

Xylanase thermal stability was determined by incubating the enzyme in a water bath adjusted to 30, 40, 50, 60 or 70 °C. Aliquots were removed at times ranging from 0 to 4 h, and immediately stored in an ice bath for subsequent activity assay (according to item 2.7). Results for enzyme thermal stability were expressed as residual activity (%).

In order to assess xylanase stability at different pH levels, crude extracts were incubated for 4 h in a universal buffer (Britton and Robinson, 1931) with different pH values (3 − 11). Fractions were collected and the residual activity of the enzyme was measured by the standard test (item 2.7).

2.9. HPLC analysis of methylxanthine

The methylxanthines (theobromine and caffeine) of the cocoa product were determined in the laboratory. Three grams of cocoa meal was extracted following the procedure by Alves and Bragagnolo (Alves and Bragagnolo, 2002). The extracts were filtered (0.45 µm) and analyzed on an Agilent Technologies series 1200 high-performance liquid chromatograph (HPLC) equipped with a reverse-phase column (Kromasil ODS 5 µm, 4.6 × 250 mm), protected with an ODS RP18 guard column. The samples were eluted with an 80:20 water/methanol solution with a flow rate of 1 ml/min and monitored at 280 nm in a Diode Array Detector (Agilent Technologies). Twenty microliters of extract was injected into the HPLC system. The analysis was calibrated against a standard curve obtained from theobromine solutions of 0–400 µg/ml.

2.10. Experimental design

A central composite face-centered experimental design (CCFC) (α = 1) was used to evaluate the effect of incubation temperature (T), inoculum concentration (IC), feather meal concentration (FM) and urea concentration (U) on xylanase production and theobromine and caffeine reduction. Fermentation samples (whole trays) were analyzed after 48 h of fermentation. All measurements were done in triplicate. Statistical analysis of the results was performed using Statistica 7.0 software.

3. Results and discussion

3.1. Chemical composition of cocoa meal and feather meal

The composition of the raw material is one of the most important