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Ethanol biosynthesis by fast hydrolysis of cassava bagasse using fungal amylases produced in optimized conditions

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ABSTRACT

The search for a renewable platform to produce high-value biochemicals and energy that are environmentally correct has been a current concern. A fast and inexpensive bioprocess for amylase production, able to hydrolyze complex residues in fermentable sugars to be used for ethanol production was developed. High titer amylase from *Rhizopus oligosporus* in solid state fermentation (SSF) was obtained by optimizing the medium supplementation using agro-industrial waste as substrate. Statistical experimental design and partial least square (PLS) regression were used to establish a relation between added chemical compounds and enzyme production, showing that urea was the most important nutrient. Crude amylase extract had competitive performance features giving higher productivities in starch hydrolysis than a commercial glucoamylase. The amylase produced was applied in the proportion of 15 U/g dry cassava bagasse to obtain cassava bagasse hydrolysate (CBH). More than 42% conversion in reducing sugars was achieved with an efficient 10 h single-step hydrolysis at 55 °C in a bioreactor. The concentrated CBH was subsequently used in fed batch process producing 89.2% ethanol yield. Furthermore, comparing just the cost of the raw materials sugarcane and CHB, the latter demonstrated to be a lower-cost feedstock for ethanol fermentation.

1. Introduction

Bio-ethanol can be produced from various renewable feedstock such as sugarcane (*Saccharum* spp.), corn (*Zea mays*), wheat (*Triticum* spp.), cassava (*Manihot* spp.), and cellulose biomass (Baeyens et al., 2015). In addition, starchy residues hydrolysis for ethanol production is a very promising technology that can become extensively adopted at large scale (Cinelli et al., 2015). In the US, world's largest ethanol producer (USDA, 2014), corn starch has been used as source of biofuel for decades, with the use of corn grain balanced for food and feed, and ethanol production (Mumm et al., 2014).

Cassava production has been increasing annually in developing countries like Nigeria, the largest producer worldwide (FAOSTAT, 2016), and for these countries is an important source of food (Anyanwu et al., 2015). This crop constitutes a staple food in Sub-Saharan Africa, so much that 29 million tons of the annual Nigerian production are demanded as food (Anyanwu et al., 2015). Cassava shows the highest yield of carbohydrates per hectare after sugarcane and sugar beet (*Beta* spp.), and one of the best water-footprints especially on relatively low fertility soils, in drought conditions, requiring low agrochemical input and adapts well in all ecological zones (Okudoh et al., 2014). Advances in the use of wastes from cassava industry can contribute to the development of these countries and supply of food and other compounds to the world. Between 2000 and 2013, Africa continent was the largest cassava producer with about 54% of the production, followed by Asian with 31% and Americas with 15%. The Brazilian production of cassava was the fourth largest in 2014 with 23.36 million tons (FAOSTAT, 2016), justifying the large amounts of cassava processing waste in the country.

For each 250–300 tons of cassava root processed, about 280 tons of bagasse with 85% moisture is produced (Pandey et al., 2000). Cassava bagasse is used as feed and has also been successfully assessed in the production of interest biomolecules demonstrating its fermentability as *n*-butanol (Lu et al., 2012), succinic acid (Shi et al., 2014), fatty acid, and neutral lipid (Chen et al., 2015). Currently, the majority of the cassava bagasse is discarded as waste, however, this residue can be

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considered as an excellent source of fermentation for higher-value biochemicals.

Currently, the starch hydrolysis processes employ two classes of enzymes, α-amylase, and glucoamylase, at hydrolysis times that typically exceed 24 h (Białas et al., 2010; Cinelli et al., 2015). Amylases include an important enzyme class with many industrial and biotechnological applications, representing 25% of the enzymes market (Kumar et al., 2012). They belong to the class of hydrolases and catalyze the hydrolysis of starch and its derivatives, releasing various products, including progressively smaller dextrins and glucose polymers (Gupta et al., 2003).

There is a wide range of applications for the hydrolysis of starch and ethanol production, such as in the food, detergent, paper, textiles, baking, chemical, and pharmaceutical industries. (Gupta et al., 2003; Castro et al., 2010; Baeyens et al., 2015). Due to increasing demand, there is a huge interest in the discovery of enzymes with improved properties on starch degradation, as well as developing techniques that reduce the cost of production of amylolytic derivatives. Potential of fungi, such as *Aspergillus* and *Rhizopus* among others, are known to produce enzymes including amylases (Vaidya et al., 2015). Fungi are especially valued as excellent producers of enzymes of industrial interest. *Rhizopus* has been highlighted for being a good producer of amylases and its ability to metabolize complex mixtures of organic compounds in most organic residues (Jin et al., 2002; Peixoto-Nogueira et al., 2008; Freitas et al., 2014). In addition, *Rhizopus oligosporus* is considered safe, which is traditionally used in the food industry (Bourdichon et al., 2012).

Solid-state fermentation (SSF) has gained attention over the past 20 years in the development of industrial bioprocesses, due to several advantages over the conventional method of submerged fermentation, such as the particular physiology presented by fungi in this mode of cultivation and particularly due to the lower energy requirement, higher product yields, and less wastewater production with lesser risk of contamination (Barrios-González, 2012; Thomas et al., 2013).

The search for economical bioprocesses for the production of enzymes able to hydrolyze complex carbohydrates, such as the combination of starch and lignocellulosic compounds, has been a current concern. This fact is due to the need for better use of waste as a renewable platform to produce chemicals, food, and biofuels, considering that oil and its derivatives are non-renewable, and expansion of agricultural land is limited. In this context, the objective of this study was to develop a rapid and low cost bioprocess using natural, robust, and safe microorganism for the production of enzymes able to use agro-industrial residues hydrolysis to produce fermentable sugars which can be used for ethanol production or another bioprocess.

2. Materials and methods

2.1. Microorganism and inoculum preparation

Rhizopus oligosporus (CCT 3762) was obtained from Fundação Tropical de Pesquisas e Tecnologia “André Tosello”, Campinas, SP, Brazil. The fungal strain was grown on potato dextrose agar (PDA) (Difco, USA) for 7 days at 30 °C. Viable spores were harvested from the culture by washing with 0.01% (v/v) Tween 80 water solution and the spore suspension was used as inoculum for SSF.

2.2. Solid state fermentation (SSF)

Tests for SSF were conducted in 250 mL Erlenmeyer flasks containing 10 g of wheat bran (Moinho Nacional, Assis, SP, Brazil). To determine the effect of nutrient supplementation on amylase production was carried out in a preliminary experiment to define the concentration range to be used in the central composite design (CCD). The standard solution comprised: 9.3% w/w $(NH_4)_2SO_4$; 4.75% w/w KH_2PO_4; 2.3% w/w urea (Soccol et al., 1994) was compared with trials without supplementation and intermediate concentrations. The substrates were prepared with 60% moisture content, sterilized at 121 °C for 20 min, inoculated with 1.10^6 spores per gram of dry substrate and incubated at 30 ± 0.5 °C (Fanem Ltda Mod.002 CB, São Paulo, Brazil) for 120 h (Escaramboni and Oliva-Neto, 2014). The amylase was extracted from the fermented substrates with 5 mL/g distilled water at 30 °C on an orbital shaker (Tecnal TE-421, São Paulo State, Brazil) at 180 rpm for 30 min. The enzymatic extract was separated by filtration and used for amylase activity assay.

Table 1
Experimental range and levels (including star points*) of each factor (nutrient concentration) in the CCD.

Nutrient[a] concentration (%, w/w)	Coded levels				
	−1.68*	−1	0	1	1.68*
X_1: ammonium sulfate	0	0.5	1.25	2	2.5
X_2: potassium phosphate	0	0.25	0.63	1	1.25
X_3: urea	0	0.25	0.63	1	1.25

[a] Ammonium sulfate – $(NH_4)_2SO_4$ Pure for analysis –American Chemical Society (P.A.-A.C.S.) (Catalog number: 01S1051.01.AH, Synth, Brazil); Monobasic potassium phosphate – KH_2PO_4 P.A. (Catalog number: 01F2002.01.AG, Synth, Brazil); Urea P.A. (Catalog number: 01U1001.01.AH, Synth, Brazil).

2.3. Central composite design (CCD)

Response surface methodology (RSM) was used to determine the mutual interactions among the selected variables and their corresponding optimum concentrations for maximizing the amylase production. Three variables (X_1: ammonium sulfate; X_2: monobasic potassium phosphate; and X_3: urea) were analyzed. A CCD having five coded levels (-1.68, −1, 0, +1, +1.68), eight points associated to 2^3 factorial design, six axial points and three replicates at the center point with a total number of 17 runs was formulated. The experimental range and the levels of each variable are summarized in Table 1.

The second-order polynomial model equation was fit for response variables, y (amylase activity, U/g), was given below:

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where y is the predicted response, β_0 is the offset term, β_i the linear coefficients, β_{ii} the quadratic coefficients and βij the interaction effect. The optimum levels of variables for maximal amylase production were obtained by analysis the response surface and contour plots.

2.4. Statistical analysis

The statistical software STATISTICA 8.0 trial version software (Statsoft Inc., Tulsa, OK, USA) was used to define CCD experimental design as well as analyze statistically the derived data and fit polynomial model for response variable under consideration (amylase activity). Means multiple comparisons in other experiments were performed by ANOVA and subsequently Tukey test (if necessary) in BioEstat 5.0 free software (Mamirauá Institute, Tefé, AM, Brazil).

2.5. Analytical methods

Amylase activity was determined using 0.5% (w/v) soluble starch (Sigma-Aldrich, USA) as substrate in 0.05 M sodium acetate buffer, pH 5.5 as a described method previously (Bernfeld, 1955) with some modifications. The reducing sugar concentration was estimated by the 3,5-dinitrosalicylic acid (DNS) method according to Miller (1959). The reaction mixture containing 100 µL of crude enzyme and 650 µL of substrate was incubated for 10 min at 60 ± 0.1 °C (Marconi MA 127, Piracicaba, SP, Brazil). The reaction was stopped by adding 500 µL of DNS solution followed by heating it in a boiling water bath for 5 min,