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Improvement of bacterial α-amylase production and application using two steps statistical factorial design

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ABSTRACT

Five agro-industrial wastes (AIW) were selected to verify their potential as low cost substrate to produce α-amylase enzyme by bacterial strains using solid state fermentation (SSF) and submerged state fermentation (SMF). Among the AIW used strawberry leaves and watermelon rind produced the maximum α-amylase production under SMF by *B.subtilis* and *B. licheniformis*, respectively. Co-culture of *B.subtilis* and *B. licheniformis* (1:1) on watermelon rind (*Citrullus lanatus*) improved α-amylase production by 176.3% and 329.2%, respectively compared to mono culture of *B.subtilis* and *B. licheniformis*. Decreasing agitation speed to 100 rpm increased the production by 58.1%. In addition, the pretreatment of WMR with different methods before fermentation inhibited the enzyme production. Using two steps of statistical factorial design (Plackett-Burman and Central Composite) to optimize the production medium enhanced α-amylase production by 10.3-fold. The produced α-amylase could effectively hydrolyze AIW, however the highest saccharification yield with the highest reducing sugar (75% and 15 mg/ml) were obtained using cantaloupe peel (*Cucumis melo*). It is superior to use cantaloupe peel as a novel AIW for saccharification produced glucose (78.3%) and uronic acid (21.3%). Moreover, using Central Composite design to optimize saccharification condition of cantaloupe peel enhanced the yield of reducing sugar by 3.73-fold.

1. Introduction

Enzymes are biological catalysis used in various sectors of industry. α-amylase (*E.C.* 3.2.1.1) has been in increasing demand due to its vital role in starch hydrolysis into low molecular weight sugars and its applications (Sundarram and Murthy, 2014). α-amylase has been used in other processes, removing environmental pollutant, bakery, detergent, paper, alcohol and desizing of textiles industries (Sundarram and Murthy, 2014; Singh et al., 2015; Saini et al., 2016). Many strategies have been adopted to increase enzyme production and decrease its production costs including use of microbial co-cultures, optimization of growth conditions, application of crude enzyme extracts and using AIW (Ali et al., 2016). AIW have become the subject of intensive research as it rich in moisture, carbohydrate, protein, in some cases, antioxidants and other bioactive compounds (Panda et al., 2016). Besides, it contains around 60–75% (w/w) starch, hydrolysable to glucose offers a good resource in fermentation processes (Soni et al., 2003). In South Africa it has been estimated that agriculture wastes contribute around 4.2 million tons per annum (Oelofse and Nahman, 2013). Microbial bioprocessing of AIW has been proven to be a potential tool for cleaning up the environment beside production of value added products (Panda et al., 2016). *Bacillus* sp. is popular strain for producing enzymes such as α-amylase (Mussatto et al., 2012). Cheaper substrate selection influences reduction of the enzyme production cost with removing environmental pollutant. Selection of substrate is based on its suitability for microorganism, availability, operational cost, conversion efficiency and non toxic nature (Panda et al., 2016). Production of α-amylase may be classified SSF and SMF. SMF technique is suitable for microorganisms such as bacteria which require high moisture content for their growth (Sundarram and Murthy, 2014). Moreover, the use of statistical designs such as Plackett-Burman and Central Composite has been carried out for α-amylase production (Duque et al., 2016; Panda et al., 2016). These advance statistical approaches offer a design that allows the study of several factors that requires minimum number of runs, thus, saving time and resources. These also allow the screening of critical factors, provide information regarding optimized levels of each factor and knowledge of the interactions between factors and its effect on amylase production. Co-culture is aerobic or an aerobic incubation of different microbial strains under aseptic conditions. Degradation of substrates occurs by the combined metabolic activity of these strains. To have a stable co-culture, the strains must be compatible and able to grow together (Panda et al., 2016). Co-culture fermentation involve two

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or more organisms which lead to better utilization of substrate, increased resistance to contamination, and productivity as compared to mono cultures (Saini et al., 2016).

This study evaluated the production of α-amylase by mono and co-culture of bacterial strains utilizing cheap AIW. Besides, using two steps statistical factorial designed to optimize the production and the application process of biomass hydrolysis by crude α-amylase for biofuel purpose.

2. Material and methods

2.1. Preparation of substrate

Agro- industrial wastes such as orange peel (OP), watermelon rind (WMR), strawberry leave (SL), pomegranate peel (PoP), egg shill (ES), lemon peel (LP), wheat bran (WB), pea peel (PP) and cantaloupe peel (CP) were collected from local market of Giza, Egypt. They were washed with tap water to remove dirt and impurities. The washed substrates were then dried in an oven at 50 °C for 24 h, grinded in a laboratory grinder to ~0.5 cm particle size.

2.2. Microbial cultures and inoculum development

B. licheniformis, B. circulans, B. macerans 314*, B. megaterium, B. amyloliquefaciens, B. subtilis,* and *B. sterothermophilus* were obtained from the Culture Collection of the National Research Centre, Dokki, Cairo, Egypt. *B. licheniformis* ATCC 21415 was obtained from American Type Culture Collection, USA. All bacterial strains were maintained on nutrient agar slants (NAS) at 35 °C and transferred weekly. To develop a homogenous inoculum suspension, a growth of each strain (2 slants of 24 h old $O.D_{600}$~0.11) was transferred into 50 ml of sterilized distilled H_2O and was incubated in a shaking incubator at 150 rpm and 35 °C for 2 h. For the develop a homogenous inoculum suspension for co-culture, 2.5 ml from *B. subtilis* inoculum was mixed with 2.5 ml from *B. licheniformis* inoculum and was added to each production flask.

2.3. Screening the ability to produce α-amylase enzyme

2.3.1. Qualitative screening

The bacterial strains were screened for their abilities to produced α-amylase by starch agar plates test. The sterilized medium containing starch (10%) and agar (12%) was used on sterilized Petri plates. Pure single colony of each bacterial strain was streaked on the culture media and was allowed to grow for 24 and 48 h at 35 °C. Starch agar plates were flooded with iodine solution (1 g iodine dissolved in an aqueous solution of potassium iodide 2%) for 10 min. Positive reaction due to starch hydrolysis is indicated by a clear zone around the bacterial growth. Blue black color on agar plate indicates negative test (Sajjad and Choudhry, 2012).

2.3.2. Quantitative screening

This screening was done by determination of α-amylase activity according to Sajjad and Choudhry (2012) by adding 0.5 ml of clear culture filtrate (crude enzyme) to 0.5 ml of 1% starch solution prepared in acetate buffer (0.05 M, pH 5.0). The mixture was incubated for 20 min at 40 °C and the released reducing sugars were determined by Somogyi method (1952). All the cultures were triplicates and the results are the mean.

2.4. Chemical analysis

The moisture content was determined by drying the sample to a constant weight at 105 °C. The ash content was estimated according to Arumugam and Manikandan (2011) by heating the residue of moisture determination at 550 °C till constant weight. Total carbohydrate was estimated by phenol sulfuric acid using glucose as standard and the color density was measured at 490 nm (Dubois et al., 1956).

2.5. Hydrolysis of some AIW using crude α-amylase

2.5.1. Qualitative examination of the hydrolysis products

These were performed by paper chromatography of the hydrolysates on Whatman No.1 filter paper (Jayme and Knolle, 1956) using the solvent system: *n*-butanol-acetone-water (4:5:1, v/v/v). For a comparison, authentic samples of galactose, glucose, manose, arabinose and xylose were co-chromatographed as reference substances. After chromatographic separation, the chromatogram was air dried and sprayed with 40–50 ml of the aniline-phthalate reagent (Partridge, 1949), air dried and then heated at 105 °C for 10 min in an oven for developing the spots.

2.5.2. Quantitative determination of the hydrolysis products

The detected spots in the chromatogram were cut off, divided into small strips and dropped into test tubes. Eluting agent (4.0 ml) was added to each tube and shaked for complete elution. The absorbance of the resulting colored solutions were determined in a BAUSCH, LOMB spectronic 2000 spectrophotometer at 390 and 360 nm for hexoses and pentoses, respectively. The quantities of sugars were determined by comparison to appropriate standard curves constructed under the same conditions.

2.6. Substrate pretreatment

WMR the best substrate for maximum α-amylase production was pretreated with different methods and the carbohydrate content was determined for each pretreated samples before using for enzyme production.

2.6.1. Liquid hot water pretreatment (LHW)

The fresh WMR were slurried with distilled water in a ratio 1:10 (solid: liquid) and autoclaved at 121 °C for 1 h (Arumugam and Manikandan, 2011). The pretreated sample was cooled to room temperature followed by centrifugation at 5000 × *g* for 20 min and dried at 50 °C in an oven for 24 h.

2.6.2. Wet oxidation pretreatment

In wet oxidation, the WMR was treated with distilled water in a ratio 1:2 (solid: liquid) in flask fully opening and was heated for 15 min at 121 °C (Sarkar et al., 2012). After that the pretreated WMR was cooled to ~25 °C and dried in an oven for 24 h at 50 °C.

2.6.3. Diluted alkaline pretreatment

Watermelon rind was assessed using 0.5 M NaOH with solid: liquid ratio 1:20 (w/v) at 30 °C for 1 h (Begum and Alimon, 2011). The pretreated WMR was washed with acetate buffer (0.02 M, pH 4.3) and washed with distilled H_2O until pH of the sample reached neutral. Sample was dried over night in an oven at 50 °C.

2.6.4. Diluted acid pretreatment

This method was performed by adding diluted H_2SO_4 (0.05 N) to WMR in a ratio solid-liquid 1:20 (w/v) and incubating at 30 °C and 150 rpm for 1 h (Begum and Alimon, 2011). The acid pretreated sample was cooled and the pH was adjusted to 6.0 with NaOH and dried at 50 °C over night.

2.6.5. Microwave oven pretreatment

Pretreatment of WMR in a microwave oven is also a feasible method and easy to operate (Sarkar et al., 2012). In this method, 1.0 g of each pretreated WMR with previous methods (LHW, wet oxidation, dilution H_2SO_4 and dilution NaOH) was subjected to microwave radiation power for 5 min. Samples were screened for enzyme production process.