Production of enzymes by *Bacillus subtilis* using cassava wastewater as substrate.

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ABSTRACT

Bacillus subtilis produces a variety of extracellular enzymes including proteases, amylases and lipolytic enzymes of great importance in industrial processes, such as pharmaceutical, leather, laundry, food and waste processing industries. However, to obtain enzymes for commercial uses, optimized process and reduction of production costs are necessary. The culture media are, besides the energy cost, the largest sources of direct variable costs. Thus, the use of agroindustrial wastes is an alternative due to their low costs, high complexity and nutritional value. The aim of this work was to compare the production of protease, alkaline lipase and α-amylase of a strain of *Bacillus subtilis* LB5a cultivated in a synthetic medium with inductor and in cassava wastewater. The culture was inoculated into 50mL of cassava wastewater or synthetic medium with inductor and incubated aerobically at 30ºC and 150rpm on a reciprocal shaker. Samples were taken periodically until 65h, centrifuged and their enzymatic activities measured. *B. subtilis* was able to produce the three enzymes in both media. The production in cassava wastewater showed a steady increase and at 65h the amylolytic activity was more than four times higher in the waste than the one in synthetic medium. Protease activity increased during the fermentation time in synthetic medium, however in cassava wastewater proteolytic activity has achieved maximum at 50h, which is more than three times higher than in the other medium at the same time. Only lipolytic activity was higher in synthetic medium than in cassava wastewater, which can be easily explained by the presence of inductor only in the fomulated medium. Therefore, it can be stated that cassava wastewater has great potential as alternative substrate to obtain amylase and protease by *Bacillus subtilis*, thus providing a way to reduce production costs.

Keywords: lipase; amylase; cassava wastewater; Bacillus subtilis; protease

INTRODUCTION

The microorganisms of *Bacillus* genus are known to be one of the most important sources of enzymes and other biomolecules of industrial interest, being responsible for the supply of about 50% of the market for enzymes [1]. The world market for enzymes is estimated at 1.6 billion dollars, 29% for the food industry, animal feed 15% and 56% in other applications [2].

Bacillus subtilis produces a variety of extracellular enzymes including proteases, amylases and lipolytic enzymes of great importance in industrial processes, such as pharmaceutical, leather, laundry, food and waste processing industries [1, 3].

Among the different categories, the hydrolase enzymes are among the largest of industrial application and, among these, the alpha-amylase and beta-galactosidase have received special attention [4]. These enzymes catalyze the hydrolysis of starch and are produced by a wide variety of microorganisms, however, for commercial applications they are basically derived from the genus *Bacillus* [1]. The major amylase produced by Bacillus are heat resistant, which is commercially interesting because many processes require high temperatures, so the thermosensibility ceases to be a limiting factor [4].

Another important group is the protease, which represents about 30% of the total sold worldwide enzymes. The thermostable proteases produced by *Bacillus* sp are among the most industrially important [6].

Finally, lipases catalyze the hydrolysis of triacylglycerols and are widely used in organic chemistry due to its high selectivity and specificity and, therefore, receive much attention because of its potential use in industrial processes [6]. *Bacillus subtilis* secretes different types of lipases, which may vary according to different growth conditions, environmental factors such as pH and amino acid supply [7].

Cassava wastewater is a carbohydrate-rich residue generated at large amounts during the processing of cassava flour and starch. The disposable of this residue causes environmental problems due to its rich composition in macro and micronutrients; however, it is a very attractive substrate for biotechnological processes [8].

Thus, the aim of this work was to test the viability of production of protease, alkaline lipase and α-amylase by a strain of *Bacillus subtilis* LB5a using cassava wastewater as substrate instead of a synthetic medium with inductor.

MATERIALS & METHODS

Microorganism and inoculum preparation

A strain of *B. subtilis*, previously isolated and identified as LB5a, pertaining to the culture collection of the Bioflavors Laboratory of DCA/FEA/Unicamp, was used. A loop of culture growth taken from a Petri dish was transferred to a conical flask containing 150 mL of nutrient broth and maintained at 30 °C for 24 h in a rotary shaker bath at a speed of 150 rpm. The inoculum was standardized by measuring the optical density at λ= 660 nm. A volume of inoculum sufficient for the culture to reach the initial concentration of $2x10^7$ CFU/mL was taken. Sterile nutrient broth, prepared under the same conditions, was used as the blank, and a parallel standard count was carried out.

Preparation and characterization of substrates

Experiments were carried out using two different substrates for each enzymatic test: cassava wastewater and synthetic medium with inductor.

Cassava wastewater was collected from a cassava flour factory (Plaza LTDA, Santa Maria da Serra, SP, Brazil) and transported to the place of processing at room temperature. It was homogenized, boiled, cooled, centrifuged at 100 rpm for 20 min and stored frozen until used. A volume of 50 mL of the previously treated cassava wastewater was poured into conical flasks and sterilized at 121 °C for 20 min. This substrate was characterized by analyses for total nitrogen, total and reducing carbohydrates, the mineral fraction (P, K, Ca, Mg, S, Al, B, Cu, Fe, Mn, Zn, Cd, Cr, Ni and Pb), ammonia, nitrate and pH. All experiments were carried out with the same substrate.

Synthetic media were prepared from a basal medium with the following composition in $g.L^{-1}$ in distilled water: yeast extract 1,0; KH_2PO_4 2,0; $(NH_4)_2SO_4$ 5,0; Sodium citrate 1,0; $MgSO_4.7H_2O$ 0,2; $CaCl_2.2H_2O$ 0,01; 0,001 $FeCl_3$; $MnSO_4.7H_2O$ 0,001; $ZnSO_4.7H_2O$ 0,001. Therefore, the medium was supplemented with 1 % (w/v) of olive oil, for lipolytic activity, soluble starch, for amylase activity, and glucose, for proteolytic activity. A volume of 50 mL of each medium was poured in conical flasks and sterilized at 121 °C for 20 min.

Fermentative process

The inoculum, using an amount calculated previously in the standardization phase, was added to the culture media. Fermentation was carried out in reciprocal shaker at 30 °C and agitation 150 rpm. Media samples were collected at regular intervals, until 65 h of experiment, centrifuged for 10 minutes at 10.000 rpm and 5 °C and the supernatants enzymatic activities were determined.

Proteolytic activity

Protease activity was based in the capacity of the extracts to promote casein hydrolysis. Thus, 0,5 mL of each extract was added to 5 mL casein 1,2% (w/v) and incubated in thermostatic bath at 37 °C for 30 minutes. After incubation, the reaction was paralyzed by adding 4 mL 200 mM acetate buffer (pH 4,0), then cooled in ice bath and filtered. To 1 mL of filtered extract were added 3 mL NaOH 1M and 0,5 mL Folin-Ciocalteau reagent diluted 1:1 in distilled water. Samples absorbance were measured at 660 nm in spectrophotometer against blank containing distilled water instead of the extracts. Enzymatic activity was measured using a tyrosine standard curve. [9]

Amylolytic activity

Amylase activity was determined considering the extracts capacity to hydrolyze soluble starch. Therefore, 0,5 mL of each extract was added to 5 mL starch 1 % (w/v) in 200 mM phosphate buffer (pH 7,0) and then incubated at 37 °C for 10 minutes. After incubation, 5 mL HCl 0,1 M was added to paralyze the reaction. To 0,5 mL of this solution, 5 mL I_2-KI 0,5 : 5,0 % (w/v) was added, the resulting solution was diluted in 200 mM phosphate buffer (pH 7,0) 1 : 9 [10].

Lipolytic activity

Lipolytic activity was based in the capacity of the extracts to promote hydrolysis of olive oil triglycerides. Thus, 1 mL of each extract was added to flasks containing 4 mL 200 mM fosfate buffer (pH 9,0), 1 mL CaCl2 110 mM, 5 mL emulsion 25% (v/v) of olive oil in Arabic gum 7% (w/v). The flasks were incubated

reciprocal bath for 20 min at 30°C and 160 rpm. After the incubation, the reaction was paralyzed by adding 15 mL acetone:ethanol (1:1). Fatty acids released were titrated with NaOH 0,05 M using phenolphthalein as indicator [11].

RESULTS & DISCUSSION

The cassava wastewater presented a good level of carbohydrates and several important micronutrients characterizing it as a good substrate for the development of microorganisms as well as for the production of biosurfactants [12]. The composition of this waste used in the experiments, is shown in Table 1, below.

Table 1. Cassava wastewater composition

Component	Concentration	Component	Concentration
Total carbohydrates	16,9 g/L	Manganese	2.7 mg/L
Reducing sugars	15,8 g/L	Zinc	0.6 mg/L
Total nitrogen	0.7 mg/L	Aluminium	< 0.01 mg/L
Phosphorous	0.4 mg/L	Organic Carbon	14.3 mg/L
Potassium	2.8 mg/L	Ammonium	54.3 mg/L
Calcium	0.3 mg/L	Nitrate	43.4 mg/L
Magnesium	0.7 mg/L	pH	6.0
Sulfur	0.1 mg/L		
Boron	0.7 mg/L		
Cooper	0.4 mg/L		
Iron	6.7 mg/L		

The strain of B. subtilis was capable to produce the three kinds of enzymes tested. These results are shown in Figures 1, 2 and 3 below.

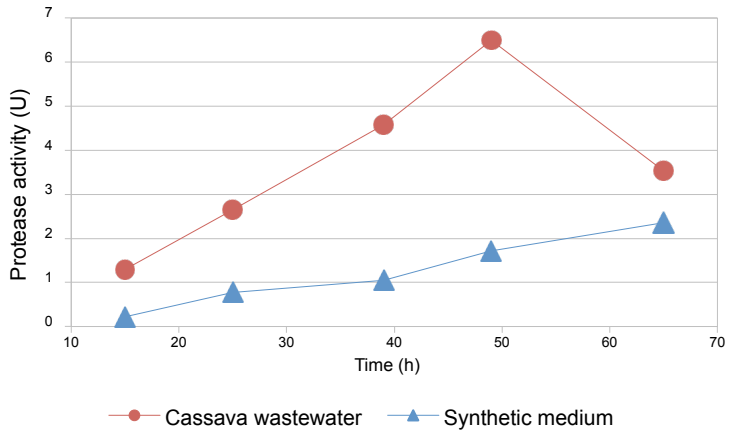

Figure 1. Proteolytic activity of the extracts.

Proteolytic activity (U = µmols of tyrosine released mL^{-1} h^{-1}) increased constantly during the fermentation in synthetic medium but always showed inferior values in comparison with cassava wastewater. In this medium, protease production has achieved its maximum value (6,5 U) at 50 h of fermentation, a result more than three times higher than the maximum found in the synthetic medium (1,7 U at 65 h).

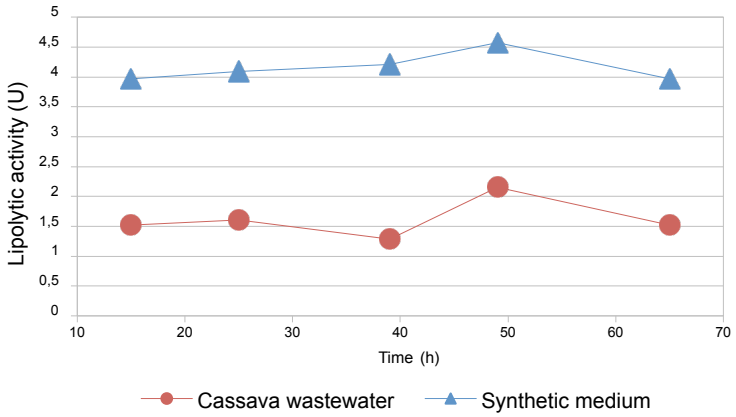

Figure 2. Lipolytic activity of the extracts.

Lipolytic activity (U = µmols of fatty acid released mL^{-1} min^{-1}) was higher in the synthetic medium than in cassava wastewater, which can be explained by the addition of olive oil only to the formulated medium. Activity values were practically constant during all the study in both media.

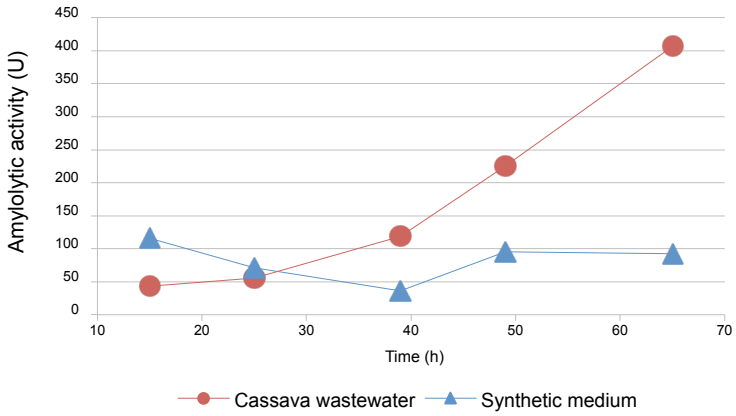

Figure 3. Amylolytic activity of the extracts.

Amylase activity (U = reduction of 0,1 mg of starch during 10 min per 0,5 mL of enzyme) in the residue showed a constant increase during all the fermentation, while the activity in the formulated medium did not show much variation. At 65 h, the amylolytic activity in the extract of the waste medium reached a value of 405,1 U, which is almost four times bigger than the best activity found in the synthetic medium (115,3 U at 15 h).

CONCLUSION

Amylase and protease production were approximately four and three times higher, respectively, in the medium composed of cassava wastewater than the synthetic medium with inducers. Therefore, it can be said that cassava wastewater presents a great potential as an alternative substrate to the production of amylase and protease by *Bacillus subilis* Lb5a, and its utilization could reduce the production costs of these enzymes, besides promoting the application of a highly polluting residue.

The strain of *Bacillus subilis* tested was not a good producer of alkaline lipases in cassava wastewater, probably due to its lack of a triglyceride acting as an inducer.

ACKNOWLEDGEMENT

The authors are thankful to CNPq and FAPESP.

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