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Short Communication

Use of a new *Trichoderma harzianum* strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production

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ABSTRACT

The on-site production of cellulases is an important strategy for the development of sustainable second-generation ethanol production processes. This study concerns the use of a specific cellulolytic enzyme complex for hydrolysis of pretreated sugar cane bagasse. Glycosyl hydrolases (FPase, xylanase, and β-glucosidase) were produced using a new strain of *Trichoderma harzianum*, isolated from the Amazon rainforest and cultivated under different conditions. The influence of the carbon source was first investigated using shake-flask cultures. Selected carbon sources were then further studied under different pH conditions using a stirred tank bioreactor. Enzymatic activities up to 121 FPU/g, 8000 IU/g, and 1730 IU/g of delignified steam-exploded bagasse + sucrose were achieved for cellulase, xylanase and β-glucosidase, respectively. This enzymatic complex was used to hydrolyze pretreated sugar cane bagasse. A comparative evaluation, using an enzymatic extract from *Trichoderma reesei* RUTC30, indicated similar performance of the *T. harzianum* enzyme complex, being a potential candidate for on-site production of enzymes.

1. Introduction

Cellulases are glycosyl hydrolases (GH) that play an important role in the bioconversion of cellulosic materials into biofuels. The major bottleneck for a wider application of cellulases in second-generation ethanol production is their cost, especially because large quantities of the enzymes are required (Zhang et al., 2006). Despite the reduction in costs of commercial enzymes (claimed by the industry), the production know-how remains a strategic issue to be considered during the development of a sustainable process for ethanol production from biomass. Besides, the effectiveness of the enzymatic mixture for hydrolysis is highly dependent on the feedstock and the pretreatment that it has received. It is therefore logical to try to produce, on-site, tailor-made enzymatic mixtures that are optimized for the specific pretreated feedstock to be processed. Furthermore, the use of the same material for enzyme production and hydrolysis could reduce the production costs of second-generation ethanol, since both processes could be co-located and share infrastructure and utilities. Moreover, cultivation of the microorganisms using the same lignocellulosic material that is intended to be hydrolyzed could be a way of selecting for the production of enzymes that are optimal for the hydrolysis of that specific material (Jorgensen and Olsson, 2006; Sorensen et al., 2011).

For on-site cellulase production, the selection of fungal strains possessing high expression capacities and a diversity of cellulolytic enzymes with high specific activity is essential in order to obtain enzyme complexes able to hydrolyze plant biomass at reduced cost (Lynd et al., 2002). In addition to the selection of fungal strain, the level of expression of cellulases is determined by the composition of the medium and the growth conditions. The metabolic processes of the microorganisms are influenced to a great extent by changes of temperature, pH, substrate and aeration, as well as by the inoculum concentration, and optimal culture conditions vary widely between species of the same organism.

The objective of the present work was to develop a procedure for on-site production of a highly effective cellulolytic enzyme complex to be used in the hydrolysis of pretreated sugar cane bagasse. The production of glycosyl hydrolases (FPase, xylanase, and β-glucosidase) was studied using a new strain of *Trichoderma harzianum*, isolated from the Amazon rainforest and cultivated under different process conditions. The influence of the type of carbon source was investigated using shake flask cultures, after which the selected substrates were studied further under different pH conditions using a stirred tank bioreactor. The enzymatic complex was then used to hydrolyze pretreated sugar cane bagasse.

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Table 1
Influence of different carbon sources and their combinations on the production of FPase, xylanase, β-glucosidase and total protein by *T. harzianum* P49P11 during submerged fermentation in flasks. The optimum cultivation times are indicated.

Substrate	FPase (FPU/mL h)	Xylanase (IU/mL h)	β-Glucosidase (IU/mL h)	Total protein (mg/mL h)
SB	0.78–96	36.96–96	9.18–96	0.56–120
DSB	0.50–72	5.69–96	1.21–72	0.53–96
Sucrose	0.42–96	22.24–72	5.48–120	0.55–72
Glycerol	0.12 -120	4.34–96	1.25–120	0.27–120
Lactose	0.22–72	23.47–72	1.73–120	0.29–120
FOS	0.43–96	23.27–72	4.11–72	0.65–72
SB + sucrose	0.85–72	33.13–96	7.37–72	0.50–96
SB + glycerol	0.22–48	5.32–120	0.38–72	0.26–96
SB + lactose	0.35–72	17.92–96	4.84–120	0.45–120
SB + FOS	0.69–48	24.02–72	0.44–72	0.31–120
DSB + sucrose	0.46–96	15.02–72	1.50–96	0.27–96
DSB + glycerol	0.32–96	7.22–96	0,50–72	0.29–120
DSB + lactose	0.48–72	14.20–72	1.09–72	0.32–120
DSB + FOS	0.60–72	17.53–72	0.88–96	0.27–96

SB: steam-pretreated bagasse; DSB: bagasse pretreated using steam followed by delignification with NaOH; FOS: fructooligosaccharide.

Table 2
Production of FPase, xylanase, β-glucosidase and total protein by *T. harzianum* P49P11 using submerged fermentation in a bioreactor. The pH was fixed (at 5.0 or 6.0) or allowed to fluctuate.

Substrate/pH	FPase (FPU/mL)	Xylanase (IU/mL)	β-Glucosidase (IU/mL)	Total protein (mg/mL)
SB				
pH 5.0	0.73	29.69	5.22	0.45
pH 6.0	0.80	63.41	10.21	0.43
LTF	0.57	20.82	4.82	0.52
DSB				
pH 5.0	0.80	58.00	15.65	0.59
pH 6.0	0.95	51.00	11.33	0.54
LTF	0.98	12.49	9.42	0.74
SB + sucrose				
pH 5.0	0.82	34.72	10.09	0.57
pH 6.0	0.84	83.00	15.56	0.52
LTF	0.89	36.73	8.45	0.64
DSB + sucrose				
pH 5.0	1.21	80.00	17.32	0.83
pH 6.0	1.01	86.00	16.83	0.71
LTF	1.02	30.11	15.04	0.76

LTF: "left to float" fluctuating pH procedure; SB: steam-pretreated bagasse; DSB: bagasse pretreated using steam followed by delignification with NaOH.

2. Methods

2.1. Microorganisms

The *T. harzianum* strain (P49P11) was isolated by screening 100 soil and decomposed wood samples from the Amazon forest reserve of Embrapa (Belém, PA – Brazil) using crystalline cellulose (Avicel) (Sigma, St. Louis, USA) as the only source of carbon. The *Trichoderma reesei* RUT C30 strain utilized was from the American Type Culture Collection. Stock cultures were stored at 4 °C on potato dextrose agar (PDA) (Difco™, Detroit, USA) slants. The fungi were grown on PDA plates (90 × 15 mm) at 29 °C for 7 days.

2.2. Pre-culture and production media

The composition of the pre-culture medium was adapted from Mandels and Weber (1969) using 10 g/L of glucose as carbon source. The pH was adjusted to 5.0 and the culture medium was sterilized at 121 °C for 20 min. The composition of the production medium was the same as that of the pre-culture medium, except for the type of carbon source. Two different insoluble carbon sources were evaluated: sugar cane bagasse pretreated using steam (designated here as SB) and delignified sugar cane bagasse (designated as DSB), which were prepared and characterized according to Rocha et al. (2012). The sugar cane bagasse was from a local mill (Usina Vale do Rosário, Orlândia, SP, Brazil). The compositions of the materials in terms of cellulose, hemicellulose and lignin were, respectively, 51.7 ± 0.6, 8.9 ± 0.1, and 34.3 ± 0.3 for SB and 89.5 ± 1.6, 3.4 ± 0.3, and 5.5 ± 0.2 for DSB. The two types of pretreated bagasse were combined with the following soluble carbon sources: sucrose, lactose, glycerol, and a fructooligosaccharide (FOS) (Nutraflora®, Corn Products, Brazil). All other chemicals used were of at least analytical grade.

2.3. Shake flask cultures

A conidia suspension, prepared by adding 20 mL of sterilized distilled water and Tween 80 to the grown PDA plates, was transferred to Erlenmeyer flasks containing 50 mL of pre-culture medium and incubated for 72 h at 29 °C on a rotary shaker at 250 rpm. A volume of 20 mL of this pre-culture was transferred to 500 mL Erlenmeyer flasks containing 200 mL of the production medium. Two sets of experiments were performed. The carbon sources (SB, DSB, sucrose, glycerol, lactose, and FOS) were first used individually, after which a second set of experiments were carried out using mixtures of SB or DSB with either