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ORIGINAL ARTICLE

Bacillus subtilis with endocellulase and exocellulase activities isolated in the thermophilic phase from composting with coffee residues

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Abstract The goal of this study was to isolate, select and characterize bacteria with cellulolytic activity from two different coffee residue composting piles, one of which had an internal temperature of 57 °C and pH 5.5 and the other, a temperature of 61 °C, and pH 9.3. Culture media were manipulated with carboxymethylcellulose and crystalline cellulose as sole carbon sources. The enzyme activity was assessed by hydrolysis halo formation, reducing sugar production and zymograms. Three out of twenty isolated strains showed higher enzymatic activity and were identified as *Bacillus subtilis* according to their morphological, physiological, biochemical characteristics and based on the sequence analysis of 16S rDNA regions. The enzymatic extracts of the three selected strains showed exocellulase and endocellulase maximum activity of 0.254 and 0.519 U/ml, respectively; the activity of these enzymes was maintained even in acid pH (4.8) and basic (9.3) and at temperatures of up to 60 °C. The enzymatic activities observed in this study are within the highest reported for cellulose produced by bacteria of the genus *Bacillus*. Endocellulase activity was shown in the zymograms from 24 h until 144 h of incubation. Furthermore, the pH effect on the endocellulase activity is reported for the first time by zymograms. The findings in this study entail the possibility to use these enzymes in the procurement of fermentable substrates for the production of energy from the large amount of residues generated by the coffee agroindustry.

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PALABRAS CLAVE
Bacterias;
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Celulasas

Bacillus subtilis con actividades de endocelulasa y exocelulasa aislado en la fase termofílica del compostaje con residuos de café

Resumen El objetivo de este estudio fue aislar, seleccionar y caracterizar bacterias con actividad celulolítica a partir de 2 diferentes pilas de compostaje de residuos de café, una con temperatura interna de 57 °C y pH 5,5; la otra con temperatura interna de 61 °C y pH 9,3. Se utilizaron medios de cultivo con carboximetilcelulosa y celulosa cristalina como únicas fuentes de carbono. La actividad enzimática fue evaluada por formación de halos de hidrólisis, producción de azúcares reductores y zimogramas. De 20 cepas aisladas, 3 presentaron mayor actividad enzimática y fueron identificadas como *Bacillus subtilis* sobre la base de sus características morfológicas, fisiológicas y bioquímicas y del análisis de las secuencias de la región 16S del ADNr. Los extractos enzimáticos de las 3 cepas seleccionadas presentaron actividad de exocelulasa y de endocelulasa, con máximos de 0,254 y 0,519 U/ml, respectivamente; la actividad de estas enzimas se mantuvo incluso a pH ácido (4,8) o básico (9,3) y a temperaturas de hasta 60 °C. Las actividades enzimáticas halladas en este estudio se ubican dentro de las más altas reportadas para celulasas producidas por bacterias del género *Bacillus*. En los zimogramas se demostró actividad de endocelulasa desde las 24 h hasta las 144 h de incubación. Asimismo, se reporta por primera vez el efecto del pH sobre la actividad de endocelulasa observado por zimogramas. Los resultados de este estudio abren la posibilidad de hacer uso de estas enzimas en la obtención de sustratos fermentables para la producción de energía a partir de los residuos generados en grandes cantidades por la agroindustria del café.

Introduction

The production of commercial microbial cellulases has a worldwide market. Cellulases are widely used in research and in the food, paper, textile, chemical and energy industries[9,47]. Most cellulases are derived from fungi because they have shown high activity and produced cellulases in large amounts. In recent years, the need for more robust cellulases in complex substrates, such as agricultural residues with high cellulose content, has increased due to their uses as potentially fermentable substrates in the production of biofuels[20,30]. One option for this would be bacterial cellulases, which form synergistic multienzymatic complexes with increased activity[43]. Moreover, bacterial cellulases are active at extreme pH, salinity and temperature conditions, such as those encountered in composting processes[41], including high temperature environments where the presence of thermostable enzymes increase bioconversion rates[1]. The bacterial species that dominate the thermophilic phase of compost processes belong to the genus *Bacillus*. They play an important role in the degradation of complex substrates, such as cellulose[10]. There are several reports of bacterial cellulases with activities at extreme pH (acidic or basic), high salinity and temperatures[3,6,17,18,24]. These cellulases have been isolated from several environments, such as termite guts[5], soil contaminated with paper industry residues[37], mangrove soil[7], degraded lignocellulosic biomass[49] and compost systems of kitchen and garden residues, municipal solid wastes, coffee residues and other agricultural residues[16,29,34,39,42]. These cellulosic residues are complex, and each of them has characteristics conferring certain specificity. Residues generated by the coffee industry also contain caffeine, gallic acid, caffeic acid, chlorogenic acid, and phenolic compounds[8] and have high acidity. In the Soconusco region, Chiapas, Mexico, large amounts of residues are generated in wet coffee processing (approximately 7200 tons), equivalent to 80% of the annual coffee production of 9000 tons[46]. These residues can be used to obtain fermentable substrates for the sustainable production of ethanol in coffee-producing zones. Thermostable and acidophilic enzymes are required to take advantage of the cellulose of complex substrates, such as those from this agricultural residue. Therefore, the aim of this study was to isolate, select and characterize bacterial strains with cellulolytic capacity from the composting process with coffee residues.

Materials and methods

Isolation of cellulolytic bacteria from composting with coffee residues

Samples were obtained from the middle area of two composting piles, each one containing coffee residues (pulp and husk) and cow manure at a ratio of 3:1 (v/v). The piles showed different characteristics: pile 1: nine days of composting, with an internal temperature of 57 °C and pH 5.5; and pile 2: 36 days of composting with an internal temperature of 61 °C and pH 9.3.

For the isolation of cellulose degrading bacteria, about 1 g of sample was added to 9 ml NaCl 0.9% and serially diluted and pour plate and spread plate techniques were done using mineral medium (g/l): 1.4 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.3 urea, 0.3 $CaCl_2 \cdot 2H_2O$, 0.3 $MgSO_4 \cdot 4H_2O$, 0.005 $FeSO_4 \cdot 7H_2O$, 0.0016

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