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Selection and molecular characterization of cellulolytic–xylanolytic fungi from surface soil-biomass mixtures from Black Belt sites

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ABSTRACT

Plant biomass is an abundant renewable natural resource that can be transformed into chemical feedstocks. Enzymes used in saccharification of lignocellulosic biomass are a major part of biofuel production costs. A cocktail of cellulolytic and xylanolytic enzymes are required for optimal saccharification of biomass. Accordingly, thirty-two fungal pure cultures were obtained from surface soil-biomass mixtures collected from Black Belt sites in Alabama by culturing on lignocellulosic biomass medium. The fungal strains were screened for the coproduction of cellulolytic and xylanolytic enzymes. Strains that displayed promising levels of cellulolytic and xylanolytic enzymes were characterized by molecular analysis of DNA sequences from the large subunit and internal transcribed spacer (ITS) of their ribosomal RNA gene. Nucleotide sequence analysis revealed that two most promising isolates FS22A and FS5A were most similar to *Penicillium janthinellum* and *Trichoderma virens*. Production dynamics of cellulolytic and xylanolytic enzymes from these two strains were explored in submerged fermentation. Volumetric productivity after 120 h incubation was 121.08 units/L/h and 348 units/L/h for the filter paper cellulase and xylanase of strain FS22A, and 90.83 units/L/h and 359 units/L/h, respectively for strain FS5A. Assays with 10 times dilution of enzymes revealed that the activities were much higher than that observed in the crude culture supernatant. Additionally, both FS22A and FS5A also produced amylase in lignocellulose medium. The enzyme profiles of these strains and their activities suggest potential applications in cost effective biomass conversion and biodegradation.

1. Introduction

Plant biomass is largely composed of lignocellulose which is the most abundant renewable natural resource in the biosphere. Lignocellulosic biomass encompasses 40–50% cellulose, 25–30% hemicellulose and 15–20% lignin (Wiselogel et al. 1996). Cellulose is the largest constituent of lignocelluloses and as much as 180 billion tons of cellulose is produced annually by plants (Park et al. 2011). The amount of cellulose synthesized by plants in relation to human population has been estimated to be about 70 kg per person per day (Lutzen et al. 1983) and has been referred to as 'biological currency' (Himmel et al. 1999). Xylan is a major hemicellulose and is the second most abundant renewable constituent of plant materials (Yang et al. 2011; Yan et al. 2009).

Cellulose is an unbranched polymer of glucose units, which can be broken down to glucose, cellobiose and cellooligosaccharides by biological and physicochemical processes. Cellulolytic enzymes, including endocellulase or endoglucanase (EC 3.2.1.4), exocellulase or exoglucanase or cellobiohydrolase (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) act in concert to breakdown cellulose polymer (Guo et al. 2008; Thongekkaew et al. 2008). Xylan is a heterogenous polysaccharide with a backbone consisting of linear xylose sequence along with side chain constituents (Kulkarni et al. 1999). Its heterogeneous nature demands a diverse array of xylanolytic enzymes for complete hydrolysis (Collins et al. 2005). Endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) degrade the main chain, whereas α-glucuronidase, α-L-arabinofuranosidase and acetylxylan esterase sever the side chain components (Biely 1985; Ryabova et al. 2009).

Depletion of fossil fuel reserves (Deswal et al. 2011) and their high price as well as concerns for energy security and the environment (Ruffell et al. 2010) have rekindled worldwide interest in renewable fuel from bioresources such as lignocellulose. Moreover, lignocellulosic materials are energy-rich, relatively inexpensive

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and available worldwide (Zhang et al. 2012). Approximately 340 million cubic meters of ethanol fuel could be produced from the available U.S. lignocellulosic material per year; and up to 40% of U.S. fuel demand could potentially be satisfied by lignocellulosic biofuels (Perlack et al. 2005).

Efficient enzymatic hydrolysis or saccharification is central to production of renewable biomass chemicals and fuel. Fungi are the primary sources of lignocellulose hydrolyzing enzymes (Bakri et al. 2003; de Vries and Visser 2001; Li et al. 2010; Maheshwari et al. 1994; Okeke 2014; Pedersen et al. 2009; Seyis and Aksoz 2003; Wen et al. 2005). Presently, cellulase production employs the mutant *Trichoderma reesei* Rut C30 (Lo et al. 2010). However, the amount of β-glucosidase secreted by *T. reesei* strain is insufficient for productive saccharification of biomass to glucose (Sternberg et al. 1977). Thus, individual enzymes originating from different fungal species produced in independent fermentations are mixed to make an optimal cocktail of cellulolytic and xylanolytic enzymes for efficient conversion of lignocellulose biomass to sugars. Multiple processing steps like producing individual enzymes from different microbes consequently contribute to high cost of enzymes for large-scale biomass saccharification. It would be ideal to find microbes that can produce all the necessary cellulolytic and xylanolytic enzymes to enable production of an ideal cocktail with a single fermentation to reduce the cost of enzymes and consequently the cost of biomass conversion and biofuel production.

Overall, the efficiency of biomass conversion to sugars and to fuel and chemicals is still a challenge (Lin et al. 2011) and demands more research including the search for more efficient microbes, enzymes and pretreatment methods. The Black Belt counties of Alabama are important agricultural areas and presumed to have accumulated high quantities of lignocellulosic biomass over numerous years, potentially hosting novel lignocellulose degrading microorganisms. In a quest for fungal strains that produce a cocktail of cellulolytic and xyalonlytic enzymes for biomass hydrolysis, this study aimed to (i) sample lignocellulose rich soils from representative Black Belt sites for isolation of potent cellulolytic–xylanolytic fungi, (ii) molecularly characterize isolates, and (iii) assess production of the enzymes by promising isolates.

2. Materials and methods

2.1. Site selection and collection of biomass-rich soil samples

The collection of biomass-rich soil samples was undertaken in order to seek and culture high enzyme yielding fungal isolates. Thus, potential high species diversity and relatively high levels of decomposing cellulosic material at the surface were deemed to be important criteria in selecting sample locations. Undisturbed forest sites are relatively rich in microbial diversity (Thoms et al. 2010), and soils from undisturbed pastures and pine forests have higher catabolic diversity than cropped soils (Degens et al. 2000). It also seemed reasonable that sites used for composting such materials, or with substantial amounts of surface litter might give a high probability of finding novel isolates. So we selected representative agricultural sites with visible remaining surface litter, reasoning that those sites would harbor microorganisms adept at decomposing agricultural waste. Forested areas in the Alabama Black Belt counties (see Table 1 and Fig. 1) were chosen based on the visual appearance of being relatively undisturbed. Specific criteria included the absence of recent cutting or clearing, the absence of evidence of recent fire, a robust litter layer, no nearby structures, and with a sufficiently closed canopy to provide shade. Forested sites were either mixed species forest or pine forests. Agricultural sites (corn, cotton, and soybean fields) were chosen by visually determining that there was substantial organic biomass cover like burrs, husks and hulls covering the soil. Most of these sites had been untilled for at least two harvesting seasons. A few sites containing long-term compost piles were sampled and these included compost from landscaping waste and composite compost piles from manure, grass clippings and crop surplus. The procedure for collecting soil and surface biomass (SSB) was as follows: a small area of approximately 100 cm^2 was cleared of all large loose debris, such as logs and limbs and the top litter layer including dead leaves and small twigs. Decaying organic matter (O layer) was collected by scraping down to the soil surface across the entire area and placing it into a sterile plastic bag. This was followed by obtaining four to six soil core samples from 10 to 20 cm of depth and composited into the same bag. Ancillary data including the latitude and longitude (by GPS) and a general description of the sample location were recorded. Map of sites was generated using Google Fusion Tables API. Samples were promptly refrigerated and stored at 4 °C. Samples were thoroughly mixed prior to inoculation of enrichment medium.

2.2. Enrichment of lignocelluloses-degrading fungi

The enrichment basal medium composed (in g/L): KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.2 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; $CaCl_2$, 0.1 g; $FeSO_4{\cdot}7H_2O$, 0.003 g; yeast extract, 0.2 g; Tween 80, 0.5 g (Okeke 2014) and 2 mL trace elements solution (Focht 1994). About 50 mL of enrichment basal medium, 0.25 g sawdust, and 0.25 g shredded paper were placed into 250 mL flasks and autoclaved at 121 °C for 20 min. Upon cooling to room temperature, 2.5 g of each composited SSB sample was placed into the sterilized medium. Cultures were incubated in an orbital shaker at 30 °C for 3 days. After 3 days, 2.5 mL of each culture was transferred into a fresh isolation medium and further incubated for approximately 3.5 days. All enrichment cultures were set up in duplicates.

2.3. Isolation of cellulolytic and xylanolytic pure cultures from enrichment cultures

The enrichment culture was diluted serially from 10^{-1} to 10^{-5} in sterile saline (0.9% NaCl). Diluted enrichment samples (10^{-2} to 10^{-5}) were plated on the enrichment basal medium to which sawdust (4 g/L), carboxymethyl (CM)-cellulose (1 g/L), chloroamphenicol (75 mg/L) and agar (20 g/L) were added. Cultures were incubated at 30 °C for 7 days. Discrete fungal colonies growing luxuriantly on the enrichment basal medium were plated on potato dextrose agar and incubated at 30 °C for 3 days. Cultures were preserved at 4 °C until assessed for production of cellulase and xylanase.

2.4. Screening of fungal isolates for the production of cellulolytic and xylanolytic enzymes

Thirty-two potential cellulolytic and xylanolytic fungal isolates were screened for enzyme production in submerged medium. The medium comprised (per liter) of KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.2 g; $MgSO_4{\cdot}7H_2O$, 0.5 g; $CaCl_2$, 0.1 g; $FeSO_4{\cdot}7H_2O$, 0.003 g and 2 mL of trace elements solution (Focht 1994). In independent parallel duplicate experiments, sterile 50 mL of the enrichment medium in 250 mL Erlenmeyer flasks were inoculated with two 1.25 cm agar discs obtained from potato dextrose agar plate cultures of each isolate and incubated at 30 °C for 3 days. Cultures were incubated with orbital shaking (200 rpm) at 30 °C for 7 days. Fungal biomass was removed by centrifugation (5000 rpm for10 min) and the supernatant was assessed for filter paper cellulase and xylanase activities. Nine fungi selected based on cellulase and xylanase