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Research Paper

Thermomyces lanuginosus STm: A source of thermostable hydrolytic enzymes for novel application in extraction of high-quality natural rubber from *Taraxacum kok-saghyz* (Rubber dandelion)

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ABSTRACT

Hydrolytic enzymes from a newly isolated strain of the thermophilic fungus *Thermomyces lanuginosus* were used to extract rubber from *Taraxacum kok-saghyz* commonly known as rubber (or Russian or Kazak(h)) dandelion. The fungus was isolated from garden soil and identified as *Thermomyces lanuginosus* STm based on 18S rRNA gene sequence analysis. The isolate produced considerable amounts of extracellular hydrolytic enzymes on lignocellulosic substrates at 55 °C incubated for 8 days in 150 mL shake flask experiments. The maximum enzyme activities on wheat straw and guayule bagasse were: xylanase (167.41; 130.1 U/mg), inulinase (69.8; 34.1 U/mg), cellulase (carboxymethyl cellulase) (16.7; 4.8 U/mg), filter paper assay (FPase) (14.2; 5.5 FPU g^{-1}) and pectinase (7.2; 3.2 U/mg), respectively. In addition, alkali-pretreated roots of *Taraxacum kok-saghyz* (TK), incubated with crude enzyme extracts from *T. lanuginosus* STm grown on guayule bagasse, subsequently yielded more natural rubber (90 mg/g dry TK root) than previously established protocols, Eskew process (24 mg/g) and commercial-enzyme-combination process (45 mg/g). Rubber purity in the *T. lanuginosus* STm treatment was 71.7%, greater than the Eskew process at 37.5%. However, the crude *T. lanuginosus* STm enzyme treatment at 91.6% rubber purity approached the purity of the commercial-enzyme-combination process at 94.1% purity.

1. Introduction

Microbial based enzymes have multifaceted applications in biofuel, pulp and paper, and food and feed (van den Brink and de Vries, 2011). Generally, agricultural wastes and forest residues (soft and hardwoods) are the major source of lignocellulosic biomass worldwide (Chaudhary et al., 2012). The basic biochemical nature of lignocellulosic biomass provides simple biobased value-added products such as biofuels and base chemicals (Maitan-Alfenas et al., 2015). Degradation of lignocellulose is mainly distributed among bacteria and fungi. Hundreds of different species of fungi are able to degrade lignocellulose, and the most commonly isolated fungi are *Aspergillus*, *Penicillium*, *Rhizopus*, *Trichoderma* and *Thermomyces*.

A major barrier to industrial application of lignocellulosic wastes (LCW) is the structure of lignocellulose, which has evolved to resist degradation by the crosslinking of cellulose, hemicellulose and lignin fibers (Lin and Tanaka, 2006; Xiao et al., 2007). The goal of any pretreatment process, therefore, is to alter or remove structural and compositional hindrance to enzymatic hydrolysis to increase the yield of desired products (Hendriks and Zeeman, 2009). Compared to other pretreatment methods, such as physical and acidic methods, alkaline pretreatment proves to be cost-effective, least energy intensive, and most effective on different feedstocks (Cheng et al., 2010; Xu et al., 2010). Biodegradation of cellulose and hemicellulose is achieved through the concerted activities of cellulases, hemicellulases, ligninases and xylanases (Liu et al., 2006). The growing demand for these enzymes by bio-refineries has intensified the search for microorganisms with greater capacity to produce

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highly active enzymes in abundant amounts, using cheap and readily available feedstocks as substrates.

Saprophytic, wood-rotting fungi are highly efficient and ubiquitous lignocellulose degraders due to their capacity to produce a variety of hydrolytic and oxidative enzymes (Baldrian and Valášková, 2008; Highley and Dashek, 1998). These fungi largely gain nutrition by degrading dead plant biomass; hence, they are particularly important producers of cell wall-degrading hydrolytic enzymes (van den Brink and de Vries, 2011). The selection of appropriate lignocellulosic substrates for fungus growth and target enzymes are considerably important for development of efficient biotechnological procedures (Singh et al., 2009).

T. lanuginosus is a thermophilic fungus belonging to the class Deuteromycetes (Cooney and Emerson, 1964), and commonly isolated from self-heating masses of organic debris (Emerson, 1968). *T. lanuginosus* secretes a range of enzymes such as α-amylase, glucoamylase (Nguyen et al., 2002), pectinase (Puchart et al., 1999), protease (Hasnain et al., 1992) and lipase (Berg et al., 1998). These enzymes have been studied to a limited extent although they are reported to be thermostable catalysts.

Research conducted in the present work was aimed at enhancing our knowledge of production of extracellular hydrolytic enzymes by *T. lanuginosus* STm enzymes on different lignocellulosic substrates. In addition, we have applied these enzymes to a recalcitrant problem associated with the aqueous extraction of natural rubber from the rubber-producing dandelion *Taraxacum kok-saghyz* (TK). TK contains significant amounts of high-quality natural rubber comparable to that from *Hevea brasiliensis* (Buranov et al., 2005). The earliest published aqueous extraction process (Eskew and Edwards, 1946) resulted in low yields of rubber contaminated with tightly bound lignocellulosic debris. Unbound rubber can be purified from the bound form by dissolution in strong organic solvents, followed by filtration, and solvent stripping, but this additional process is a poor fit with our goal of environmentally friendly processing. Therefore, we tested the ability of *T. lanuginosus* STm, and its exogenous enzymes, produced on different substrates, to release the TK rubber from the bound lignocellulosic debris.

2. Materials and methods

2.1. Isolation of thermophilic fungal strain

Garden soil samples were collected from Multan City, Pakistan. The samples were incubated at different temperatures ranging from 40 °C to 55 °C in order to acclimatize and isolate thermophilic fungi. The soil (10 g) was initially kept at 30 °C for 3 days and then at 40 °C for the next 7 days, and finally at 55 °C for 5 days. After acclimatization, 1 g of soil was added to a 250 mL flask containing 150 mL of potato dextrose broth (PDB) with chloramphenicol and ampicillin (0.2 g/L each) to inhibit bacterial growth and incubated at 55 °C in a shaker incubator at 130 rpm for 7 days of incubation time. Then, 2 mL inoculum from the flask was added to 150 mL of PDB and incubated at 55 °C for 4 days. When dense growth appeared in the flask after 7 days, the fungus was transferred to potato dextrose agar (PDA) plates and incubated at 55 °C for 4 days. Fungal cultures were maintained on PDA slants and stored in a refrigerator at 4 °C till further use.

2.2. Molecular identification of fungus

2.2.1. DNA extraction and PCR amplification of ITS region

Isolated fungus was identified based on their 18S rRNA gene sequence. For this purpose, fungus was grown on PDB medium for 4 days at 55 °C. After incubation, fungal culture was freeze dried before DNA extraction. Fungal DNA was extracted using the Fungal Genomic DNA Isolation Kit (Norgen, Biotek Corporation, Ontario, Canada) following manufacturer's instructions. The extracted DNA was confirmed by visualizing it as bands using gel (0.8% agarose with ethidium bromide) electrophoresis and finally stored at 4 °C until PCR analysis. Amplification of the ITS region (ITS1, ITS2, and 5.8 S rRNA) was performed using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The PCR reaction mixture (25 μL) was prepared using 0.3 μM of forward and reverse primers each, 2 μL template DNA and 1X GoTaq® Green Master Mix (Promega, USA) in PCR grade water. PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation for 30 s at 94 °C, annealing at 55 °C for 30 s and extension at 72 °C for 1 min with a final extension at 72 °C for 10 min. The resultant PCR product was visualized on a 1% agarose gel containing ethidium bromide before sequencing.

2.2.2. DNA sequencing and phylogenetic analysis

PCR products were sequenced by Macrogen, Seoul, South Korea. The sequence was compared to the available fungal sequences on the NCBI database (GenBank) using the Blastn program (Altschul et al., 1997) and the fungus identified as *Thermomyces lanuginosus*-STm. Sequence alignments were performed using Clustal X 2.1 (Thompson et al., 1994). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). A bootstrap analysis with 500 replicates was carried out to check the robustness of the trees. Finally, the phylogenetic trees were plotted using the NJ plot program (Perriere and Gouy, 1996).

2.3. Pretreatment of biomass for enzyme production

Guayule bagasse was collected in plastic bags after latex extraction, whereas, wheat straw was collected as a raw form by researchers at OARDC, The Ohio State University, Wooster, USA. Both substrates were stored at room temperature. Guayule bagasse and wheat straw (400 g) were added separately to 4 L of 0.1% NaOH (constituted with distilled water) and incubated at room temperature (26 °C) overnight. After 24 h, the substrates were washed with tap water until neutral pH was attained. Pretreated guayule bagasse and wheat straw were then dried at 80 °C in an oven for 48 h.

2.4. Cellulase, inulinase, pectinase and xylanase production

Alkali pretreated guayule bagasse and wheat straw (3 g) taken separately in 150 mL of Mandel's medium in 250 Erlenmeyer flask [composition (g/L): $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2; Urea, 0.3; $CaCl_2$, 0.3; $MgSO_4{\cdot}7H_2O$, 0.3; peptone, 1; lactose, 1 and 0.1 mL of trace metals solution (mg/mL): $FeSO_4{\cdot}7H_2O$, 4.6; $MnSO_4{\cdot}H_2O$, 0.93; $ZnCl_2$, 0.83; $COCl_2{\cdot}6H_20$, 1.83, tween 80, 2; (Mandels et al., 1981)] were inoculated with [1.5 mL (1 mL = 0.54×10^6/mL)] mycelial suspension of *T. lanuginosus* STm. Inoculated flasks were incubated at 55 °C in a shaker incubator (Innova 4000) at 150 rpm for 8 days. Supernatants from the flasks were filtered using a Whatman No. 5 filter paper and then the filtrate was centrifuged (Thermo Scientific Sorvall Legeno Micro 21 R) at 10,000 rpm for 12 min at 4 °C. The clear supernatant contained crude extracellular enzymes including cellulase, pectinase, inulinase and xylanase. Enzyme activities and protein content were assayed every 24 h for 8 days.

2.5. Enzyme assays

Enzyme activities of cellulase (based on filter paper unit, FPU), endoglucanase, CMCase (carboxymethyl cellulase) (Adsul et al., 2007), inulinase, xylanase and pectinase activities, respectively, and reducing sugars were quantified according to Miller (1959).