

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)



Shomaila Sikandar^{a,c}, Victor C. Ujor^d, Thaddeus C. Ezeji^d, Jesse L. Rossington^a, Frederick C. Michel Jr.^a, Colleen M. McMahan^e, Naeem Ali^{c,**}, Katrina Cornish^{a,b,*}

^a Food, Agricultural and Biological Engineering, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH, USA

^b Horticulture and Crop Science, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH, USA

^c Microbiology Research Laboratory, Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan

^d Animal Sciences, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, OH, USA

^e USDA-ARS, Western Regional Research Center, Albany, CA, USA

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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 FPUG 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.

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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

* Corresponding author at: Williams Hall, 1680 Madison Avenue, Wooster, OH 44691, USA.

** Corresponding author.

E-mail addresses: naeemali95@gmail.com (N. Ali), [\(K. Cornish\).](mailto:cornish.19@osu.edu)

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* (Burakov 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, Bitek 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'-TCCGTAGGTGAACCTGCCG-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): (NH₄)₂SO₄, 1.4; KH₂PO₄, 2; Urea, 0.3; CaCl₂, 0.3; MgSO₄·7H₂O, 0.3; peptone, 1; lactose, 1 and 0.1 mL of trace metals solution (mg/mL): FeSO₄·7H₂O, 4.6; MnSO₄·H₂O, 0.93; ZnCl₂, 0.83; COCl₂·6H₂O, 1.83, tween 80, 2; (Mandels et al., 1981)] were inoculated with [1.5 mL (1 mL = 0.54 × 10⁶/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).

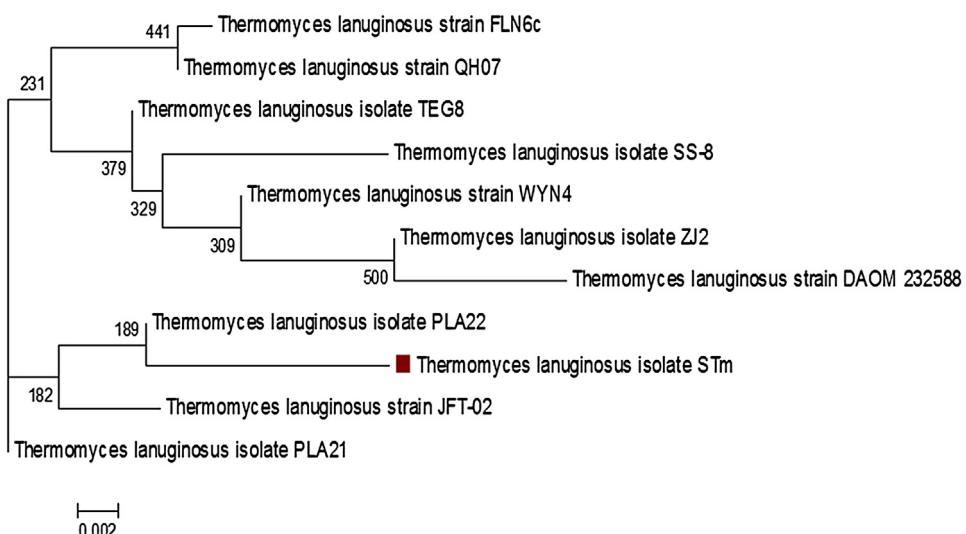


Fig. 1. Phylogenetic tree of the *Thermomyces lanuginosus* strain STm isolated from garden compost based on Neighbour-Joining method. Branch points show bootstrap percentages (500 replicated). The bar 0.002 indicates changes per nucleotide position.

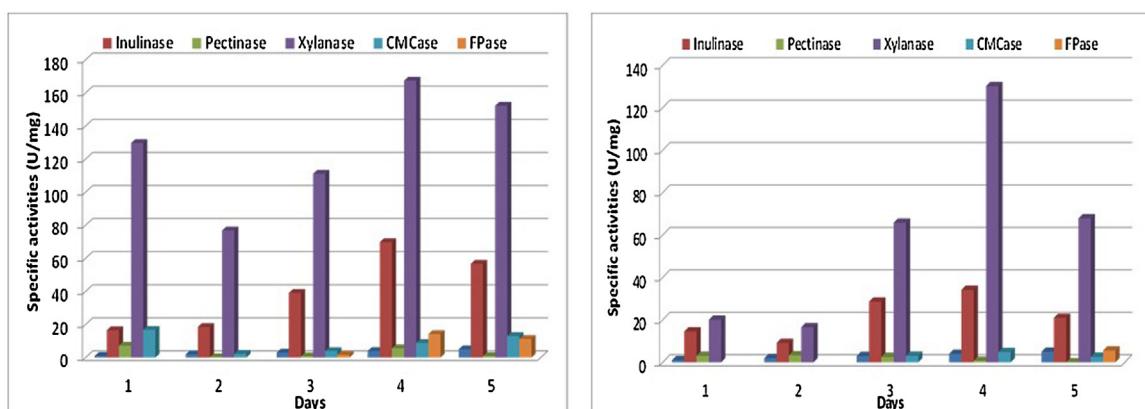


Fig. 2. Hydrolytic activities of different enzymes using lignocellulosic biomass (left panel) wheat straw; (right panel) guayule bagasse. CMCase is carboxymethyl cellulase, FPase is cellulase.

One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 $\mu\text{mol}/\text{min}$ of glucose, fructose, xylose or galacturonic acid from the appropriate substrates under the standard assay conditions.

2.6. Protein determination

Protein content was quantified using the Bio-Rad Protein Assay kit (Bio-Rad, California, USA) according to the manufacturer's protocol. Protein concentration present in crude extract of wheat straw and guayule bagasse were 37.1 and 40.2 mg/mL, respectively.

2.7. Alkaline pretreatment of Taraxacum kok-saghyz (TK) roots

Roots of TK were harvested from an OARDC, Wooster, OH field, placed in plastic bags, and stored in a cold room at 4 °C until used. Roots were washed, cut into small pieces (20–40 mm) and dried in an oven at 45 °C. TK roots (50 g) were mixed with 500 mL of 10% NaOH (pH 7) and boiled in a water bath at 100 °C for 35 min to loosen the lignocellulosic component and to extract inulin. After boiling, the floating TK roots were filtered, rinsed, replaced in the flask with 500 mL distilled water and boiled for 20 min. This step was repeated. The roots were filtered and rinsed with distilled water.

2.8. Enzymatic and fungal treatment of alkali-treated TK roots

Two different approaches were used for the extraction and purification of TK natural rubber from alkali pretreated TK roots using crude enzymes (including cellulases, inulinases, pectinases and xylanases) produced from *T. lanuginosus* STm growing on guayule bagasse and wheat straw. In the first set of experiments, named as T1 (enzyme process), 10 g of alkali treated TK roots were added to 250 mL flasks containing 15 mL of crude enzyme extract produced during 8 days of growth on guayule bagasse or wheat straw and 5 mL of citrate buffer (pH 4.8), and then incubated at 40 °C for 3 days. In another set of experiments, named as T1-STm (fungal treatment), *T. lanuginosus* STm was directly inoculated on the alkali pretreated TK roots by inoculating 5 g of alkali pretreated TK roots with 12 mL (1 mL = $1.08 \times 10^6/\text{ml}$) of mycelial suspension of *T. lanuginosus* STm and 5 mL of citrate buffer (pH 4.8) and then incubated at 40 °C for 3 days.

2.9. Extraction and purification of natural rubber

Following incubation of alkali pretreated TK roots with the T1 and T1-STm processes, the rubber, which was aggregated on top of the liquid medium, was filtered, rinsed and added to a 250 mL flask containing 100 mL distilled water and 7–10 glass beads (0.75 mm).



Fig. 3. Rubber produced by treatment of *Taraxacum kok-saghyz* roots with: (left panel) crude enzymes produced using wheat straw; (right panel) crude enzymes produced using guayule bagasse.



Fig. 4. Natural rubber extracted from *Taraxacum kok-saghyz* roots by growing *Thermomyces lanuginosus* STm on hydrolyzed root bagasse. Left panel: washed rubber in flask; upper right panel: rubber from flask lightly stretched indicating low contamination with plant material; lower right panel: unstretched rubber.

The flask was incubated in a shaker incubator for 20 min at 40 °C, allowed to settle, and the floating rubber was filtered and weighed. The filtered rubber was then oven dried at 40 °C for 12 h and weighed again.

2.10. Analysis of rubber impurities

The impurities in the extracted TK rubber from both processes (T1 and T1-STm), and from TK rubber samples collected from the Eskew process and the commercial enzymes process, were quantified as follows: the dried rubber samples were cut into small pieces (5–10 mm) and put into a 100 mL flask. 50 mL of turpentine oil (Crown brand Turpentine, Lowe's) was added and the flasks were placed in an oven at 130–135 °C for 2–3 h to dissolve the rubber polymers. The mixture of dissolved rubber and insoluble impurities were filtered using a wire cloth screen with a mesh size of 45 µm (Grainger). The impurities collected on the screen were quantified gravimetrically. Rubber yield was calculated using this formula:

$$\text{Rubber yield}(\text{mg/g}) = [\text{rubber}(\text{mg})/\text{dry weight of TK roots(g)}]$$

2.11. Analysis of molecular weight

The molecular weights and their distributions of the rubber samples were determined by gel permeation chromatography. Approximately a 3 mg dried rubber sample was solubilized in 3 mL

tetrahydrofuran (THF) overnight with gentle (~100 rpm) shaking (Multi-Purpose Rotator, Thermo Scientific). The rubber solution was syringe-filtered through a 1.6 µm glass microfiber GF/A filter (Whatman, GE Healthcare). For each sample, a 50 µL aliquot was injected into a Hewlett-Packard 1100 series, model G1314A, HPLC (1.0 mL/min flow rate, THF continuous phase) and separated at 35 °C by two Agilent PL gel 10 µm Mixed-B size exclusion columns in series. Column output was analyzed using 1) a multi-angle laser light scattering detector (DAWN Heleos-II, Wyatt Technology, Santa Barbara, CA), 2) a refractive index detector (Agilent 1260 Infinity, dn/dc = 0.129), and 3) a variable wavelength UV-vis detector (HP 1100 series, model G1314A, at 254 nm). The rubber molecular weights were calculated by ASTRA software from baseline to baseline of refractive index and light scattering peaks. Light scattering provides an absolute measurement for determination of molecular mass (Wyatt, 1993), therefore molecular weight standards were not used. The scattered light signal is proportional to cMw, where c is the concentration, determined simultaneously by the Refractive Index detector applying the appropriate dn/dc value (0.129).

2.12. Scanning electron microscopic (SEM) analysis

Scanning electron microscopy (SEM) was used to study the morphological features and surface characteristics of guayule bagasse and wheat straw after alkaline pretreatment. SEM was also used

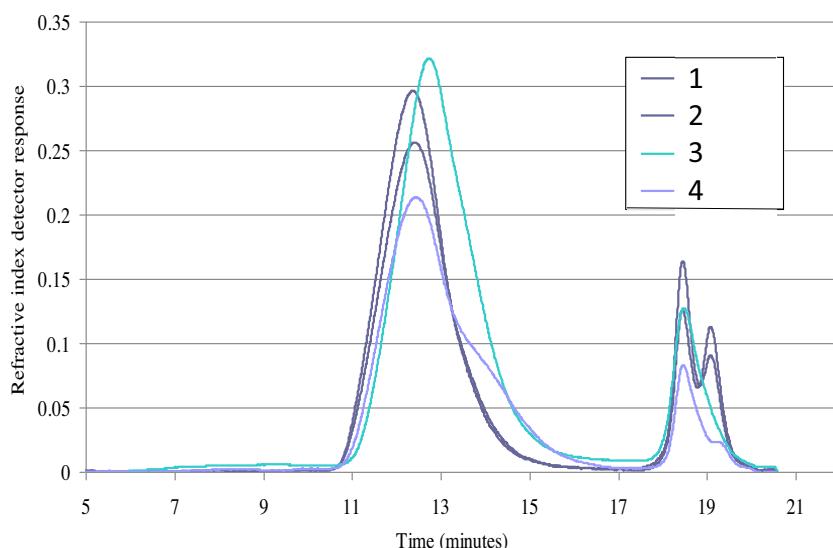


Fig. 5. Gel permeation chromatograms of rubber extracted from different processes; 1, *Taraxacum kok-saghyz* rubber from the exogenous *Thermomyces lanuginosus* enzyme treatment; 2, *Taraxacum kok-saghyz* rubber extracted using the Eskew process; 3, Hevea reference; 4. Guayule reference.

Table 1

Yield and purity of natural rubber extracted from TK using crude enzymes (T1-enzyme process), direct application of *T. lanuginosus* STm (T1-STm fungus process), and the commercial enzyme process. The *T. lanuginosus* enzymes (T1 enzyme process) were produced by growing *T. lanuginosus* on guayule bagasse. The characterization of Hevea and guayule reference samples is included.

Serial no#	TK rubber process	Rubber yield (mg/g dry TK root)	Rubber Purity (%)	Molecular weight (kg mol ⁻¹)	Polydispersity w/n
1	Eskew	24	37	1643	2.01
2	T1-STm fungus processed rubber	90	71	—	—
3	T1 enzyme processed rubber	77	91	1387	2.34
4	Commercial enzymes	45	94	—	—
5	Hevea – ref	—	—	1203	2.65
6	Guayule – Sac	—	—	1059	1.96

to compare rubber samples obtained from crude enzyme treatment and from the rubber obtained from the Eskew process (Eskew and Edwards, 1946) used at the Natural Rubber Alternatives and Bioproducts Research and Bioprocessing pilot facility, in Wooster, Ohio. This facility is associated with PENRA (Program of Excellence in Natural Rubber Alternatives) (<http://u.osu.edu/penna/>) and its name is abbreviated to the PENRA pilot plant in this report.

3. Results

3.1. Identification of the fungal strain and phylogenetic analysis

The thermophilic fungus obtained from garden soil compost was identified on a molecular basis using 18S ribosomal RNA gene using ITS1 and ITS4 sequences. The 1017 bp partial 18S rRNA gene sequence of the isolated *T. lanuginosus* STm fungal strain was obtained and submitted to GenBank database. The gene sequence was also submitted to GenBank under the accession number ID: KJ432867. Based on the results of BLASTN search, using the 18S rRNA sequence data, the new fungal isolate was found to be *T. lanuginosus* with 99% similarity index (Fig. 1).

3.2. Cellulases, inulinases, pectinases and xylanases production

T. lanuginosus STm culture grown in Mandel's medium at 55 °C for 8 days exhibited varied enzymatic activities at different times and substrates (Fig. 2). Maximum activities in supernatants from cultures grown on wheat straw were 167.4, 69.8, 16.7, and 7.2 (U/mg) and 14.2 FPU g⁻¹ compared with guayule bagasse, 130.1, 34.1, 4.8, 3.2 (U/mg) and 5.5 FPU g⁻¹ for xylanase, inulinase,

CMCase, pectinase and FPase, respectively. Overall, wheat straw was the better substrate for the production of cellulase, pectinase, inulinase and xylanase. Exogenous xylanases had greater activity than the other assayed enzymes on both substrates.

3.3. Enzymatic rubber extraction and molecular characterization

Natural rubber was extracted from alkali treated TK roots using crude enzyme extracts (T1 enzyme process) and by directly applying fungus *T. lanuginosus* (T1-STm fungus process (Fig. 4)). The rubber collected after using crude enzymes from guayule bagasse contained less biomass and was more pure (Fig. 3 right) than the rubber collected after using wheat straw crude enzymes (Fig. 3 left). Also, a greater yield of 77 mg rubber/g dry root was obtained by enzymatic extracts from guayule bagasse than from wheat straw (Table 1, Fig. 3). Maximum rubber purity was achieved (91 and 71%) when the inulin-extracted roots after alkaline treatment were treated with crude enzyme extracts of *T. lanuginosus* (T1 enzyme process) and after inoculation with *T. lanuginosus* (T1-STm fungus process) (Table 1). Higher yield (90 mg/g) than the rubber obtained from Eskew process (Eskew and Edwards, 1946) also was achieved after inoculation with *T. lanuginosus* (T1-STm fungus process), as well as by using crude enzymes (T1 enzyme process) (77 mg/g). Molecular weight analysis (Fig. 5, Table 1) indicated that weight-average molecular weight of fungal enzyme treated TK rubber (T1 enzyme process) (1,387,000 g mol⁻¹) was comparable with that of TK rubber from the PENRA pilot plant (1,642,500 g mol⁻¹), and to that of reference samples, tested at the same time, for: 1) guayule natural rubber produced in the 1980's from the Firestone pilot plant at Sacaton, Arizona (1,058,500 g mol⁻¹) (archival sample from

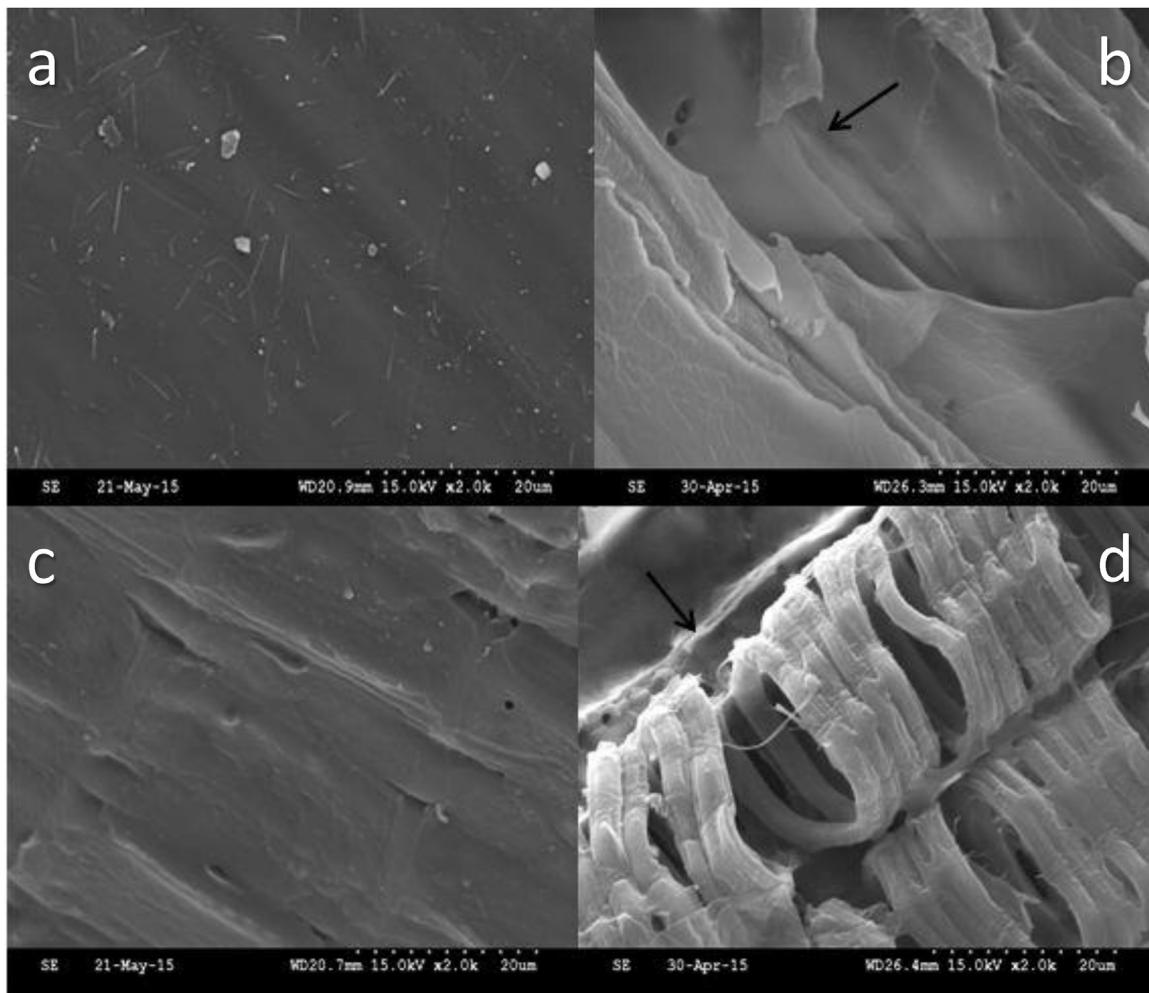


Fig. 6. Scanning electron micrographs (2000 \times) of untreated guayule bagasse and wheat straw (a, c); and alkaline (NaOH) pretreated guayule bagasse and wheat straw (b, d), arrows indicating the distorted structures of the pretreated substrate (b) and a xylem vessel (d).

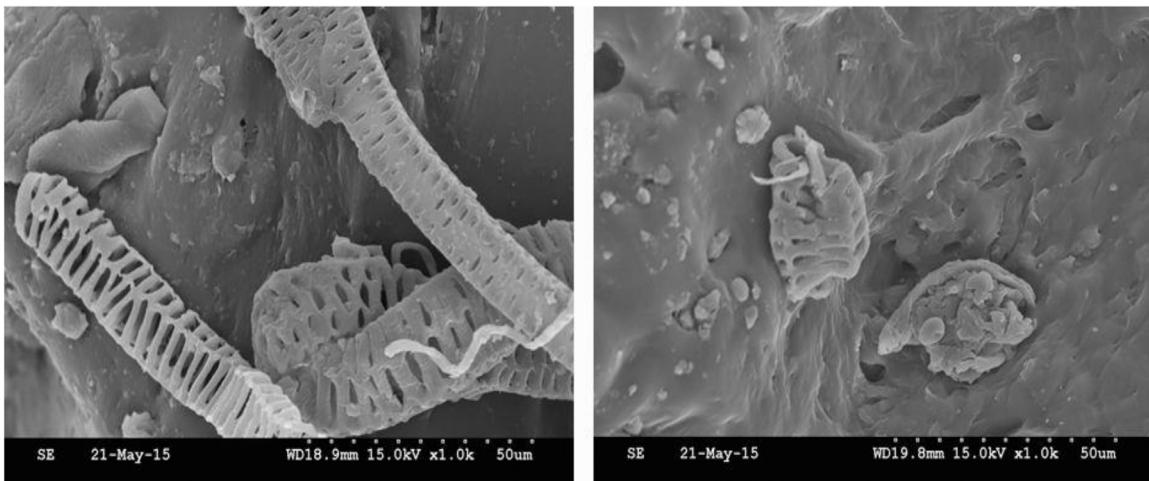


Fig. 7. Scanning electron micrographs (1000 \times) of natural rubber extracted (left panel) by the commercial enzymes process, (right panel) the crude enzymes from *T. lanuginosus* STm grown on guayule bagasse.

Schloman, 2005) and 2) a Hevea commercial TSR-10 control sample (1,203,000) (5).

The TK rubber samples had more of a monomodal distribution in the main peak, lacking the shoulder observed in the Hevea reference sample. A two way analysis of variance indicated no significant

\bar{M}_w differences among the four samples ($P=0.10$) but a significant difference in polydispersity (\bar{M}_w/\bar{M}_n) was observed ($P<0.001$). (\bar{M}_w/\bar{M}_n) was highest in the Hevea sample (mean = 2.649), followed by enzyme-treated TK (mean = 2.343) and PENRA extracted TK (mean = 2.01), with guayule rubber having the lowest poly-

dispersity (mean = 1.959). A second set of samples in which *T. lanuginosus* enzyme treated TK rubber was compared with PENRA process treated TK rubber also were analyzed and no differences were found between the two rubber samples ($P = 0.664$).

3.4. SEM analysis of alkaline pretreatment of substrates and extracted rubber

SEM imaging demonstrated morphological features and surface characteristics of guayule bagasse and wheat straw that were significantly altered after alkali (NaOH) pretreatment (Fig. 6). Initially, the untreated guayule bagasse was coarse and stiff, but pretreatment darkened the color and broke down the bagasse. Similarly, wheat straw initially was yellow and rigid, but pretreatment changed the color to light brown with considerable loss of rigidity. SEM analysis showed a disruption of substrate surfaces and the fibers of pretreated materials appeared to be distorted and separated from the initial connected structure (Fig. 6b, d), while the external surface area and the porosity increased. SEM imaging also demonstrated a loosening of the internal structure of xylem vessels in the wheat straw.

SEM analysis of TK rubber produced by the Eskew process (Eskew and Edwards, 1946) and by using crude fungal enzyme extracts (cellulases, inulinases, pectinases and xylanases) from *T. lanuginosus* STm (Fig. 7) showed significant variations in surface morphology. Rubber obtained from the Eskew process (Eskew and Edwards, 1946) contained more impurities from undigested TK biomass than rubber extracted by then enzymatic process.

4. Discussion

The use of pretreated lignocellulosic waste biomasses as substrates for the production of enzymes would have both economic and environmental advantages. Enzymes that are more thermostable (up to 70 °C) use shorter reaction times for maximum saccharification of plant polymers compared to enzymes produced by mesophilic fungi *Trichoderma* and *Aspergillus*. The study evaluated the potential use of *T. lanuginosus* STm for efficient biomass conversion and thereby extraction of value added products such as thermostable enzymes (xylanases, cellulases, pectinases and inulinases). Cellulase production has never previously been reported by any strain of *T. lanuginosus*. However, *T. lanuginosus* STm did show considerable ability to produce cellulase along with other hydrolytic enzymes. Moreover, the synergistic action of FPase and CMCase has been considered vital to the biodegradation of crystalline cellulose. However, greater cellulolytic activity depended upon the types of lignocellulosic biomass and the fungus (microbe) used (Singh et al., 2009). Previously, reports have indicated production of thermostable hemicellulases from different strains of *T. lanuginosus*. *T. lanuginosus* has been shown in numerous studies to produce extremely high levels of xylanases and cellulose-free beta-xylanase (Gomes et al., 1993; Jensen et al., 1987; Li et al., 1997).

Incubation temperature has been reported to play a significant role in production rate and activities of xylanase, pectinase and cellulase (Ramesh and Lonsane, 1987). *T. lanuginosus* STm rapidly grew on complex biomasses at temperatures above 50 °C but was unable to grow at 30 °C and 40 °C (data not shown). In the present work, *T. lanuginosus* STm produced high levels of xylanases (985.3 and 766.5 U/mL) on wheat straw and guayule bagasse substrates. Puchart et al. (1999) reported maximum xylanase activity of only 1.0–1.5 U/mL produced by *T. lanuginosus* on corncobs medium. Differences in xylanase activities might be due to the different substrates or the different strains. Several strains of *T. lanuginosus* have been found to be hyper producers of extracellular xylanase but several other studies have also shown that different strains

of this fungus vary in their expression of xylanases, mannanases and other glycosyl hydrolases. In contrast, Cesar and MrSa (1996) reported that their *T. lanuginosus* strain produced endoxylanase with no other hydrolytic activity and had highest activity in the temperature range of 60–70 °C. Damaso et al. (2000) reported that lignocellulosic biomass is a better substrate for producing xylan degrading enzymes than xylan itself. The growth of *T. lanuginosus* STm on pretreated lignocellulosic biomass at 55 °C suggests that this fungus could efficiently degrade other types of plant biomass.

The content and structure of lignocellulosic substrates changed noticeably during alkaline (NaOH) treatment (Fig. 6). NaOH pretreatment caused substrate modifications from exterior and interior, exposing the cell internal structure (xylem vessels), and generating irregular cracks and pores, as reported previously (Hu and Wen, 2008). Pretreatment is vital to altering the structure of cellulosic biomass by breaking hydrogen bonds and allowing accessibility of hydrolytic enzymes (Mosier et al., 2005). The potential production and stability of a range of hydrolytic enzymes make *T. lanuginosus* STm a potential candidate in bio-processing technology such as saccharification of lignocellulosic biomass, starch processing, biofuel production, paper pulp bleaching, chemicals synthesis (Vieille and Zeikus, 2001) and extraction of value-added products (Agbor et al., 2011). The hydrolytic enzymes (cellulases, xylanases and pectinases and others) produced by *T. lanuginosus* STm using lignocellulosic wastes could be used in the bioconversion of cellulosic biomass into value added products. Previous work reported the saccharification of different agro-wastes employing enzymes from different microorganisms including bacteria and fungi (Baig et al., 2004; Van Wyk and Leogale, 2001). Thermostable enzymes offer potential benefits in high rate mineralization of lignocellulosic substrates (Viikari et al., 2007) in industrial processes.

The present study reports, for the first time, extraction of natural rubber from TK by means of hydrolytic enzymes produced by *T. lanuginosus* STm using guayule bagasse as a substrate. A high yield of rubber with low impurities was obtained (Table 1, Fig. 3). We believe that, because enzymes from guayule bagasse were induced in the presence of residual rubber and high levels of potentially inhibitory terpenes, and in the presence of lignin, that this substrate might induce enzymes particularly efficacious in a rubber-based extraction system. Wheat straw does not contain rubber and terpene resins and the lignin from this monocotyledonous species is significantly different to that from the dicotyledonous guayule, and *T. kok-saghyz*, and these differences may explain its less efficacious exogenous enzyme pool. Attempts to grow *T. lanuginosus* STm directly on TK roots were much less effective than treatment with enzymes produced on guayule bagasse.

To reduce the cost of the enzymes needed for the rubber extraction, in-house production of hydrolytic enzymes may be beneficial. Enzymatic hydrolysis of TK roots not only promotes safe and efficient biotechnological procedures of production of value-added products but it will be environmentally friendly and may reduce processing cost. The efficacy of *T. lanuginosus* STm and its enzymes as processing aids in rubber extraction from TK roots is likely due to a combination of physical and biochemical actions. Alkali pretreatment of lignocellulosic biomass results in partial solubilization of organic carbon and proteins, reducing fiber content (Hu and Wen, 2008) and thereby releasing rubber from root tissues. However, the increased yield of rubber by the enzyme cocktail of *Thermomyces lanuginosus* STm compared to the commercial enzymes process (Table 1) indicates the presence of additional enzymatic activities, which although uncharacterized, seem to be responsible for breaking linkages between the rubber and lignocellulosic biomass that persist during root drying, chopping, inulin extraction and milling. This is likely due to the unique enzymatic fingerprint generated by the fungus, wherein the particular combination of excreted enzymes is capable of disrupting the tight linkages (likely covalent)

between the biomass and the rubber that might have prevented the dried rubber from being fully extracted by other methods (Fig. 3, Table 1).

5. Conclusion

The thermophilic fungus *T. lanuginosus* STm produces hydrolytic enzymes including cellulase, xylanase, inulinase and pectinase when grown on different lignocellulosic biomasses. This report demonstrates for the first time that extracted natural rubber yield from TK roots was considerably enhanced using hydrolytic enzymes produced on guayule bagasse compared to any other process previously used. Moreover, this bio-process is likely to be relatively cost-effective, and in combination with the commercial-enzymes-process may lead to development of a fully aqueous rubber bio-processing system for TK roots in the future.

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