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Extraction of nanolignin from coconut fibers by controlled microbial hydrolysis

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ABSTRACT

Biodegradable nanomaterials derived from biopolymers like cellulose, lignin, chitin and starch are hypothesized to support the development of eco-friendly functional nanosystem. In this work, nanolignin was extracted from coconut fibers by a controlled microbial hydrolysis process and compared with the nanolignins prepared by high shear homogenization and ultrasonication processes. The bulk lignin extracted from coconut fibers by soda pulping process was subjected to controlled hydrolysis by a lignin degrading fungal isolate, *Aspergillus* sp. The obtained nanolignin was characterized for size by DLS particle size analyzer, morphology by AFM and FE-SEM, chemical nature by FTIR, glass transition temperature by DSC and crystallite size by XRD. The microbial process yielded 58.4% nanolignin while homogenization and ultrasonication processes yielded 81.4% and 64.3%, respectively. Consequently, nanolignin, a value added compound can be extracted from coconut fibers to be used in textile, biomedical and environmental applications among others.

1. Introduction

While synthetic polymers have replaced the traditional metals and glass based materials for diversified applications due to low cost and ease of processing, increasing environmental concerns pave way for the emergence of biopolymers. There is a great potential of the biopolymer based nanomaterials to be used in nano-medicine due to their biocompatibility and biodegradability (Huang et al., 2015). Lignin is the most available biopolymer next only to cellulose and constitutes 25–30% of the non-fossil organic molecules on Earth. Its potential areas of applications include fillers in polymer composites, stabilizing agents, lubricants, coating additives, plasticizers and surfactants (Morandim-Giannetti et al., 2012; Thakur et al., 2014). While technologies are well established for conversion of carbohydrates to value-added products like pulp, sugar monomers and ethanol, lignin valorization process is less-developed and technical lignin are almost burnt for generation of heat and steam (Azadi et al., 2013). Currently, the pulp and paper industry liberates approximately 50 million tons of degraded lignin as part of the 130 million tons of kraft pulp produced annually (Bruijnincx et al., 2015).

Recently, nanolignin is gaining importance due to the ever-increasing demand for bio-based & bio-active nanomaterial fillers for use in bio-degradable composites. Though lignin in its native form is used as filler to increase the resistance of natural rubber vulcanizates to thermo-oxidative degradation in air (Košíková et al., 2007) and functions like a chain extender and cross-linking agent for biopolyurethane molecules (Luo et al., 2013), nanolignin increased the thermal stability when blended with polyvinyl alcohol (PVA) more effectively, when compared to native lignin/PVA blends (Nair et al., 2014). Similarly, the incorporation of nanolignin in wheat gluten based bio-nanocomposites resulted in increased mechanical performance (tensile strength and modulus), improved thermal stability and reduced water sensitivity and present an excellent UV resistance (Yang et al., 2015). Also, nanolignin application for finishing process of linen fabrics helps to obtain multifunctional textile products having UV barrier, antibacterial, antistatic properties guaranteeing positive effect on human physiology (Zimniewska et al., 2008). Nanolignin infused with silver ions and coated with a cationic polyelectrolyte layer form a biodegradable and green alternative to silver nanoparticles. The polyelectrolyte layer promotes the adhesion of the particles to bacterial cell membranes and, together with silver ions, can kill a broad spectrum of bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa* and quaternary-amine-resistant *Ralstonia* sp (Richter et al., 2015). Nanolignin preparation by acidic precipitation method was reported to result in biodegradable nanoparticles from lignin that are apparently non-toxic for microalgae and yeast (Frangville et al., 2012).

The recent methods explored for preparation of nanolignin include high shear homogenization (Nair et al., 2014), ultrasonic treatment (Zimniewska et al., 2008) and precipitation from an ethylene glycol solution by using dilute acidic aqueous solutions (Frangville et al.,

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2012). For application oriented synthesis, the lignin has been chemically modified by the esterification of its hydroxyl groups in order to both change the lignin solubility and increase the interaction with the polymer matrix in the nanocomposite (Nevárez et al., 2011). While the high shear homogenization and ultrasonic treatment protocols are energy intensive, chemical process releases toxic effluent in addition to modifying the surface chemistry of lignin. To the best of our knowledge, no reports appeared on the preparation of nanolignin by microbial process.

White-rot fungi are the most efficient degraders of lignin due to secretion of ligninolytic extracellular oxidative enzymes. Two major groups of enzymes involved in lignin degradation are peroxidases and laccase (EC 1.10.3.2). Three peroxidases involved in lignin degradation are produced by white-rot fungi: Lignin peroxidase (LiP, EC 1.11.1.14), characterized by oxidation of high redox-potential aromatic compounds (including veratryl alcohol); manganese peroxidase (MnP, EC 1.11.1.13), that requires Mn^{2+} to complete the catalytic cycle and forms Mn^{3+} chelates acting as diffusing oxidizers; and, Versatile peroxidase (VP, EC 1.11.1.16) that oxidize Mn^{2+} as well as non-phenolic aromatic compounds, phenols and dyes (Martínez, 2002). MnP is the most common lignin-modifying peroxidase enzyme produced by almost all wood-colonizing basidiomycetes causing white-rot and various soil-colonizing litter-decomposing fungi (Hofrichter, 2002). But, very few research work reported the ability of *Aspergillus* sp. to synthesize hydrolytic enzymes of lignin (Zhang et al., 2015).

In this work, we have reported the preparation of nanolignin from coconut fibers by controlled microbial hydrolysis (fungal isolate: *Aspergillus* sp.) and their complete characterization. For comparison, nanolignin was prepared by two reported protocols, homogenization and ultrasonication.

2. Materials and methods

2.1. Lignin extraction

Extraction of lignin from coconut fiber was carried out by soda pulping process. Here, the coconut fibers were treated with sodium hydroxide at 170 °C followed by acid precipitation at pH 2.0. Lignin estimation was done as given in the standard TAPPI T-222 om-02. Thus extracted lignin is referred as 'bulk lignin' in further work.

2.2. Isolation of lignin degrading fungus

Compost samples prepared from cotton stalks (ICAR-Central Institute for Research on Cotton Technology, Mumbai, India) were used for isolation of lignin degrading microorganism. For enrichment, 2.5 g compost sample was added in 50 mL of sterile distilled water and kept in shaker for 1 min. The resulting suspension (0.5 mL) was added to 20 mL of modified mineral salts medium containing, $NaNO_3$, 1.5 g; $MgSO_4{\cdot}7H_2O$, 0.25 g; KH_2PO_4, 0.5 g; KC1, 0.25 g added with 0.01% (w/v) bulk lignin as sole carbon source and incubated at 27 °C for 7 days at 100 rpm (Henderson, 1961). The resulting fungal colonies were isolated and purified by single spore isolation method and sub cultured in Rose Bengal agar medium for further studies. Lactophenol cotton blue was used for fungal staining and based on the morphological observation, its characteristic conidiophore, the genus was identified to be *Aspergillus* sp.

2.3. Nanolignin extraction

Bulk lignin in aqueous suspension (7% concentration) was subjected to high shear homogenization at 10,000 rpm for 60 min in IKA T 25 digital ULTRA-TURRAX®. The resultant sample was allowed to settle for 1 h and only the supernatant was taken for analysis of nanolignin. For ultrasonication, the aqueous suspension of bulk lignin (7% concentration) was sonicated in an ultrasonic water bath (ELMA®, India) with 30 W power and 37 kHz frequency for 60 min. Subsequently, the sonicated sample was allowed to settle for 1 h and only the supernatant was taken for analysis of nanolignin. During both the homogenization and ultrasonication processes, the samples were kept in an ice bath to avoid the rise in temperature.

For microbial process, bulk lignin was used as the sole carbon source in basal medium (Tien and Kirk, 1988) for the fungal isolate *Aspergillus* sp., under shaking condition at 110 rpm for 31 °C for 15 days. The process was monitored on alternate days and the samples taken were analyzed. The samples were centrifuged at 1000 rpm for 15 min to remove biomass and un-hydrolyzed lignin. The supernatant was filtered through 1 μm filter and the filtrate containing nanolignin was taken for further analysis.

2.4. Lignin peroxidase (LiP) assay

The methylene blue reaction was used for analysis of LiP activity in the culture supernatant. For this purpose, the 2.7 mL of assay mixture contained 2.2 mL of supernatant, 0.1 mL of 1 mM methylene blue, 0.3 mL of 0.5 M sodium tartrate buffer (pH 4.0). The oxidative reaction was started by the addition of 0.1 mL of 4.5 mM H_2O_2. The colour that developed in the presence of LiP was compared to a blank assay, wherein double distilled water was used to replace the supernatant (Magalhães et al., 1996). Mixtures with LiP showed a change of colour from blue to greenish blue and then to purple blue.

2.5. Size and zeta potential analyses

The particle size distribution and zeta potential of nanolignin were measured using Nicomp™ 380 ZLS size analyzer by dynamic light scattering (DLS) principle. This instrument employs a design, which permits both multi-angle particle size analysis by DLS and low-angle zeta potential analysis by electrophoretic light scattering (ELS). Size calibration was carried out using 90 nm size polystyrene latex spheres and zeta potential calibration by using 491 nm polystyrene latex spheres. The size distribution was obtained based on the dynamic light scattering and autocorrelation principle. The mean diameter of the particles was calculated from their Brownian motion via the Stokes–Einstein equation. For this, HeNe laser (632.8 nm) was used and the scattering intensity was analysed by Avalanche photodiode detector at 90° orientation. To obtain the average size of bulk lignin, laser diffraction particle size analyzer (Microtrac S3500®) was used.

2.6. FTIR and XRD characterization

FTIR analysis was carried out in IRPrestige-21® in transmission mode using KBr pellets. Freeze dried nanolignin was pressed with KBr to form pellets and a total of 64 scans were taken per sample with a resolution of 4 cm^{-1}. Wide angle X-ray diffraction patterns of nanolignin were obtained using a Philips® PW 1710 X-ray diffractometer with nickel filtered Cu Kα (λ = 1.54 Å) radiation and analyzed using automatic powder diffraction (APD) software. The diffracted intensities were recorded from 5° to 80° 2θ angles. The size of the crystallites in lignin can be calculated using the Scherrer formula given in Eq. (1).

$$\text{Mean size of crystallites} = \frac{k\lambda}{\beta \cos\theta} \quad (1)$$

Where, k is the shape factor and taken to be 0.9 for nanolignin, λ is the wavelength of X-ray (0.154 nm), β is the line broadening at half the maximum intensity (FWHM), after subtracting the instrumental line broadening, in radians and θ is the Bragg angle (in degrees) (Goudarzi et al., 2014).

2.7. FEG-SEM, TEM and AFM analyses

Field Emission Gun − Scanning Electron Microscope (JEOL JSM-