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BIOPROSPECTING ANTIMICROBIAL POTENTIAL OF LIGNIN STREAM OF PADDY STRAW AGAINST FOOD-BORNE PATHOGENS

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Abstract

The present study was carried out to exploit the antimicrobial potential of lignin extracts obtained from four varieties of paddy straw i.e. PUSA-44, PR-111, PR-114, PR-122 against Aeromonas hydrophila (MTCC 1739), Klebsiella pneumoniae (MTCC 7028), Escherichia coli (MTCC 739) and Staphylococcus aureus (MTCC 96). Antimicrobial activity was tested both qualitatively w.r.t. zone of inhibition (ZOI) and quantitatively w.r.t. minimum inhibitory concentration (MIC). The best results were shown by lignin obtained from PR-122, where ZOI (mm) and MIC (µg/ml) for A. hydrophila, K. pneumoniae, E. coli and S. aureus were found to be 25 and 50, 19.3 and 50, 20 and 100, 18.6 and 100, respectively. Further, the lignin extracts were characterized using FT-IR spectrum and zeta potential. The present study exploits the immense potential of valorization of lignin-stream obtained from paddy straw in food and pharma industries.
Keywords: lignin valorization; stubble paddy straw burning; food-borne pathogens; minimum inhibitory concentratio.

Introduction

The current challenges of energy crisis and greenhouse gas emissions (Kotia *et al*., 2019; Kotia *et al*., 2017a) has led to the exploitation of second generation raw materials for bioenergy production and other applications in biorefinery manner (Arora *et al*., 2015; Chahartaghi *et al*., 2019; Jilte *et al.,* 2019; Kumar *et al.,* 2018; Bhardwaj *et al.,* 2014; Duran *et al.,* 2015; Sharma *et al*., 2019; Kotia *et al*., 2016a; Churasia *et al*., 2016; Ghazvini *et al*., 2020; Kumar *et al*., 2003; Kumar and Kumar, 2018; Kotia *et al*., 2018). However, out of the three major components (cellulose, hemicellulose and lignin) of lignocellulosic raw materials, valorization of lignin needs much attention. The materialization of the biorefinery is currently focused as the area of interest that transforms lignocellulosic biomass to products of potential uses (Sharma *et al.,* 2018; Vyas *et al.,* 2018; Kotia *et al*., 2018; Kotia *et al*., 2016b; Chauhan *et al*., 2015; Patel *et al.,* 2017; Jha *et al.,* 2019; Priyadarshi *et al.,* 2019; Jha *et al.,* 2019; Kotia and Ghosh, 2015; Kotia *et al*., 2016c). Lignin is an amorphous polymer crosslinked by phenolic units which furnishes structural integrity in the plants (Brethauer and Studer, 2015; Calvo-Flores and Dobado, 2010; Singh *et al*., 2013; Gupta *et al.,* 2012; Kalra and Kumar, 2018; Yadav *et al*., 2011; Vyas *et al.,* 2010). Due to the inherent property of lignin as inexhaustible and inexpensive to produce various biomaterials, lignin's substitution prospective expands to replace products derived from contrasting mediums (Watkins *et al*., 2015; Pramanil and Maji, 2015; Pramanik and Padan 2016). The flexibility in lignin monomer composition has been proven to be useful for enhancing the production of biomaterials (Ragauskas *et al*., 2014). On the other hand, many foodborne pathogens have become resistant to the synthetic drugs (Arora *et al*., 2012; Korekar *et al*., 2011). Hence, plant-based drugs and antimicrobial products are extensively used as traditional antibiotics attributable to their effectiveness and prevention of emergence of new diseases (Beisl *et al*., 2017). Three basic phenolic derivatives including sinapyl alcohol (S) p-coumaryl alcohol (H) and coniferyl alcohol (G) have been reported in the literature with high antimicrobial activity. Antimicrobial activities of phenol ring is due to its property to delocalize and stabilize the unpaired electrons (Cheetangdee, 2019; Anup *et al.,* 2000; Arora *et al.,* 2015; Chowdary *et al.,* 2019; Chilana *et al.,* 2015; Sharma *et al*., 2014; Manna *et al.,* 2017). The hydroxyl group present in the phenolic moiety of lignin interacts with cell membrane of microbes (Kumar *et al*., 2013; Gupta *et al*., 2013), thereby destructing the lipid bilayer and increases the permeability of cell membrane causing leakage of the cell components (Sriroth and Sunthornvarabhas, 2018; Spasojevic *et al.,* 2016; Papuc *et al.,* 2017). The usage of phenolic compounds aids in food preservation and food processing because of the raised awareness among the consumers of natural based food products and the increasing antibiotic resistance (Dong *et al.,* 2011; Cein-Karaka and Newman, 2015; Kumar *et al*., 2020; Kaur *et al.,* 2014; Kaur *et al.,* 2019; Sangma *et al.,* 2019). Moreover, the exploration of phenolic compounds can provide extra benefits on the food packaging as well as on the health care services (Espinoza-Acosta *et al.,* 2016). Presence of coumaric acid in lignin has also observed to exhibit free radical scavenging activity (Kaur and Uppal, 2015; Upton and Kasko, 2015; Fernandes *et al.,* 2013) which is helpful in curing cancer (Ugartonda *et al.,* 2008; Kumar and Mistri, 2019) and cardiovascular diseases (Boz, 2015). Thus, biodegradability, abundance, neutrality to harmful gases, cost efficient, eco-friendly and biocidal properties of lignin exhibit promising applications (Thakur *et al.,* 2014).

The present study was carried out with the objective to valorize the lignin stream obtained from paddy straw and to exploit its antimicrobial potential, both qualitatively and quantitively, against food-borne pathogens viz. *Aeromonas hydrophila* (MTCC 1739), *Klebsiella pneumoniae* (MTCC

7028), *Escherichia coli* (MTCC 739) and *Staphylococcus aureus* (MTCC 96). To the finest of our information, this is the leading study to exploit and compare the antimicrobial potential of lignin stream obtained from four different varieties of paddy straw against food-borne pathogens.

Materials and Methods

Procurement of Paddy Straw

Samples of paddy straw of varieties PUSA-44 and PR-111 were procured from Kot Ise Khan village, Moga, Punjab (30.9522° N, 75.1291° E), whereas, PR-114 and PR-122 were procured from Kaderwala village, Moga, Punjab (30.9984° N, 75.1290° E). The straws were separated from the roots using a sterilized cutter and were dusted off manually and were collected in the dried form directly from the fields. The collected samples were then properly washed using clean water to remove the dust particles and other surface impurities. Then, the samples were sun-dried to constant weight and stored in air-tight packs at 4 °C for further analysis (Kaur and Phutela, 2016).

Preparation of the samples

The straws of different varieties were reduced to the size of 1-2 cm pieces by cutting it with scissor on cleansed dry surface and analyzed for moisture content in duplicates by drying the samples in hot air oven at a temperature of 105 ^{o}C for duration of 4 h (Sluiter *et al.,* 2008). Equation (1) illustrates the calculation of moisture in paddy straw (Sameni *et al.*, 2017)

$$\textbf{Moisture content (\%)} = \frac{W_1 - W_2}{W_2} \textbf{ X 100} \quad (1)$$

Where W_1 and W_2 are initial and dry weights of paddy straw, respectively.

Extraction of Lignin

The extraction of lignin was done using TAPPI-T22 method (TAPPI, 2006). Samples were processed with ice cold treatment in which 1 g of chopped, oven-dried sample was treated with 15 ml of 72 % H_2SO_4 with continuous stirring until thick black slurry was obtained. The slurry obtained was incubated at 20 °C for 2 h and further diluted with distilled water to 3 % concentration of H_2SO_4 and subjected to boiling for 4 h on a hot plate. The slurry volume was maintained up to the level of 575 ml with addition of distilled water at regular intervals as per the protocol.

Further, after boiling for 4 h, the flask was kept in an inclined position overnight to allow the solid particles settled down. Finally, the supernatant and pellet were separated out using Whatman filter paper. The settled portion of the slurry was the acid insoluble lignin and the liquid portion contained acid soluble lignin. The solid residues obtained over the filter paper were then washed carefully with hot water twice followed by washing with tap water to make it impurity free and pH was set to 6.5-7.0. Further, the solid residue was crushed to fine powder, autoclaved and stored in an air-tight vial for further use.

Solubilization of lignin

About 0.2 g of extracted lignin was mixed in 10 ml of DMSO and sonicated for 20 min. Further, it was filtered through Whatman filter paper to remove debris, if any.

Selection of foodborne pathogens

Four foodborne pathogens, *Aeromonas hydrophila* (MTCC 1739), *Klebsiella pneumoniae* (MTCC 7028), *Escherichia coli* (MTCC 739) and *Staphylococcus aureus* (MTCC 96) were procured from Microbial Type Culture Collection, Chandigarh. The cultures were revived in nutrient broth with composition (in g l^{-1}) beef extract, 1; yeast extract, 2; Peptone, 5; sodium chloride, 5; pH was maintained at 7.0 and incubated at 37^{o}C for 24 h.

Determination of Zone of Inhibition (ZOI)

Prior to analysis, the turbidity due to growth of microbial cultures in nutrient broth was adjusted to 0.5 McFarland Standard (Arora *et al.,* 2012). Antimicrobial activity was evaluated by measuring the ZOI using well diffusion method. The microbial cultures were inoculated onto petri plates containing MHA medium (composition (g l^{-1}) Beef extract, 2; acid hydrolysate of casein, 17.50; starch, 1.50; agar, 17.0; pH 7.3) using spread plate technique. Further, wells of 5 mm were punched in the middle of petri plates and DMSO solubilized lignin obtained from all the four varieties of paddy straw were loaded in the wells separately. Positive and negative controls were DMSO and gentamicin (GEN 10) antibiotic disc, respectively. The plates were then incubated at 37 °C for 24 h. The test was performed in triplicates and ZOI (in mm) was measured (Arora *et al.,* 2012).

Determination of minimum inhibitory concentration (MIC)

The samples which showed positive result in ZOI test were further subjected to MIC analysis. The experiment was performed in 96 well microtitre plates in duplicates. Two-fold dilutions (1000-12.5 µg ml^{-1}) of the samples were prepared and 10 µl of diluted samples were loaded in the plate alongwith 100 µl of the inoculum. DMSO solubilized lignin without bacterial culture was kept as negative control, gentamicin as positive control and bacterial culture without DMSO solubilized lignin was kept as growth control. The microtitre plates were incubated for 24 h at 37 °C and absorbance was measured at 600 nm using Microtitre Plate Reader (Arora *et al.,* 2012).

Characterization of Lignin Sample by Analytical Techniques

Lignin samples were characterized w.r.t. Fourier transform infrared spectroscopy (FTIR) and zeta potential. FTIR analysis was done in the range of 4000-400 cm^{-1} using Shimadzu FTIR 8400S spectrometer with the detector at 4 cm-1 resolution and 12 scans per sample were used. Moreover, to determine the electrokinetic properties of lignin particles, the zeta potential was determined at 25 °C with concentration of 10 mg ml^{-1}, with Zetasizer Nano ZS (Malvern Panalytical, GNDU, Amritsar). The sample was prepared by dissolving dry powder in DMSO with the concentration 1g ml^{-1} and was sonicated at room temperature for 10 min, to obtain a good colloidal dispersion.

Results and Discussion

Antimicrobial activity

Acid insoluble lignin obtained was found to be 18 % (for all the four varieties), respectively. Similar results has been reported in the literature (Bakker *et al.,* 2012; Dinh *et al.,* 2017). Lignin obtained from four varieties of paddy straw (PUSA-44, PR-111, PR-114 and PR-122) were tested for ZOI against gram negative bacteria *A. hydrophila*, *E. coli* and *K. pneumoniae*, and gram positive bacteria *S. aureus*. Table 1 shows the ZOI against the selected pathogens. PR-122 showed remarkable ZOI against all four pathogens, while PR-111 showed very small ZOI against *E. coli* and there was no ZOI observed by PUSA-44 and PR-114. The maximum size of ZOI i.e. 25 mm was observed for *A. hydrophila* by lignin from PR-122, while the performance of same lignin was almost similar for *E. coli*, *K. pneumoniae* and *S. aureus* with ZOI ranging between 18.6 mm and 20 mm (Fig. 1 A-D). The inhibitory effect of lignin is attributed to the presence of double bond in the Cα=Cβ position of the side chain and a methyl group in the γ-position (Espinoza-Acosta *et al.*, 2016). However, the difference in antimicrobial properties of lignin extracted from various varieties of paddy straw is due to variable genetic makeup. The polyphenolic compounds present in lignin moieties causes damage to the cell membrane and subsequent lysis of bacteria due to leakage of cell constituents (Barber *et al.,* 2000).

Further, on the basis of results of ZOI, lignin extract obtained from PR122 was selected for MIC analysis. The results of MIC against all the four pathogens are shown in Table 2. The maximum inhibitory effect on *A.hydrophila* and *K.pneumoniae was observed* at the lignin concentration of 100 µg ml^{-1} whereas the MIC against *E.coli* and *S. aureus* it was calculated at 50 µg ml^{-1}. Comparable results have been described by Alzagameem *et al,* 2019.

Characterization of lignin by FTIR analysis and Zeta potential

The FTIR analysis of raw paddy straw PR-122 and lignin extracted from PR-122 variety was done using Shimadzu FTIR 8400S spectrometer. Fig. 1 (A) and (B) shows the FTIR spectrum of raw sample and extracted lignin. The stretching of O–H in methyl groups for lignin is reflected in the band of 3308 and 3323 cm^{-1}, respectively for raw and extracted samples. The presence of guaiacyl and syringyl groups was confirmed by the appearance of peaks in range of 1330–1375 cm^{-1}. Moreover, the C–O or C–H groups were confirmed by the peaks in the region of 1031 cm^{-1} in both raw and extracted samples. The aromatic rings in the samples were confirmed by the peaks at 1635 cm^{-1} (Sunthornvarabhas *et al*., 2017).

To determine the electro-kinetic property of the extracts, zeta potential was determined, which showed that charge of lignin and its tendency to precipitate. The zeta potential of lignin extracted from PR-122 was found to be 0.596 with zeta deviation (mV) of 4.88 which indicates that the sample was not pure and had less stability (Fig. 2). The peak represents that the compound can be rapidly coagulated or flocculated (Surina *et al*., 2015). A negative zeta potential aids in stability and enhanced enzymatic hydrolysis after pretreatment (Huang *et al*., 2017; Tian *et al*., 2017). However, in the present study a positive zeta potential was obtained which indicates that the lignin should be separated prior to enzymatic hydrolysis.

Table 1: Antimicrobial activities of lignin from obtained from different varieties of paddy straw against foodborne pathogens

Microorganisms	Zone of Inhibition (mm)			
	PS111	PS 122	PS 114	PS 44
Aeromonas hydrophila	-	25.0±0.2	-	-
E. coli	1.0±0.4	20.0± 0.4	-	-
Klebsiella pneumoniae	-	19.3± 0.4	-	-
Staphylococcus aureus	-	18.6± 0.2	-	-

Table 2: MIC for the growth inhibition of bacteria

Organism	MIC against PS 122 (µg/ml)
Aeromonas hydrophila (MTCC 1739)	50
E. coli (MTCC 739)	100
Klebsiella pneumoniae (MTCC 7028)	50
Staphylococcus aureus (MTCC 96)	100

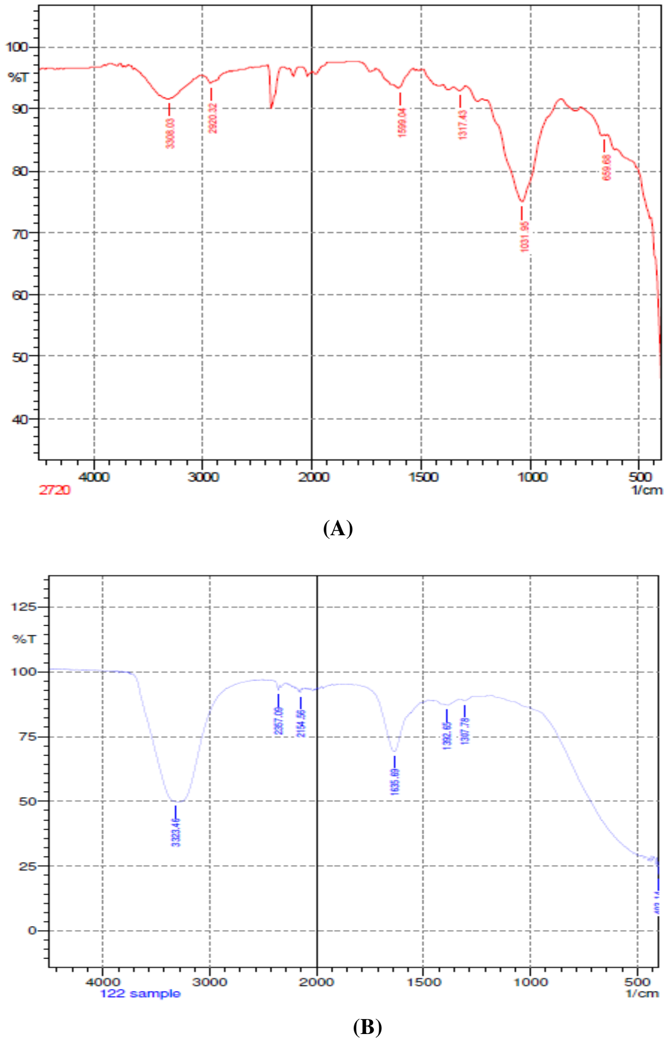

Fig. 1: FTIR spectrum of (A) raw rice straw PR122 and (B) extracted lignin from rice straw PR122

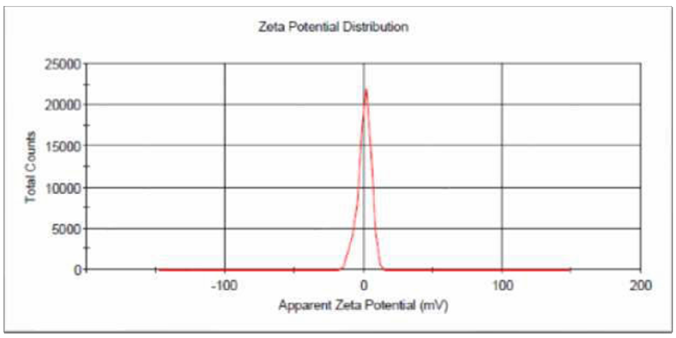

Fig. 2: Zeta potential of lignin obtained from PR122

Conclusions

Pure lignin or lignin derivatives can offer a good substitute for the development of plant- based antibiotics. Lignin samples extracted from different varieties of paddy straw (PR-111, PUSA-44, PR-114, PR-122) were used to determine the antimicrobial activity against food-borne pathogens. The present study shows immense potential of such bio-based products for food industry with lignin valorization. This can resolve the issue of stubble burning of paddy straw, and aid in the development of sustainable biorefineries. However, further studies are required to characterize the bioactive compounds having good antimicrobial activities.

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