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RESEARCH PAPER

TITLE

EVALUATING THE LIGNIN DEGRADING POTENTIAL OF INDIGENOUSLY ISOLATED FUNGAL STRAIN 81 FROM PAPER AND PULP INDUSTRY WASTE WATER

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EVALUATING THE LIGNIN DEGRADING POTENTIAL OF INDIGENOUSLY ISOLATED FUNGAL STRAIN 81 FROM PAPER AND PULP INDUSTRY WASTE WATER

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Abstract

Lignin is a phenolic biopolymer and a complex recalcitrant compound found in the wastewater effluent of the pulp and paper industry. Wastewater generated by the pulp and paper industry is having lignin as a major part in the form of black liquor, which poses serious hazards for the ecosystem along with health risks. Biological methods including the use of micro-organisms to degrade lignin by the enzymatic battery of various bacteria and especially fungi proved out to be effective. The present study includes the application of indigenously isolated fungal strains 81 from the effluent wastewater of a local pulp and paper industry of Pakistan. The strain was grown on lignin amended minimal salt media for the screening of best lignin degrader for determining their capability of lignin degradation and color reduction. Absorbance for lignin degradation and color reduction was measured at 280nm and 465nm respectively on the spectrophotometer. The results proclaim fungal strain 81 identified as Aspergillus fumigatus, after molecular characterization (accession no. MW132910 in NCBI data base) possess the potential to degrade lignin. Hence, it could help in the removal of lignin from toxic industrial wastewater.

Key words: Lignin, black liquor, degradation, color reduction

Introduction:

The pulp and paper industry is the most resource-intensive in terms of energy and water consumption. Paper manufacturing produces indicative quantities of wastewater, i.e. a ton of paper produces $60m^3$ of wastewater (Scholes, 2021). The effluent wastewater, also called black liquor from pulp-and-paper mills contains a significant amount of pollutants appertaining to high biochemical oxygen demand (BOD), chemical oxygen demand (COD), and dissolved solids, mainly due to high alkali–lignin ratios and poses serious threats to the environment (Shankar et al., 2020). Lignin is the generic term for a large group of aromatic rigid and hermetic polymers resulting from the oxidative coupling of 4-hydroxy phenylpropanoids and is the main component of woody plants. Not only the chemical nature is malefic but it's dark coloration also presents significant harm to aquatic life. The color and toxicity of wastewater are due to high-molecular-weight lignin and its derivatives. The chemical or physical degradation of lignin is very difficult due to the presence of recalcitrant and non-hydrolysable carbon-carbon linkages and aryl ether bonds (Mohanan et al., 2020).

Lignin is the second most abundant polymer after cellulose and, annually, approximately 5×10^6 metric tons of lignin is produced industrially (Banwell et al., 2021). Lignin is a cross-linked macromolecular material derived from oxidative coupling of monolignols, mainly hydroxy cinnamyl alcohols with the three main components as p-coumaryl, synapyl and coniferyl alcohols (Kai et al., 2018). Chemically, it consists of various functional groups e.g., methoxy group (CH3O), carboxylic group (–COO−), and carbonyl group (C = O), which make it a complex structure. Almost all annual plants on the earth contains phenylpropane units of lignin (Tribot et al., 2019; Yousuf et al., 2020). Two families of ligninolytic enzymes play the major role in the degradation of lignin including laccases and peroxidases i.e lignin peroxidase and manganese peroxidase (Thapa et al., 2020). Several methods including chemical coagulation/flocculation of lignin using synthetic or natural coagulants, adsorption of organic compounds on activated carbon and polymer resin, photochemical UV/TiO2 oxidation, catalytic wet air oxidation and incineration have already been employed for removing organic pollutants from wastewater (Akhter et al., 2019). However, along with the loss of sugars during chemical treatment these treatment methods require high energy consumption thus, increasing the cost of treatment. However, to eliminate organic pollutants of pulp-and-paper wastewater, biological treatments are found as a better substitute against the conventional methods (Madan et al., 2018).

In nature, micro-organisms are known for their biodegradation ability and exhibit excellent potential in waste water treatment (Iram et al., 2019). Various ligninolytic micro-organisms like bacteria actinomycetes, and white and soft rot fungi are found to have lignin degradation potential due to presence of specific enzymes and can be utilized for lignin biological treatment in wastewaters (Ayeronfe et al., 2018; Singh et al., 2021, Kour et al., 2021; Lenka, 2022)). Microbiological methods are found more striking due to their ecological significance and cost effectiveness (Kour et al., 2021; Li & Zheng, 2020; Parmar & Shukla, 2018). However, the exploitation of their potential is mainly based on the development of an integrated waste treatment process with high efficiency of reducing BOD and COD (Di Fraia et al., 2018). Therefore, present research would be an initiative towards the selection of candidate micro-organisms along with its potential of lignin-degradation and better metabolic traits to remove the pollutant from pulp and paper effluent.

At present, in Pakistan, there are about 100 operational pulp and paper manufacturing units with an annual production capacity of 650 thousand tons. Production units are involved in making chipboards, white duplex coated, un-coated boards along with writing, printing, and packing papers. This industry discharges about 15-200 m^3/ton of highly alkaline wastewater in the form of black liquor, with major constituent as lignin. The major environmental impacts of this waste include contamination of the freshwater resources. Considering the negative impact and health repercussions, the treatment of pulp and paper industry effluent is a growing concern all over the world (Chowdhary et al., 2020; Del Rio et al., 2022; Deshwal et al.,

2019). Per capita water availability of Pakistan has shown decline from 1,299 m^3 in 1996-97 to 1,100 m^3 in 2006 and predicted less than 700m^3 per capita by 2025. Available water resources could not be able to meet up the demands in future and this industry consumes a lot of water and produce highly polluted wastewater which is needed to be treated. Focus of our research is to address these issues.

Thorough research has shown that white rot fungi are having varying potential of lignin degradation and are the most efficient ones. The presence of powerful lignin-degrading enzymes is the main reason behind this extra-ordinary activity. Cellulose is to be used by White-rot wood fungi as a carbon source and to gain access to cellulose fraction, it degrades lignin. Moreover, using white rot fungi consume less environmental damage and less energy consumption. Basidiomycetes possesses extracellular oxidative enzymes namely peroxidases and laccases which give them high degradation potential. Present study is designed to look into the biological waste water treatment of pulp and paper industry using fungal strains. For this purpose, already isolated strains are used and screening and molecular characterization is performed. In addition, optimization of parameters such as temperature, pH is also done to determine optimum values for lignin degradation and decolorization are also a part of the study.

Methodology:

Isolation of selected strains:

Strain 81 isolated from effluent waste water sample of Bulley Shah Packaging, Kasur Pakistan. The strain was purified on Sabouraud dextrose agar medium and incubated at 30°C for three to five days.

Two step screening:

Three different concentrations of lignin i.e., 0.5%, 1% and 1.5% were used in Czapeck dox agar medium containing monopotassium phosphate, $MgSO_4$., KCl, $FeSO_4$, $NaNO_3$, 2% technical agar and 2% lignin in 100mg/ml. After inoculation, plates were incubated at 30°C for a period of 8 days and were observed at regular intervals for the formation of zones in the form of visible growth. In the next step Czapeck broth media was used. The prepared media was then inoculated with strain and placed in shaking incubator at 150 rpm for 9 days. All the experiments were performed in triplicate.

Samples from broth were taken after 2 days to determine the growth as a standard. Then, biomass was determined thrice on 3^{rd}, $6^{th,}$ and 9^{th} day of incubation. For this purpose, samples were taken in 2ml Eppendorf tubes and absorbance was measured at 600nm.

Determination of color reduction:

The color unit (CU) of broths was determined according to the Canadian Pulp and paper Association Standard Method. For measurement of color reduction, broths were centrifuged at 8000×g for 15 min in centrifuge to separate suspended solids then pH was adjusted to 7.92 and absorbance was measured spectrophotometrically at PtCob. 0.1214 standard solution at 465 nm.

Determination of lignin degradation:

For measurement of lignin degradation, in this method, 4.5 mL of 0.55% (w/v) NaOH was added to 0.5 mL of broth. The sample was centrifuged at 8000×g for 30 min. Pellet was discarded and supernatant was diluted with 3.0 mL phosphate buffer and absorbance was measured at 280 nm. The degraded lignin percentage was then calculated by using initial and final values. The same procedure was repeated on 6^{th} and 9^{th} days to determine change in the extent of lignin degradation.

Determination of enzyme activity:

For measurement of enzyme activity, the sample was centrifuged at 3000rpm for 10 minutes. The supernatant was collected in another falcon and pallet was discarded. Then, reaction mixture was prepared using 0.5ml of that crude enzyme extract, 0.5ml of guaiacol and 1.5 ml of sodium acetate buffer. The mixture was then incubated at 30°C for 10 minutes then the absorbance was measured at 450nm.The same procedure was repeated on 6^{th} and 9^{th} day to determine changes in enzyme activity.

Molecular characterization:

Extraction of DNA:

For DNA extraction, cultures were first obtained on fresh plates. Then 3 to 5 mg freeze dry mycelia was taken from the fresh plates and was thoroughly mixed with 500µl CTAB buffer in vials. The mixture was ground to make slurry with sterile pestle and mortar and stored in freezer for 30 minutes. For heat shock treatment, tubes were then placed in water bath set at 90°C. In the next step, tubes were taken out from water bath and 10µl proteinase K, 10µl lysozyme and 10% SDS about 50µl was added tubes which were then incubated at 37°C for one hour. After incubation, 500µl CTAB and 5M NaOH was added up to 100µl. The mixture was again incubated at 65°C for 30 minutes and after adding 500µl PCI incubated at 10,000rpm for 20 minutes at 4°C. Supernatant was shifted to another vial and was centrifuged at 10,000rpm at 4°C for 10 minutes after adding 500µl PCI. Supernatant was taken in new vial and added with 500µl iso-propanol and 300µl sodium acetate. Then vials were kept in freezer overnight to be proceeded on next day. Centrifugation was done again at 10,000 rpm for about 5 to 6 minutes. Supernatant was discarded and 200µl of 70% ethanol was added and centrifuged at same temperature and rpm for about 2-3 minutes. Ethanol was discarded and 50-100 µl TE buffer was added and pellet was dissolved in it and stored at -20°C before analysis on Nanodrop.

DNA Quantification by Nanodrop:

DNA concentration was evaluated using Nanodrop Spectrophotometer. Firstly, 2µl of blank or TE buffer was placed on optical surface of Nanodrop and then 2µl of extracted DNA was placed on it. Nanodrop gives DNA concentration at absorbance of 260nm and purity of sample at 280nm.

For Gel Electrophoresis, Agarose gel (IX) was prepared by adding 0.3g of agarose gel and 3ml 10XTBE (Tris Boric acid EDTA) buffer in 27ml of distilled water. To get the transparent solution, gel was heated in oven and Ethidium Bromide was added to it afterwards. Solution was poured into gel tray

and comb of suitable size was placed into gel. After solidification, gel was placed in electrophoresis chamber containing IX TBE buffer. 3µl of DNA sample was mixed with 3µl of loading dye and loaded into wells of solidified Agarose gel. Voltage, current and time was adjusted as 110 volts, 500mA, and 30 minutes respectively.

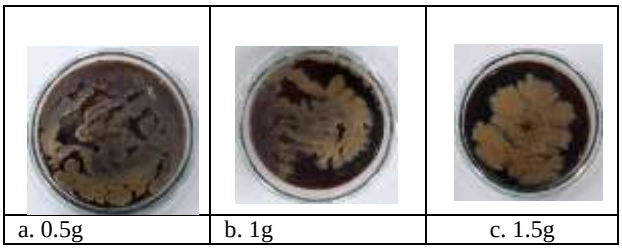
Gel was visualized under UV trans-illuminator at low and high resolutions for detection of DNA bands. Photographs were taken using Digital Camera DC 290 (Kodak, New York, USA) to analyze DNA presence and quality.

After obtaining DNA samples of appropriate size, samples were sent for 18S rRNA gene sequencing for molecular identification of fungal strains. Sequences of strain 81 were taken from online sequencing site and were saved in WORD document. BLAST was opened at official site of National Center for Bioinformatics (NCBI). Nucleotide BLAST was selected and sequence of both strains was pasted there. The sequences were BLAST to find the homologous sequences present already online. The data of various partial homologous sequences was downloaded and saved.

Figure 1. Isolated fungal strain 81

Screening (Agar Plate Assay):

Fungal strain 81 were inoculated in plates with three different concentrations of lignin i.e 0.5%,1 % and 1.5%. Both strains showed growth in the form of visible colonial zones. The zone diameter with 0.5% lignin concentration was 29mm. With 1% lignin the diameter was 35mm and with 1.5% lignin the zones were found out to be 39mm. Overall, 81 strain with 1.5% concentration of lignin showed the best zone i.e., 39mm. Then both strains were subjected to secondary screening for biomass and lignin degradation.



a. 0.5g	b. 1g	c. 1.5g

Figure 2. Agar plate assay of Fungal Strain 81 with different concentrations of lignin

Secondary screening:

Biomass, lignin degradation, enzyme activity and color reduction assay:

Fungal strains were then proceeded to secondary screening with MSM broth containing 2% lignin and incubated at 30°C at 150 rpm in shaking incubator and observed from 3 to 9 days. The reading for growth was obtained by measuring absorbance at 600nm

on spectrophotometer against distilled water as a blank. Maximum growth by SD5 and 81 was recorded on 3rd day which was 0.703nm and 0.801nm respectively.

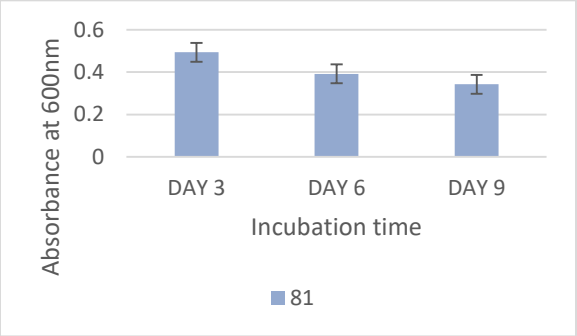


Figure 3. Absorbance values showing Growth of Fungal Strain 81

In addition to that the initial color unit reading of black liquor was 4.832nm which provided the color units in waste water as 19901.153 recorded against the standard (Pt.Cob 0.1214) with reference to distilled water as blank. Since the color of lignin is pH dependent so the pH of supernatant after centrifuging the sample was adjusted at pH 7.6 by phosphate buffer. After incubation of three, six- and nine-days readings for color reduction was obtained by measuring absorbance at 465nm on spectrophotometer. Strain showed maximum color reduction on 3rd day of incubation which was 59% and 61% respectively. The percentage of color reduction started decreasing on 6th and 9th day of incubation.

Figure 4: Visual representation of color reduction by Fungal Strain 81

Control **81**

The degradation trend of 81 strains is shown in the following graph.

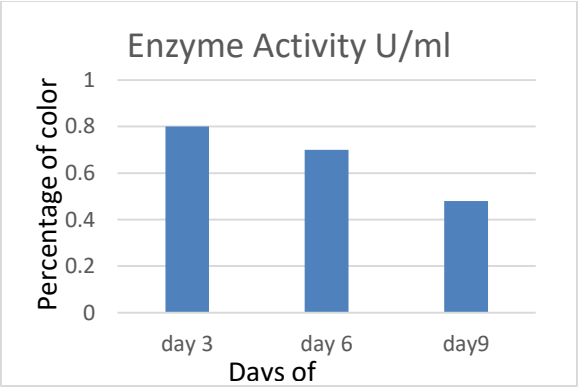
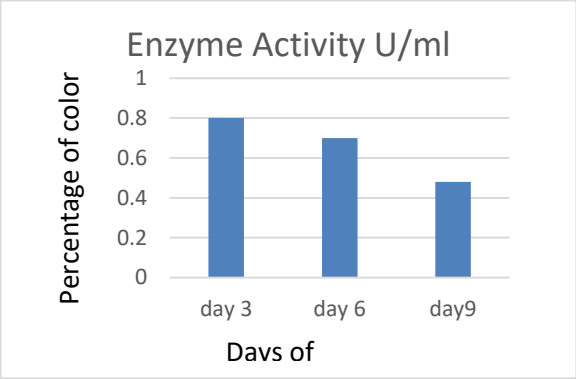


Figure 5. Color reduction by Fungal Strain 81

Furthermore, lignin degradation was measured in the similar manner on three different days of incubation from 3 to 9th day for both strains. The maximum lignin was degraded by SD5 on day 3 which was 20.5% and by 81 was 17.58%.



Then, enzyme activity of the crude extract was also determined by preparing reaction mixture specific for determining laccase activity and then checking absorbance at 450nm and then on putting values of absorbance in formula. The maximum enzyme activity was observed at day 3 which was 0.801U/ml by Fungal Strain 81.

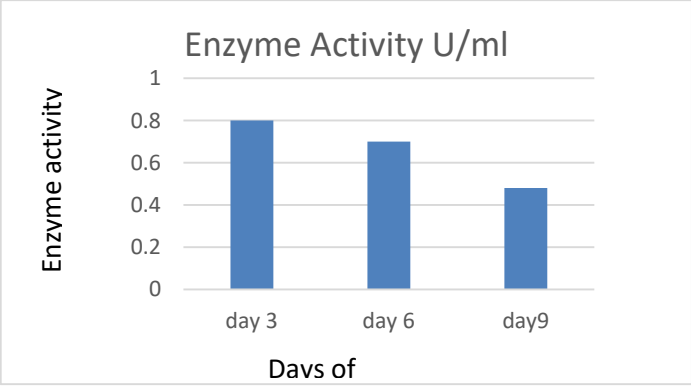


Figure 7. Enzyme activity of Fungal Strain 81

Molecular characterization:

Extraction of DNA:

From 72 hours fresh fungal culture, genomic DNA was extracted using Phenol Chloroform technique. Purified DNA was then added to 50µl T.E buffer and was stored at 4°C in freezer.

DNA Quantification by Nanodrop:

DNA concentration was evaluated using Spectrophotometer 7415 NANO JEN0003/ Version 1.0. Nanodrop gives DNA concentration, absorbance at 260nm and purity of sample at 280nm.

Sample	**Concentration**	**λ_1 260nm**	**λ_2 280nm**
Fungal strain 81	270	5.400	3.280

Gel Electrophoresis:

After DNA extraction, gel electrophoresis was performed to check the extracted DNA of **fungal strain 81**. DNA sample was mixed with loading dye and loaded in wells in gel. After which gel tank was connected to 110 volts electricity voltage with 400 AMP current. Terminals were disconnected after 30 minutes. DNA bands were visualized under UV trans-illuminator.

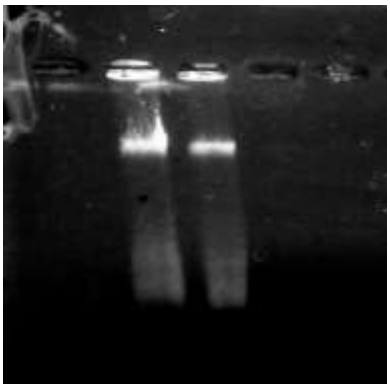


Figure 8. DNA bands showing SD5 and 81

4.3.4 DNA Sequencing of Fungal strain 81:

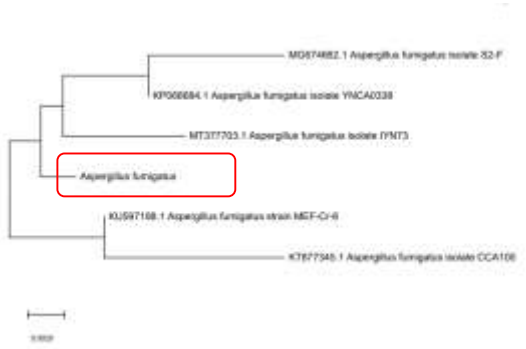


Figure 9. 16sRNA Phylogenetic sequence of fungal strain 81 identified as Aspergillus fumigatus

#Accession	Sequence ID	Release Date
MW132910	Seq5_S81	Nov 01, 2021

Discussion:

Industrialization with technological advancement proved to be a bane as well as a boon. It is a bane due to its limitations to meet sustainability in the environment. These industries pose threats to the environment causing pollution. Among the industrial sectors, pulp and paper industry being an important part, utilizes enormous quantity of chemicals, lignocellulosic constituents of plants and water during manufacturing and releases immense harmful wastewater. For per ton paper production, about 60,000–1,00,000 gallons of water are used, and for 1 Kg of paper production, around 300-400 l brown colored effluents are discharged into the environment along with 200–250 g of alkali Kraft lignin. These effluents from paper and pulp industry also known as black liquor are having high-molecular-weight lignin and its derivatives and tannins which gives the effluent its dark color, toxicity, and higher COD values. Post-industrial lignin poses serious threats to the aquatic environment due to its dark color, which inhibits the light penetration, leading to the death of photoautotrophs and increase of heterotrophs along with developing anaerobic conditions, disturbing the water micro and macro fauna in aquatic environments. So, the colored effluents must be decolorized before its discharge. Chemical and physical treatments can decolorize the effluents, still toxic chemicals persists after treatment (Cherian & Siddiqua, 2019).

For example, chlorine decolorization of lignin-rich effluents in the pulp and paper industry leads to the formation of colorless but toxic and carcinogenic chlorolignins (Bohacz & Korniłłowicz-Kowalska, 2020; Wołowicz & Wawrzkiewicz, 2021). The widely used precipitation of lignins from lignocellulosic raw materials in the sulfite and sulfate pulping process leaves organic and inorganic contaminants in the effluent (Naseer et al., 2019). The methods of biological decolorization and detoxification of brown-colored sewage rich in post-industrial lignin are regarded to be environmentally friendly and cheaper than the physicochemical methods. For these

purposes, certain bacterial species and some white-rot basidiomycetes have already been used in practice because if lignin remains undegraded, it imparts toxic properties to water.

A study was conducted for wastewater treatment using fungal strains. To achieve this, the samples were collected from **Bulleh Shah Packaging, Kasur, Pakistan**. The already isolated fungal strains were enriched and purified on fresh SDA Plates. Then, two fungal strains were selected and tested for the ability to utilize lignin as a carbon source. So, media plates containing 0.5%, 1% and 1.5% lignin were prepared and inoculated with both fungal strains. Plates were placed at 30^0C incubator for 2 weeks and were observed at regular intervals for the formation of visible colonial zones. Both the strains found out to be lignin degraders as they showed growth on the surface of agar plates containing lignin. Overall, fungal strain 81 with 1.5% lignin showed best growth and zone of it was 39mm. The isolated fungal strain 81 was then processed for secondary screening. For this purpose, MSM was prepared along with 2% lignin and inoculated with strains to observe the changes in growth, color reduction, lignin degradation and enzyme activity. Samples were taken every 3rd day till the 9th day for the measurement of above-mentioned factors. Maximum absorbance for growth shown by fungal strain 81 on 3rd day of incubation which was 0.801nm. Maximum color reduction was shown by fungal strain 81 was 61%. Fungal strain 81 showed maximum lignin degradation on 3rd day of incubation which was 17.58% and maximum enzyme activity on 3rd day of incubation which was 0.521U/ml.

Although, bacteria possess the capability to degrade the lignin and decolorize lignin, but fungi especially white rot fungi possess versatile enzymatic battery which possess the ability to efficiently degrade the lignin and remove color from wastewaters. Hence, fungi are found to be more efficient in employing the pollutants from pulp and paper industry effluents.

Conclusion:

Present study concluded that fungal strain 81 identified as *Aspergillus fumigatus* after molecular characterization (accession no. MW132910 submitted in NCBI data base) if provided appropriate conditions, their ability of lignin degradation could be increased manifolds. The strain is harmless to humans. It is concluded that it can be employed in pulp and paper industries for treating lignin degrading and decolorizing agents.

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This work was conducted in environmental and applied microbiology lab, department of microbiology, Quaid-i-Azam university.

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