INDIGENOUS LACCASE PRODUCING MICROBES: A SUSTAINABLE GATEWAY TO CLEANER PRODUCTION IN HANDMADE PAPER INDUSTRY IN JAIPUR REGION

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Abstract: With advent of industrial infiltration, the state of Rajasthan has witnessed tremendous growth in Medium and Small Scale industrial hubs. Handmade paper industry is considered as an important industry in terms of Jaipur's scenario since decades, famously known as "Sanganeri hand made paper". Mechanical, chemical or a combinatorial approach of pulping which is not supposedly cost intensive, is highly energy consuming and pollutes the environment by virtue of different chemicals used. Considering this fact, we proposed an eco friendly technology for cleaner production based on exploration of indigenous micro flora. For this study, soil and waste water samples were collected in triplicates in aseptic containers from Kalpana Handmade Paper Industry located at Sanganer, Jaipur. The samples were screened for bacterial isolates capable of producing laccase, an important enzyme responsible for delignification. **Laccases** (EC 1.10.3.2) are copper-containing oxidase enzymes found in many plants, fungi, and microorganisms. Three bacterial isolates, based on their biochemical properties were characterised as *Alcaligenes sp., Pseudomonas sp., and Klebsiella sp.* and fungal isolates which were screened in presence of Tannic Acid (TA) belonged to genera *Aspergillus sp.* and *Fusarium* sp. Laccase activity as monitored in Cell Free Extract (CFE), was found to be maximum of 33.8U/ml for *Aspergillus sp*

Keywords: Biopulping, cleaner production, handmade paper industry, laccase, microbes

Introduction: Pulp and paper mills are categorized as a core sector industry and contribute significantly to industrial water pollution. As per the Ministry of Environment and Forest (MoEF), Government of India, the pulp and paper sector has been placed in the "Red Category" which indicates severe polluting potential of the industry. Adherence to standards prescribed by Central Pollution Control Board (CPCB) for effluent disposal has become mandatory (Tewari *et al.,* 2009). Processing of paper involves two main processes, pulping and bleaching. The conventional pulping process involves removal of lignin either by chemical or mechanical means, which otherwise neither cost efffective, nor ecofriendly, low yield, toxic by-products are produced (Narkhede and Vidhale, 2005). To circumvent this "non-eco friendly" approach, microbial systems needs to be explored which essentially makes use of biocatalysts to achieve delignification without depolymerisation of cellulosic fibres (Singhal *et al*., 2005). The use of enzymes for the treatment or the removal of environmental and industrial pollutants has attracted increasing attention because of their high efficiency, high selectivity, and environmentally benign reactions (Vishwanath *et al*., 2014). Laccases are multicopper enzymes belonging to the blue oxidases group of enzymes (Figure-1) which widely exist in nature and are defined as nomenclature wise oxidoreductases type according to the Enzyme Commission (EC) which oxidize diphenols and allied substances (Kiiskinen et al., 2004). The higher plants and fungi predominantly contains laccases (Mayer and Staples, 2002). Fungal Laccases have been implicated in degradation of lignin and protection from toxic phenolic monomers of polyphenols. Laccases are helpful for number of industrial applications such as prevention of wine discoloration, paper processing and oxidation of dye, detoxification of environmental pollutants and chemicals production from lignin (Ahmed and Siddiqui, 2015).

Materials and methods

Study Area: Sanganer is a town situated 16 km south of Jaipur, the capital of Indian state of Rajasthan. It is famous for textile printing, handmade paper industry

Collection of samples: Soil and waste water samples were aseptically collected (triplicates), transported and processed from Kalpna Handmade Paper Industry, Sanganer Jaipur (Grab Methodology, APHA, 2000).

Screening, Isolation and identification of indigenous Laccase Producing Microbes (Abraham and Singh, 2013 ; Demissie and Kumar, 2014): For microbiological investigations, to screen for potential laccase producing microbes, soil and waste water samples were processed as per the layout (Figure 1). Briefly, soil and waste water samples were serially diluted up to 10^{-8} in 0.1% saline and were aseptically plated on respective culture media and incubated at 37°C for 24-48 hours under static conditions. For primary screening of laccase producing bacteria, culture media was amended with 0.2g/l bromo phenol blue following which 2, 20-azino-bis-[3-ethyl benzothiazoline-6-sulfonic acid] (ATBS) was used as a confirmatory substrate. Likewise, for fungal isolates, serially diluted soil samples were spread on Saboraud Dextrose Agar supplemented with 1% tannic acid. Following inoculation, plates were incubated for 4-5 days at

28±2º C and observed for development of yellowish brown halos around the mycelia mesh. The screened bacterial isolates were subjected to a series of biochemical tests for identification (Cappucino and Shermann, 2001). Likewise, fungal isolates were morphologically and microscopically characterised based on Lactophenol Cotton Blue staining.

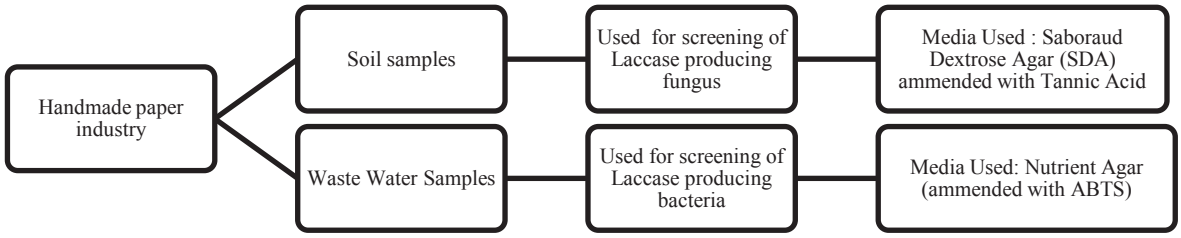

Figure 1: Schema for screening of Laccase producing microbes

Secondary Screening concomitant with Laccase Production in Laccase Production Media (LPM)

Screening by plate assay: For bacterial isolates, bromo phenol blue (0.2g/l) was used and actively growing strains ($O.D._{600}$- 0.6) were linearly streaked (single). The fungal colonies obtained in SDA plates were further screened by point inoculation in presence of tannic acid and pure individual colony were obtained.

Production of Laccase by submerged fermentation (For Fungal isolates only) (Desai *et al.*, 2011): The potent fungal strains were cultivated in Sabaroud Dextrose broth and kept in shaking incubator at 100 rpm for 7 days at 30 C. 3 ml mycelia suspension taken from SDB and inoculated in altered basal medium which consisted of (all in g/L) 0.2 KH_2PO_4, 0.5 $MgSO_4$, 0.5 $NH_4H_2PO_4$, 0.1 Yeast-extract and 10.0 glucose with 0.1% stock mineral medium containing (all in g/L) 1.4 $ZnSO4.7H_2O$ and 1 $FeSO_4$.7H2O supplemented with sole carbon source glucose (l%, w/v), L-glutamic acid [0.07% (w/v); neutralized with KOH] as nitrogen source, and thiamin (0.2 mg 1-l) and biotin (0.02 mg 1-l) to fulfil vitamin requirements. These cultures were grown for 2 weeks, then the mycelia mesh was removed and spent medium was filtered through whatmann filter paper to measure laccase activity.

Laccase Assay (Arora and Sandhu, 1985): Guaiacol has been reported as efficient substrate for laccase assay. The reaction mixture contained 3ml acetate buffer, 1ml guaiacol and 1ml enzyme source and enzyme blank contained 1ml of distilled water instead of enzyme source. The mixture was incubated at 30ºC for 15min and absorbance was read at 450nm blank using UV spectrophotometer (Jadhav *et al.*, 2009). Enzyme activity was expressed as International Units (IU), where 1 IU is defined as amount of enzyme required to oxidize 1micromole of guaiacol per min. The laccase activity in U/ml is calculated using the extinction coefficient of guaiacol (12,100 M^{-1} cm^{-1}) at 450 nm.

Results and Discussion: Identification and screening of Laccase producing microbes: In our study we report 3 bacterial isolates *Alcaligenes sp., Klebsiella sp.*and *Pseudomonas sp.* from waste water released from industry. The recombinant strain produced a high level of laccase compared to the wild type. *Streptomyces sp.* has been isolated from soil (Demissie and Kumar, 2012). *Klebsiella aerogenes* NCIM 2098 *(K. aerogenes)* of family *Enterobacteriaceae* was found to be effective in lignin removal (Jha *et al.*, 2002a). Some bacterial species such as *S.lavendulae*, *S.cyaneus*, and *Marinomonas mediterranea* are identified showing laccase distribution (Diamantidis *et al.* 2000).Figure 2 represents pure culture of screened isolates in presence of ABTS. Table 1 represents the biochemical attributes of bacterial isolates

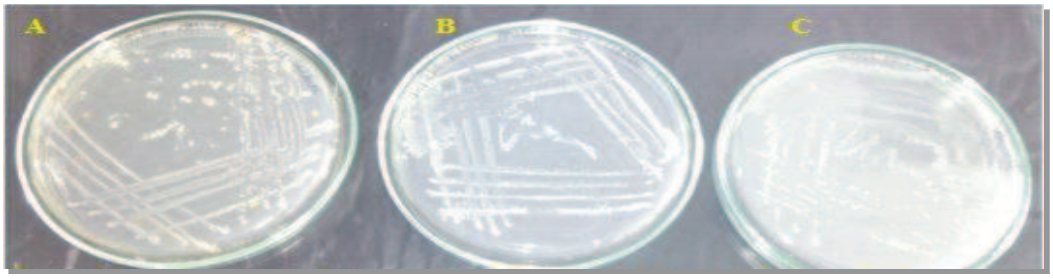

Figure 2: Pure Culture of Laccase producing bacteria

Table 1: Biochemical characteristics of Laccase producing bacterial strains

Strains	TESTS FOR IDENTIFICATION OF BACTERIAL STRAINS UPTO GENERIC LEVEL											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
A	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
B	-ve	Acid +ve	Acid +ve	Acid+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	+ve
C	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve
A: *Alcalignes sp.*				B: *Klebsiella sp.*					C: *Pseudomonas sp.*			

Where, I= Gram staining; II= Lactose; III=Dextrose; IV=Sucrose; V=Catalase; VI: Indole; VII=Methyl Red; VIII=Voges Praukauer; IX=Citrate; X=Starch; XI=Gelatin; XII= Urease

Secondary Screening of isolates (Plate Assay/Point Inoculation Assay) For bacterial isolates, bromophenol blue was used as a primary substrate for laccase induction. Other research groups have used copper sulphate as an inducer (Abraham and Singh, 2015). Figure 3 represents halos around linear steak indicative of extracellular laccase production in bacteria. Figure 4 represents screening of fungi in presence of Tannic Acid.

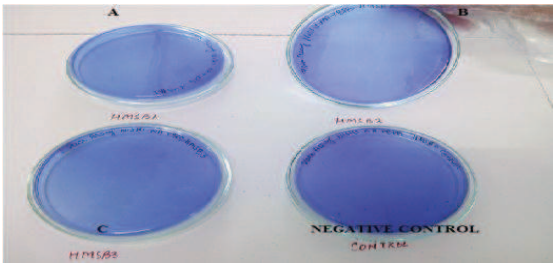

Figure 3: Plate Assay for bacteria

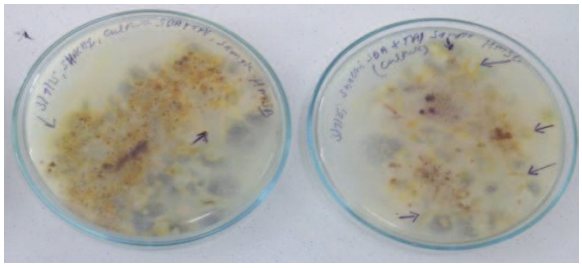

Figure 4: Point inoculation Assay for fungi

Laccase production (Only fungi) by submerged fermentation Submerged and solid-state modes of fermentation are used mainly for the production of laccase. Wild-type filamentous fungi are used for large-scale production of laccase in different cultivation techniques (Kapoor and Pannu, 2014). In our pilot study, submerged fermentation utilising Production media proved to be fastidious in nature specially for *Aspergillus sp.* Mycelium formation during growth of fungal cells can also impede impeller action causing blockades resulting in oxygen and mass transfer limitations; however, this has been overcome by immobilization. Sedarati *et al.* (2003) compared the free cell cultures of *T. versicolor* with immobilized cultures using nylon mesh for the bioremediation of pentachlorophenol (PCP) and 2,4-dichlorophenol (2,4 DCP). Christie and Shanmugam , 2012 by virtue of submerged fermentation , yielded laccase from *Alternaria arborescence* and *Fusarium oxysporium.* Similarly, Gochev and Krastano, 2007, isolated laccase producing *Trichoderma sp.*through submerged fermentation.

Laccase Assay: Laccase activity was measured as expressed as International Units (IU), where 1 IU is defined as amount of enzyme required to oxidize 1micromole of guaiacol per min. The laccase activity in U/ml is calculated using the extinction coefficient of guaiacol (12,100 M^{-1} cm^{-1}) at 450 nm. It ranged from 27.7U/ml- 33.8U/ml for *Fusarium sp.* and *Aspergillus sp.* respectively at 25ºC-30 ºC at pH 6.8. A significant increase ($p<0.05$) in laccase activity when contrasted with abiotic control. Laccase enzyme extracted from *Trametes versicolor* exhibited high enzyme activity at a temperature of 50ºC (Garcia *et al.*, 2009). Enzyme activity as high as that of *Alternaria arborescence* showed maximum production of enzyme (800U/l) at 300 C in 4.5 pH followed by *Fusarium oxysporium* (JQ 950134) with 600 U/l at 450 C in 5 pH after 15 days of incubation (Christie and Shanmugam, 2012).

Conclusion: Screening of potential microbes, indigenous to specific habitats are key to bioprospecting. This pilot study led to characterization of microflora capable of producing laccases. Laccases are ubiquitous in nature belongs to multicopper oxidase which catalyze oxidation reaction coupled to water formation on four electron reduction of molecular oxygen. They are presumed to be potential tool of biopulping thereby reflecting

their enormous potential in paper and pulp industries. The devised study which is selectively and specifically based at bioprospective strategy would play a pivotal role in generation of an ecofriendly process which would lead to development of a cleaner production technology thereby obliterating mechanical and chemical processes which are energy and cost intensive.

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