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Synergistic Effect of Bacterial Consortium for Enhanced Laccase Production by Submerged Fermentation

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Abstract

Industrialisation is rapidly changing the pace of economy by leaps and bounds. At the same time, the effects of pollution are evident in terms of infiltration and accumulation of hazardous substances in environment at large. Rajasthan has witnessed tremendous growth in small scale industries, one of them being the handmade paper industry. Globally, the finished product famously known as "Sanganeri handmade paper" is being appreciated for its ethnic hues and multi-usage. The current practices of paper manufacturing rely on intensive mechanical pulping process utilising an array of raw materials finally leading to enormous volumes of effluent. A combination of mechanical and chemical pulping process has certain identifiable gaps in the form of high production cost, high energy consumption and generation of large volumes of solid waste and effluents rich in high BOD,COD, synthetic dyes, heavy metals, bleaching agents, lignins and diversified range of xenobiotic compounds; thereby posing an environmental threat. Considering this fact, we proposed a pilot study aimed at cleaner and greener production of handmade paper by bioprospecting of indigenous micro flora. For this study, soil samples were collected in accordance with standard procedures from local handmade paper industry located at Sanganer, Jaipur. Preliminarily, 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. Furthermore, synergistic effect of bacterial consortium was explored for enhanced laccase production through submerged fermentation. Laccase activity as monitored in Cell Free Extract (CFE), was found to be maximum of 60.9 U/ml for bacterial consortium and was highly significant ($p<0.05$) with respect to abiotic control. This pilot study suggested the role of autochthonous micro flora in delignification of raw materials thereby obliterating the energy and cost intensive chemico-mechanical pulping process.

Keywords: Bacterial consortium; Cleaner production; Handmade paper industry; Laccase

Introduction

Pulp and paper mills are categorized as a core sector industry and 5th largest contributor of industrial water pollution. This industry utilise large volumes of ligno cellulosic components and water during different unit operations and release intensely coloured black liquors chlorinated lingo sulphonic acids, chlorinated resin acids, chlorinated phenols and chlorinated compounds including chlorolignins, chlorophenols, chloroguiacols and chloroaliphatics [1] in the effluent [2]. These compounds are acutely toxic being mutagenic and genotoxic in nature [3]. 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. The in-house unit operations of a paper mill is schematically represented in Figure 1 indicating different pollutants being released in a sequential manner [4].

The conventional pulping process involves removal of lignin either by chemical or mechanical means, which otherwise neither cost effective, nor ecofriendly, low yield, toxic by-products are produced [5].

Microbial biodegradation is carried out by different organisms like bacteria, fungus, and algae [6,7]. Effective Microorganism (EM) is the consortia of valuable and naturally occurring microorganisms which extra cellularly release organic acids and enzymes for utilization and degradation of anthropogenic compounds [8]. Ligninolytic enzymes have found immense usage in many industrial and biotechnological applications [9] including delignification of lingo cellulosic biomass for fuel (ethanol) production; food, brewery, and wine; animal feed; denim stone washing; laundry detergents; paper and pulp industries; and bioremediation of chemical pollutants [10]. Owing to the well-established fact of bioefficacy of lignolytic enzymes, the search for efficient production systems has been explored in recent past utilizing mushroom *Stereum ostea* [11] marine fungi [12] *Trichoderma* sp. [13]. The production system should be cost effective which can be accomplished by using cheaper raw materials and optimizing the fermentation process for scale up is need of an hour [14,15]. Because of the diverse applications of ligninolytic enzymes in industrial processes, there is a wide interest in the induction, enhancement, and stabilization of these enzymes. The production of ligninolytic enzymes can be stimulated by the presence of a wide variety of inducing substrates mainly aromatic or phenolic compounds related to lignin or lignin derivatives such as ferulic acid, 2,5-xylidine, and veratryl alcohol [16]. Copper as a micronutrient has a key role as a metal activator, induces both laccase transcription, and plays an important role in laccase production [17]. Surfactants can stimulate the growth of spores and increase the bioavailability of less soluble substrates for the fungus. Laccases are multicopper enzymes belonging to the blue oxidases group of enzymes which widely exist in nature and are defined as nomenclature wise oxido reductases type according to the Enzyme Commission (EC) which oxidize diphenols and allied substances [18]. The higher plants and fungi predominantly contain laccases [19].

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This technology of using microbial systems as potential source of lignolytic enzymes has gained momentum in recent past. It has been envisaged that the current practices of manufacturing handmade paper has identifiable gaps not only in terms of commercial aspect but also environmental benignity [20]. Plausibly, *microbial* and plant systems offer sustainable options based at *clean and green technology* of handmade paper processing. With the growing trend of environmental friendliness, demand of handmade papers made out of natural fibers is rising. Moreover, the rising cost of traditionally used cellulosic raw materials like cotton rags and hosiery waste, being used in handmade papermaking is also forcing the industry to search for additional cellulosic raw materials for production of handmade paper and board which are available as waste biomass in different parts of the world. This should help in providing more opportunities for cost effective, locally available lingo cellulosic raw materials / agro residues like Banana, Ankara, Pineapple, etc. there by addressing the problem of environment and the issue of global warming in a right prospective [21]. Taking into consideration the fact of utilising wastes into value added products, agro-residues rich in lingo-cellulose have been considered as potential bio pulping tool [22]. Noteworthy is the fact that agro-industrial residues have also been used to produce Laccases through Solid State Fermentation (SSF) [23].

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 (Figure 2).

Collection of sample

Soil samples were aseptically collected (triplicates) from a local handmade paper industry, Sanganer Jaipur, transported and processed as per the standard procedures [24].

Screening, isolation and identification of indigenous laccase producing microbes

For microbiological investigations, to screen for potential laccase producing bacteria, soil samples from the vicinity of handmade paper industry were processed as per the layout (Figure 3) [25-27].

Briefly, soil samples were serially diluted up to 10^{-8} in 0.1% saline and were aseptically streaked (line steak) on nutrient agar amended

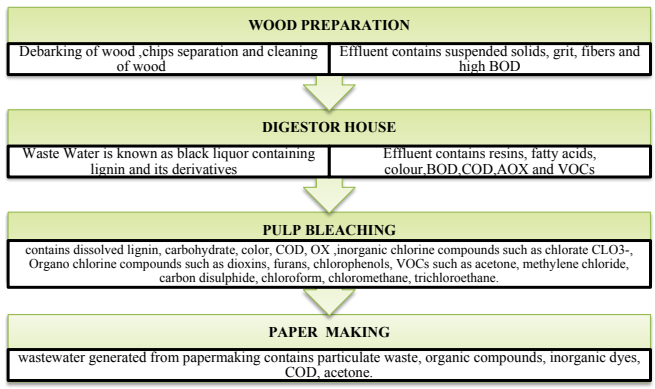

Figure 1: Schematic representation of unit processes in manufacturing of paper and the toxicants /pollutants released at different stages.

Figure 2: Study area.

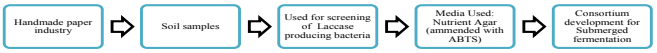

Figure 3: Schema of proposed study.

with 0.2 g/l bromo phenol blue and incubated at 37°C for 24-48 hours under static conditions for development of halo around the streak. The screened bacterial isolates were subjected to a series of biochemical tests for identification [28].

Preparation of bacterial consortia

A consortium was prepared in nutrient broth medium seeded with equal aliquots of overnight grown broth culture of screened and enriched bacterial isolates (monoculture); incubated at 28˚C for 24 hours. The broth was then used as seed or starter culture for laccase enzyme production [29].

Enhanced Production of Laccase by Bacterial Consortium in a Submerged Fermentation

Laccase production was carried out under submerged condition in Laccase Production Medium (LPM) comprising of Tryptone 10 gl^{-1}, Yeast Extract 5 gl^{-1}, Sodium Chloride 10 gl^{-1}, Fructose 0.1 gl^{-1} and Copper Sulphate 0.001 gl^{-1}). Actively growing bacterial consortium (24 hour old) was seeded (1% v/v) in freshly prepared LPM and incubated at 30°C under agitating conditions at 120 rpm [30].

Laccase Assay

Guaiacol has been reported as efficient substrate for laccase assay [31]. The reaction mixture contained 3ml acetate buffer, 1 ml Guaiacol and 1 ml enzyme source (Extra Cellular Fraction/Crude Filtrate) and enzyme blank (Control) contained 1 ml of distilled water instead of enzyme source. The mixture was incubated at 30°C for 15min and absorbance was read at 450 nm blank using UV spectrophotometer. 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 by the formula:

$$E.A = (A^* V) / (t^* e * v)$$

Where, E.A = Enzyme Activity (U/ml), A = Absorbance at 450nm, V = Total volume of reaction mixture (ml), v = enzyme volume (ml), t = Incubation time (min) and e = Extinction Coefficient ($M^{-1}cm^{-1}$).

Statistical Analysis

All the samples were collected in triplicates. The data (experimental and abiotic controls) was statiscally validated by One Way ANOVA

using SPSS version 20. Significance levels were corroborated at $p<0.05$ levels.

Results and Discussion

Screening, isolation and identification of indigenous laccase producing microbes and consortium development

In our study we reported 3 bacterial isolates *Alcaligenes sp., Klebsiella sp.*and *Pseudomonas sp.*capable of producing laccase; from soil samples from vicinity of handmade paper industry. Screening of wood decaying microbes have been phenomenal in exploring their properties of releasing extracellular enzymes which are responsible for delignification [32] isolated 58 bacterial isolates from effluent and soil sediment samples from chemically contaminated sites of Surat to screen for potential laccase producers [29] attempted to screen, optimize, production and partially purify laccase enzyme produced from consortium of laccase producing *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. White rot fungi have also been reported to possess lignolytic properties and notably effect of different inducers has also been explored for optimal laccase production [11,33]. Selvam et al. [34]. Isolated three wood rot fungi *Polyporus hirsutus*, *Daedalea flavida*, *Phellinus* sp from Western Ghats of India, for bioremediation of Paper and Pulp Mill Effluent (PMPE) Synergistic effect of lignolytic bacterial fungal consortium utilizing *Bacillus subtilis* and *Micrococcus luteus*, and fungi *Phanerochaete crysosporium* isolated from pulp and paper mills effluent has been studied to reduce lignin content by 97.4% within 9 days [35]. Genetically engineered microorganisms and enzymes can displace many of the environmentally adverse practices used in pulp processing. Fungi such as *O. piliferum* can degrade lignin and is used in a fermentation process. This is applied before carrying out the normal mechanical or chemical pulping. It reduces the overall energy requirements for mechanical process and reduces the quantum of chemicals for chemical pulping [36]. The recombinant strain produced a high level of laccase compared to the wild type. *Streptomyces sp.* has been isolated from soil [37]. Figure 4 represents pure culture of screened isolates in presence of ABTS. Table 1 represents the biochemical attributes of bacterial isolates. Figure 5 represents primary screening in presence of bromo-phenol blue.

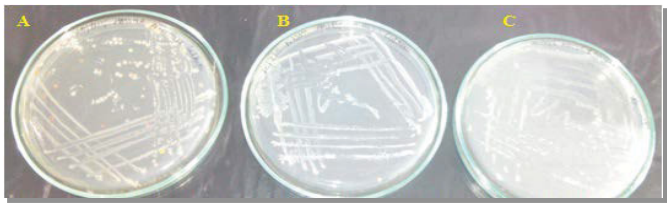

Figure 4: Screened bacterial isolates in pure culture.

	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.***			

Note: 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

Table 1: Biochemical characteristics of laccase producing bacterial strains.

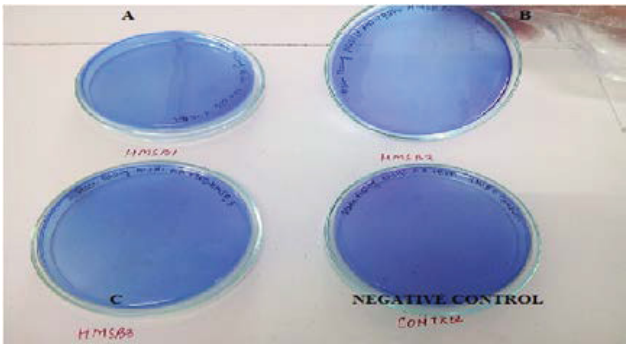

Figure 5: Bromophenol/plate assay for laccase producers.

Laccase production by bacterial consortia through 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 [35]. Selected *Trichoderma* species for the production of laccase in submerged fermentation [13]. In our previous study, submerged fermentation was carried out for fungal isolates *Aspergillus sp.* and *Fusarium sp* [25]. Usha has studied the diversified effect of white rot fungi *Stereum ostrea* in their response to supplemented inducers, surfactants, and copper sulphate in solid state fermentation [11]. Copper as a micronutrient has a key role as a metal activator, induces both laccase transcription, and plays an important role in laccase production [17]. Enhanced production of laccase in the presence of industrial effluents by wood decaying fungus has been reported thus indicating the microbial remediation process [12]. Every industrial process relies on cost- effective production and this can be ascertained by using cheaper raw materials and optimizing the fermentation process [14,33]. As contrasted by submerged fermentation, fairly viable technique for this purpose is the production of these enzymes by solid state fermentation (SSF) technique using agro industrial wastes as a support substrate. Most of such wastes are rich in soluble carbohydrates and also contain inducers of laccase synthesis, ensuring an efficient production of these enzymes Solid state fermentation utilizing natural lignin containing substrates such as rice bran, wheat bran, coir dust, potato peel, etc. [34]. Figure 6 represents submerged fermentation for Laccase production by *Alcaligenes sp* (A)., *Klebsiella sp* (B)., *Pseudomonas sp.*(C), and bacterial consortium (D).

Laccase Production

Laccase assay

Enzymatic systems are catalytic, highly specific, and operable under stable physiological conditions of temperature and pH. Noteworthy is the fact that enzymes by virtue of their specificity can modify the surface characteristics of fiber, improve fibre, improves the runnability of the pulping, bleaching and papermaking processes [38-40]. Laccases are one such group of lignolytic enzymes which have found immense usage in diverdified industrial sectors. Biochemically, crude filtrate of cell is used to monitor laccase activity by virtue of it's extracellular nature. Laccase activity was measured as expressed as International Units (IU), where 1 IU is defined as amount of enzyme required to

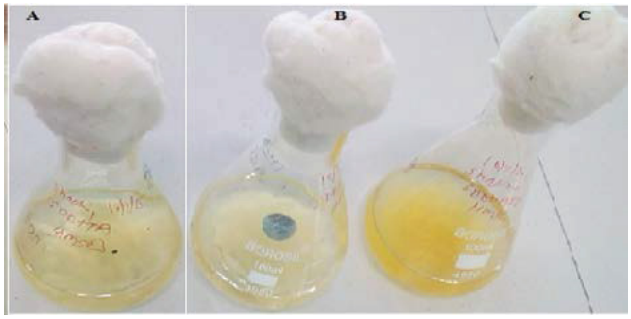

Figure 6: Submerged fermentation by monoculture and bacterial consortium for laccase production.

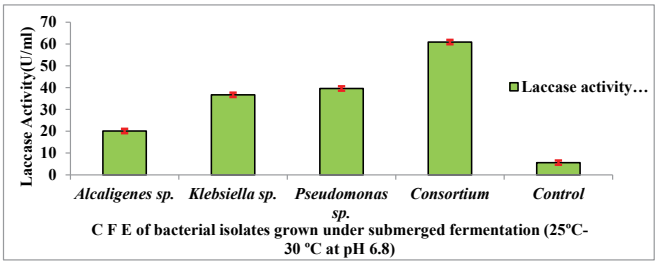

Figure 7: Laccase activity of bacterial strains and consortium as observed in cell free extract obtained after submerged fermentation at (25ºC - 30ºC at pH 6.8) ($p<0.05$).

oxidize 1micromole of guaiacol per min [41]. The laccase activity in U/ml is calculated using the extinction coefficient of guaiacol (12,100 M^{-1} cm^{-1}) at 450 nm. Phenolic and aromatic compounds such as guaiacol, veratryl alcohol, and ABTS have been widely employed to improve the production of ligninolytic enzymes by several fungal species [41]. The effect of copper has shown to enhance laccase activity as reported by some research groups [19]. Shanmugam S et al. [39] and Ding Z et al. [42] suugested various process parameters to attain enhanced laccase production under submerged fermentation conditions. Our findings revealed that laccase activity ranged from 20.1 U/ml- 60.9.8 U/ml for *Alcaligenes sp.* and consortium respectively 25ºC-30ºC at pH 6.8. A significant increase ($p<0.05$) in laccase activity was observed when contrasted with abiotic control (5.6 U/ml) [43] maximum laccase production at 30°C temperature by *Streptomyces lavendulae*. The bacterial isolate SB1 showed the maximum laccase activity of 0.351 U/L at incubation temperature of 30°C [31]. The highest laccase activity was observed at 40ºC (0.0388 U/ml) while slight decrease in enzyme activity was observed at 50ºC (0.0382 U/ml) [29]. Figure 7 represents laccase activity in C F E of bacterial isolates grown under submerged fermentation (25ºC-30ºC at pH 6.8) ($p<0.05$). Molecular biology tools have been proved in cloning and expression of novel laccase genes from white-rot fungus Polyporus grammocephalus in TR16 for enhanced production in *Pischia pastoris* [44].

Conclusion

Screening of potential microbes, indigenous to specific habitats are key to bio prospecting. This pilot study led to characterization of microflora capable of producing laccases in monoculture as well as consortium. Synergistic effect of screened isolates exhibited highly significant laccase activity. 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 bio pulping thereby reflecting their enormous potential not only in paper and pulp industries but also textile, fertilizers and other allied industrial sectors. The devised study which is selectively and specifically based at bio prospective strategy would play a pivotal role in generation of an eco-friendly process which would lead to development of a cleaner production technology thereby obliterating mechanical and chemical processes which are energy and cost intensive. Further insights may be based on metagenomics based analysis to screen for otherwise unexplored microbes which may culminate into potential microbial –factories.

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