ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

ENHANCED PRODUCTION OF LACCASE BY MICROPARTICLE INDUCED CULTIVATION OF BASIDIOMYCETES AND EVALUATION OF ITS EFFICIENCY AS DEINKING AGENT

Shraddha Prabhu*, Samia A. Palat Tharayil

Department of Microbiology, Sophia College for Women, Bhulabhai Desai Road, Opp. Breach Candy Hospital, Mumbai, Maharashtra, India

**Corresponding author: shraresearch@gmail.com*

ABSTRACT

Recycling paper is worthy alternative to cut down the dependence on trees thereby preventing the hazards like deforestation. Deinking is an important step in the recycling process which involves the removal of ink particles from fiber surface and decolorization of the dislodged ink. Enzymes such as laccases may prove eco-friendly cost effective alternatives to the conventional chemicals methods used for the recycling process. However, the commercial application of this enzyme is limited by insufficient production and less stability. Hence, in this study, the laccase produced by basidiomycetes species, *P. ostreatus* was evaluated for its potential as a deinking agent. *P. ostreatus* was able to grow on many cheap substrates and when grown in the medium containing sawdust showed significant laccase production. Addition of 5g/L of Al_2O_3 microparticles (microparticle enhanced cultivation) improved the production of laccase. The maximum activity of *P. ostreatus* laccase was observed at pH 3 and at 28°C. Activity was further stabilized by incorporation of Copper ions and immobilization in Ca-alginate beads. The enzyme was capable of decolorizing ink from color printer cartridge and from low quality coloured papers such as bus tickets. The brightness of the handsheets made from pulp was more pronounced than those treated with chemicals.

Keywords: Laccase, Microparticle enhanced cultivation, Basidiomycetes, Deinking

1. INTRODUCTION

Laccase is p-diphenol: dioxygen oxidoreductase belonging to the family of multi-copper proteins, and is widely distributed in higher plants, bacteria, fungi, and insects. It has the ability to oxidize a wide range of aromatic and non-aromatic compounds such as substituted phenols, some inorganic ions, and variety of other non-phenolic compounds. As it requires molecular oxygen (O_2) as the only co-substrate for bio-catalysis and reduces it to water by one-electron oxidation of substrate, it is regarded as the "Green Tool". This has attracted considerable attention in different environ-mental, industrial, and biotechnological sectors. However under harsh conditions, it is not able to exhibit maximum efficiency. Other major limitation is commercialization of the enzyme due to insufficient stocks. Therefore, novel strains which can tolerate harsh conditions or give stable enzyme production with minimum cost are in huge demand. Hence, research must be diverted towards improvement of enzyme production, activity and stability. Morphology engineering and immobilization may help to obtain more robust and active enzyme which will boost its exploitation, reutilization, and production, thereby enabling commercialization [1].

Traditional approaches consisted of manipulation of spore concentration, pH-shifting and mechanical stress exerted by stirring and aeration. However, these methods proved to be insufficient. Hence, modern technique such as addition of mineral microparticles to the media (microparticle-enhanced cultivation, MPEC) is one possible option. MPEC is especially useful for the fungi which have an agglomerative character of pellet formation from asexual spores. It is known that the method works by decreasing the diameter of macropellets, ultimately changing the morphology into dispersed hyphae on addition of microparticles like Al_2O_3 or talc, to improve the production of metabolites and enzymes. The relatively low cost of the method is of great relevance for large scale applications [2].

Recycling of wastepaper has gained momentum over the past decades due to the demand of green plants being imposed by the paper industry throughout the world. Deinking is an important step in the recycling process

and involves the dislodgement of ink particles from fiber surface and then removal of the detached ink particles by flotation, washing etc. The developments in the deinking process have immensely helped the utilization of secondary fiber such as old newsprint, xeroxed papers and laser/inkjet printed papers for making white grade papers. The conventional methods of deinking utilize chemicals such as sodium hydroxide, sodium silicate, hydrogen peroxide, chlorine based chemicals and other chelating agents which are environmentally hazardous [3]. In addition to that, several organic composites present in the pulp and paper industrial effluent have been classified as mutagenic or carcinogenic. On the other hand, chemical and physical techniques like incineration and coagulation have been reported as the common methods to reduce the toxicity and color of the pulp and effluent of paper recycling. However, these methods are cost intensive. Hence, to overcome the demerits of deinking by toxic chemical process, the enzymatic approach has drawn enough attention as it can detach ink particles from fibers lowering the process cost and making it eco-friendly [4]. Different enzymes like cellulases, xylanases, pectinases, amylases, lipases, esterases and laccases are employed for deinking [3]. Although de-inking of waste paper using cellulase and hemicellulase has been carried out at industrial scale, the physical properties of the de-inked pulp were not found to be good as compared to chemically de-inked pulp as reported by Zhang in 2008 and Bansal *et al.* in 2018. It is, therefore, desirable to explore other enzymes for de-inking [5]. The role of laccases, in particular, has been well established in the delignification of pulps resulting in an increase in viscosity. Hence, laccases could be proposed as interesting alternative for deinking of different types of papers which chiefly contains lignin rich mechanical pulp, as they enable detachment of ink particles as well as contaminants. Enzyme was found to enhance brightness and reduce the residual ink concentration of the treated paper [3]. But several challenges like high production cost, less stability and requirement of mediators has hindered the complete exploitation of the enzyme limiting its application in the industry. The recent studies on microbial laccases suggest new opportunities for this enzyme as good candidate for commercial applications in paper recycling. Hence, the aim of this study was to assess the potential of laccase obtained from basidiomycetes for its use as an eco-friendly, biological treatment for ink decolorization of low quality paper pulp while enhancing its production and evaluating its activity in the immobilized form.

2. MATERIAL AND METHODS

2.1. Collection and maintenance of fungal culture

In present study, ability of basidiomycetes to produce laccase was investigated. Three different basidiomycetes, *Pleurotus ostreatus* (bought from a local market), Bracket mushroom (obtained from garden tree) and *Pleurotus sajor-caju* (purchased from directorate of mushroom research, Solan) were collected and a piece from fruiting body was inoculated on Malt extract agar (MEA) plate supplemented with chloramphenicol (composition g/L: Malt extract: 20g, Yeast extract: 2g, Agar: 15g, Distilled water: 1L. chloramphenicol: 0.1g, pH: 5.5±0.3 at 25°C). It was incubated at 28°C for 4-5 days. All the cultures were maintained by subculturing weekly and stored at 4 °C. Wet mount was performed using lactophenol cotton blue and morphology was studied under high power 40x objective of light microscope. Cultures grown on MEA plates were further screened for laccase activity.

2.2. Screening of fungi for laccase production

The preliminary screening for laccase production was done by laccase plate assay. For this, half of the plate was inoculated with fungal culture and after 2 days growth, a well of 6 mm diameter was bored in other half. To this well, 100 µL of 1% α-Naphthol solution prepared in 95% (v/v) ethanol was added followed by incubation at 28°C for 24 to 48 hours [6].

2.3. Laccase production in liquid media

Laccase positive fungal strains were grown on Malt extract agar as matt growth and two mycelial plugs were bored from the plate for inoculation in yeast extract medium (composition g/L: Yeast extract: 27.5g, Distilled water: 1L) containing different lignin rich substrates such as coconut husk, saw dust, bagasse (0.5 g of each of them to 25 mL of the above mentioned growth medium in 150 mL Erlenmeyer flasks) [7]. After incubating at RT on a rotatory shaker for 4 to 5 days, fungal mycelium was transferred to separate flasks containing 15ml of Malt Extract broth and incubated further. Aliquots were withdrawn at regular intervals to determine extracellular laccase activity.

2.4. Extracellular laccase activity

Laccase activity was estimated by the oxidation of substrate (ABTS) 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). For this, the medium was filtered through Whatman no.1 filter paper and 500 µL of filtrate

(crude enzyme) was added to 500 µL of 2mM of ABTS solution (prepared in 0.1M of citrate phosphate buffer (pH 3). The final volume was adjusted to 3ml with 0.1M of sodium acetate buffer, pH 5.0. Oxidation of ABTS was determined using spectrophotometer at 420 nm after 5 minutes of incubation. One unit of enzyme activity was expressed as the amount of enzyme oxidizing 1 mM of ABTS per minute per mL of enzyme extract [8].

2.5. Effect of temperature and pH on fungal growth in liquid medium.

Mycelium plugs were inoculated in 150ml of Erlenmeyer flask with 25ml of yeast extract medium (composition g/L: Yeast extract: 27.5g, Saw dust: 20g, Distilled water: 1L) and incubated at 28°C and 37°C. To study the effect of pH on fungal growth, pH of the medium was adjusted to 3.0 and 5.0, respectively.

2.6. Optimization of parameters for enzyme activity

To study the effect of pH, laccase activity was tested by performing enzyme assay using citrate phosphate buffer (pH 3), sodium acetate buffer (pH 4 and 5) and sodium phosphate buffer (pH 6 and 7) [9]. To study the effect of temperature, the laccase activity was tested by performing enzyme assay at pH 3, incubating the tubes at temperatures (4-100°C) for 10 minutes. To study the effect of substrate concentration, the laccase activity was tested by performing enzyme assay at pH 3, at 28°C, using substrate (ABTS) concentrations ranging from 2-20 mM. To study the effect of Cu, the laccase activity was tested by performing enzyme assay at pH 3, 28°C, using $CuSO_4$ (solution prepared Citrate-Phosphate buffer, pH 3.0) concentrations ranging from (0.1-50 mM) [10]. Standard Graph of ABTS vs. Absorbance was used to determine enzyme activity (in terms of concentration of product formed) in all the experiments for optimization.

2.7. Entrapment in Calcium Alginate beads

For enzyme immobilization, sodium alginate solution (4%) and crude enzyme were mixed in a ratio of 1:1 (v/v) using magnetic stirrer. This mixture was dropped using syringe into 50 ml of calcium chloride (2%). The beads formed were then incubated overnight at 4°C in $CaCl_2$ [11]. They were then washed and stored at room temperature till use.

2.8. Cultivation with micro particles

For the MPEC experiments, Aluminum oxide (Al_2O_3) microparticles (53-149 µm) obtained as dry powder (Himedia) was sterilized separately and added in the following concentration: 5g, 15g and 30 g of Al_2O_3 g/L, to Malt extract broth flasks. Mycelial plugs were inoculated in 25 ml of medium and incubated at room temperature on a rotatory shaker for 3 days. Control was also set up, where Al_2O_3 microparticles were not added. Medium was filtered through Whatman No.1 paper and the filtrate obtained was monitored for the laccase activity [2].

2.9. Morphological characterization

Morphological analysis of mycelia grown in medium supplemented with Al_2O_3 microparticles was conducted by addition of pellets formed in broth into a Petri plate containing 5 mL of distilled water. Images were captured using a phone camera and further analyzed by imageJ software [12].

2.10. Ink decolorization Pilot assay

Pilot ink decolorization assay was performed with colour ink (obtained from printer cartridge) where, the ink was incubated with enzyme on shaker (150 rpm) for one hour, at room temperature. Untreated ink sample served as control. Absorbance was measured using Colorimeter at absorption maxima of the ink (490nm). Decolorization was determined by comparing absorbance (OD) of enzyme treated ink sample with control and by calculating decolorization efficiency [13].

Decolorization efficiency = (OD control- OD test / OD control) x 100

2.11. Pulp Preparation

Used BEST bus tickets were first sorted based on colour, and then soaked overnight in water at room temperature and subsequently blended to obtain soft cottony pulp. Pulp was then squeezed to remove absorbed water and oven dried for 1 hour at 156°C [3]. Oven dried pulp was used for further experiments.

2.12. Sequences for deinking the pulp

A standard deinking sequence (P-C) was performed for oven dried pulp. Here, P referred to the pulp preparation stage and C referred to chemical bleaching stage.

For chemical bleaching stage, 10g pulp obtained (5% consistency) was treated with sodium hydroxide (2%), tween 80 (0.2%), EDTA (0.5%), sodium bisulphate (8%) and H_2O_2 (1%) at 50°C with continuous shaking at 200 rpm for 15 min. For Enzymatic treatment, laccase in free (8%) and immobilized form (Ca-alginate beads) was

incorporated after pulping stage (P-L). Treatment was carried out at 200 rpm for 1 hour, at pH 3, 28°C. Untreated pulp served as a control [4]. The treated pulps were then washed thoroughly with distilled water and recovered for testing.

2.13. Preliminary determination of efficiency of deinking

Absorbance of supernatant obtained all three types of treatments of pulp was measured at 540nm for orange, green and at 420nm for black-blue tickets. Lower absorbance indicates better efficiency of deinking [13].

2.14. Preparation of hand sheets and residual ink measurement

The slurry of the treated pulp was pressed on absorbent sheet and dried at room temperature for 24 hours. Brightness of the handsheets was determined visually. Residual ink present in the hand sheets were measured by incubating a piece (1cm^2) of the same with acetone for 5-10 minutes and measuring the absorbance at absorption maxima of the ink released. Lower absorbance indicates lower residual ink and better de-inking efficiency

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of laccase positive basidiomycetes

Laccase plate assay showed that both *P. ostreatus* and *P. sajorcaju* gave positive reaction with α-Naphthol solution, giving a purple coloured halo on the plate. *P. ostreatus* showed more pronounced color around the well compared to *P. sajor-caju* (Fig. 1).

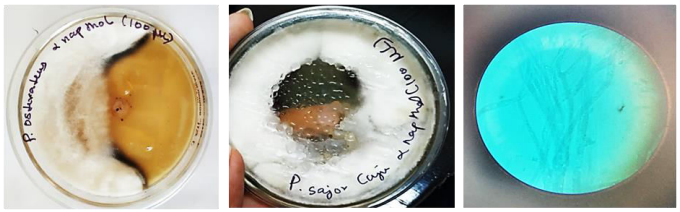


Fig. 1: a) Qualitative laccase Plate assay (purple color around growth) *P.ostreatus*, and b) *P. sajor-caju,* c) wet mount of *P. ostreatus*

3.2. Effect of temperature and pH on fungal growth in liquid medium

Both temperature and pH had significant impact on fungal growth. The ability of the fungal strain to grow in liquid media at different temperature and pH was tested. It was found that room temperature (28°C) and pH 5, supported fungal growth and the growth was inhibited below pH 3. At warmer temperature of 37°C, no significant fungal growth was observed. Further studies were carried out with cultures grown at optimum temperature (28°C) and pH 5. It was also observed during our study that fungal growth and subsequent laccase production was very sensitive to climatic conditions and other factors like production of metabolic end products. It was difficult to maintain the fungal strains as it ceased to grow and went into the dormant stage, when pH of the liquid medium post mycelial growth dropped below 3.

3.3. Effect of different natural substrate on fungal growth and production of laccase

When liquid medium was supplemented with different natural substrates and inoculated with mycelial plugs for both the laccase positive *Pleurotus* species, it was observed that *P. ostreatus* grew in medium containing variety of different substrates; whereas *P. sajor-caju* grew well only in medium containing coconut husk. Furthermore, when qualitative test for laccase was done using 2mM ABTS and 10mM guaiacol, positive results were obtained only for *P. ostreatus* grown in medium supplemented with sawdust. It was reported in a study that, laccase activity reached its maximal value before *P. ostreatus* growth ended [14]. Similar findings were also observed in present study, where laccase activity was obtained only when *P. ostreatus* was actively growing on both plate and in liquid medium. *P. ostreatus*, grown on sawdust tested positive for laccase production with both substrates (2Mm ABTS and 10mM guaiacol) in qualitative assay (Fig.2).

Fig. 2: Qualitative testing with a. 2mM ABTS and b. 10mM guaiacol for laccase obtained from *P. ostreatus*

Further experiments were performed using laccase obtained from *P. ostreatus*. Moreover, since laccase was only produced during growth phase of the culture, changing the medium at regular intervals was an essential

requirement for consistent laccase production. It was also observed that production of laccase continued for a week after its initial production and ceased within 14 days of fungal growth.

3.4. Optimization using different parameters

Subsequently, enzyme laccase obtained was subjected to optimization studies. For temperature, the highest enzyme activity of 144mmol/min was observed at 28°C (Fig. 3b). Laccase activity greatly reduced above 37°C. Highest laccase activity of 295.5 mmol/min was obtained up to pH 3 and sharp drop was observed above pH 5 (Fig.3a). Previous study done with *P. ostreatus* strain *10969* reported that when the pH was below 2.5 or above 5, only 40% of the maximum laccase activity was reached. Increasing of temperature above 50°C, the enzyme activity reduces sharply [15]. Enzyme activity increased linearly up on increasing the substrate ABTS concentration till 20 mM concentration. Km and Vmax value as determined from Michealis Menton graph was found to be 6.23mmol/L and 274.75mmol/min, respectively. (Fig.3c).This was similar to values of Km and Vmax, 0.31mmol/L and 303.25 mmol/min, respectively as reported in another study [15]. It has been reported previously that incubation with the metal-supplemented extracts exhibited in general higher laccase activity [10]. This study showed that laccase activity increased linearly with increase in $CuSO_4$ concentration, highest enzyme activity of 340mmol/min obtained at 50 mM (Fig 3d). Copper thus positively affects the activity and of the enzyme.

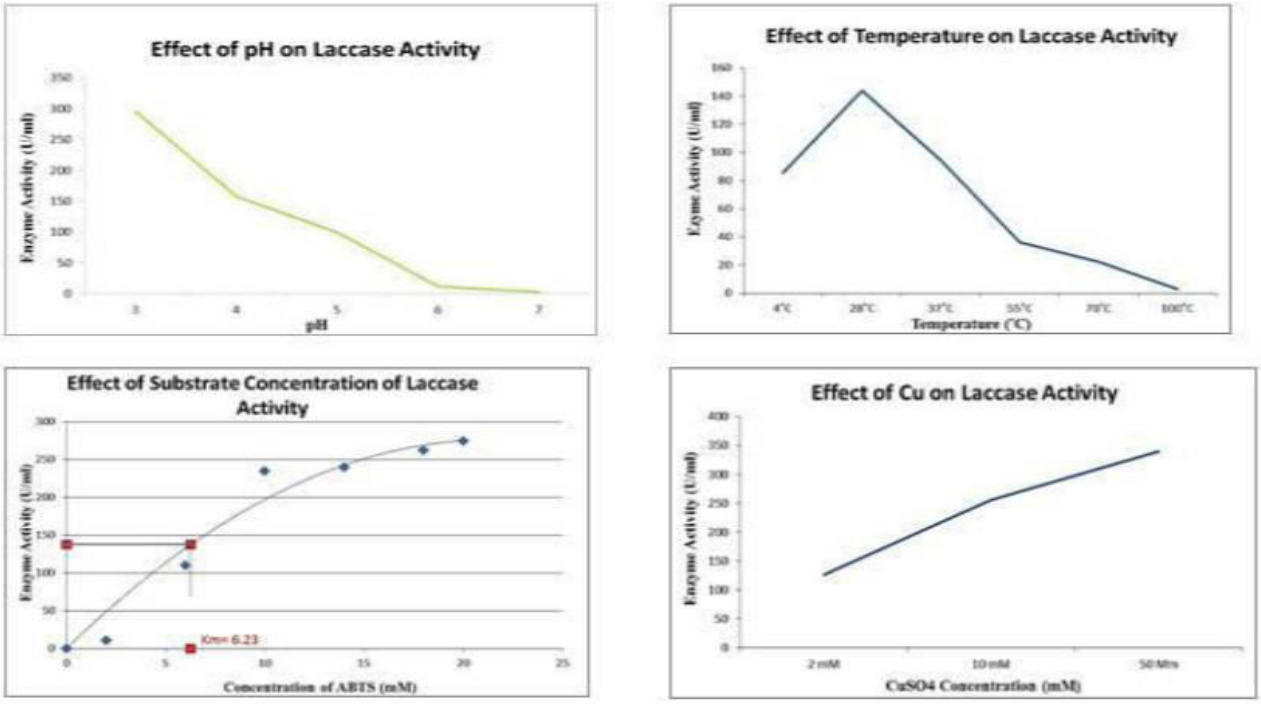


Fig. 3: Effect of different parameters on laccase activity a) pH b) temperature c) ABTS concentration d) Cu ions

3.5. Effect of immobilization on laccase activity

The laccase activity was determined for immobilized enzyme in Ca-alginate beads and compared with free enzyme. The activity was lower compared to free enzyme. This may be due to slower diffusion of oxygen in the immobilized beads during the short incubation time of assay (Fig.4).

3.6. Effect of Al_2O_3 microparticles on laccase production and fungal morphology

A previous study for microparticle enhanced cultivation experiment (MPEC), on *P. sapidus* reported that 15 g/L of Al_2O_3 increased the laccase activity by two-fold and decreased the pellet size [2]. The effect of microparticles on laccase production was studied by addition of 5-30 g/L of Al_2O_3 on the 3rd day of

incubation, and the maximum activity obtained was 388.5 U/ml (Fig.5e and f).

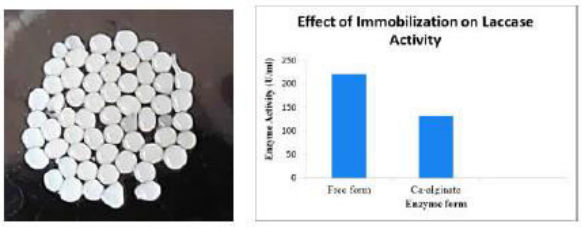


Fig. 4: a. Immobilization of laccase in Ca alginate beads b. Comparison of laccase activity in free and immobilized form

The size of the pellets formed in all the flasks were determined by ImageJ software. It was found that the pellets formed in control were bigger with average size around 4.6 to 5.0 mm. Shape descriptors indicated morphology as circular. Unlike control, pellets formed in the presence of 5 g/L of Al_2O_3 are of average size 2-4 mm, and more in number. Morphology was slightly elongated. With further increase in the Al_2O_3 concentration, there was overall considerable decrease in the size (Fig.5a-d). However with further increase in the concentration of Al_2O_3 microparticles *i.e.*10, 15 and 30 g/L had detrimental effect on laccase production. Addition of Al_2O_3 microparticles had reduced the pellet size and increased number, indicating that fungal morphology is tightly linked to improving enzyme production. Hence, 5g/L of Al_2O_3 was considered for enhancing laccase production. The laccase obtained was then subjected to deinking studies.

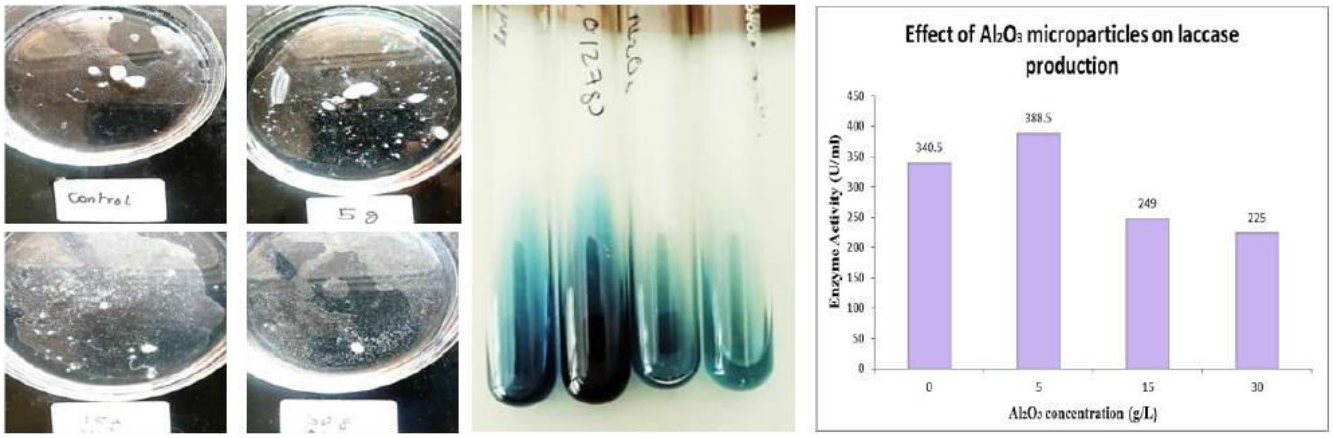


Fig. 5: a) Effect of addition of Al_2O_3 microparticles on fungal morphology: i.0g/L, ii.5g/L, iii.15 g/L, iv.30 g/L. b) Amount of Laccase production indicated by blue color. c) Maximum production of laccase seen at 5 g/L of Al_2O_3

3.7. Ink decolorization efficiency

Assay was performed using colour ink obtained from printer cartridge. Results of pilot ink
decolorization assay revealed that both free and immobilized form of enzyme were capable of decolorizing ink. However, immobilized form showed higher efficiency than free form.

Table 1: Ink decolorization efficiency of free and immobilized form of laccase.

Ink	Absorbance	% decolorization
Control	0.88	-
Free Enzyme	0.50	43%
Immobilized	0.40	54%

3.8. Preliminary determination of efficiency of deinking

Subsequently, standard deinking protocol was followed for coloured bus ticket pulp. Lowest OD in all cases was obtained for laccase enzyme treatment, as shown in (Table 2).

For bus tickets pulp, the positive effect of laccase treatment could also be visually detected in the color of the sheets, as compared to chemically treated sheets and control. Handsheets of pulp prepared for bus tickets are as shown in Fig.6. As reported in previous studies laccase is not only used in deinking by removing ink particles by delignification of paper fiber, but also decolorizing the ink which was transferred to the filtrate [13].Visually, bus tickets pulp treated with laccase

appeared lighter as compared to its chemically treated counterpart. Green ticket showed highest deinking efficiency post enzymatic treatment visually, followed by black and orange. It was noted that for black ticket there was decolorization, though not as prominent as green ticket.

Table 2: Efficiency of deinking for colored pulp of bus tickets

Treatment	P(control)	P-C		P-L	
		OD units	% reduction	OD units	% reduction
Orange	0.66	0.42	36	0.13	80
Green	0.67	0.34	50	0.10	85
Black	0.80	0.52	35	0.18	77
Treatment	P(control)	P-C		P-L	
		OD units	% reduction	OD units	% reduction
Orange	0.66	0.42	36	0.13	80
Green	0.67	0.34	50	0.10	85
Black	0.80	0.52	35	0.18	77

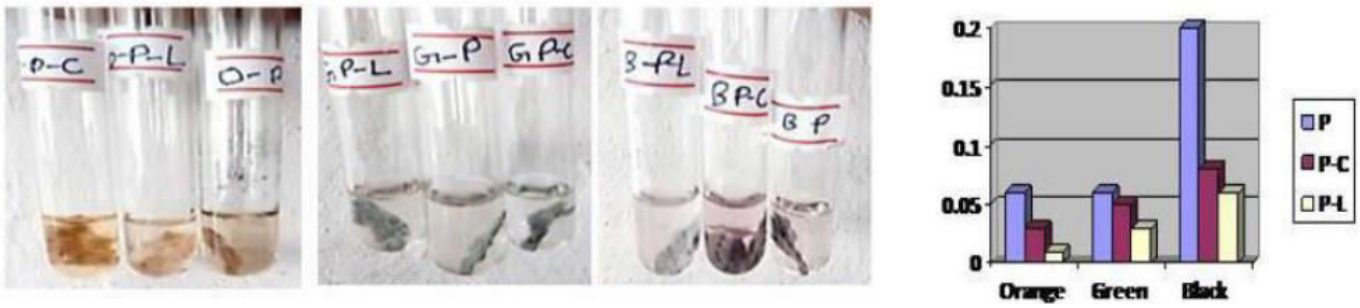
P: Pulp, P-C: pulp + chemical treatment, P-L: pulp + laccase treatment

Fig. 6: handsheets prepared from bus tickets pulp a) just after treatment, b) After air-drying for 48 hours. control (left), P-C (middle), P-L (right)

3.9. Residual ink measurement of handsheets

Residual ink present in dried handsheets were determined by measuring absorbance at 420 nm for green and black bus ticket pulp and 540 nm for orange bus ticket. Enzyme treatment was effective in reducing residual ink (lower absorbance of supernatant compared to control and chemical treated) in all three pulps.

This has added benefit of providing no treatment to the effluent generated during the deinking process, thereby lowering the COD content, and being less harmful to the environment [16].



a. orange b. green c. black d. absorbance of residual ink for each handmade sheet

Fig. 7: Comparison of Residual ink for different colored bus tickets pulp after treatment

4. CONCLUSION

One of the main limitations that must be overcome in order to use fungal laccases for deinking applications is the relatively slow growth of the fungi and low production and stability under harsh conditions. This study showed that addition of Al_2O_3 microparticles improved the production of laccase and the activity of enzyme could be enhanced by addition of Copper ions. It was evident that enzymatic treatment was more effective in dislodging and decolorizing ink from the colored paper pulp compared to chemical treatment and immobilization of the enzyme improved its activity and stability. This would consequently reduce the cost of chemicals otherwise needed for pulp treatment. Removal of ink from paper pulp using laccase represents a potentially commercially viable, effective and

environment- friendly alternative to traditional physicochemical methods which require both high energy and investment.

5. ACKNOWLEDGEMENTS

Author is thankful to Department of microbiology, Sophia College for Women, for providing necessary facilities for this research.

6. REFERENCES

1. Agrawal K, Chaturvedi V, Verma. *Bioresour. Bioprocess*, 2018; **5**:4**.**
2. Antecka A, Blatkiewicz M, Bizukojć M, Ledakowicz S. *Biotechnology Letters*, 2016; **38**:667-672.
3. Virk AP, Puri M, Gupta V, Capalash N et al. *PLoS ONE*, 2013; **8(8):**e72346.
4. Singh G, Arya SK, Gupta V, Sharma P. *J Mole Biol Tech*, 2017; **2(1):**105.
5. Shankar S, Shikha, Bhan C, Chandra R et al. *Environmental Sustainability*, 2018; **1**:233-244.
6. Stalpers JA. *Studies in Mycology*, 1978; **16**:1-248.
7. Sahay R, Yadav RSS, Yadav KDS. *Chinese Journal of Biotechnology*, 2008; **24**(12):2068-2073.
8. Wolfenden BS, Willson RL. *Journal Chemical Society Perkin Transactions*, 1982; **2**:805-812.
9. Atalla, M Mabrouk R, Hamed Eman A, Youssry Amani et al. *Saudi journal of Biological Sciences,* 2013; **20(4):**373-381.
10. Baldrian P, Gabriel J. *FEMS Microbiology Letters*, 2002; **206**:69-74.
11. Zabin KB, Sikandar IM, Harichandra ZN. *Journal of Genetic Engineering and Biotechnology*, 2017; **15(1):**139-150.
12. Yatmaz E, Karahalil E, Germec M, Ilgin M et al. *Bioprocess Biosyst Eng.*, 2016; **39**:1391-1399.
13. Nyman K, Hakala T. *Bioresources*, 2011; **6**:1336-1350.
14. Tinoco R, Acevedo A, Galindo E, Serrano-Carreon L. *J Ind Microbiol Biotechnol*, 2011; **38**:531-540.
15. Lihua Liu, Zhiwei Lin, Teng Zheng, Ling Lin et al. *Enzyme and Microbial Technology*, 2009; **44(7):**426-433.
16. Lee CK, Ibrahim D, Omar IC, Wan Rosli WD. *Bioresources*, 2011; **6(4):**3859-3875.