JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 1441-1446

Kinetics and Thermodynamics of Crude Laccase Mediated Decolorization of Acid Red 131 Obtained by Growing *A.niger* over *Limonia acidissima* Shell as Solid Support

S. Miruthula and S. Sridhar*

[1]Department of Biotechnology, Jeppiaar Engineering College, Chennai, India.

(Received: 13 March 2015; accepted: 18 April 2015)

Synthetic Dyes from leather industries contain recalcitrant substances/complex pollutants which are difficult to degrade and may impact human health is a major health concern. Enzymes from microbes offer an attractive option for removal of synthetic dyes at low cost, better technology transfer and are eco-friendly. Solid-state fermentation uses inexpensive and widely available agricultural residues as substrates for enzyme production. Among various solid substrates, *Limonia acidissima* (Wood Apple) shell has been found to be a suitable substrate for producing enzyme by solid-state fermentation. The present study was undertaken to produce crude laccase on wood apple shell powder by using *Aspergillus niger* in solid-state fermentation. Decolorization of Acid Red 131 was studied using crude laccase. Various Physico-chemical parameters such as pH, temperature, Metal Ion and salts were studied. Maximum laccase production reached 108.4U/µg at 40% moisture. The decolorization studies reported that maximum decolorization was observed at dye concentration of 50mg/L after an incubation time of 60 minutes. The thermodynamic studies on decolorization follow first order kinetics and the reaction is exoergonic, ΔG of the reaction is negative which supports the fast reactivity at the transition state.

Key words: Laccase, *Limonia acidissima*, Physico-chemical parameters, Decolorization, Thermodynamic studies.

Discharge and percolation of wastewater from various manufacturing and processing industries is a major threat to eco-system. Tannery wastes are prime source of pollution to agricultural lands and water bodies, as they form a plume containing consortia of chemical components ranging from inorganic salts to organic solvents, which makes them stable and difficult to remediate by a waste water treatment plant[1, 2]. Contaminated wastewater represents high biological oxygen demand (BOD) and chemical oxygen demand (COD), thereby affecting aquatic flora and fauna, creating lack of oxygen, resulting in development of dead zones lacking aquatic life[3,4]. Human welfare has forced the monitoring and regulating bodies to have continuous vigil over the discharges made by industries, thereby mandating proper treatment before waste effluent being released into environment. Despite of various physico-chemical methodologies such as chemical precipitation, ion-exchange resins, reverse osmosis, oxidation processes, filtration and electro-coagulation have been tried to treat the toxic effects of dye-containing waste water[5]. Higher operational costs, generation of primary and secondary sludges, technical snags during scaling up and technology transfer during real-time operations are potential primary factors acting as hindrance for various physical and chemical methodologies[6]. Moreover, no single

* To whom all correspondence should be addressed.
E-mail: sridhar@jeppiaarcollege.org

methodologies are available to combat pollution efficiently, compelling involvement of more than one approach to remediate these point pollutants. Microorganisms (Bacteria/Fungi) have developed /adapted it to survive from environmental stress caused by these synthetic organics released from the industry. Bio-mediated approaches have been gaining focus towards fighting pollution in an ecological and eco-efficient manner. Among bio-mediated techniques, enzymatic remediation approaches have been gaining significant attention from various sectors as they are environmental friendly, cost-effective and they do not produce sludge[7]. Enzymes from various microbial sources such as bacteria, fungi and plants have been reported in playing a pivotal role in bioremediation of various toxic synthetic organic pollutants released into the environment[8]. Laccases are multi-copper oxidoreductases involved in oxidizing a wide variety of organic compounds based on one-electron oxidation and four-electron reduction of molecular oxygen to water[9]. There is a paradigm transition from utilization of whole organisms to cell-free systems (extracellular fraction-enzymes) from its application spanning from biosynthesis to bioremediation[10]. The process economics involved in production of enzymes is a robust process, as it involves various inter-related factors involving physic-chemical parameters, economics, scale-up, technical snags during real-time application and technology transfer. Approaches for enhanced production of enzymes are carried out using various fermentation techniques. Among fermentation techniques, solid State fermentation is considered as primary approach, utilizing various organic agricultural, forestry and food industrial waste to produce enzymes, yielding higher titres of enzymes, with high temperature and pH stability[11, 12, 13]. The use of such wastes not only provides cheap substrates, but metabolites present within these substrates, act as an internal mediator, inducing and secreting extracellular enzymes efficiently[14]. *Limonia acidissima* (wood apple) fruit belongs to family Rutaceae, much research has been focused on wood apple shell is focused towards adsorption because of their abundant availability at low or no cost, efficient dye removal from aqueous solutions as well as minimum volume of sludge to be disposed[15]. We are reporting for the first of its kind for production of crude laccase enzyme isolated from *Aspergillus* niger using *Limonia acidissima* shell as solid support towards decolorization of prototypical leather dye Acid Red 131.

MATERIALS AND METHODS

Chemicals

All the chemicals were purchased from HI- Media. Dye (Acid red 131) used was gifted by KH SHOES, Vellore, TamilNadu, India.

Culture collection and Maintenance

The microorganism used in this study was *Aspergillus niger*, was gifted by Marina labs, Chennai, India and was maintained in Potato Dextrose Agar slants at 4 Degree Celsius (^{0}C) and subcultured every 2 days using nutrient broth.

Solid State fermentation

Aspergillus niger was grown on 250 milliLitre (mL) Erlenmeyer flask containing 5gram(g) of *Limonia acidissima* shell as a solid-support substrate and appropriate amount of basal media (pH 6.5) consists (g-gram/L-litre): K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.5; KCL, 0.5; yeast, 0.25; peptone, 0.25; glucose, 12.5 was added to maintain 40 Percentage (%) moisture after drying of substrate. The substrate and the media used for SSF were sterilized in autoclave during 15 minutes (min) at 121^{0}C. The culture conditions were maintained at room temperature approximately at 27±2°C under static conditions for seven days. After seven days of incubation, the enzyme was harvested by the addition of 50ml double distilled water and filtered using Whatmann filter paper grade No1. The filtrates are centrifuged at 5000(g) for 15min. The supernatant obtained after centrifugation acts as a sole source of biocatalyst (crude enzyme).

Enzyme and protein assay

Laccase enzyme activity was carried out using 1milliMolar (mM) Guaiacol as substrate, in 100mM sodium acetate buffer (pH 6.5) with total reaction volume maintaining at 5 mL as described in Mansur et al 2003[16]. 1 Unit (U) of enzyme activity was defined as the amount of enzyme that elicited an increase in A465 (μ = 12

000 M^{-1} cm^{-1}) of 0.1 absorbance unit per minute. Enzyme activity (Equation 01) was calculated by using the formula,

$$\text{Enzyme activity}\left(\frac{U}{mL}\right) = \frac{[A_t - A_0] \times \text{Dilution factor} \times \text{Total volume} \times 1000}{\text{M.E.C} \times \text{Enzyme volume} \times \text{Time}} \quad ...(1)$$

Where

A_0 is the initial absorbance immediately after the enzyme addition

A_t is the final absorbance of reaction mixture after incubation

M.E.C is the molar extension coefficient of guaiacol.

Protein assay was performed using standard Bradford method (1976) using bovine serum albumin as a standard protein (Bradford 1976)[17].

Effect of Physicochemical parameters on enzyme activity

Four physico-chemical parameters such as pH, temperature, metal ion concentration and salt concentration were studied using a standard procedure with few modifications[18]. The reaction mixture consists of incubating 100 microLitre (µL) of crude laccase sample and 900 µL of buffer of different pH values (2, 4, 6, 8 & 10) for 120 minutes and the residual activity were measured. The effect of temperature is studied by incubating crude laccase sample for 120 minutes at different temperatures (30, 40, 50, 60 &70^{0}C) at optimum pH. After heat treatment, the samples were cooled in ice water and the residual laccase activities were measured using Guaiacol as standard substrate. The effect of metal ion (1mM of Na_2So_4, KI, & $ZnCl_2$) and salt concentration (0.1mM of NaCl & KCl) towards crude enzyme stability were studied by incubating 100 µL of crude enzyme, 1mM of 100 µL of metal ion/0.1mM of salt concentration and 800 µL of sodium citrate buffer (pH 6.0). The residual activities were expressed in terms of enzyme activities. All reaction procedures were carried out in triplicates and standard deviation was found to be below 5% confidence limit.

Enzyme-based decolorization

Decolorization of Acid Red 131 was studied using crude enzyme obtained by growing *Aspergillus niger* on the solid support without further processing and purification. The reaction mixture for all decolorization studies consists of 0.1M sodium acetate buffer, 0.5ml of 50 milligram/Litre (mg/L) dye Weight/Volume (W/V) and 0.5mL of crude enzyme, making final volume of the reaction mixture to 5ml[2,19]. Samples were aliquoted at every 60 minutes and decolorization was studied by monitoring the decrease in the absorbance at 550 nanometer (nm). The percentage of decolorization was calculated using the following equation (Equation 02).

$$\% \text{ Decolorization} = \frac{(\text{Initial absorbance}) - (\text{Final absorbance})}{\text{Initial absorbance}} \times 100 \quad ...(2)$$

Effect of Physicochemical parameter on dye decolorization

The decrease in the spectral absorbance during decolorization of Acid Red 131 was recorded using UV-Visible Spectrophotometer (Elico, India). The dye removal efficiency was expressed as percentage of decolorization as expressed in Equation 02. The reaction mixture for all decolorization studies consists of 0.1M sodium acetate buffer, 0.5mL of 50mg/L dye (W/V) and 0.5mL of crude enzyme, making final volume of the reaction mixture to 5mL[2,19]. The effect of pH was studied at different pH range (pH 4,5,6,7 and 8). The influence of temperature towards decolorization of dyes was studied at optimum pH at different temperature range (30, 40, 50 & 60^{0}C). The optimal dye concentration was determined by incubating the reaction mixture at four dye concentrations (25, 50, 75, 100mg/L) at optimal pH under standard conditions as described above. The effect of metal ion (1mM of Na_2SO_4, $MgSO_4$, KI & $CuSO_4$) and salt concentration (0.1mM of NaCl, KCl, Na_2CO_3 & Na_2SO_3) towards dye decolorization were studied by incubating 500 µL of crude enzyme, 1mM of 100µL of metal ion/0.1mM of salt concentration and 400µL of buffer at optimal pH making total reaction volume to be 5mL. Kinetics and thermodynamic parameters such as Entropy (ΔS), Enthalpy (ΔH) and Gibbs free energy (ΔG) of dye decolorization by crude laccase enzyme was calculated using Eyring-Polanyi plot.

RESULTS AND DISCUSSION

Maximum crude laccase specific activity was found to be 108.4U/µg, when *A.niger* was grown on *Limonia acidissima* shell as solid support. Moisture is key parameter to control the growth of microorganism and metabolite production in SSF. Increase in moisture content decreased laccase enzyme activity[20].

Effect of Physicochemical parameters on enzyme activity

The optimum pH for crude enzyme was found to be pH 6, when Guaiacol was used as standard substrate for laccase assay. The optimal pH laccase obtained from various fungal sources varies with the type of the substrate utilized, redox potential of the isolated enzyme, presence of mediators and molecular oxygen affects the optimal pH[21]. The effect of temperature on crude enzyme activity was investigated by recording the change in absorbance using spectrophotometrically at 470 nm using guaiacol as the substrate. The optimum temperature was found to be 60°C. The optimum temperature varies from fungal strain to strain. The effect of metal ion (1mM concentration of Na_2SO_4, kI & $ZnCl_2$) and salts (0.1mM concentration of NaCl & KCl) on enzyme activity was investigated. The salt tolerability of laccase enzyme showed that chloride salts (NaCl & KCl) enhances the enzyme activity (0.0263 & 0.0238 U/mL) when compared to 0.021U/mL without salt even after 120 minutes of incubation time. This shows that laccase enzyme was highly stable in the presence of chloride salts. After 60 minutes incubation with the metal ions such as Na_2SO_4, kI & $ZnCl_2$ at 1mM concentration, kI metal ion showed activity of 0.01002U/ml when compared to 0.0165U/ml without metal ion. Previous studies reported that Laccase activity of *P. ostreatus* was enhanced by 8%, 6%, 6%, 4%, and 4% in Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+}. Also they reported laccase activity was not dependent on metal ion. No change in laccase activity was estimated in the presence of Zn^{2+} in *A. niger*[22]. It is clear from that the laccase activity was not dependent on the metal ions.

Effect of physicochemical parameter on dye decolorization

The optimal pH for crude enzyme mediated dye decolorization was found to be pH5. The effect of temperature towards the decolorization of Acid Red 131 reported maximum decolorization at 30°C. Decolorization decreased beyond optimal temperature indicating the fact that on increase in temperature, the rate of inactivation of enzyme (Crude laccase) is faster than enzyme mediated remediation[23,24]. The effect of metal ion (1mM concentration of Na_2SO_4, Mg, kI, & $CuSO_4$) and salts (0.1mM concentration of NaCl, Kcl, Na_2CO_3 & Na_2SO_3) on dye (50mg/L) decolorization was studied. Among the metal ions KI reported 16.73% decolorization when compared to 18.82% without metal ion. In the case of salts KCl showed the decolorization of 22.96% when compared to 23.52% without salt even after 120 minutes. Our reports indicate that these metal ions and salts play a pivotal role in inhibition of decolorization. The dye concentration at which maximum decolorization was found to be 50mg/L dye with the incubation of 60 minutes. Increase in dye concentration resulted in a significant change in percentage decolorization as well as the time required for decolorization. The time required for decolorization was gradually increased up to 45 min for 2,500 mg/L with decolorization efficiency reduced to 85 %[25].

Table 1. Equations for the linear best fit and rate constants (k) obtained using the kinetic equation for first-order kinetics at 30, 40, 50 and 60^{0}C for the biodegradation of Acid Red 131 dye

Temp. (^{0}C)	Linear Equation	R	kI × 10^{-2} h^{-1}
30	1.937x + 0.062	1	193.7
40	1.864x + 0.136	1	186.4
50	1.810x + 0.189	1	180
60	1.932x + 0.067	1	193.2

Table 2. Thermodynamic data ΔH, ΔS, and ΔG for the bio- degradation of dye by crude enzyme mediated decolorization

Thermodynamic data	Acid Red dye (SSF)
ΔH(J/mol)	-2164.134
ΔS(J/mol)	186.484
ΔG (kJ/mol)	-58.668

Thermodynamic studies on dye decolorization

Thermodynamic factors such as free energy, energy of activation, rate and kinetics of crude laccase mediated decolorization were addressed. Acid Red 131 decolorization/ degradation followed first-order kinetics. It was observed that the kinetic constants (k) for the biodegradation of Acid red 131 increased as the temperature was decreased from 30 to 50^{0}C, and

then decreased for 60^0C. The decreases in the rates of degradation could be because of structural changes in the active sites of the dyes that prevent the formation of the enzyme–dye complexes, or because of the absence of some enzyme mediator acting together with the laccase. By applying the Eyring–Polanyi equation (03), the thermodynamic data and kinetic constants at three temperatures were obtained.

$$Ln\frac{k}{T} = \frac{-\Delta H}{R} \times \frac{1}{T} + \ln\frac{k_E}{h} + \frac{\Delta S}{R} \quad ...(3)$$

The straight line obtained for dye degradation followed the equation: y = 260.3x-1.330 (R = 0.823) and the thermodynamic data were obtained by using the Eyring- Polanyi equation. The Gibbs free energy of activation (ΔG) was calculated by use of the equation ΔG = ΔH – TΔS. Table 1 represents the thermodynamic data for crude enzyme catalyzed decolorization of the prototypical dye. Crude enzyme catalyzed bio-degradation reaction were found to be enthalpy-driven (high negative ΔH values), with positive entropy (ΔS) indicating that in the transition state, less complex structures were formed (Table 2). The high values of ”H obtained show the reactions are enthalpy- driven and the positive values of ΔS indicate the activated complexes are less structured than the reactants. The high values of ΔG explain the low reaction rates during biodegradation of the reactive dyes[26]. Results indicate that the reaction is exoergonic by nature, where ”G of the reaction is negative and supports fast reactivity at the transition state.

CONCLUSION

This study reported the isolation of crude laccase produced by solid state fermentation using *Limonia acidissima* shell solid support substrate. The effect of various process parameters such as temperature, pH, metal ion and salt on laccase production using solid-state fermentation was studied. The biological role played by crude enzyme towards decolorization was studied using preliminary parameters pH, temperature, Metal ion, Salt, dye concentration and incubation time and was warranted by thermodynamic studies. The results suggest the potential of Laccase from *A.niger* for future application towards degradation of dye using Solid state fermentation.

ACKNOWLEDGEMENTS

The authors are thankful to the Department of Biotechnology, Jeppiaar Engineering College, Chennai, TamilNadu (India) for providing the laboratory and technical facilities.

REFERENCES

1. Jayanthi, M., Kanchana, D., Saranraj, P., Sujitha, D. Bioadsorption of Chromium by *Penicillium chrysogenum* and *Aspergillus niger* Isolated from Tannery Effluent. *International Journal of Microbiological Research.*, 2014; **5**(1): 40-47.
2. Jagadeesan Vijayaraghavan, S.J., Sardhar Basha, Josephraj Jegan. A review on efficacious methods to decolorize reactive azo dye. *Journal of Urban and Environmental Engineering.*, 2013; **7**: 30-47.
3. Sivaranjani, A., Madhan, B., Barathidasan, K. Decolourization of Acid Red 131 by using *Shigella sp*. Isolated from Tannery Effluent. *International Journal of Pharmaceutical & Biological Archives.*, 2013; **3**(5): 142-146.
4. Drinan, J.E., Spellan, F. Water and Wastewater Treatment: A Guide for the Non-engineering Professional, Second Edition. CRC Press. Technology & Engineering, 2012.
5. Daassi, D., Belbahri, L., Vallat, A., Woodward, S., Nasri, M., Mechichi, T. Enhanced reduction of phenol content and toxicity in olive mill wastewaters by a newly isolated strain of *Coriolopsis gallica*. *Environment Science Pollution Research.*, 2014; **21**: 1746-1758.
6. Dalel Daassi, Tahar Mechichi, Moncef Nasri, Rodriguez-couto. Decolorization of the metal textile dye Lanaset Grey G by immobilized white-rot fungi. *Journal of Environmental Management.*, 2013; **129**: 324-332.
7. Saratale, R.G., Saratale, G.D., Chang, J.S., Govindwar, S.P. Bacterial decolorization and degradation of azo dyes: a review. *J. Taiwan Inst. Chem. Eng.*, 2011; **42**: 138–157.
8. Karigar, C.S. Rao, S.S. Role of Microbial Enzymes in the Bioremediation of Pollutants: A Review. *Enzyme Research.*, 2011; Article ID 805187, DOI:10.4061/2011/805187.
9. Fokina, O., Eipper, J., Winandy, L., Kerzenmacher, S., Fischer, R. Improving the

performance of a biofuel cell cathode with laccase-containing culture supernatant from *Pycnoporus sanguineus*. *Bioresource Technology.*, 2015; **175**: 445–453.

10. Sridhar, S., Chinnathambi, V., Arumugam, P., Suresh, P.K. Insilico & Invitro physicochemical screening of *Rigidosporus sp*. crude laccase assisted decolorization of synthetic dyes-Approaches for a cost effective enzyme –based remediation methodology. *Applied biochemistry & biotechnology*., 2013; **169**(3): 911-922.
11. Kalogeris, E., Iniotaki, F., Topakas, E., Christakopoulos, P., Kekos, D., Macris, B.J. Performance of an intermittent agitation rotating drum type bioreactor for solid-state fermentation of wheat straw. *Bioresource Technology.*, 2003; **86**: 207-213.
12. Toca-Herrera, J.L., Osma, J.F., Rodriguez Couto. Potential of solid-state fermentation for laccase production. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology.*, 2007; 391-400.
13. Langemaat, J.V., pyle, D.L. Solid state fermentation and bioremediation: Development of a continuous process for the production of fungal tannase. *Chem. Eng. J*., 2001; **84**: 115-123.
14. Papinutti, V.L., Diorio, L.A., Forchiassin, F. Production of laccase and manganese peroxidase by *Fomes sclerodermeus* grown on wheat bran. *J. Ind. Microb. Biotechnol*., 2003*;* **30**: 157-160.
15. Sartape, A.S., Mandhare, A.M., Jadhav, V.V., Raut, P.D., Anuse, M.A., Kolekar, S.S. Removal of malachite green dye from aqueous solution with adsorption technique using *Limonia acidissima* (wood apple) shell as low cost adsorbent. *Arabian Journal of Chemistry*., 2014; http://dx.doi.org/10.1016/j.arabjc.2013.12.019.
16. Mansur, M., Arias, M.E., Copa-Patino, J.L., Flardh, M., Gonzalez, A.E. The white-rot fungus *Pleurotus ostreatus* secretes laccase isozymes with different substrate specificities. *Mycologia.*, 2003; **95**(6): 1013–1020.
17. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*., 1976; **72**: 248-254.
18. Li, L., Dai, W., Yu, P., Zhao, J., Qu, Y. Decolorisation of synthetic dyes by crude laccase from *Rigidoporus lignosus* W1. *Journal of Chemical Technology and Biotechnology.*, 2009; **84**: 399–404.
19. Margarida S. Roriz, Johann F. Osma, Jose A. Teixeria, Rodriguez Couto. Application of Response Surface Methodological approach to optimize reactive black5 decoloration by crude laccase from *T.pubescens*. *Journal of Hazardous materials*, 2009; **169**: 691-696.
20. Abdulkareem Jasim Hashim. Determination of optimal conditions for laccase production by *Pleurotus ostreatus* using sawdust as solid medium and its use in phenol degradation. *J. Baghdad for Sci.*, 2012; **9**(3): 491-499.
21. Desai, S.S., Tennali, G.B., Channur, N., Anup, A.C., Deshpande, G., Azhar Murtuza, B.O. Isolation of laccase producing fungi and partial characterization of laccase. *Biotechnology Bioinformatics and Bioengineering.*, 2011; **1**(4): 543-549.
22. Sidra Ilyas, Skinder Sultan, Abdul Rehman. Decolourization and degradation of azo Dye, Synozol Red HF6BN, by *Pleurotus ostreatus*. *African Journal of Biotechnology*., 2012; **11**(88): 15422-15429.
23. Nyanhongo, G.S., Gomes, J., Gubitz, G., Zvauya, R., Read, J., Steiner, W. Decolorization of textile dyes by laccases froma newly isolated strain of *Trametes modesta*. *Water Research.*, 2002; **36**: 1449–1456.
24. Dawkar, V.V., Jadhav, U.U., Tamboli, D.P., Govindwar, S.P. Efûcient industrial dye decolorization by *Bacillus sp*. VUS with its enzyme system. *Ecotoxicol Environ Saf.*, 2010; **73**: 1696–1703.
25. Swati, V., Surwase, Krutika, K., Deshpande, Swapnil, S., Phugare, Jyoti, P., Jadhav. Biotransformation studies of textile dye Remazol Orange 3R. *3 Biotech.*, 2012; DOI 10.1007/s13205-012-0093-1.
26. Wagner, J., Barreto, Suely, M.O., Doi, Renata Afonso, Nathalia D. Bernardino. A kinetic study of the biodegradation of textile dye mixtures by *Pleurotus ostreatus* fungus. *Monatsh Chem*., 2012; **143**: 1389–1395.