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Production Characteristics, Activity Patterns and Biodecolourisation Applications of Thermostable Laccases from *Corynebacterium Efficiens* and *Enterobacter Ludwigii*

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The production conditions, characteristics and dye decolourisation potentials of two purified bacterial laccases from Corynebacterium efficiens and Enterobacter ludwigii were investigated. Optimum production pH was 3.0 and 4.0 respectively for E. ludwigii and C. efficiens. Khaya sp. and Gmelina arborea wood shavings, $(NH_4)_2SO_4$ (30.2µM/ml) and K^+ maximally supported laccase production in both organisms. The Vmax and specificity constants for both purified laccases were 44.6µM/mg; 1858.4S^{-1}µ$M^{-1}$and 23.15µM/mg; 1015.4S^{-1}µM^{-1} respectively. Maximum laccase activity was between 80°C and 90°C and pH 7.0. In both laccases, Fe^{3+} induced higher activity and red Dylon dye was 69% and 64% decolourised. The properties exhibited by these laccases could enhance their suitability in diverse biotechnological applications.

Keywords: *Corynebacterium Efficiens*, *Enterobacter ludwigii*, Media Optimisation, Laccase Characterisation, Dye-Decolourisation

Introduction

Laccases are phenol-oxidizing, copper-containing dimeric or tetrameric glycoproteins composed of hexoamines, glucose, mannose, galactose, fucose and arabinose[1]. The enzyme degrades dyes and a range of inorganic and organic substrates with concomitant four-electron reduction of oxygen to water[2,3]. Since laccases are constitutively consistent on molecular oxygen as an electron acceptor and do not crucially require of H_2O_2 or Mn^{2+} or any other co-substrate to oxidise substrates, they are the most industrially promising oxidoreductase group of enzymes[4,5] and have an edge over other ligninolytic enzymes[1]. Laccases possess properties of diverse biotechnological and industrial relevance such as medical care biosensor, lignocellulose delignification, waste water bioremediation, textile dye transformation and food technological uses[6,7,8]. Literature has reported more instances of laccase distribution in plants and higher fungi[9,10,11,12,13,14] than from bacteria, hence this present addition to the growing pool of information on bacterial laccases. The eco-friendliness of laccases makes the search for new and high-yielding laccase producing bacterial strains a veritable goal. This work therefore investigates the laccase production patterns of two bacterial species, the effect of physicochemical parameters on production, characterization of the laccase and their application in decolourisation of dyes.

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Materials and method

Sample collection

Soil and decaying wood samples were variously collected at different locations within the University premises. Wood shavings: Melina (*Gmelina arborea*), Mahogany (*Khaya* sp.), Iroko (*Melicia excelsa*) and Ire (*Celtis occidentalis*) and agricultural residues (rice bran, wheat bran, corn stover and sugarcane bagasse) were bought at a saw mill and market located at Bodija, Ibadan, while all chemical reagents used were analytical grade.

Culture collection and inoculum preparation

Laccase-producing bacteria and previously isolated from maize rhizosphere and decaying wood respectively were collected from the culture collection of our work at the Department of Microbiology, University of Ibadan. They were molecularly identified using 16S rRNA sequence analysis[15, 16] as *Enterobacter ludwigii* BOT4 with ascension number KX057746 and *Corynebacterium efficiens* PSB4 with ascension number KX057747 from National Center for Biotechnology Information (NCBI).

Fresh streaking of the pure bacteria cultures was made from stock agar slants and 24 hour old streaked colonies were subcultured into sterile nutrient broth. One milliliter (1ml) of culture broth equivalent to 10^8 colony forming unit was used as inoculum load in subsequent studies.

Laccase production

The production medium (25ml) containing (w/v) glucose: 1; Yeast Extract: 0.3; K_2HPO_4: 0.4; KH_2PO_4: 0.6; $MgSO_4$: 0.5; $CuSO_4$: 0.01; $FeSO_4$: 0.005; and $CaCl_2$: 0.1 in pH 7.0 citrate phosphate buffer was sterilised and inoculated with 10^8cfu/ml bacteria cultures. The conical flasks containing the inoculated broth were incubated at 30±2°C for 72 hrs. Every 24 hours, 3ml of the fermentation medium was aseptically harvested and centrifuged along with 5% w/v sterile glass beads using micro-centrifuge (MIKRO 220R, Hettich) at 13,000rpm for 15 minutes at 4°C[17]. The supernatant was kept for further studies.

Effect of pH, carbon and nitrogen sources, cations and surfactants on laccase production

The effect of pH (3.0-11.0) on laccase production was investigated using buffer solutions to adjust the medium pH. Membrane-filtered, 1% (w/v) additions of carbon sources (arabinose, galactose, fructose, sucrose and lactose) were included in each production broth while glucose served as the control. Gelatin, peptone, ammonium sulphate, sodium nitrate and urea were added to nitrogen source-free production media at the concentration of 0.3% (w/v); Yeast extract was used as the control. The effect of supplementing 100mM of different cations and surfactants (chloride salts of K^+, Na^+, Fe^{3+}, Hg2+, Li^+, EDTA and Benzoic acid) on laccase production was investigated by adding these singly into the production broth, inoculating with 10^8cfu/ml isolates and incubating over 72 hours. The production broth served as control. In all tests, triplicate samples were aseptically withdrawn after each 24 hour incubation period, centrifuged and the supernatant used to determine the extent of laccase production.

Effect of lignocellulosic materials as sole carbon sources on laccase production

Agro wastes: wheat and rice brans, sugarcane bagasse, maize stover, corn cob and different indigenous wood dusts: Melina (*Gmelina arborea*), Mahogany (*Khaya* sp.), Iroko (*Melicia excelsa*) and Ire (*Celtis occidentalis*) at 1% w/v were substituted for glucose in the production medium at a mesh size of 120µm and set-up in 25ml broths in conical flasks. The flasks were autoclaved after which they were inoculated with 10^8cfu/ml isolates and then incubated between 3-12 days. The broths were centrifuged after each production period and the supernatant used to quantify laccase production.

Laccase assay

The laccase activity (from triplicate experiments) of the cell-free production broth was spectrophotometrically determined by measuring the oxidation of 2, 2'azino-bis (3-ethyl benzthiazoline-6-sulfonic acid) (ABTS; Sigma, St. Louis, USA) at 30°C according to modified method of Mongkolthanaruk *et al.*[17]. The reaction mixture contained 600µL enzyme extract, 400µL citrate phosphate buffer pH 5.0 (0.2M) and 120µL ABTS solution (1mM). The reaction was terminated using 40µl of 5% Trichloroacetic acid (TCA) (Merck) after 10minutes. The assay mixture was made up to 3.6ml with sterile water. In each assay (except otherwise stated), the blank contained all the reaction mixture components except the enzyme which was replaced with the requisite volume of distilled water. Oxidation was monitored by determining the increase in absorbance at 420 nm (ε420 = 36,000 M-1 cm-1) using JENWAY, 6405 UV/VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1µmol of ABTS per minute[17].

Characterization of crude laccase

Effect of temperature on laccase activity and stability

Temperature effects on laccase activity was examined over a range of 25°C-90°C by incubating 300µl laccase, 200 µl citrate-phosphate buffer and 60µl ABTS at the various temperature values using water-bath (Mrc, England) for 10 minutes where the reaction was stopped with 20µl 5% TCA. Thermostability was determined over 60 minutes at the following intervals: 10, 15, 30, 45 and 60 minutes using ABTS as substrate at pH 7.0 (citrate-phosphate) over a temperature range of 30° to 90°C[17].

Effect of pH on laccase activity and stability

Laccase activity was evaluated over a pH range of 3.0 to 9.0 using ABTS as substrate. The assay mixture which contained 300µl of the crude enzyme and 300µl of each buffer at various pH values was incubated at room temperature for 60 minutes, and then 120µl of ABTS was added. The reaction was stopped after 10 minutes with 40µl of 5% TCA. The reaction

mixture was then made up to 3.6ml with sterile distilled water and laccase activity determined. To investigate the effect of pH on enzyme stability, equal volume of the crude enzyme and each of the buffers of various pH values (150µl) was incubated at room temperature over 10, 15, 30, 45 and 60 minutes. The residual laccase activity was determined using 60µl of ABTS as substrate[17,18]. Effect of Different Cations and Surfactant Concentrations on Laccase.

Activity

The effect of varying concentrations (0.1mM, 0.5mM, 1mM and 5mM) of cations and surfactants on laccase activity was investigated. Mixtures of equal volume (150µl) of crude laccases with the metal ions and surfactants were incubated at room temperature for 60 minutes[19]. The blank contained 150µl distilled water instead of the enzyme. The residual laccase activity was determined as above.

Purification of laccase

The crude laccase was initially purified using 40% and 80% $(NH_4)_2SO_4$ precipitation and incubating overnight at 4°C. The mixture was cold-centrifuged at 13,000rpm for 10minutes. The precipitate obtained was dissolved in 50mM (pH 5.0) citrate phosphate buffer and dialyzed under refrigeration against 3 volumes of the same buffer. The dialysate was loaded on 24.5cm × 1.25cm Sephadex G-100 (Sigma) filtration gel column and the absorbed proteins eluted with 0.2M NaCl in citrate phosphate buffer at a flow rate of 0.5 ml/min[18]. Laccase activity and the total protein content of the enzyme was done at each stage of purification (i.e. the crude, ammonium sulphate precipitated, dialysed and the column purified enzymes) to determine the effect of the purification step on the enzyme[20].

Determination of kinetic properties of laccase

Varying concentrations of ABTS (0.1-1.0mM), was added to equal volumes (300µl) of the purified enzyme. A blank was prepared similarly by substituting the enzyme with deionised water. The V*max*, K_m and K_{cat} values were determined using a Lineweaver-Burk plot.

Determination of dye decolourisation potentials of *corynebacterium efficiens* and *enterobacter ludwigii* laccases

Different concentrations (0.05, 0.075 and 0.10%) of dye in distilled water were prepared. 100 millilitres of the different dye concentration mixtures was dispensed into appropriately labeled screw-capped tubes, sterilized and aseptically mixed with 5ml of the enzyme. The control however contained water instead of the bacterial laccase. This was gently mixed and aliquots were withdrawn from the experimental setup every 24 hours over 96 hours to read absorbance at different dye wavelengths depending on the dye (Madonna Blue Dylon® 18 at 405nm, Jungle Green Dylon® 26 at 570nm and Scarlet Red Dylon® 32 at 660nm)[21]. The percentage decolourization was calculated as follows:

$$\frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance} \times 100$$

Statistical analysis

Experiments were conducted in triplicates and the means determined.

Result and Discussion

The lignin barrier of agricultural biomass can be microbially attacked through the secretion of ligninolytic enzymes[18]. *C. efficiens* and *E. ludwigii* laccase were well-favoured at pH ranges 3 – 6 . pH 3 and pH 4 were optimal for laccase yield in both isolates, while a decline in yield occurred from pH 8 to pH 11. Nyanhongo *et al.*[23] reported an initial pH of 7.0 was the best for optimal growth and laccase production by *Trametes modesta.* The substitution of glucose by other sugars enhanced laccase yield by both bacteria Table 1a. Fructose and galactose maximally enhanced production at 48hours with the highest yield of 47.4 µM/ml and 47 µM/ml respectively. $NaNO_3$ was the most effective sole nitrogen source yielding 33.2 µM/ml in *E. ludwigii* Table 1b. A ten-fold production increase was reported when glucose was substituted with galactose[24] while a medium with $(NH_4)_2SO_4$ had highest laccase activity in *Cerrena unicolor*[25]. Cations and EDTA additions led to a high laccase production in *C. efficiens* with both K^+ and EDTA yielding 38.2 µM/ml over 48 hrs Table 1c, 1d. Metal ions have been reported to stimulate laccase production in an actively growing culture[26]. Agro-industrial wastes enhanced laccase production above that of glucose, increasing with incubation period Table 2a, maximum in *C. efficiens* upon Rice bran and Corn stover (48.4µM/ml) and in Corn cob for *E. ludwigii* laccase (54.2µM/ml) and Corn stover (53.8µM/ml) medium. In a similar work, wheat bran and corn stover enhanced laccase yield[24] and banana peel afforded a laccase yield 2 folds short of the control[18]. Lignin, cellulose, and hemicelluloses may be utilized as support substrates[27] to increase

Table 1 — Effect of media Carbon, Nitrogen, Cation and Surfactant modification on laccase production (µM/mL) by *C. efficiens* and *E. ludwigii*

(a) Carbon Source

	C. efficiens			*E. ludwigii*		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
Glucose	14.2	12.8	13.8	13.6	17.2	6.2
Arabinose	16	32	11.8	8	29.8	10.6
Fructose	12	47.4	14.2	13.6	35.8	14.2
Galactose	13.8	43.6	8.4	18.8	47	13.2
Sucrose	19.2	28.6	9.4	6.4	22	15.8
Lactose	8.4	24.4	11.6	11.2	26.8	11.6

(b) Nitrogen Source

	C. efficiens			*E. ludwigii*		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
Yeast Extract	14.2	12.8	13.8	13.6	17.2	6.2
$(NH_4)_2SO_4$	5.6	23.6	22.2	7.2	30.2	14
Peptone	11	27	9.4	5.2	10.4	10.2
$NaNO_3$	7.8	28.2	14	12.4	25	33.2
Urea	12.4	21.4	12.4	9.4	26.4	12.4
Gelatin	12.4	19.2	11.6	15	16.2	10.8

(c) Metal ions

	C. efficiens			*E. ludwigii*		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
Control	14.2	12.8	13.8	13.6	17.2	6.2
K^+	35	38.2	6.8	48.2	49.4	9.8
Na^+	19.2	20	9.6	10.4	8.4	18
Fe^{3+}	5.4	22.2	8.8	15.6	22.2	5.6
Hg^{2+}	29.2	30.4	11.6	16	16.8	5.8
Li^+	34	14.8	16	48.8	48.8	11.4

(d) Surfactant Source

	C. efficiens			*E. ludwigii*		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
Control	14.2	12.8	13.8	13.6	17.2	6.2
EDTA	38.2	38.2	9.2	8.8	13.4	8
Benzoic acid	5.8	10	10.4	27.8	23.6	7.6

laccase production in comparison to glucose[28]. The utilization of cheap or waste substrates for enzyme production contributes to the economy of industrial production. Melina (*G. arborea*) and Mahogany (*Khaya* sp.) enhanced maximum yield in *C. efficiens* (104.6 µM/ml) and *E. ludwigii* (108.4 µM/ml) respectively on the 9th day Table 2b. Saw dust has been reported to elicit high laccase production[24,29]. The very high laccase yield obtained in the wood shavings production medium compared to that in agro residues is a clear indication that the two bacteria *C. efficiens* and *E. ludwigii* may be effectively applied in biopulping studies. The optimum *C. efficiens* and *E. ludwigii* laccase activity was observed at 70°C (47µM/ml and 58.6µM/ml respectively) Figure 1a. Farnet *et al*.[30] reported increased laccase activity with pre-incubation of enzymes at 40°C and 50°C. Temperature stability was best in *E. ludwigii*, (70°C - 90°C) and stabilized with increasing temperature (60°C through 90°C) in *C. efficiens* Figure 1b. Lac II enzyme produced from *Cerrena unicolor*[31] had a half life of 90 minutes at 70°C. As thermostable laccases, they may have biotechnological applications in high temperature operations[32]. The pH profile of *C. efficiens* and *E. ludwigii* laccase exhibited the highest increase at an alkaline regime of pH 8 – pH 9 Figure 2a. *C. efficiens* and *E. ludwigii* respectively demonstrated increased stability at pH ranges 6 to 9 Figure 2b an outcome disagreeing with Xu[33] and Arias *et al.*[34] who recorded a decrease in laccase activity at neutral to alkaline pH regimes. Ferric ions (5mM) increased both laccase activities, a finding contrary to the inhibitory

Table 2 — Effect of Lignocellulose-based agricultural wastes on laccase production (µM/mL) by *C. efficiens* and *E. ludwigii*

(a) Agricultural wastes

	C. efficiens				*E. ludwigii*			
	3 days	6 days	9 days	12 days	3 days	6 days	9 days	12 days
Rice bran	26.4	39.2	48.4	40	20.4	41.2	51.6	39
Wheat bran	25.8	36.8	46.8	38.4	20.8	44.8	50.6	37.6
Corn stover	21.2	33.6	48.4	40.8	17.2	33.2	53.8	43
Cane bagasse	8.6	31.2	46.4	42.6	5	25.6	44.6	43.6

(b) Wood dusts

	C. efficiens				*E. ludwigii*			
	3 days	6 days	9 days	12 days	3 days	6 days	9 days	12 days
Mahogany	56.6	82.6	97.8	73.4	59.2	89.2	108.4	87.6
Iroko	49.2	77.8	92.8	80.6	59.6	86.4	101.4	74.6
Melina	48	75.2	104.6	79.2	55.4	86.8	107.8	88.6
Ire	51.2	77.6	94.6	70.8	52.6	81.4	98.6	77.2

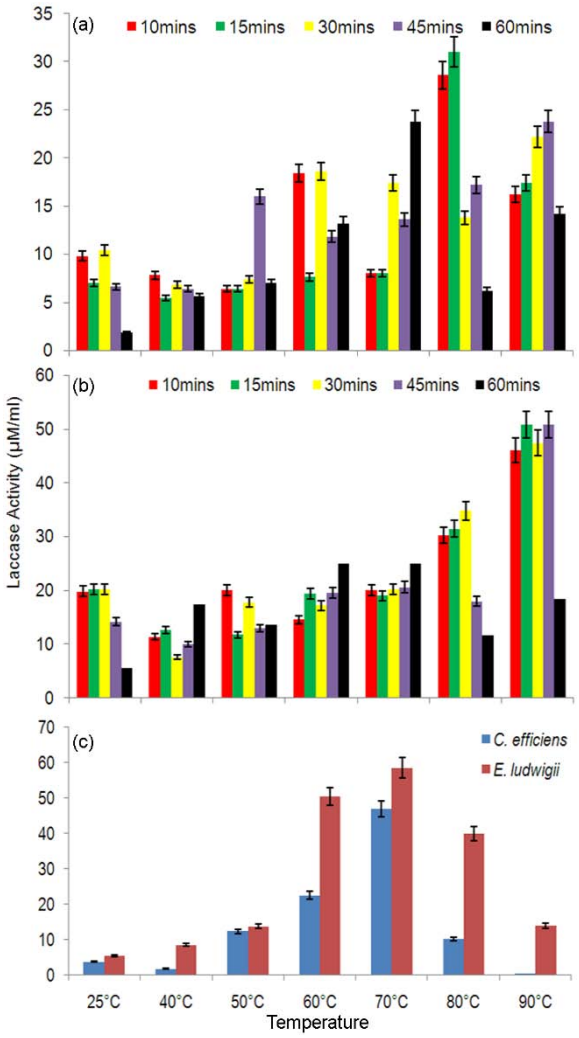

Fig. 1 — Effect of Temperature on (a) Activity and (b) Stability of *C. efficiens* and *E. ludiwigii* laccase

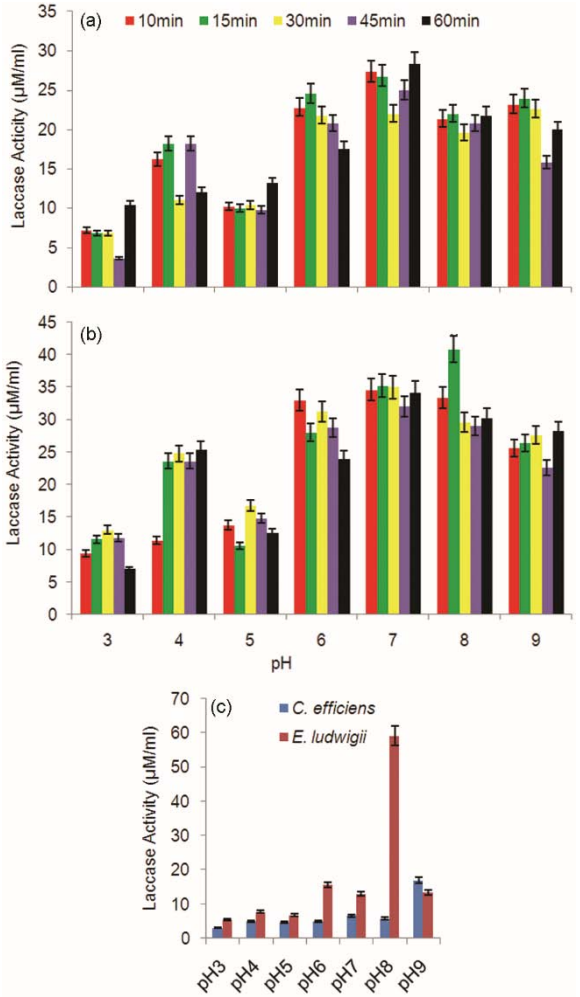

Fig. 2 — Effect of pH on (a) Activity and (b) Stability of *C. efficiens* and *E. ludiwigii* laccase

Table 3 — Partial purification of laccase from *C. effficiens* and *E. ludwigii*

(a) *C. effficiens* Laccase

Purification step	Total activity (µM/ml)	Total protein (mg)	Specific activity (µM/mg)	Purification fold	Yield (%)
Crude enzyme	0.240	0.183	1.31	1	100
$(NH_4)_2SO_4$ precipitation	0.206	0.128	1.61	1.23	95
Daialysis	0.178	0.062	2.87	2.19	77.78
Sephadex G-100	0.108	0.022	4.9	3.74	45

(b) *E. ludwigii* Laccase

Purification step	Total activity (µM/ml)	Total protein (mg)	Specific activity (µM/mg)	Purification fold	Yield (%)
Crude enzyme	0.387	0.290	1.33	1	100
$(NH_4)_2SO_4$ precipitation	0.306	0.168	1.82	1.37	95
Daialysis	0.248	0.088	3.02	2.27	83.33
Sephadex G-100	0.196	0.036	5.44	4.1	63.75

effect of Fe^{3+} reported by Hatvani and Mecs[35]. Na^+ and K^+ had inhibitory effects while other cations and surfactants did not affect laccase activities. *C. efficiens* laccase was purified 3.74 fold with specific activity 4.9µmol/mg Table 3. Its elution profile exhibited peaked at fractions 8 (45.2 µM/ml) (results not shown) while *E. ludwigii* laccase exhibited a double peak in fractions 14 (57 µM/ml) and 17 (55.8 µM/ml) respectively. Increase in specific activity occurred after Sephadex G-100 chromatographic step, as also reported by other researchers[36,37]. The Vmax, K_m, K*cat* and specificity constant (K*cat*/K_m) for *C. efficiens* laccase obtained from the Lineweaver-Burk plot (r^2= 0.856) were 23.15µM/mg; 0.076µM; 77.17 S^{-1} and 1015.4$S^{-1}mM^{-1}$ respectively while that for *E. ludwigii* laccase (r^2 = 0.936) were 44.6µM/mg; 0.08 µM, 148.67S^{-1} and 1858.4$S^{-1}µM^{-1}$, respectively. Enzyme constants vary based on the source and type of substrate and laccases from different source organisms may have different preferences[38]. The K_m values of these two laccases were low which indicates a high substrate affinity for the enzyme. Laccase-based dye decolourization is an efficient method which has attracted much interest from industries[5]. Dylon 32, Dylon 18 and Dylon 26 dyes having typical Azo, triphenlymethane and anthraquinone chromophores were decolourised. Manganese peroxidase and laccase have been reported to decolourize some synthetic dyes[39]. Rai *et al.*[40] reported that although isolation of aerobic dye-degrading bacterial strains had been carried out for more than two decades; their potential for this purpose has rarely been addressed[8]. *C. efficiens* laccase elicited maximum decolourization (68.41%) of the Scarlet Red Dylon 32 Figure 3a at 750mg/L after 96hrs incubation, Jungle Green Dylon 26 was least decolourized. This is probably because while dyes containing –NH2 and –OH groups are most vulnerable to laccase attack, steric hindrances in the green Dylon 26 may reduce the accessibility of the –NH2 and –OH groups to enzymes[41]. A more efficient trend was recorded in *E. ludwigii* laccase Dylon Red decolourisation Figure 3b yielding 70.68% at

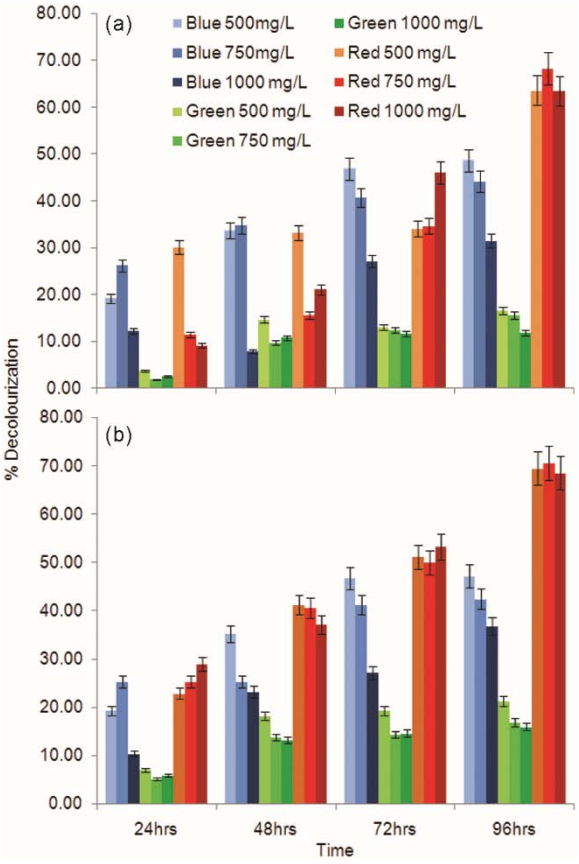

Fig. 3 — Decolourization pattern of different textile dyes by (a) *C. efficiens* and (b) *E. ludwigii* laccase at different time intervals

750mg/L. These values support the hypothesis that the decolourization potential of different laccases on the same dye varies and depends on the organism source, dye structure and the redox-potential of the enzyme[23,42].

Conclusion

Laccase production by *E. ludwigii* BOT4 KX057746 and *C. efficiens* PSB4 KX057747 was supported by acidic pH, fructose and galactose, sodium nitrate, 1% agro-industrial wastes and wood shavings, K^+, Li^+ and EDTA. However, *E. ludwigii* responded better to cultural parameters and presented higher laccase yield. Wood shavings should be encouraged for large-scale production of laccases. The laccases were thermostable having high activity at mildly alkaline pH. This will be beneficial in industrial processes that operate at almost extreme conditions and their large-scale production could contribute to the reduction of environmental pollution caused by untreated phenolic effluents and agricultural residues.

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