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Biopulping of sugarcane bagasse and decolorization of kraft liquor by the laccase produced by *Klebsiella aerogenes* NCIM 2098

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ABSTRACT

Aims: Laccase, a copper-containing enzyme, oxidizes variety of aromatic compounds. Since laccase is essential for lignin degradation, it can be used for lignin removal in the pulp and paper industry (biopulping). Laccase is also employed as a dechlorinating agent (biobleaching), along with the removal of phenolic and other aromatic pollutants. In the present investigation it was aimed to employ the laccase produced by the bacterium *Klebsiella aerogenes* along with the bacterium itself in biopulping of sugarcane bagasse and biobleaching of kraft liquor effluent.
Methodology and results: A laccase was isolated from the bacterium *K. aerogenes,* purified to homogeneity and characterized. The enzyme was purified by conventional techniques following salt precipitation, ion exchange chromatography, and affinity chromatography on Con A sepharose. The purified laccase was found to be monomeric glycoprotein with a Mr of 64 kDa when measured by Sephadex G-200 gel chromatography and SDS-PAGE. The V_{max} and K_m of laccase towards the substrate guaiacol was determined. The optimum pH of the laccase was found to be 5.0. biopulping and biobleaching activities were determined by TAPPI standard methods. Treatment of sugarcane baggase by *K. aerogenes* also significantly reduced lignin content of the bagasse.
Conclusion, significance and impact of study: The bacterium *K. aerogenes* and a laccase produced by it were used separately for biopulping of sugarcane bagasse and biobleaching of kraft liquor effluent. Treatment with both brought significant reduction in lignin content and kappa number of the pulp. The handsheets prepared from the treated pulp showed improved brightness without affecting the strength properties of paper. The bacterium and the laccase efficiently decolorized the kraft liquor proving to have biobleaching potential.

Keywords: Biopulping, bioleaching, laccase, *Klebsiella aerogenes*

INTRODUCTION

Klebsiella aerogenes NCIM 2098 of family *Enterobacteriaceae* was found to be effective in lignin removal (Jha *et al*., 2002a; 2002b). *K. aerogenes* was further tested for presence of laccase to ascertain its possible role in lignin degradation.

Laccase was shown to be used for bleaching in the pulp and paper industry and as a stabilizer during wine processing (Agellis *et al*., 2002), as a dechlorinating agent (Ahn *et al.*, 2002), for biodegradation of the organochlorine pesticide lindane (Tekere *et al.*, 2002), removal of phenolic and other aromatic pollutants like lignin present in natural and industrial waste waters (Hatakka, 1994; Leonowicz *et al.*, 2001; Tsioulpas *et al.*, 2002). Applications of laccases in industries as well as their potential extension in nanobiotechnology have been reviewed by Rodriguez and Herrera (2006).

Until recently laccases were found only in eukaryotes, e.g., fungi, plants and insects (Arora and Rampal, 2002), but now their existence in prokaryotes is also been proved. Corresponding genes have been found in Gram positive and Gram negative bacteria such as *Marinomonas mediterranea*, *Azospirillum lipoferum* and *Bacillus sphaericus* (Alexandre and Zhulin, 2000; Claus, 2003). The spore protein Cot A of *Bacillus subtilis* has been recognized to be laccase with Mr of 65 kDa (Engita *et al*., 2002). In the present research paper some properties of purified laccase from *K. aerogenes* and its effect in biopulping of sugarcane bagasse and decolorization of kraft liquor effluent have been presented The results reveal that laccase produced by *K. aerogenes* brought significant reduction in kappa number and lignin content of the pulp with remarkable decolorizing ability towards kraft liquor.

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MATERIALS AND METHODS

Microorganisms

Klebsiella aerogenes NCIM 2098 [Family-*Enterobacteriaceae*] was procured from the National Collection of Industrial Microorganism (NCIM), National chemical laboratory (NCL), Pune, India. The culture was maintained on nutrient agar slants with periodic transfer.

Chemicals

DEAE cellulose, Con-A Sepharose, guaiacol, N-bromosuccinimide, EDTA, NADH, NADPH, NAD^+, Molecular weight markers, Sephadex G-200 were of Sigma chemicals. All other chemicals were of AR grade.

Production of laccase

K. aerogenes was cultivated in a medium containing 1 g glucose, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.015 g KH_2PO_4, 0.005 g NH_4Cl, 0.1 g KCl, 0.005 g $FeSO_4 \cdot 7H_2O$ and 0.5 g peptone in 100 mL distilled water. Before autoclaving at 15 psi steam pressure for 15 min, pH of the medium was adjusted to 7.0 with 1N NaOH or 0.1N HCl.

Conical flasks of 500 mL capacity containing 100 mL sterilized medium were inoculated with 5 mL nutrient broth with actively growing *K. aerogenes* containing approximately 10^8 cells. Inoculated flasks were kept on a rotary shaker at 120 rpm and incubated at 37 °C for further 24 h. Cultures were induced by addition of 1 mL of filter sterilized 1% kraft liquor in distilled water. The flasks were allowed to grow for another 24 h and were harvested by centrifugation at 8000 g for 10 min at 4 °C in C-24 Remi cold centrifuge. Clarified supernatant was referred to as crude culture fraction (CCF) and used for further studies.

Laccase assay

Assay of the enzyme laccase was performed by the method of Das *et al.* (1997). Reaction mixture contained 10 mM guaiacol in 0.1 M sodium acetate buffer (SAB) pH 5.0 with 10% acetone (v/v), and 100 µg protein as source of laccase in total volume of 2 mL at 30 °C. The change in absorbance of the reaction mixture containing guaiacol was monitored at 470 nm for 3 min.

One unit of laccase was considered as that amount of enzyme, which forms 1 µM of product under experimental conditions (Das *et al.*, 1997). Specific activity was calculated with the extinction coefficient for the product of 6740/Mole/cm and expressed in units per milligram protein.

Laccase purification and characterization

Ammonium sulphate precipitation

To the clarified CCF ammonium sulphate was added to 30% saturation with constant stirring. Change in pH was adjusted by addition of cold ammonia solution intermittently. The precipitated proteins were collected by centrifugation at 10,000 *g* for 20 min. and solubilized in 50 mM SAB, pH 5.0 and dialyzed against the same for 24 h and designated as ammonium sulphate fraction (ASF).

DEAE cellulose chromatography

ASF was loaded on previously equilibrated DEAE cellulose column (25 x 1 cm) with 20 mM sodium phosphate buffer (SPB) pH 7.0. Enzyme was allowed to bind to the column for 1 h. The bound enzyme was eluted with 0.05-0.4 M sodium chloride gradient in SPB. Fractions of 1 mL each were collected at the rate of 20 mL/h with the help of automatic fraction collector (LKB). Eluted fractions were read at 280 nm for protein and 614 nm for laccase (copper containing protein). The fractions, where the ratio of 614/280 was high, were pooled and selected for further purification.

Affinity purification by Concanavalin A-Sepharose

The pooled fractions from DEAE cellulose column were concentrated using cellulose as absorbent and were allowed to bind for 4 h with Concanavalin A-Sepharose in 20 mM SAB, pH 5.0 in presence of 1 mM $CaCl_2$ and 1mM $MgCl_2$ (Dalvia *et al.*, 1990). The beads were carefully washed eight times with SAB till the reading at 280 nm was similar to that of control SAB. The bound proteins with Con A Sepharose were eluted by 250 mM N-acetyl glucosamine in SAB. Fractions of 1 mL each were collected, read at 280 nm and those with higher OD were designated as AFCF and selected for further procedure.

Test of homogeneity

Molecular exclusion chromatography: The AFCF with high laccase activity were again concentrated using cellulose as absorbent and loaded on Sephadex G-200 column (30 x 1 cm) previously swelled for 24 h in SAB (50mM pH 5.0) and proteins were eluted by the same in fractions of 1mL each. Molecular weight (Mr) of the enzyme was determined by comparing with standard proteins run on the same column using BSA dimer 132 kDa, BSA monomer 66 kDa, pepsin 34.7 kDa, trypsinogen 24 kDa and lysozyme 14.3 kDa.

SDS polyacrylamide gel electrophoresis (Laemmli, 1970): Mr of purified laccase was determined by SDS polyacrylamide gel electrophoresis also [12.5% .w/v. polyacrylamide in 0.2 M Tris/glycine buffer at pH 8.3. under denaturing conditions. (10 w/v SDS, 0.5 M Tris – HCl, pH 8.89, distilled water 0.5 mL, glycerol 2.5 mL and 2-mercaptoethanol – 0.25 mL). Proteins were strained with a solution of 0.1% coomassie brilliant blue R 250 in 5:1:5 (acetic acid: methanol: distilled water.). The Mr of laccase was estimated with reference to the lysozyme, 14.3 kDa, trypsinogen 24 kDa, ovalbumin 45 kDa, BSA 66 kDa and phosphorylase B 97.4 kDa.

Protein and carbohydrate measurement: Proteins were measured by the methods of Lowry *et al.* (1951) and Bradford (1976) using lipid free BSA as a standard protein. Carbohydrate content of purified laccase was determined by phenol-sulphuric acid method (Hounsell *et al.*, 1996).

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Measurement of optimum pH

The optimum pH was determined by measuring the activity of laccase by using SAB and SPB of different pH in the reaction mixture. Temperature stability was also determined by exposing the laccase at optimum pH to different temperatures for 1 h before the activity was measured at 37 °C.

Treatment with metal ion chelator

Purified laccase (about 25 µg) was mixed with 1 mM EDTA and left at 37 °C for 1 h. EDTA was removed by dialysis against SAB (pH 5.0) for 10 h at 4 °C. The residual activity was measured as described above.

Effect of metal ions

The EDTA treated laccase was used for determination of effect of metal ions on activity. The reaction mixture contained 2.5 µg EDTA treated laccase along with 0.5 mM solution of metal ion prepared in sterile distilled water keeping other contents and conditions of reaction mixture same for measurement of activity.

Effect of N-bromosuccinimide and sodium azide

N-bromosuccinimide or sodium azide (5 mM) in 10 mM SAB (pH 5.0) and 5 µg of purified laccase was mixed and left at 37 °C for 1 h. Residual activity was measured by addition of other contents of reaction mixture as mentioned above.

Effect of coenzymes

The reaction mixture contained 2 mM coenzymes (NAD^+, NADH and NADPH) and 5 µg of purified laccase. The activity was measured after one hour as described above.

Kinetic analysis of laccase

The apparent V_{max} and K_m of laccase for guaiacol was calculated from linear regression analysis of Lineweaver-Burk plot using varying concentrations of guaiacol in the range of 1 µM to 100 µM.

Biopulping studies were carried out at laboratory scale in 2.5 L conical flasks containing sugarcane bagasse as raw material at 10% w/v in modified Czapek Dox medium (1% glucose, 0.05% $MgSO_4 \cdot 7H_2O$, 0.015% KH_2PO_4, 0.5% $NaNO_3$, 0.1% KCl and 0.005% $FeSO_4 \cdot 7H_2O$). Initial pH was adjusted to 7.2. Incubation temperature was 37 ± 2 °C. Inoculum was added at 10% v/v to sterilized submerged medium. After 7 days of incubation the bagasse was used for KAPPA number determination.

Black liquor was collected from workshop of Department of Pulp and paper Technology Laxminarayan Institute of Technology, Nagpur University, Nagpur (India). Evaluation of *K. aerogenes* to determine the optimum conditions of lignin degradation and decolorization of black liquor was conducted in 250 mL conical flask containing 50 mL black liquor, supplemented with 1% glucose, 0.05% $MgSO_4 \cdot 7H_2O$, 0.015% KH_2PO_4, 0.5% $NaNO_3$, 0.1% KCl and 0.005% $FeSO_4 \cdot 7H_2O$. Initial pH was adjusted to 7.2.

Handsheets were prepared by passing the uniform diluted *K. aerogenes* and laccase treated and untreated pulp through a sieve; the pulp was retained on the sieve. This pulp was then pressed by rollers and then allowed to dry. The hand sheets were further tested for strength properties. Handsheets were prepared by passing the uniform diluted *K. aerogenes* and laccase treated and untreated pulp through a sieve; the pulp was retained on the sieve. This pulp was then pressed by rollers and then allowed to dry. The hand sheets were further tested for strength properties.

RESULTS AND DISCUSSION

Laccase was eluted in the 0.1 M gradient of from DEAE cellulose column (Figure 1). Fractions showing highest ratio at 614/280 were pooled and concentrated. The concentrated enzyme was loaded on to Sephadex G-200 column. Relative molecular weight (Mr) of the laccase was found to be approximately 64 kDa when compared with the standards as shown in Figure 2. Details of the purification scheme is provided in Table 1.

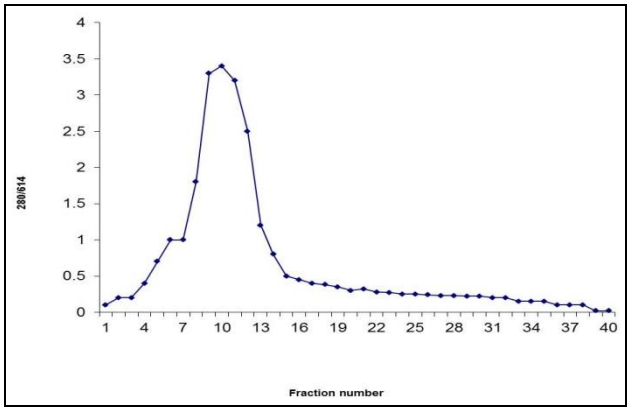
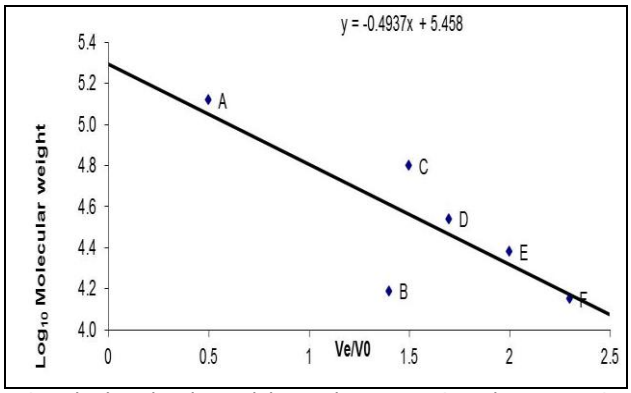


Figure 1: DEAE cellulose chromatography of laccase of *K. aerogenes*.



* Standard Molecular weight markers: A: BSA Dimer, B: BSA monomer, C: Laccase, D: Pepsin, E: Trypsinogen, F: Lysozyme.

Figure 2: Sephadex G-200 gel filtration chromatography for molecular weight determination of *K. aerogenes* laccase.

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Table 1: Scheme of purification of laccase produced by *K. aerogenes*.

Step no.	Purification step	Volume (mL)	Enzyme activity (Units/mL)	Total activity (units/mL X Vol)	Total protein content (mg)	Specific activity (Units/mg protein)	Fold purification (Sp. activity/ Initial sp. activity)	Yield (% of original total activity)
1	Initial preparation (CCF)	1000	2.94	2940	3000	0.98	1.0	100
2	$(NH_4)_2SO_4$ fraction (ASF)	30	53.28	1598.40	54	29.59	30.19	54.36
3	DEAE cellulose fraction (AFCF)	9	39.99	359.91	1.30	276.85	282.50	12.24
4	Con-A Sepharose fraction	3	30	90	0.29	310.35	316.68	3.06

Laccase produced by *K. aerogenes* was found to be monomeric as established by the presence of single band in SDS-PAGE (Figure 5). Mr was also found to be approximately 64 kDa even by SDS-PAGE when compared with standard markers. Laccase is a glycoprotein as it was established by its positive reaction with phenol sulphuric acid reagent. The protein is also recognized and bound by concanavalin A, thereby indicating the presence of oligosaccharide chain. Monomeric laccase with and 64 kDa has also been reported for *Trametes multicolor (*Hess *et al*., 2002*)* and laccase with Mr 66 kDa was mentioned for two isozymes of *Coriolopsis rigida* by Sapparrat *et al.* (2002). The apparent V_{max} and K_m of laccase for guaiacol were found to be 136 units/min and 55 µM, respectively. The pH dependence of enzyme was tested between pH 3.0 to 7.0, purified laccase showed maximum activity at pH 5.0 as presented in Figure 3. These results are found to be similar to those reported for laccase of *Trametes multicolor* (Hess *et al.*, 2002).

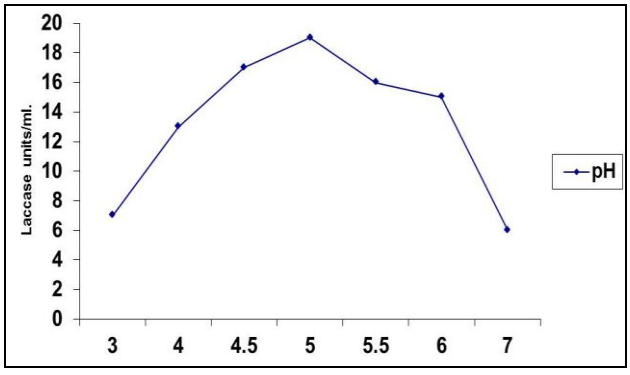

Figure 3: Effect of pH on laccase activity using guaiacol as substrate.

Laccases are inhibited by metal ion chelators (Bollag and Leonowicz, 1998). The results presented in Figure 4 indicate that EDTA inhibited the enzyme activity, similarly the fraction obtained after the ion exchange chromatography also showed a decline in the activity which may be due to the loss of metal ions required for the activity. Supplementation of Cu^{2+} restored the laccase activity thereby suggesting dependence of laccase on the copper ions. N-bromosuccinimide reacts with tryptophan decreasing activity of laccase, Sequence alignment of fungal laccases has shown that the copper binding domains are highly conserved (Cullen, 1997). The conserved copper coordinated sites in different laccases have the sequence His–Trp–His, His–Leu–His, and His–Cys–His localized near the N and C termini, suggesting the pivotal role of the tryptophan residue for activity (Cullen, 1997; Palmmieri *et al.*, 2000).

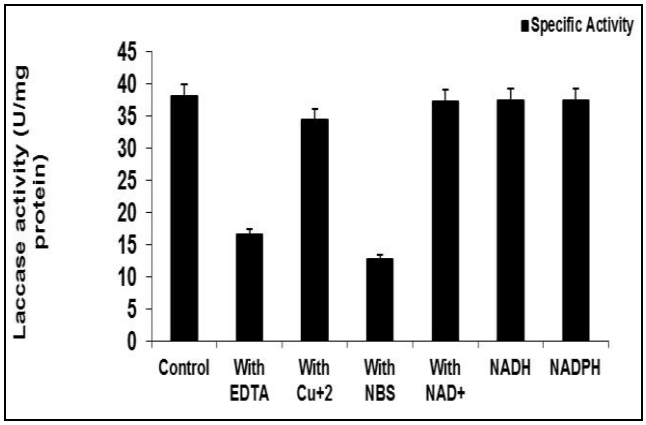

Figure 4: Effect of additives on laccase activity.

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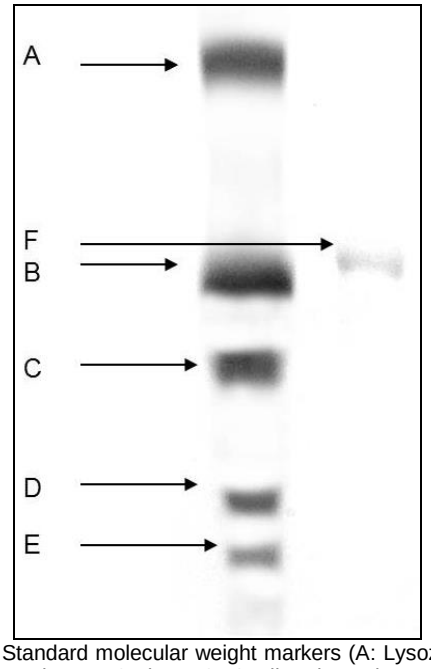

* Lane 1: Standard molecular weight markers (A: Lysozyme 14.3 kDa, B: Trypsinogen 24 kDa, C: Ovalbumin 45 kDa D: BSA 66 kDa and E: Phosphorylase B 97.4 kDa); Lane 2 F: Laccase

Figure 5: SDS-PAGE of laccase of *K. aerogenes.*

Presence of NAD^+, NADP+ and NADPH could not bring any significant change in activity of *K. aerogenes* laccase (Higuchi, 2004).

Laccase degrades lignin with the help of a mediator. The reaction mechanism mediated by the mediators such as ABTS [2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)], HBT [1-Hydroxybenzotriazole], HNNS [4-hydroxy-3-nitroso-1-naphtalene sulphonic acid], NNDS [1-nitroso-2-naphthol-3, 6-disulfonic acid] and RBB [Remazol brilliant blue]. The reaction seems to proceed as oxygen activates laccase and further in turn the enzyme oxidizes the mediator during reaction. The oxidized mediator diffuses into the pulp and further oxidizes the lignin disrupting it into smaller fragments, which are easily removed from the pulp by alkaline extraction (Call and Mucke, 1997). Treatment of sugarcane bagasse with *K. aerogenes* for biopulping experiment has brought a significant decrease in lignin content and kappa number of sugarcane bagasse (Table 2). This lower lignin content prior to pulping leads to a pulp with lesser residual lignin and thus reducing the cost of chemicals and energy required to remove the lignin during bleaching processes.

Figure 6 summarizes *in situ* lignin degradation by *K. aerogenes* laccase after treatment with pulp *K. aerogenes* laccase also successfully reduced kappa number of pulp thereby indicating removal of residual lignin from pulp also.

Table 2: Delignification of bagasse by *K. aerogenes*.

Samples	Kappa no. before treatment	Kappa no. after treatment	Kappa no. after digestion.
Untreated bagasse	35	35	20
K. aerogenes treated bagasse	35	26	16.4

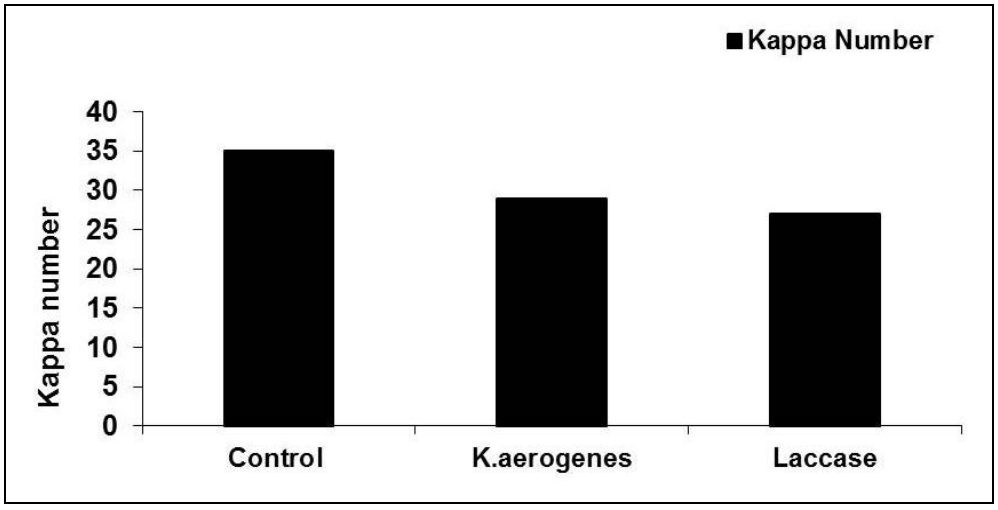

Figure 6: Effect of *K. aerogenes* laccase treatment on delignification of pulp.

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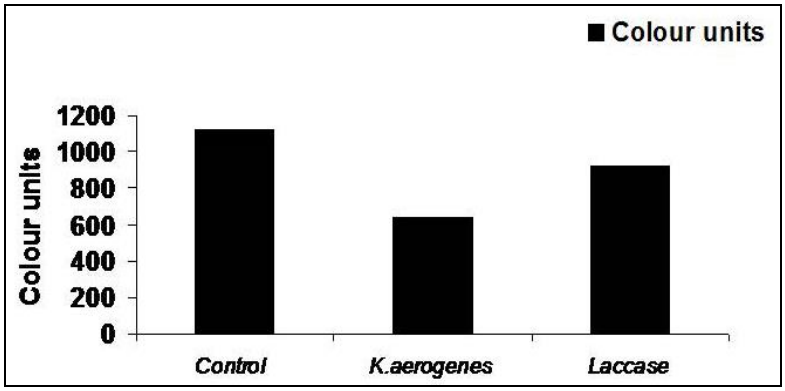

Figure 7: Effect of laccase treatment on decolorization of black liquor.

Removal of residual lignin by *K. aerogenes* laccase improved the brightness properties of the paper without affecting its strength, as presented in Table 3. These results are similar with the reported use of laccase for kraft pulp delignification by Call and Mucke (1997); Bollag and Leonowicz (1998). *K. aerogenes* had brought up to 80% reduction in color of kraft liquor as reported earlier by Jha *et al.* (2002b). As shown in Figure 7, there is substantial decrease in the color of kraft liquor by the action of *K. aerogenes* laccase, which is comparable to the activity as demonstrated by *Trametess modesta* and *Trametes versicolor* (Lorenzo *et al.*, 2002). *Paenibacillus* sp. (AY952466), *Aneurinibacillus aneurinilyticus* (AY856831) and *Bacillus* sp. (AY952465) also degraded the lignin of kraft liquor by 37%, 33% and 30%, respectively as reported by Chandra *et al.* (2007), Bourbonnais and Paice (1996) .

Laccase contains all the 4 copper atoms in the 2^+ oxidation state in the active site (Bertrand *et al.*, 2002; Piontek *et al*., 2002). It oxidizes variety of aromatic hydrogen donors. It catalyzes the removal of an electron and a proton from phenolic hydroxyl or aromatic amino groups to form free phenoxy radicals and amino radicals, respectively (Hess *et al.*, 2002). It not only oxidizes phenolic and methoxyphenolic compounds but also decarboxylates them with low molecular weight mediator (Bourbonnais *et al.*, 1997a; 1997b). Laccase is essential for lignin degradation by the white rot fungus *Pycnoporus cinnabarinus* (Eggert *et al.*, 1997). The *K. aerogenes* and the laccase produced by it is also a copper ion dependent enzyme having capability of lignin degradation as demonstrated for other microorganisms thus the bacterium *K. aerogenes* and laccase produced by it find application in biopulping and biobleaching of kraft liquor in pulp and paper industries.

CONCLUSION

Laccase from the bacterium *K. aerogenes* was isolated, purified to homogeneity by ammonium sulphate precipitation, DEAE-cellulose chromatography and affinity chromatography. The purified laccase appears to be monomeric glycoprotein with a Mr of 64 kDa when measured by Sephadex G-200 Gel exclusion chromatography and SDS-PAGE. The V_{max} and K_m of laccase towards the guaiacol was 136 units/min and 55 µM respectively. The laccase was used for biopulping of sugarcane bagasse and biobleaching of kraft liquor effluent. The laccase brought significant reduction in kappa number and lignin content of the pulp. The hand sheets prepared from the treated pulp showed improved brightness without affecting the strength and other properties of paper. The laccase when used for biobleaching of kraft liquor proved to have decolorizing potential.

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