Chapter-6

Biopulping of Bagasse and Eucalyptus
by *Cryptococcus albidus*
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**Introduction**

The pulp and paper sector represents one of the energy intensive and highly polluting sectors within the Indian economy and is therefore of particular interest in the context of both local and global environmental discussions. There are two strategies for managing industrial pollution; end of the pipe treatment like effluent treatment and process modification. Process modification involves changes in manufacturing processes, such that, water and energy consumption decrease and fewer and less toxic pollutants are generated.

In pulp and paper industry one of the process modifications is biopulping. Biopulping involves the treatment of lignocellulose with a fungus having lignolytic enzyme system and the subsequent processing of material by mechanical or chemical pulping (Saad et al., 2008). Messner and Srebotnik (1994) reported a 23% reduction in pulp-refining energy required by incubating spruce and pine chips for 2 weeks with the white-rot fungus *Phanerochaete chrysosporium* at 35–40°C. In chemical pulping, the wood biotreatment can increase pulp yield and reduce alkali requirements during kraft pulping (Hakala et al., 2004; Mendonca et al., 2004). Mendonca et al., (2002) showed that few days of pretreatment are enough to cause modifications in the lignin structure, favoring the delignification. Therefore, it is not necessary to reach high lignin losses during the pretreatment, but just to promote alterations in the macromolecule of the lignin (Saad et al., 2008).

The concept of biopulping is based on the ability of fungi to colonize and degrade lignin selectively in wood thereby leaving cellulose relatively intact (Ferraz et al., 2008). Some fungal species remove lignin more efficiently than other wood components; such degradation pattern is known as selective lignin degradation or delignification. The selective delignification of wood facilitates its softening in a subsequent pulping process. However, the extent of lignin removal during fungal pretreatment is not related to the energy savings in biomechanical pulping or to the increase in delignification rates observed in organosolv and kraft pulping. Data
obtained from a single fungal species or from several species considered altogether indicate that there is no clear correlation between the biopulping efficiency and the wood weight or component losses (Ferraz et al., 2008). This shows that the process of biopulping is poorly understood.

Wood cell walls are made up mostly of cellulose, hemicellulose and lignin. The tensile strength of wood fibers is primarily determined by cellulose and hemicelluloses, while lignin mediates adhesion among the fibers. Lignin is an extremely complex, three dimensional heteropolymer made up primarily of phenyl propane units. Due to the large size of lignin, degradation should take place in an extracellular fashion. Lignin requires aerobic conditions for degradation as carbon-carbon and ether bonds joining subunits together must be cleaved via an oxidative mechanism. Thus complex but non-specific enzyme system is required for lignin degradation (Breen and Singleton, 1999). The ligninolytic enzyme system of most of the fungi comprises lignin peroxidase, manganese peroxidase, laccase and xylanase (Shah et al., 2005; Vicentim et al., 2007).

This study focuses on the use of *C. albidus* isolated in chapter 3 for biopulping. *C. albidus* is a very good producer of laccase, an important lignolytic enzyme. Screening is done to find the most suitable substrate among bagasse and eucalyptus. The process of biopulping was studied by analyzing the enzymes produced, substrate colonization by SEM analysis and chemical modifications by FT-IR. The presence of *C. albidus* was tracked during biopulping by DGGE. Finally the temperature and amount of chemicals required for chemical pulping were optimized.

**Materials and Methods**

Fungal strain *C. albidus* isolated in chapter 3 was used for biopulping as it showed maximum laccase production.

**Screening of substrate for biopulping**

Two types of substrate, Bagasse and eucalyptus wood were tested. 25 g each of bagasse and eucalyptus was taken, steamed for 30 min to reduce indigenous microbial population, and then inoculated with 20% fungal inoculum. 25 ml of PDB was added to help fungus establish on substrate. The flasks were kept at 30°C and 60% moisture content. An uninoculated control was also kept. Sampling was done on 15th day, 30th day and 60th day. Enzyme estimation and DGGE analysis was done in
all the samples. Scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR) was conducted in the end of the experiments i.e. 60th day.

**Ligno-cellulolytic enzymes analysis**

For enzyme analysis, 2 gm substrate was removed aseptically and enzymes were extracted using 10 ml of 50mM sodium acetate buffer having pH 5. This crude filtrate was used for estimating LiP, MnP, Laccase, xylanase, CMCase and FPase. The enzyme analysis for lignin peroxidase (Tien and Kirk, 1983), manganese peroxidase (Glenn et al., 1986) and laccase (Niku-Paavola et al., 1988) are given in chapter 3. Enzyme activity for all the enzymes studied was calculated as μM change in substrate concentration by the enzyme produced per gm of wood.

**Xylanase**

1 % Oat xylan solution was prepared in citrate buffer (50mM, pH 4.5). 0.9 ml enzyme and 0.1 ml xylan solution were mixed and incubated at 50°C for 30 min. Sugar released was estimated using DNS method (Adsul et al., 2004). One unit (U/ml) of activity was defined as 1 μmol of xylose liberated per min by 1 ml of crude filtrate under assay conditions.

**Exoglucanase: FPase assay**

Citrate buffer (50mM, pH 4.5) was prepared. Strips of Whatman No 1 filter paper 50mg or 1 x 3 cm² were used as substrate. Enzyme (0.1) was incubated along with buffer (1.9 ml) and paper strip at 50°C for 60 min. Sugar released was estimated using DNS method (Adsul et al., 2004). One unit (U/ml) of activity was defined as 1 μmol of glucose liberated per min by 1 ml of crude filtrate under assay conditions.

**Endoglucanase: CMCase assay**

For estimating CMCase activity, 1 % CMC solution was prepared in citrate buffer (50mM, pH 4.5). 0.5 ml enzyme and 0.5 ml buffer were mixed and incubated at 50°C for 30 min. Sugar released can be estimated using DNS method (Adsul et al., 2004). One unit (U/ml) of activity was defined as 1 μmol of glucose liberated per min by 1 ml of crude filtrate under assay conditions.

**DNS sugar estimation**

The glucose liberated during the enzyme assay of xylanase, CMCase and FPase was estimated by DNS sugar estimation method. 3 ml of Dinitrosalicylic acid (DNS) reagent is added to 3 ml of sample, mixed, covered and heated at 90°C for 5-15 min. 40% potassium sodium tartrate is added to preserve colour and spectrophotometer reading at 575 nm is taken. Standard curve is made and the
concentration of sugar is calculated by using standard curve. Amount of sugar released gives the estimation of enzyme activity (Adsul et al., 2004).

**SEM, FT-IR and DGGE analysis**

The 60th day bagasse and eucalyptus samples along with control samples were analyzed by SEM (Srivastava and Thakur, 2006; Srivastava and Thakur, 2007) and FT-IR (Pandey and Pitman, 2003) as per method given in chapter 5. ITS1 region was used for tracking the presence of *C. albidus* during the process of biopulping as per methods given in chapter 4 (Doyle and Doyle, 1987; Hortal et al., 2006; Michaelsen et al., 2006).

**Optimization of temperature and chemical requirement for biopulping**

Bagasse was mixed with 20% fungal inoculum and kept for 1 month (temperature 30°C, moisture 60%). After 1 month the biopulp was collected, washed and dried. Further, the biopulp was subjected to different chemical and temperature treatments for 1 ½ hrs each. Chemicals (white liquor) added were 6.5g NaOH and 1.5g Na₂S in 100 ml. White liquor was added in the ratio pulp:white liquor 1:10. Finally eight different types of pulp were analyzed for kappa number and viscosity.

1. Chemical pulp: Temp- 200°C, Chemical 100% (CP200-100)
2. Biopulp (BP)
3. Biopulp: Temp- 150°C, Chemical 50% (BP150-50)
4. Biopulp: Temp- 150°C, Chemical 75% (BP150-75)
5. Biopulp: Temp- 150°C, Chemical 100% (BP150-100)
6. Biopulp: Temp- 175°C, Chemical 50% (BP175-50)
7. Biopulp: Temp- 175°C, Chemical 75% (BP175-75)
8. Biopulp: Temp- 175°C, Chemical 100% (BP175-100)

**Estimation of Kappa number**

Kappa number was analyzed according to the TAPPI Historical Method T236 hm-85 (TAPPI, 1991). An air-dried sample was weighed to an equal of 2.50 g of oven-dried test specimens and was mixed with 25.00 ml of 4.00 N H₂SO₄ and 25.00 ml of 0.10 N KMnO₄ solutions. The reaction was allowed to proceed for 10 minutes, and then treated with 5.00 ml of 1.00 N KI to stop the reaction. The reaction temperature was recorded at 5 minutes intervals. The reaction was titrated with standard solution 0.10 N Na₂S₂O₃ with starch as indicator, added towards the end of the reaction. The Kappa number was calculated as follows:
\[ K = \frac{pf}{w}1 + 0.013(25 - t) \]

where \( K \) = Kappa number, \( p \) = percentage of \( \text{KMnO}_4 \) consumed, %, \( f \) = correction factor for different percent of \( \text{KMnO}_4 \) consumed, \( t \) = reaction temperature, \( ^\circ\text{C} \) and \( w \) = amount of moisture-free pulp in the specimen, g.

**Estimation of Viscosity**

Viscosity was tested according to Tappi method T230 (TAPPI, 1991). An air-dried sample was weighed to an equal of 0.25 g of oven-dried test specimen to the nearest \( \pm 0.0001 \) g and placed in a bottle. A 25 mL of de-ionized water and 8 to 10 beads were added into bottle and shaked for at least 20 minutes until the fibers were completely dispersed. A 25 mL of CED (Cupri Ethylene Diamine) solution was added into the bottle and shake for at least 30 minutes until the fibers were completely dissolved. The sample solution was transferred into the viscometer and was placed in a water bath at constant temperature 25.0\( ^\circ\text{C} \) \( \pm \) 0.1. The time required for the sample solution to flow from the upper mark to the lower mark of the viscosity tube was recorded and the viscosity was calculated as follows:

\[ V = C \times t \times d \]

where \( V \) = viscosity, \( C \) = viscometer constant, \( t \) = efflux time, s and \( d \) = density of the pulp solution, \( \text{g/cm}^3 \) (=1.353)

**Results**

**Screening of substrate for biopulping**

Two substrate bagasse and eucalyptus were tested for biopulping by *Cryptococcus albidus*. In both the substrates, dominant enzyme was laccase followed by xylanase. MnP was also produced in very less amount. Further, enzymes CMCase and FPase were produced in negligible amounts and lignin peroxidase was not detected in bagasse as well as eucalyptus. Among the two substrates, bagasse and eucalyptus, more enzyme production was observed in bagasse (Figure 6.1.) with 61 U/g of wood (after 2 months) for laccase and 34 U/g of wood (after 1 month) for xylanase.
Scanning electron microscope (SEM) studies

The bagasse and eucalyptus were viewed at high magnifications using scanning electron microscope (SEM) to see the colonization of these two substrates by *Cryptococcus albidus* (Figure 6.2a and b; Figure 6.3a and b.), respectively. White colour arrow shows the colonization of substrate by fungi and black colour arrow shows the pits created by the growing fungi. The fungal colonization is more extensive in bagasse as compared to eucalyptus.

Evaluation of chemical modification of substrate by FTIR studies

The control and treated bagasse and eucalyptus were studied using FT-IR. The FT-IR spectra of both bagasse (Figure 6.4a and b) and eucalyptus Figure 6.5a and b.) show the characteristic peaks of lignin and carbohydrates. The samples of both bagasse and eucalyptus show the characteristic spectra of wood with many peaks in the region 1800-700 cm\(^{-1}\). The region from 1200-900 cm\(^{-1}\) is for polysaccharides present in the wood (Pandey and Pitman, 2003). The lignin of hemp, jute and flax show peaks at 2917 and 2847 cm\(^{-1}\). Both the peaks were also present in case of bagasse and eucalyptus. Other peaks signifying lignin were present at 1599, 1511.1 1426, 1370 and 1140 cm\(^{-1}\) in bagasse and at 1591.6, 1511 1426, 1370 and 1140 cm\(^{-1}\) in eucalyptus (Boeriu et al., 2004; Pandey and Pitman, 2003). The degradation of lignin in both bagasse and eucalyptus was evident with the increase in the intensity of 1646 cm\(^{-1}\) and decrease in the intensity of 1599 and 1511 cm\(^{-1}\) (Faix et al., 1991).
Figure 6.2. Surface colonization of bagasse by Cryptococcus albidus
(a) control (b) treated.
Figure 6.3. Surface colonization of eucalyptus by *Cryptococcus albidus* (a) control (b) treated.
Figure 6.4a. FT-IR spectra of untreated bagasse.
Figure 6.4 b. FT-IR spectra of bagasse treated by *C. albidus*. 
Figure 6.5a. FT-IR spectra of untreated eucalyptus.
Figure 6.5b. FT-IR spectra of eucalyptus treated by *C. albidus*. 

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**DGGE analysis for tracking C. albidus**

The strain introduced for biopulping has to compete with the natural population of microorganisms existing to establish itself. To facilitate the establishment of *C. albidus*, the bagasse and eucalyptus were steamed for 30 min before inoculation and aseptic conditions were maintained throughout the experiment. From figure 6.6 it can be suggested that steam treatment was effective in case of bagasse but not in case of eucalyptus. Further analyzing the microbial community on 30th and 60th day’s showed that in case of bagasse *C. albidus* exists as the dominant fungal strain while in eucalyptus rich diversity exists (Figure 6.6.). Though in both the cases *C. albidus* has established itself and exists throughout the duration of experiment, in eucalyptus it is not the dominant species.

![DGGE analysis](image)

Figure 6.6. DGGE analysis of bagasse and eucalyptus during biopulping. Lane 1: *C. albidus*, Lane 2: bagasse (0 day), Lane 3: bagasse (30 day), Lane 4: bagasse (60 day), Lane 5: eucalyptus (0 day), Lane 6: eucalyptus (30 day) and Lane 7: Eucalyptus (60 day)

**Optimization of temperature and chemical requirement for biopulping**

In view of above results bagasse is better substrate for biopulping by *C. albidus*. It was further optimized for temperature and the amount of chemicals required for pulping. There was an increase in kappa number after biopulping (Table 6.1.). However, the viscosity also increased as a result of treatment. When the biopulp was subjected to different temperature and heat treatment minimum there was
reduction in kappa number and viscosity. The most suitable combination was biopulp treated at 175°C with 75% chemicals (Figure 6.7.).

Table 6.1. Kappa number and viscosity of bagasse treated at different temperature and chemical concentrations

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Pulp Sample</th>
<th>Kappa Number</th>
<th>Viscosity (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CP200-100</td>
<td>18 ± 0.56</td>
<td>38 ± 1.62</td>
</tr>
<tr>
<td>2.</td>
<td>BP</td>
<td>28 ± 1.23</td>
<td>69 ± 2.14</td>
</tr>
<tr>
<td>3.</td>
<td>BP150-50</td>
<td>26 ± 1.29</td>
<td>52 ± 1.45</td>
</tr>
<tr>
<td>4.</td>
<td>BP150-75</td>
<td>23 ± 0.68</td>
<td>49 ± 1.21</td>
</tr>
<tr>
<td>5.</td>
<td>BP150-100</td>
<td>22 ± 0.85</td>
<td>46 ± 0.94</td>
</tr>
<tr>
<td>6.</td>
<td>BP175-50</td>
<td>24 ± 0.91</td>
<td>48 ± 1.23</td>
</tr>
<tr>
<td>7.</td>
<td>BP175-75</td>
<td>21 ± 0.74</td>
<td>45 ± 1.75</td>
</tr>
<tr>
<td>8.</td>
<td>BP175-100</td>
<td>21 ± 0.76</td>
<td>41 ± 1.68</td>
</tr>
</tbody>
</table>

Figure 6.7. Pulp samples (a) chemical pulp (b) bio-pulp (c) bio-chemo pulp

Discussion

Biopulping is used as a pretreatment before mechanical or chemical pulping. The benefits of biopulping are reduction in chemical and energy consumption and improvement in pulp quality. Biological degradation of lignocellulosics has been increasingly emphasized (Akhtar et al., 1998) and much attention has currently been drawn toward development of new environmentally friendly technologies for pulp and paper manufacture (Mesner and Srebotnik, 1994; Reid, 1998). Increase of demand for
paper production and limited wood resources have directed researchers to look for appropriate additional resources of non-wood materials for pulp and paper manufacturing. Several kinds of non-wood lignocellulosic by-products of agricultural cultivation have been investigated (Giovannozzi-Sermanni et al., 1994; Martinez et al., 1994). Bagasse is an agro-waste and eucalyptus mainly grows in plantations. Thus the use of these two raw materials is a good alternative for woody plants.

The process of wood degradation is mediated through enzymes. These enzymes are extracellular in nature. The fungi having wood degradation capacity secrete hydrolytic enzymes such as cellulases, pectinases and xylanases, which are typically induced by their substrates. On the other hand, lignin (a polymer of phenylpropane units connected by different C–C and C–O–C linkages) is oxidized and degraded by a ligninase system made up of at least three enzyme activities: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. The degradation is oxidative in nature, thus, it is an aerobic process. For biopulping selective lignin degradation is required. Thus fungi having robust ligninase system and deficient cellulolytic activity are suitable for biopulping. Ceriporiopsis subvermispora is a good example (Aguiar and Ferraz, 2008).

The three enzymes, lignin peroxidase, manganese peroxidase and laccase work with different mechanisms. All three are glycosylated metal containing enzymes that are non-substrate specific. Among the three, manganese peroxidase and laccase are of greater significance. Manganese peroxidase mainly acts on non-phenolic component of lignin while laccase acts on phenolics (Breen and Singleton, 1999). Apart from these two enzymes, xylanases also play a crucial role in delignification. They attack hemicelluloses of wood and leads to its depolymerization (Baraznenok et al., 1999). The activity of xylanase, manganese peroxidase and laccase was detected in the biopulp of both bagasse and eucalyptus (Figure 6.1). The enzyme activity was more in case of bagasse than eucalyptus. Among the three enzymes laccase showed maximum production followed by xylanase and then manganese peroxidase. Lignin peroxidase was not detected. The production of other cellulolytic enzymes like CMCase and FPase was negligible. Thus fungi are suitable for biopulping as it has good ligninase system and poor cellulase production. From figure 6.1. we can say that the activity of xylanase and manganese peroxidase declines after 1 month though the laccase activity continue to increase. Thus 1 month seems to be an appropriate duration for biopulping.
The analysis of biopulp surface by SEM reveals that *C. albidus* has been able to colonize bagasse better than eucalyptus (Figure 6.2a,b and 6.3a,b.). This might be one possible reason of better enzyme production in bagasse biopulp. The formation of pits and tunnels by the fungi are clearly evident in the case of bagasse (Daniel, 1994). This increases the porosity of the wood and in turn reduces the chemical and energy requirements.

FT-IR analysis shows that both the substrates were chemically modified. The peaks at 2917 and 2847 cm\(^{-1}\), present in both the substrates, signifies CH stretching in aliphatic methylene group that can originate from fatty acids present in the lignin preparations (Boeriu et al., 2004). The peaks in bagasse and eucalyptus around 1600, 1511 and 1426 cm\(^{-1}\) shows aromatic ring vibration (Hoareau et al., 2004). On comparing the treated samples with control samples for bagasse (Figure 6.4a and b.) and eucalyptus (Figure 6.5a and b.), it was found that there was decrease in the peak 1600 and 1511 cm\(^{-1}\) after treatment and an increase in 1646 cm\(^{-1}\). Decrease in peaks is due to lignin degradation and carbonyl moieties are formed after degradation that cause increase in 1646 cm\(^{-1}\) (Faix et al., 1991). The FT-IR is generally used to see qualitative changes and before any quantitative measurement it requires calibration with other lignin estimation methods (Pandey and Pitman, 2003).

The results of SEM and FT-IR shows that *C. albidus* was able to colonize both the substrates and lead to their chemical modifications. However, the colonization was better on bagasse than eucalyptus. One possible reason for this might be the effectiveness of steam sterilization in case of bagasse as compared to eucalyptus (Figure 6.6). It is often observed that in studies using bioaugmentation, there is a need of repeated addition of inoculum (Boon et al., 2000; Sigler et al., 2001). However in case of *C. albidus* it has survived in both the substrates for two month without any subsequent addition. In case of bagasse it is the dominant strain.

For further optimization of temperature and chemical requirement, biopulp bagasse was used. Among all the temperature tested 175°C was most suitable with 75% chemical requirement. The chemical pulping was performed at 200°C and the kappa number was comparable (Figure 6.7, Table 6.1.). The viscosity of biopulp sample is higher than chemical pulp shows that fungal treatment has lead to the improvement in fiber quality (Maijala et al., 2008). Thus this study proves the biopulping potential of *C. albidus* especially in reference of bagasse.