

Deinking of recycled mixed office paper using two endo-glucanases, CelB and CelE, from the anaerobic fungus *Orpinomyces* PC-2

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ABSTRACT: We studied the deinking of recycled mixed office paper (MOP) using two endo-glucanases, CelB and CelE. Treatment of MOP pulp with either enzyme followed by a flotation stage significantly reduced both dirt count and residual ink area and increased brightness. CelE appeared to have deinking effects superior to those of CelB. CelB was more effective than CelE at hydrolyzing pulp cellulose. Ink particles in MOP treated with CelB tended to be smaller than those treated with CelE. Ink particles smaller than 20 μm were not efficiently removed by flotation.

Application: Two enzymes, CelE and CelB, make it easier to remove ink particles from mixed office paper, turning this type of used paper into a more suitable candidate for recycling.

The recycling rate of used paper has steadily increased in recent decades as part of the effort to preserve forest resources and reduce the costs of municipal waste treatments. Currently, mixed office paper (MOP) is one of the types of paper least often recycled. The reason MOP is not often recycled is that it is difficult to remove ink particles from it. Because conventional chemical methods do not effectively remove inks from MOP, the enzymatic treatment of MOP has been investigated as an alternative deinking method [1]. Enzymatic treatment of MOP can improve deinking efficiency and decrease the total treatment cost as well [2]. An added benefit of using an enzymatic deinking process is that it increases the freeness of the pulp, which improves its recyclability on the paper machine [1, 3].

Among the many enzymes that have been studied for deinking various recycled papers, cellulases are the most effective for deinking MOP [4, 5]. Most cellulases used in deinking MOP on laboratory, pilot-plant, and mill scales, however, are mixtures of enzymes such as endo-1,4-glucanases (E.C.3.2.1.4.), cellobiohydrolase (E.C.3.2.1.91.), and 1,4-glucosidase (E.C.3.2.1.21.). Each of these individual enzymes plays a different role in the hydrolysis of cellulose [6]. The ability of the same type of enzyme to hydrolyze cellulose can vary with the source of the enzyme. Consequently, the contributions of individual enzymes to the deinking of MOP are poorly understood, making it difficult to optimize the deinking process of MOP.

It is well established that cellulase contains a catalytic domain and a cellulose-binding domain. In efforts to determine the individual contributions of monocomponents of cellulase, researchers have shown that a pure cellulose-binding domain has negative impacts on the deinking of MOP [7] and that a single endo-glucanase can deink MOP effectively [5]. In this paper, we demonstrate that two pure endo-glucanases, CelE and CelB, effectively facilitate the deinking of MOP and that CelE has deinking effects superior to those of CelB.

MATERIALS AND METHODS

Materials

Commercial printing paper was printed with repeated lines of the letter N. Photocopies were made and used as MOP samples.

Genes coding for two endo-glucanases, CelB and CelE (Aureozyme), were cloned from the anaerobic fungus *Orpinomyces* strain PC-2 and overexpressed using the pET system in *Escherichia coli*. [8, 9]. The recombinant enzymes were partially purified from an *E. coli* culture before use. A nonionic surfactant (Kolb AG, Switzerland) was used in the flotation stage of the deinking process.

Enzyme assay

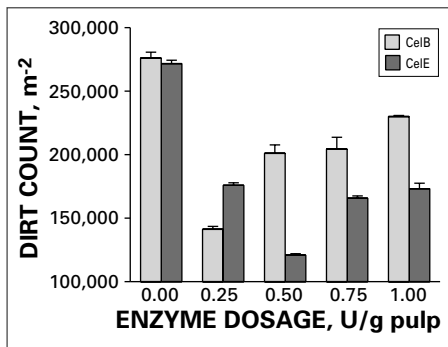
An enzyme solution (50 mL) containing either CelB or CelE was mixed with 200 mL of a carboxymethyl-cellulose (CMC) solution that contains 1.0 g of CMC and 0.15 g of calcium chloride dihydrate in

100 mL of sodium citrate-phosphate buffer (50 mM, pH 6.0). The resulting solution was incubated at 50°C for 15 min. We terminated the reaction by placing the tubes on ice and adding 500 mL of a DNS solution that contains 3,5-dinitrosalicylic acid (1.0 g), NaOH (1.6 g), and potassium sodium tartrate tetrahydrate (30 g) in water (100 mL).

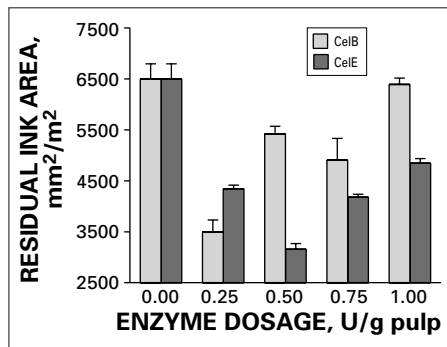
The reaction mixture was boiled for 5 min and then placed on ice. The optical absorbance at 550 nm was measured, and the corresponding glucose concentration was determined from a calibration curve of glucose standards (0–10 mM). If the corresponding glucose concentration was more than 3 mM, the assay was repeated with a more dilute enzyme sample. One unit (U) of the enzyme activity is defined as releasing 1 μmol of glucose per minute under the assay conditions. Control experiments were carried out under the same assay conditions, except that the enzyme solution was replaced with citrate-phosphate buffer (50 mM, pH 6.0).

Deinking process

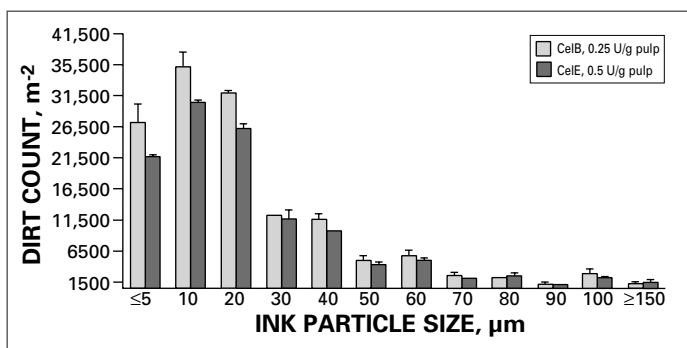
The MOP sample (4.65 g, oven-dried weight) was cut into small pieces (2.7 cm $\times$ 3.5 cm) and soaked in 170 mL of 50 mM sodium citrate buffer (pH 6.0) for 1 h at room temperature with shaking (150 rpm, Φ 18 mm). The soaked paper was pulped in an Osterizer blender for 20 s. The pulp was transferred to a 500 mL Erlenmeyer flask, and a slurry was created by adding 80 mL of 50 mM sodium citrate buffer (pH 6.0).



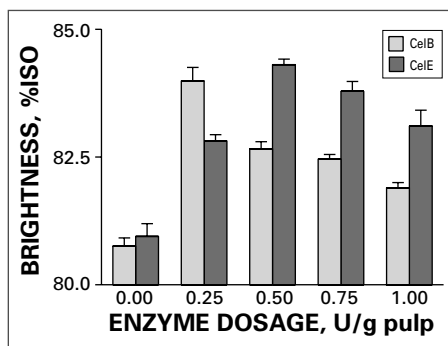
1. Effects of enzyme dosages on the number of residual ink particles.



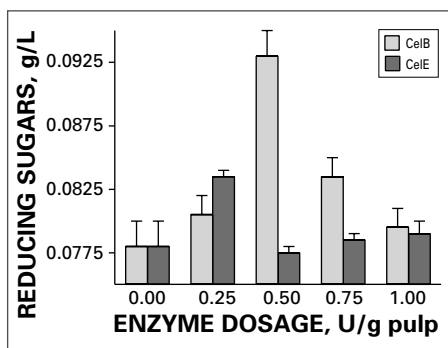
2. Effects of enzyme dosages on residual ink area.



3. The size distribution of residual ink particles after enzymatic deinking of MOP.



4. Effects of enzyme dosages on brightness.



5. Effects of enzyme dosages on the amount of reducing sugars released in treating pulp with an endo-glucanase.

Scan 2000 software, we obtained comprehensive information that included the number of ink particles (dirt count), the residual ink area, and the ink particle size distribution.

Brightness analysis

The ISO brightness of the handsheets was determined with a Carl Zeiss Elrepho Colorimeter (Germany) according to TAPPI method T 525 om-86.

Reducing-sugar analysis

After samples containing enzymes were incubated for 20 min, 5 mL portions of the pulp slurry were transferred to tubes and centrifuged for 10 min at 4000 rpm. The content of reducing sugars in the supernatant, defined as an equivalent amount of glucose in grams per liter of supernatant, was determined using the DNS method [10].

RESULTS

Compared with the controls, every dosage of CelB and CelE resulted in a decrease in dirt count. The biggest reduction in dirt count resulted from using CelE at a dosage of 0.50 U/g of pulp, which reduced approximately 2.7×10^5 ink particles in a square meter to about 1.2×10^5 . For CelB, the biggest decrease in dirt count was obtained at a dosage of 0.25 U/g of pulp. Except at 0.25 U/g of pulp, CelE was more effective than CelB at reducing dirt count. These results are shown in the bar chart of Fig. 1. (In each of the figures, the height of each bar represents the mean value of at least six replications, and the error bars represent the standard deviations.)

Compared with the controls, both CelB and CelE resulted in a reduction in residual ink area, as shown in Fig. 2. The most effective dosages for reducing the residual ink were 0.25 U/g of pulp for CelB and 0.5 U/g of pulp for CelE. Except at 0.25 U/g of pulp, CelE resulted in a greater reduction in residual ink area than obtained with CelB.

As Fig. 3 shows, CelB and CelE produced similar ink particle size distributions. Most residual ink particles were smaller than 20 μm.

Compared with the controls, pulp brightness increased after treatment with either CelB or CelE followed by a flotation process, as Fig. 4 shows. Pulp brightness after treatment with CelB was greater than that after treatment with CelE at the dosage of 0.25 U/g of pulp. At

After 1 h with the pulp at 50°C, various dosages (0.25, 0.50, 0.75, or 1.00 U/g of pulp) of either CelB or CelE enzyme were mixed with the pulp slurry and incubated at 50°C for 20 min. For a control sample, no enzyme was added to the pulp slurry.

After this final incubation with the added enzyme, samples were either used to determine the content of reducing sugars or were subjected to flotation and then made into handsheets.

Flotation

After incubation with the enzyme, 250 mL of ice water and then 0.4% of surfactant (weight percent based on oven-dried weight of MOP) were added to the pulp slurry and mixed well. The pulp slurry was transferred from the 500 mL Erlenmeyer flask to a 500 mL graduated cylinder, and a flotation was performed for 5 min. Air was passed through a Pyrex gas dispersion tube at a flow rate of 1.5 L/min.

Handsheets

After flotation, the pulp was divided into two equal parts, and two handsheets were made according to TAPPI T 205. The handsheets were dried and used for image and brightness analyses. At least six replications were made for each deinking condition.

Image analysis

Image analysis of the handsheets was performed using a PC, a scanner, and Spec Scan 2000 software (Apogee Systems). The analyzed area of the handsheets was 132.27 cm². Using the Spec

dosages of 0.5 U/g of pulp and higher, CelE led to greater brightness than did CelB.

We evaluated the content of reducing sugars in the pulp slurry after the pulp was incubated with either CelB or CelE for 20 min. **Figure 5** shows the results. The maximum amount of reducing sugars produced by CelE occurred at the dosage of 0.25 U/g of pulp, whereas the maximum amount produced by CelB was at 0.5 U/g of pulp. At the dosage of 0.25 U/g of pulp, CelE released more reducing sugars than CelB released. However, CelB hydrolyzed cellulose much faster than did CelE when the dosage was 0.5 U/g of pulp or 0.75 U/g of pulp.

We also investigated the effect of incubation time on the deinking of MOP and found that the deinking effect was slightly better at 20 min than at other incubation times (data not shown). Consequently, we chose the incubation time of 20 min for all deinking experiments.

DISCUSSION

Generally speaking, the reduction of the dirt count correlates proportionally with the reduction of residual ink area unless the distributions of ink particle sizes are significantly different from one another. In Fig. 3, we can see that both CelB and CelE led to similar distributions of ink particle sizes, which explains the close correlation between the reduction in the dirt count and the reduction in residual ink area under all deinking conditions.

From Fig. 3, we can also see that most residual ink particles were 20 μ m or smaller. Particles with the sizes of 20 μ m or smaller accounted for more than half of the dirt count when either CelB or CelE was used. These results are consistent with the finding that the flotation process is not very effective at removing ink particles smaller than 20 μ m [11]. CelB resulted in more ink particles with sizes of 20 μ m and smaller than did CelE. Apparently, the reason that CelE has superior deinking effects is that CelE treatment results in fewer small ink particles than does CelB treatment.

After 20 min of incubation with CelE or CelB, the dirt count and residual ink area of the treated pulp decreased significantly (Figs. 1 and 2). At the same time, brightness increased (Fig. 4). These

results demonstrate that both CelE and CelB can deink MOP effectively. The increase in brightness is closely correlated with the decreases in dirt count and residual ink area, suggesting that ink particles are the most important factor affecting brightness. In other words, the deinking process with the enzymatic treatment followed by a flotation process did not generate new chromophores that affect brightness.

In a previous study, we demonstrated that a cellulose binding domain alone has negative impacts on deinking efficiency [7]. Both CelB and CelE are genetically engineered enzymes. Based on similarities in amino acid sequences [12], the catalytic domains of CelB and CelE belong to the family 5 of glycosyl hydrolases. These enzymes are typical endo-glucanases and lack any cellobiohydrolase activity [8,9]. They contain neither a cellulose-binding domain nor a docking domain [8,9]. Here, we have demonstrated that endo-glucanases lacking a cellulose-binding domain could result in superior deinking effects. CelB and CelE have different optimal dosages for deinking, although they are highly homologous to each other [9].

The cellulose-binding domain plays an important role in the degradation of cellulose [13–15]. Lacking such a domain, CelB and CelE hydrolyze cellulose slowly [9]. Compared with other endo-glucanases that have a cellulose-binding domain, the amounts of reducing sugars released by CelB and CelE were relatively small under the same incubation conditions (data not shown). The lower the amount of reducing sugars released, the less pulp lost in deinking. Therefore, a weak hydrolytic ability to pulp cellulose is a desirable feature for an enzyme that is used for deinking MOP. It may be that removing the cellulose-binding domain would produce a superior endo-glucanase for deinking. However, more research has to be done before we can draw such a conclusion, because removing the cellulose-binding domain through recombinant DNA techniques could affect other properties of the enzyme as well.

Both CelE and CelB are most effective at hydrolyzing CMC (the substrate for the enzyme assay) at pH 6.0 and 50°C. When incubation with either CelE or CelB was carried out at room temperature or

when a pre-boiled enzyme was used in the incubation, dirt count and residual ink area did not differ from the control experiments (data not shown). Therefore, we carried out the pulp treatments under the optimal conditions (pH 6 and 50°C) in this study.

Many variables, especially mechanical force (i.e., pulping time), affect the efficiency of the deinking process. Because the effort in this study was to investigate whether or not a treatment of pulp with an endo-glucanase facilitates the removal of ink particles from MOP, other variables, such as pulping time and flotation time, were held constant for all treatments. **TJ**

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INSIGHTS FROM THE AUTHORS

The kind of cellulase enzyme that is currently used for deinking is a mixture of exo-glucanase and endo-glucanase. In this study, we looked at the contributions that monocomponents of cellulases make to deinking. We demonstrated that endo-glucanase had positive deinking effects, whereas exo-glucanase had a negative impact on deinking efficiency.

Mills can improve the deinking efficiency by selecting the right enzyme, and that is the purpose of this research. Our next step is to study the mechanisms by which each enzyme facilitates deinking. After that, we hope to develop an optimum enzyme formulation for a mill-scale trial.

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