# EFFECT OF ENZYME CONCOCTIONS ON FIBER SURFACE ROUGHNESS AND DEINKING EFFICIENCY OF SORTED OFFICE PAPER

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Bio-deinking of sorted office paper (SOP) by various enzyme concoctions having cellulase, xylanase, amylase and lipase was investigated. The effect of various enzyme concoctions on pulp brightness, effective residual ink concentration (ERIC), deinkability factor based on brightness ($D_B$), deinkability factor based on ERIC ($D_E$), dirt counts, strength properties and effluent characteristics was studied and compared with their respective control. Also, the effect of different enzyme concoctions on fiber surface and fiber morphological changes during the deinking process were studied by atomic force microscopy (AFM) and scanning electron microscopy (SEM). Results showed that, compared to control, a maximum improvement in brightness by 13.30% (ISO), $D_B$ 37.79%, and $D_E$ 83.0% and a reduction in ERIC and dirt counts by 68.18 and 88.04%, respectively, were achieved with a concoction of cellulase+xylanase+amylase+lipase at a dosage of 6, 3, 1.5 and 6 IU/mL, respectively. This indicated that there was a synergistic deinking effect among the concoctions of these enzymes as fiber surface roughness increased by 159% compared to control.

***Keywords***: sorted office paper, biodeinking efficiency, enzyme concoctions, fiber surface roughness

## INTRODUCTION

Consumption of paper goes hand in hand with population growth and literacy rates, the demand for pulp products continuing to be high, in spite of the general beliefs that the advancement in information technology and computerization would result in a paperless global society. Recovered paper has become an important source of fibers in pulp and paper industry, because of the scarcity of wood fibers throughout the world.[1] Deinking is an important step in the fiber recycling process for white grade papers.[2] SOP contains photocopier and laser printed papers coated with toners. These toners are copolymers of styrene and acrylate that get thermally fused with cellulosic fibers of the paper during printing.[3] Conventional chemical deinking is not a successful process for removing the ink particles from the fibers. Therefore, enzymatic deinking has attracted a great deal of attention owing to efficient dislodging of the ink particles, mitigating chemicals demand and giving a cost-effective technology with a minimum impact on the environment.[3]

Many researchers have reported the use of different enzymes during the eco-friendly deinking process, such as cellulases, hemicellulases, pectinases, amylases and ligninases.[4] The cellulose and hemicellulose hydrolysis on the surface of the fibers leads to the removal of small fibrils, a phenomenon known as "peeling of fibers", which facilitates ink detachment from the fiber surface. It is observed that the treatment with cellulase facilitates the removal of residual fibers from toner surfaces, which in turn enhances the flotation efficiencies.[5] Zollner and Schroeder have reported the use of α-amylase to deink white office papers and obtained a substantial increase in toner removal.[6] Lipases are enzymes catalyzing the hydrolysis of the oil-based binder or the resins in the ink.[7]

In the present study, four different strategies for enzymatic deinking of SOP aim at: (a) using cellulases for removing inks in an indirect way by peeling-off fibers, (b) hydrolyzing cellulose and hemicellulose on the surface of the fibers to remove small fibrils by cellulase and xylanase, (c) using amylase in concoctions of cellulase and xylanase for degrading the starch layer on the surface of the papers and (d) including lipase in the concoctions of cellulase, xylanase and amylase for hydrolyzing vegetable oil-based ink binders. In each concoction, the effect of enzyme actions on fiber surface is studied by AFM and SEM.

## EXPERIMENTAL

### Waste paper collection and characterization

SOP was collected from M/s Khatema Fiber Ltd., Khatema, District Udham Singh Nagar (U.K., India) – a recycled paper based industry. SOP consists of waste paper, as typically generated by offices, containing primarily white and colored ground-wood free paper, free from unbleached fibers, may include a small percentage of ground-wood computer printout and facsimile papers, coated with toner and laser printing, and industrial papers. The total prohibitive materials and outthrows were of 3% in SOP.[8] The ash content (TAPPI T 211 om-02 "Ash in wood, pulp, paper and paperboard: combustion at 525 °C") in SOP was of 20.72%.[9] The moisture content (TAPPI T 208 wd-98 "Moisture in wood, pulp, paper and paperboard by toluene distillation") in SOP was of 10%.[9]

### Waste paper pulping

SOP was torn into small pieces and soaked in warm water at 50 °C for 30 min. Pulping was done in a hydropulper of 500 g capacity (Weverk, A-47054, Sweden). SOP was pulped for a pulping time of 20 min, temperature of 60 °C and consistency of 12% in the presence of a surfactant (oleic acid) dose of 0.05% and pH 7.2±2. The rotor speed of the hydropulper was adjusted to 650 rpm.

### Enzyme treatment

The pulp produced under optimum conditions was treated with different doses of cellulase from *Aspergillus niger* AT-3 ($CMC_{ase}$, $FP_{ase}$ and β-glucosidase activities of 25.12, 2.23 and 0.87 IU/mL, respectively) and xylanase from *Coprinus cinereus* AT-1 (xylanase, laccase and cellulase activities of 742.47, 25.9 and 0.98 IU/mL, respectively, and protein concentration 5.8 mg/mL) isolated at Biotechnology Research Laboratory, Indian Institute of Technology Roorkee, Saharanpur Campus, Saharanpur,[10] as well as commercially available enzyme, like amylase and lipase (Advanced Enzymes, Mumbai) in combinations under the following conditions: consistency 12%, pH 5.3±2, temperature 55±2 °C, reaction time 60 min and surfactant (Tween 80) dose 0.1%. Enzymatically treated pulp was washed with tap water on a Weverk laboratory flat stationary screen, with 300 mesh wire bottom for the removal of hydrolysed chemicals and subjected to ink flotation at a flotation time of 10 min, consistency 1%, temperature 35±2 °C and pH 7.2±2. After ink flotation, the pulp was washed with warm water and pulp pad was prepared for brightness determination (TAPPI T 218 sp-02 "Forming handsheets for reflectance testing of pulp [Büchner funnel procedure]" and evaluated for brightness (TAPPI T452 om-02 "Brightness of pulp, paper and paperboard [Directional reflectance at 457 nm]") and ERIC (TAPPI T567 pm-97 "Determination of effective residual ink concentration (ERIC) by infrared reflectance measurement"). After ink flotation, the pulps were defibered in a WEVERK disintegrator and evaluated for CSF (TAPPI T 227 om-99 "Freeness of pulp [Canadian standard method]"). Laboratory handsheets (60 g/m$^2$) were prepared on a British sheet former (TAPPI T 205 sp-02 "Forming handsheets for physical tests of pulp"), conditioned at a relative humidity of 65±2% and temperature of 27±1 °C, and evaluated for burst index (TAPPI T 403 om-97 "Bursting strength of paper"), tensile index (TAPPI T 494 om-01 "Tensile properties of paper and paperboard [using constant rate of elongation apparatus]"), double fold (TAPPI T 423 cm-98 "Folding endurance of paper [Schopper type tester]"), tear index (TAPPI T 414 om-98 "Internal tearing resistance of paper [Elmendorf-type method]") and pulp viscosity (TAPPI T 230 om-04 "Viscosity of pulp [capillary viscometer method") and compared with the control.[9] Similarly, laboratory-made handsheets were evaluated for dirt counts (TAPPI T 213 om-01 "Dirt in pulp") and deinkability factors, i.e. $D_B$ (based on brightness) and $D_E$ (based on ERIC), respectively, after ink flotation, using following formula:

$$D_B, \% = \frac{B_F - B_P}{B_B - B_P} \times 100 \qquad [1]$$

where $B_P$ = Brightness after pulping, % (ISO)
$B_F$ = Brightness after flotation, % (ISO)
$B_B$ = Brightness of the sample paper without the presence of ink particles (blank), % (ISO)
$D_B$ = Deinkability factor based on brightness, % (ISO)

$$D_E, \% = \frac{E_P - E_F}{E_P - E_B} \times 100 \qquad [2]$$

where $E_B$ = ERIC value in the absence of ink particles (blank)
$E_F$ = ERIC value after flotation deinking
$E_P$ = ERIC value of the sample sheet before ink removal (after pulping)
$D_E$ = Deinkability factor based on ERIC value, %

Filtrates collected from enzymatic treatment and ink flotation stages were mixed in equal amounts and the combined effluents were analyzed for COD (IS 3025: Part 58, 2006: COD – Closed reflux titrimetric method using Thermo reactor CR2010, BOD (IS 3025:

Part 44: 2006 and colour)[11] and total solids (IS 3025: Part 15, 2003 – Total residue (total solids – dissolved and suspended solids).[12]

**Scanning electron microscopy (SEM)**

Detailed morphological investigations of pulp samples pertaining to changes in the fiber surface texture before and after enzymatic treatment were carried out by SEM (Leo 435 VP, England). Fungal mat was taken and subjected to fixation using 3% glutaraldehyde (*v*/*v*) and 2% formaldehyde (4:1) (*v*/*v*) for 24 h. Following the primary fixation, samples were washed thrice with double-distilled water. The samples were then treated with alcohol gradients of 30, 50, 70, 80, 90% and absolute alcohol (99.9%) for dehydration. The samples were kept for 15 min each in up to 70% alcohol gradient, thereafter, treated for 30 min each for subsequent alcohol gradients. After treating with absolute alcohol, the samples were air-dried and examined under SEM using the gold shadowing technique.[13] Electron photomicrographs were taken at desired magnifications.

**Atomic force microscopy**

Atomic force microscopy was used to study the morphology of cellulose and lignin substrates after enzymatic modifications and to obtain information about changes in fiber surface properties.[14] AFM measurements were made with a Nanoscope-IIIa multimode scanning probe microscope (Digital Instruments Inc., Santa Barbara, CA, USA). The images were scanned in tapping mode[15,16] in air using silicon cantilevers (Point probes, type=NCH, delivered by Nanosensors, Neuchald, Switzerland). No image processing except flattening was done and at least three areas on each sample were measured. The root mean square (rms) roughness of all samples was determined from the 1 $\mu m^2$ AFM topography images.

## RESULTS AND DISCUSSION

### Effect of enzyme concoctions on bio-deinking

When SOP pulp was treated with crude cellulase (*A. niger* AT-3) prior to ink flotation, its ERIC value and dirt counts mitigated by 61.84 and 82.29%, whereas brightness, $D_B$ and $D_E$ improved by 8.13, 23.10 and 76.30%, respectively, compared to the control (Table 1). Woodward *et al*. explained that cellulase binding on pulp fiber might result in a surface fiber alteration, which was sufficient to favour ink detachment during repulping.[17] Lee *et al*. reported that the main effect was the hydrolysis and superficial degradation of cellulose that implied ink removal from fibers.[18] Non-ionic surfactants were found to interact with cellulases to improve their action; they were often used to enhance enzyme assisted deinking.[19] Pélach *et al*. reported that cellulase improved ink detachment from old newspapers giving similar or better results when cellulase was used instead of conventional chemicals.[20]

The introduction of cellulase treatment improved all the mechanical strength properties, freeness level and pulp viscosity, except double fold numbers, compared to the control. The increase in pulp viscosity was due to the high specific surface area of the tertiary fines (generated during waste paper pulping) and the attack of cellulases was specific towards this fraction of the pulp. The improvement in strength properties might be due to the peeling effect.[21] Cellulase present in crude enzyme preparation played an important role in the reduction of refining energy, as well as in the improvement in mechanical strength properties of enzymatically treated pulps. Pulp fibrillation by cellulase was recognized as a means of enhancing strength properties by Bolaski *et al*.[22] The combined effluent generated at the end of the deinking trial under optimum conditions showed an increase in total solids, BOD and COD, which might be due to the hydrolysis of fibrils attached with ink particles and the removal of additives/contaminants added during stock preparation of SOP. Magnin *et al*. also experienced an increase in COD as a result of the enzymatic treatment, which might be due to the hydrolytic property of the enzymes. The increase in COD as a result of the enzymatic treatment was due to the release of soluble sugars from the pulp.[23]

Table 1 shows the effect of cellulase (*A. niger* AT-3) and xylanase (*C. cinereus* AT-1) at different doses on the deinking efficiency of SOP. At an equivalent dose of cellulase and xylanase (6 IU/mL each) during the enzymatic treatment in the presence of surfactant, pulp brightness, $D_B$ and $D_E$ improved by 11.20, 31.83 and 81.20%, compared to the control, and by 5.06, 8.73 and 4.9%, respectively, compared to cellulase treatment alone. Conversely, ERIC values and dirt counts reduced by 65.95 and 83.00%, compared to the control, and by 4.11 and 0.71%, respectively, when compared to cellulase treatment alone. On the other hand, all the mechanical strength properties (burst index, tensile index and double fold numbers) decreased while tear index increased as a result of cellulase and xylanase treatment. Total solids, COD and BOD of the combined effluent increased when compared to cellulase treatment alone. At the

same time, tensile and burst indexes showed an improvement, compared to the control, while double fold numbers and tear index slightly decreased. Enzyme treatment hydrolyzed xylan (low molecular weight) from the pulp and resulted in an increase in the average molecular weight of the polymer system. Therefore, tear index slightly improved, whereas other properties, like burst and tensile indexes, depending upon hydrogen bonding, decreased due to depolymerization of xylan.[24] Waste paper recycling resulted in a decrease in fiber bonding, fiber flexibility and conformability. Therefore, burst strength, zero span tensile, tensile strength and double fold decreased, whereas tear strength increased, due to the effect of drying on fiber stiffness, compared to virgin pulp.

At half dosing of cellulase and xylanase treatments (3 IU/mL each), pulp brightness, $D_B$ and $D_E$ were found to increase by 8.23, 23.39 and 74.3%, respectively, compared to the control. ERIC value and dirt count decreased by 60.48 and 82.05%, respectively. At the same time, all these parameters were found to decrease in comparison with enzyme concoctions of 6 IU/mL each. Similarly, cellulase and xylanase treatments showed a reduction in all the studied mechanical strength properties, which might be due to an incomplete removal of ink particles and a lower hydrolysis of fines. It was validated by the reduction in total solids, COD and BOD, which were lower compared to full dosing of cellulase and xylanase during deinking.

Yet, another enzymatic deinking trial was carried out using cellulase and xylanase combinations of 6 and 3 IU/mL, respectively. An increase in ERIC values, dirt counts, pulp brightness, $D_B$ and $D_E$, pulp freeness, pulp viscosity, mechanical strength properties, total solids, COD and BOD of the combined effluent generated during deinking was observed, compared to the results obtained with half dosing of the enzyme concoctions. The best results were, however, obtained with full enzyme dosing (i.e. 6 IU/mL for each, cellulase and xylanase). The enzymatically (cellulase+hemicellulase) deinked pulp gave higher brightness, improved physical properties and lower ERIC than the pulps deinked with each individual enzyme.[25] The highest deinking efficiency, of 62%, was obtained using the cellulase–hemicellulase systems during enzymatic deinking of laser printed office waste papers.[2] Taleb and Maximino investigated the effect of pergalase A-40 (a mixture of cellulase and hemicellulase) treatment on cellulosic fibers and observed that treated pulp showed higher tensile value with reduced tear index.[26]

Since starch was widely present in mixed office waste, being used both as a surface-sizing agent and wet-end additive, its degradation was very likely to aid cellulase assisted deinking. Amylase enzyme influenced the degradation of the starch layer on the surface of the papers. The toner particles adhering to the paper surface were released by the enzymatic treatment and subjected to subsequent separation from the pulp suspension *via* flotation. Following this concept, SOP pulp was treated with different doses of amylase and fixed doses of cellulase and xylanase and the effect of the treatment on deinking efficiency was studied. Cellulase, xylanase and amylase charged at 6, 3 and 6 IU/mL doses, respectively, during enzymatic treatment improved brightness, $D_B$ and $D_E$ by 11.6, 33.25 and 82.5%, compared to the control and these parameters improved by 1.90, 5.69 and 4.40%, respectively, compared to cellulase and xylanase (6 and 3 IU/mL) treatments (Table 1). Alike, ERIC values and dirt counts mitigated by 67.05 and 86.81%, respectively, compared to the control and by 5.21 and 4.52%, respectively, compared to cellulase+xylanase (6 and 3 IU/mL) treatments, respectively. All the mechanical strength properties, except tear index and effluent characteristics (total solids, COD and BOD), decreased compared to the control, as well cellulase and xylanase treatments.

In another set of experiments, the dosages of cellulase and xylanase were kept constant as above, while amylase dosage was reduced from 6 to 3 IU/mL; it showed insignificant reductions in brightness, $D_B$ and $D_E$, ERIC value, dirt count, effluent characteristics and physical strength properties. Further, a reduction in amylase dosage from 6 to 1.5 IU/mL showed reductions in all the parameters as stated above. Concoctions of three different enzymes, where cellulase and xylanase dosages were kept constant and amylase dosage reduced from 6 to 3 and 1.5 IU/mL respectively, brightness, $D_B$ and $D_E$ showed improvements, while ERIC value and dirt count showed reduction, compared to the results for concoctions of cellulase and xylanase treatment (6 and 3 IU/mL).

Zollner and Schroeder reported the use of α-amylase to deink white office papers and obtained a substantial increase in toner removal, suggesting that a new enzymatic approach could be useful.[6] Mixed office waste often contained starch and

therefore, the α-amylase increased the efficiency of the deinking treatment by degrading the starch layer on the surface of the paper. Experimental results indicated that by adding amylase enzyme to a cellulase-assisted deinking process, it was possible to improve significantly the dirt removal by flotation. Zhenying *et al.* noted that a concoction of cellulase and amylase (1:1.5) had the best deinking efficiency with 12% increment in brightness under optimal conditions of deinking, as stated in Table 1.[27]

Printing inks containing vegetable oil-based ink binders could only be degraded by lipase.[28] Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are enzymes catalyzing the hydrolysis of acyl glycerols at the interface of oil and water.[7] The deinking of other types of paper was also found to increase by a treatment with lipases and esterases, owing to an enzymatic hydrolysis of the oil-based binder or the resins in the ink. Moreover, lipases had a surfactant effect due to their amphoteric properties and thereby facilitated the deinking of recovered paper. With this objective, SOP pulp was treated with concoctions of cellulase (6 IU/mL), xylanase (3 IU/mL), amylase (1.5 IU/mL) and lipase dosages varied from 1.5 to 6 IU/mL (Table 1). Cellulase, xylanase, amylase and lipase at a dosing of 6, 3, 1.5 and 6 IU/mL, respectively, increased the pulp brightness, $D_B$ and $D_E$ by 13.3, 37.79 and 83.00%, compared to the control, and by 2.58, 7.10 and 0.7%, respectively, compared to the concoction of cellulase, xylanase and amylase in the ratio of 6, 3 and 1.5 IU/mL. Quite the reverse, ERIC values and dirt counts mitigated by 68.17 and 88.03%, compared to the control and by 1.95 and 1.39%, respectively, compared to a mixture of cellulase, xylanase and amylase.

The introduction of lipase into the mixture mitigated all the mechanical strength properties, except tear index, and on the contrary, increased total solids, COD and BOD, compared to the control, as well as the concoction of cellulase, xylanase and amylase (6, 3 and 1.5 IU/mL). Further, lipase dosages were reduced to 3 and 1.5 IU/mL, respectively, while keeping other enzyme concentrations constant. Minimum deinking efficiency was found when lipase concentration was decreased to 1.5 IU/mL. The increase in COD and BOD of the combined bleach effluent was due to the hydrolysis of xylan, cellulose, starch and fatty acids attached to ink particles by xylanase,[29,30] cellulase,[23] amylase[6] and lipase.[7]

Previous studies showed that a treatment of paper printed with soya bean oil based ink with enzyme preparations containing cellulases, xylanases and lipases, in addition to a neutral surfactant, resulted in decreased dirt counts and residual ink areas.[28] A mixture of cellulase, hemicellulase and lipase gave a deinking efficiency of 55-56%.[2] The mixture displayed the best synergistic performance observed for increased breaking length, burst index and tear index values of the deinked pulp when compared with the cellulase/xylanase deinked pulp. The increasing deinking efficiency of the enzyme concoctions used on SOP pulps was summarized as follows:

Control < C (6 IU/mL) < CX (3 and 3 IU/mL) < CX (6 and 3 IU/mL) < CX (6 and 6 IU/mL) < CXA (6, 3 and 1.5 IU/mL) < CXA (6, 3 and 3 IU/mL) < CXA (6, 3 and 6 IU/mL) < CXAL (6, 3, 1.5 and 1.5 IU/mL) < CXAL (6, 3, 1.5 and 3 IU/mL) < CXAL (6, 3, 1.5 and 6 IU/mL).

**Deinking model equations and statistical analysis**

The following empirical equations were obtained by using the experimental data from the nonlinear polynomial regression analysis program to predict the $D_B$ and $D_E$:

$Y_1 (D_B) = 0.141x^3-2.974x^2+20.33x-4.866$ [3]

$Y_2 (D_E) = 0.417\ x^3-8.903x^2+58.64x-29.32$ [4]

variables: x = Enzyme combinations, $y_1$ and $y_2$ = $D_B$ and $D_E$, respectively

In the case of $D_B$: R = 0.94306469, $R^2$ = 0.88937100, Adjustable $R^2$ = 0.84195858, Standard error of estimate = 4.1088, Durbin-Watson statistics = 2.3920, Constant variance test: (P = 0.0290), Power of performed test with α = 0.0500: 0.9988, where R is the regression coefficient.

In the case of $D_E$: R = 0.89247699, $R^2$ = 0.79651519, Adjustable $R^2$ = 0.70930741, Standard error of estimate = 13.1889, Durbin-Watson statistics = 2.0739, Constant variance Test: Passed (P = 0.3535), Power of performed test with α = 0.0500: 0.9820, where R is the regression coefficient.

As the value of $R^2$ was above 0.80 and less than 1.0, i.e. 0.889 and 0.796 for $D_B$ and $D_E$ respectively, it means that the predicted values of $D_B$ and $D_E$ gave minimum regression errors up to a 3rd order of polynomial regression analysis.

Table 1
Effect of different enzyme combinations during enzymatic deinking of sorted office paper

| **Particulars** | **Results after pulping*** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Brightness, % | 61.80±0.85 | | | | | | | | | | |
| ERIC, ppm | 283.20±6.9 | | | | | | | | | | |
| **Enzyme treatment stage | Control | Cellulase | Cellulase+Xylanase | | | Cellulase+Xylanase+Amylase | | | Cellulase+Xylanase+Amylase+Lipase | | |
| | | 100 (6 IU/mL) | 100+50 (6+3 IU/mL) | 50+50 (3+3 IU/mL) | 100+100 (6+6 IU/mL) | 100+50+25 (6+3+1.5 IU/mL) | 100+50+50 (6+3+3 IU/mL) | 100+50+100 (6+3+6 IU/mL) | 100+50+25+25 (6+3+1.5+1.5 IU/mL) | 100+50+25+50 (6+3+1.5+3 IU/mL) | 100+50+25+100 (6+3+1.5+6 IU/mL) |
| **Results after ink flotation*** | | | | | | | | | | | |
| Total pulp yield, % | 82.80±1.4 | 80.10±2.0 | 79.25±1.7 | 79.15±1.2 | 79.00±1.4 | 78.80±1.0 | 78.10±1.3 | 77.79±1.4 | 78.25±1.4 | 78.00±1.2 | 77.75±1.3 |
| Brightness, % | 64.80±0.90 | 72.93±0.96 | 74.50±1.0 | 73.03±0.98 | 76.00±1.4 | 75.52±1.1 | 75.60±1.2 | 76.4±1.3 | 76.93±1.2 | 77.03±1.4 | 78.10±1.5 |
| Deinkability ($D_B$), % | 8.52±0.15 | 31.62±0.40 | 36.08±0.44 | 31.91±0.39 | 40.35±0.48 | 38.98±0.47 | 39.21±0.46 | 41.77±0.50 | 42.99±0.52 | 43.27±0.53 | 46.31±0.55 |
| ERIC, ppm | 276.23±8.74 | 105.39±6.5 | 100.80±4.8 | 109.15±5.6 | 94.05±5.0 | 91.75±4.2 | 91.60±4.4 | 91.00±4.0 | 90.17±2.9 | 88.65±3.1 | 87.90±3.0 |
| Deinkability ($D_E$), % | 5.0±0.10 | 81.3±0.85 | 83.1±0.87 | 79.3±0.84 | 86.2±0.89 | 87.2±0.90 | 87.3±0.91 | 87.5±0.92 | 87.6±0.94 | 87.8±0.96 | 88±0.99 |
| Dirt count, $mm^2/m^2$ | 20181±65 | 3573±27 | 3360±28 | 3622±24 | 3130±26 | 2808±16 | 2714±15 | 2660±19 | 2610±20 | 2505±23 | 2414±14 |
| CSF, mL | 510±2.0 | 560±3.0 | 556±3.0 | 550±4.0 | 565±3.0 | 558±3 | 560±4.0 | 563±2 | 560±3.0 | 563±4 | 566±4 |
| Pulp viscosity, $cm^3/g$ | 422.30±5.9 | 450.20±3.9 | 480.00 | 471.4±4.6 | 500.68 | 528±5.0 | 529.2±5.1 | 530±5.3 | 532.7±6.2 | 533±4.7 | 536±4.9 |
| **Characteristics of effluent** | | | | | | | | | | | |
| Total solid, mg/L | 1.42 | 1.60 | 1.62 | 1.62 | 1.65 | 1.72 | 1.73 | 1.77 | 1.80 | 1.81 | 1.83 |
| COD, kg/tonne | 23.4 | 65.10 | 65.70 | 65.50 | 66.10 | 65.80 | 66.06 | 66.20 | 66.40 | 66.50 | 67.22 |
| BOD, kg/tonne | 8.0 | 25.66 | 25.85 | 25.10 | 26.30 | 26.10 | 26.25 | 26.33 | 26.30 | 26.35 | 26.40 |
| **Strength properties** | | | | | | | | | | | |
| Tensile index, Nm/g | 22.44±1.1 | 24.75±1.6 | 24.40±1.2 | 23.10±1.4 | 24.12±1.3 | 23.50±1.8 | 23.13±1.9 | 23.00±1.6 | 23.45±1.5 | 23.40±1.3 | 23.05±1.5 |
| Tear index, $mNm^2/g$ | 6.73±0.27 | 6.00±0.23 | 6.15±0.19 | 6.25±0.16 | 6.35±0.18 | 6.85±0.40 | 7.05±0.44 | 7.12±0.37 | 7.25±0.32 | 6.88±0.30 | 6.86±0.33 |
| Burst index, $kPam^2/g$ | 0.87±0.09 | 1.31±0.13 | 1.26±0.12 | 1.17±0.09 | 1.2 1±0.10 | 1.22±0.14 | 1.20±0.12 | 1.18±0.12 | 1.21 ±0.14 | 1.20±0.13 | 1.18±0.10 |
| Double fold, number | 7 | 6 | 5 | 6 | 7 | 5 | 8 | 6 | 7 | 7 | 6 |

± refers to standard deviation,

*Pulping conditions:

| | |
|---|---|
| Pulping time, min | = 20 |
| Surfactant (Oleic acid) dose, % | = 0.05 |
| Temperature, $^0C$ | = 65±2 |
| Consistency, % | = 12% |
| pH | = 7.2±2 |

**Enzymatic treatment:

| | |
|---|---|
| Reaction time, min | = 60 |
| Surfactant (Tween 80) dose, % | = 0.1 |
| pH | = 5.3±2 |
| Consistency, % | = 12 |
| Temperature, $^0C$ | = 55±2 |

***Flotation conditions:

| | |
|---|---|
| Consistency, % | = 1 |
| Temperature, $^0C$ | = 35±2 |
| pH | = 7.2±2 |
| Flotation time, min | = 10 |

Further, normality test was performed for the validation of results.

Normality test for $D_B$:
Enzyme combinations: W-statistics = 0.968
P = 0.870 Passed
$D_B$: W-statistics = 0.757
P = 0.632 Passed

Normality test for $D_E$:
Enzyme combinations: W-statistics = 0.968 P = 0.870 Passed
$D_E$: W-statistics = 0.442
P = 0.312 Passed
where P indicates normal distribution coefficient.

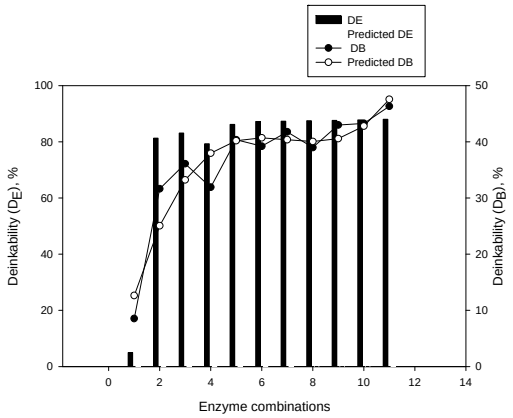


Figure 1: Effect of various enzyme doses on experimental and predicted deinkability factors ($D_E$ and $D_B$) during enzymatic deinking of SOP: 1 – control, 2 – cellulase (6 IU/mL), 3 – cellulase+xylanase (6+3 IU/mL), 4 – cellulose+xylanase (3+3 IU/mL), 5 – cellulose+xylanase (6+6 IU/mL), 6 – cellulose+xylanase+amylase (6+3+3 IU/mL), 7 – cellulose+xylanase+amylase (6+3+1.5 IU/mL), 8 – cellulase+xylanase+amylase (6+1.5+1.5 IU/mL), 9 – cellulase+xylanase+amylase+lipase (6+3+1.5+1.5 IU/mL), 10 – cellulase+xylanase+amylase+lipase (6+3+1.5+3 IU/mL) and cellulase+xylanase+amylase+lipase (6+3+1.5+6 IU/mL)]

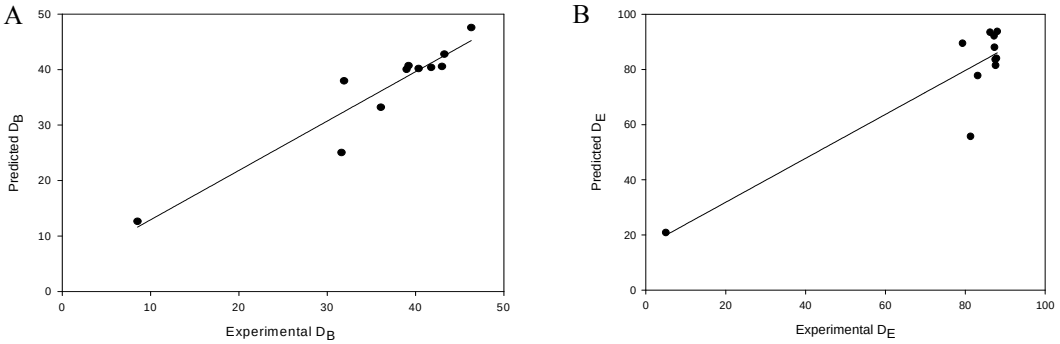


Figure 2: Plots of predicted against experimental values due to variation in mixed enzyme doses for the validation of: (A) predicted deinkability factor ($D_B$) and (B) experimental deinkability factor ($D_E$)

The lower values of P compared to W-statistics pass the normality test. The predicted values of $D_B$ and $D_E$ were plotted in Figure 1, which shows a reasonable fit for the data. The curves were plotted between the experimental values of $D_B$ against the predicted value of $D_B$ and experimental values of $D_E$ against the predicted value of $D_E$, and illustrated a linear relationship with a minimum deviation from the tangible values (Figures 2-3).

**AFM and SEM of bio-deinked pulp**

The structural changes on cellulose surface were analyzed concomitantly with the action of different enzyme combinations. One of the features obtained from AFM measurements was the degree of roughness, which was used to analyze the changes in the surface properties brought about by friction, adhesion and biocatalytic activity during pulp deinking. Ink particles, rosin and resins being hydrophobic in

nature, were marked as dark areas, while carbohydrates, i.e. cellulose, starch and hemicelluloses, being hydrophilic, were marked as bright areas in the phase imaging [Plate1A (i)]. On the surface of SOP fibers, irregular particles or granules were seen, which might be of tertiary fines (broken microfibrils), ink particles or rosin or resins [Plate1A (ii)], which were further validated by SEM, showing the presence of small particles deposited on fiber surface [Plate1A (iv)]. The surface of untreated SOP (control) fibers showed the presence of swollen fissures, surface cracks or trenches. Plate1C (iii) illustrates that the surface roughness of SOP fibers was found to lie in the range 0-50 nm.

Chemically deinked SOP pulp showed surface irregularities that appeared as a result of dislodging of ink films from the fiber surface, which were efficiently removed from the suspension during the flotation step [Plate1B (i)]. While acting on paper fibers (making them swell), NaOH contributed to ink removal as it favored the detachment and fragmentation of the adhered ink.[31] Additionally, it might also act directly on the printed ink film and weaken its structure leading to fragmentation.[32] The topographical structure of cellulose fibers showed that the removal of paper additives (rosin, resin, polymeric ink binders etc.) increased the hydrophilic area on fiber surface, i.e. reduction in dark areas in the phase imaging as a result of chemical deinking [Plate1B (ii)]. On the other hand, the surface of chemically deinked fibers became grainy, compared to the control, which might be due to the deposition of hemicelluloses, celluloses and starches (added during stock preparation as dry strength additives), as a result of peeling reactions that occurred in the presence of NaOH. SEM also revealed irregular particles or granules present on fiber surface [Plate1B (iv)]. Surface roughness of the paper increased by 10 nm, compared to SOP pulp [Plate1B (iii)].

Surface roughness of cellulose fibers began to increase after adding cellulase during enzymatic deinking of SOP [Plate1C (i)]. These observed changes were brought about by the action of cellulase, which might constitute the first direct visualization, supporting the fact that the exocellulase selectively hydrolyzed the hydrophobic faces of cellulose. The limited accessibility of the hydrophobic faces in native cellulose might contribute significantly to the rate-limiting slowness of cellulose hydrolysis. Natural cellulose was a bundle of linear 1,4-β linked glucan chains held tightly in a crystalline structure by the cumulative effect of many inter- and intra-chain hydrogen bonds. SEM [Plate1C (iv)] demonstrates how the cellulase treatment modified the fiber surface by introducing external fibrillation, cracks, swelling and peeling and thereby making the fiber surface rougher and more heterogeneous, with small microfibrils on the surface [Plate1C (ii)]. Instead, control pulps [Plate1A (i-iv)] were smoother and cleaner with no sign of fibrillation. Non-fibrous additives, which were deposited on the surface of fibers, constituted a physical barrier for the penetration of bleaching agents after deinking. Cellulase treatment was effective in opening closed cell wall pores of pulps, as a result of the cellulose hydrolysis, which caused the hydrolysis of glycosidic linkages anywhere in the cellulose chains (micro-fibrils), affected their bonding with non-fibrous additives, their elimination facilitating the flow of bleaching agents. Commercial cellulase mixtures usually contain one or more exoglucanases, such as cellobiohydrolase (CBH), which would proceed from either the reducing end or the non-reducing end of the cellulose chain and produce a shortened chain and cellobiose. The cellulase concoction might also contain several endoglucanases (EG-I, EG-II, etc), which cleaved randomly the internal 1,4-β glycosidic bonds of the cellulose chain along its length to produce free chain ends that would be acted upon by exoglucanases.[33] The treatment with cellobiohydrolase resulted in the appearance of distinct pathways or tracks along the length of the macro-fibril. The treatment with endoglucanases appeared to cause peeling and smoothening of the fiber surface.[34] The surface roughness (70 nm) of enzymatically (cellulase) deinked fibers was 20 nm higher, compared to that of SOP fibers [Plate1C (iii)]. Presumably, the hydrophobic faces consisted of more than one cellulose chain, thus the roughness change might be indicative of the fact that the cellulose chains were hydrolyzed individually.

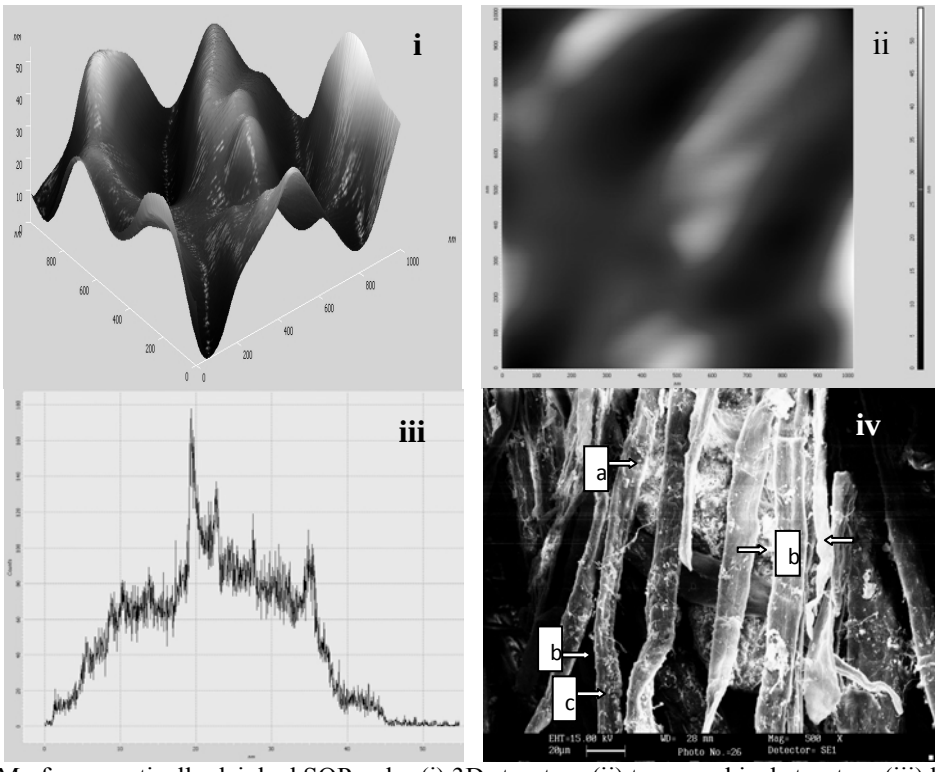


Plate 1A: AFM of enzymatically deinked SOP pulp: (i) 3D structure (ii) topographical structure (iii) histogram (iv) SEM of SOP fibers – (a) deposited non-cellulosic additives, (b) swollen fissures, (c) broken microfibrils at a magnification of 500x

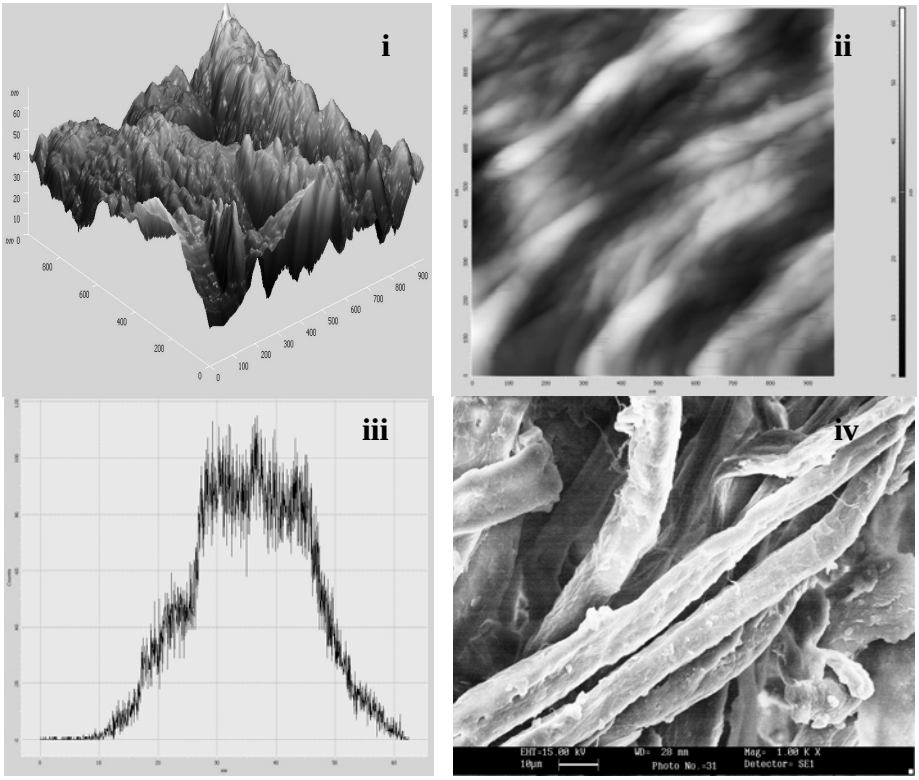


Plate 1B: AFM of chemically deinked SOP pulp: (i) 3D structure, (ii) topographical structure, (iii) histogram and (iv) SEM of chemically deinked fibers at a magnification of 1.00 KX

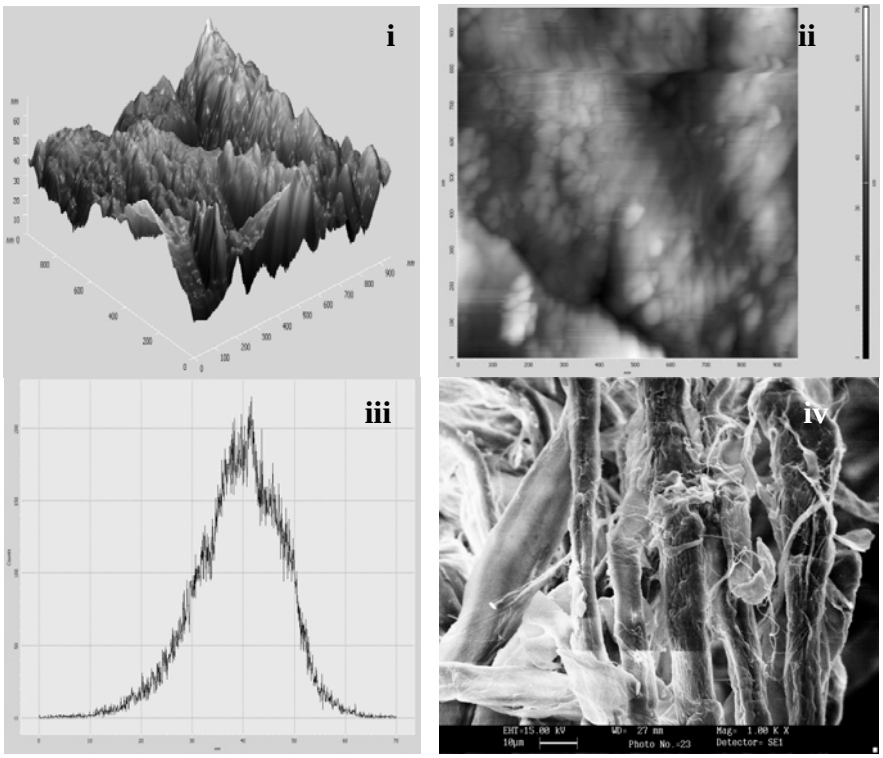


Plate 1C: AFM of cellulase deinked SOP pulp: (i) 3D structure, (ii) topographical structure, (iii) histogram and (iv) SEM of cellulase deinked fibers at a magnification of 1.00 KX

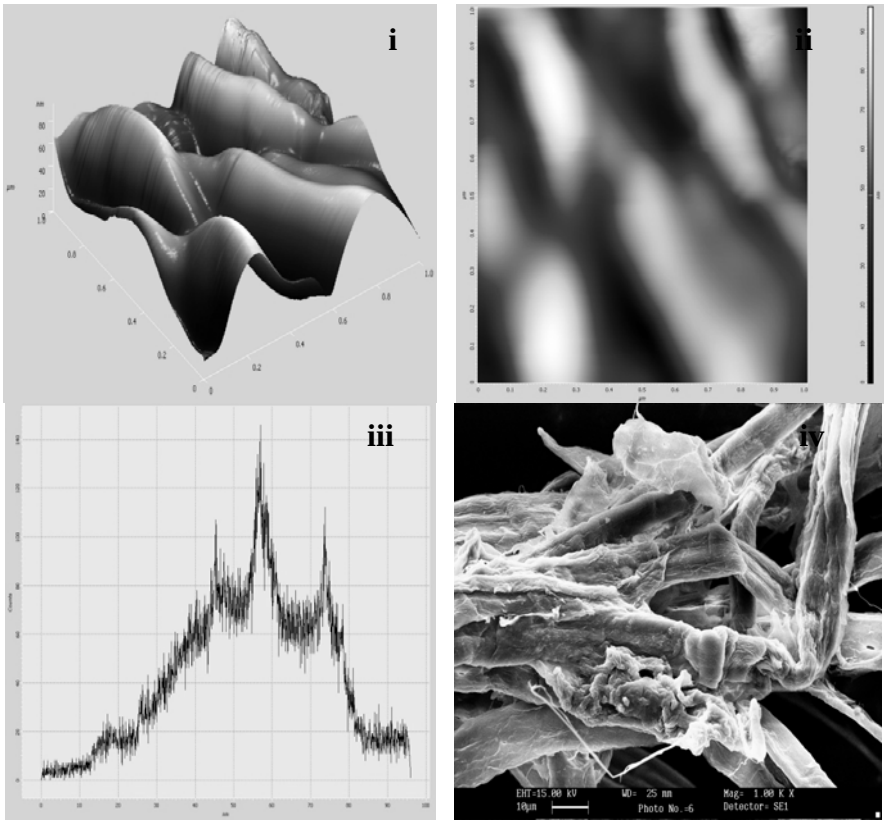


Plate 1D: AFM of enzymatically (cellulase+xylanase) deinked SOP pulp: (i) 3D structure (ii) topographical structure, (iii) histogram and (iv) SEM of cellulase+xylanase deinked fibers at a magnification of 1.00 KX

Plate 1D (i-iii) shows the AFM images of fiber surface obtained as a result of enzymatic (cellulase+xylanase) deinking of SOP pulp. Endoxylanases cleaved the internal glycosidic linkages of the heteroxylan backbone, resulting in a decreased degree of polymerization of the substrate, while β-D-xylosidases were exoglycosidases that hydrolyzed smaller xylo-oligosaccharides and xylobiose from the non-reducing ends to liberate monomeric xylose.[35] The release of xylan and the additives added during stock preparation increased the hydrophilic character of the fiber surface and the topographical structure showed many changes on the fiber surface in terms of surface roughness [Plate1D (i)], which might be due to the appearance of microfibrils on the fiber surface. A SEM study also highlights the fibrillar structure of cellulase+xylanase treated fibers and appearance of grooves and ridges, a few cracks and considerable damage to the fiber [Plate1D (iv)]. The fiber surface was less grainy [Plate1D (ii)]. Cellulase+xylanase treatment enhanced surface roughness up to 90 nm [Plate1D (iii)].

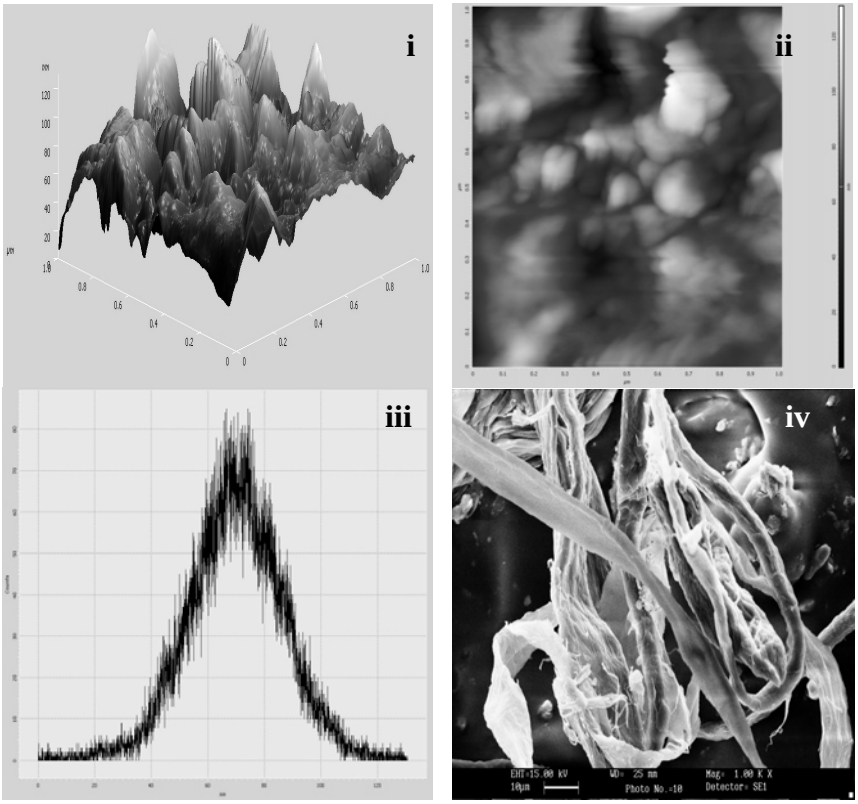


Plate 1E: AFM of enzymatically deinked SOP pulp (cellulase+xylanase+amylase): (i) 3D structure, (ii) topographical structure, (iii) histogram and (iv) SEM of cellulase+xylanase+amylase deinked fibers at a magnification of 1.00 KX

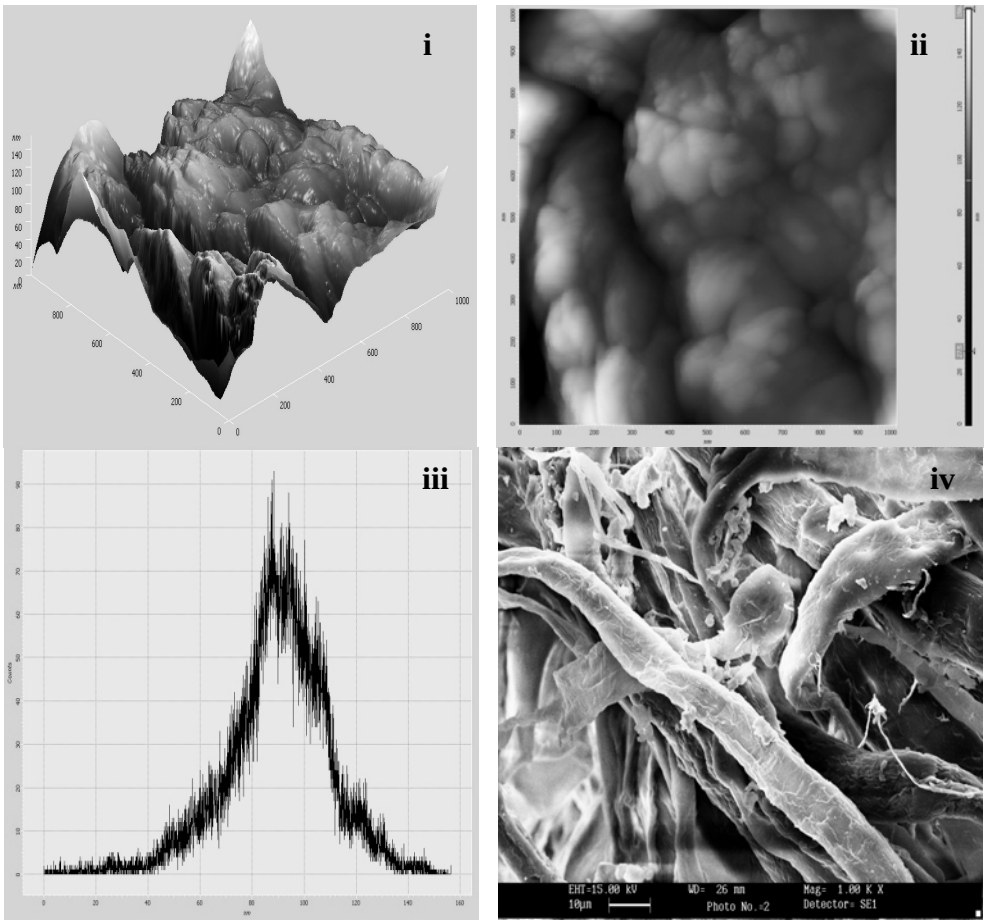


Plate 1F: AFM of enzymatically deinked SOP pulp (cellulase+xylanase+amylase+lipase): (i) 3D structure, (ii) topographical structure, (iii) histogram and (iv) SEM of cellulase+xylanase+amylase+lipase deinked fibers at a magnification of 1.00 KX

Besides cellulase and xylanase, the introduction of amylase during enzymatic deinking further led to an increase in paper roughness. Starch also formed bonds with the binders present in ink, wet strength resins, rosin, fillers, cellulose and hemicelluloses. The fiber surface was attacked by amylase to release ink particles from their surface due to solubilization of starch. α-Amylases (E.C.3.2.1.1) were endo-amylases catalyzing the hydrolysis of internal 1,4-α glycosidic linkages in starch in a random manner.[35] The AFM of cellulase, xylanase and amylase treated fibers showed that fiber surface was rough with more surface irregularities [Plate1E (i)], grainy fiber surface [Plate1E (ii)] and surface roughness increased by 120 nm [Plate1D (iii)]. SEM showed that the fibers were fibrillated with deposition of irregular particles on their surface [Plate1E (iv)]. It indicated that amylase acted in a different way than cellulase, facilitating a greater removal by flotation of smaller ink particles and showing a great deal of synergism with cellulase. Using a selected surfactant along with a cellulase/amylase mixture, the area of coverage of the residual toner particles measured by image analysis was reduced up to 96%.[36]

The introduction of lipase to the mixture of cellulase, xylanase and amylase caused the hydrolysis of oil-based binders or of the resins in the ink, thereby facilitating the deinking of recovered paperLipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) were enzymes catalyzing the hydrolysis of acyl glycerols at the interface of oil and water.[19] Plate1F (i) shows a much rougher surface containing more granular particles. It might be due to the removal of additives, which increased the hydrophilic area on the fiber surface [Plate1F (ii)]. The surface roughness increased by 159%, compared to the

control [Plate1E (iii)]. SEM confirmed the deposition of granules on the fiber surface and broken microfibrils [Plate1E (iv)].

## CONCLUSION

A concoction of cellulase, xylanase, amylase and lipase at a dosing of 6, 3, 1.5 and 6 IU/mL, respectively, increased pulp brightness, $D_B$ and $D_E$ by 13.3, 37.79 and 83.00%, compared to the control and by 5.13, 14.69 and 6.7%, respectively, compared to cellulase treatment during biodeinking of SOP. AFM and SEM studies showed a maximum surface roughness, i.e. 159% with a concoction of cellulase, xylanase, amylase and lipase, compared to the control, the surface roughness for rest of the concoctions having increased in the following descending order: control<cellulose <cellulose +xylanase< cellulose + xylanase + amylase.

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