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ORIGINAL ARTICLE

An efficient endoglucanase and lipase enzyme consortium (ELEC) for deinking of old newspaper and ultrastructural analysis of deinked pulp

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Abstract
The pulp and paper industry is looking for eco-friendly solutions in the field of enzymatic deinking for ink removal from recycled paper or pulp. In the present study, the endoglucanase and lipase enzymes obtained from *Thermomyces lanuginosus* VAPS25 were used for enzymatic deinking of old newspapers. The synergistic action of enzyme consortium on deinking efficiency was measured for the eco-friendly deinking process. The data indicated that the endoglucanase and lipase enzyme consortium (ELEC) was more effective than the individual enzyme. The deinking efficiency of 38.6% and 42.7% ISO sheet brightness of newspaper was obtained using enzyme consortium. The hand-sheet strength properties were also evidently improved. Breaking length, tensile strength, and tear index were enhanced by 10.2%, 27.6%, and 8.1%, respectively. The ultrastructural analysis of the handmade sheets provided insights into enzymes' action on pulp and paper. The enzymatically deinked pulp and paper, analyzed with the assistance of Fourier transform infrared (FTIR), Scanning electron microscopy (SEM), and X-ray diffraction (XRD) study, showed noteworthy transformation in chemical and surface structures. Using such an efficient enzyme consortium (ELEC) for deinking will help develop an eco-friendly process for waste paper recycling and its use in sustainable development of paper industry.

Keywords *Thermomyces lanuginosus* VAPS25 · Endoglucanase and lipase enzyme consortium (ELEC), Enzymatic deinking · Paper recycling · Old newspaper

1 Introduction

Waste papers are now used mainly in the pulp and paper industry for paper manufacture [1]. This waste paper recycling is now considered to reduce environmental stress [2, 3]. Additionally, recycled paper is used to sustain the environment's sustainable development and mitigate landfilling costs [4, 5]. The main concern associated with paper recycling is the residual ink trapped in the paper, which decreases the paper's brightness. Consumption of secondary fibers is amplified in recent times, and deinking is a significant step for fiber recycling [6, 7].

Many chemicals are used in the conventional chemical deinking process, leading to toxic effluents generation containing many environmental pollutants. Removing these toxic chemicals from water bodies is very costly [6, 8]. Many health problems are caused by the chemical deinking based on chlorine. Therefore, enzymes are a capable substitute for the conventional deinking process to improve these circumstances. Many researchers practice enzymatic deinking due to its better efficiency in deinking, efficient ink particle detachment, and cost-effectiveness [9, 10]. Microbial enzymes are used in biological deinking and removal of toxic elements from industrial effluents, thus making it safer for the environment [9, 11–14]. Banana peel waste was used for adsorption of methylene blue after enzymatic treatment and it was observed that the

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adsorption ratio was increased to 96.5% [13]. Cellulases have been described previously to attain deinking, reducing chemical cost, enhancing ink exclusion, advancing drainage and runnability, and diminishing BOD and COD amount in used water and waste matter [15]. Lipase is utilized in the degradation of oil-based ink residues and pitch control during paper recycling. Pitch formations deteriorate the quality of paper and affect the paper machine's functioning [16]. The deinking efficiency (DE) of 86.6% and 12.9% has been reported earlier by researchers on laser-printed paper and newspaper, respectively [17]. Microbial enzymes, including xylanases, amylases, cellulases, lipases, laccase and pectinases have been applied for deinking of waste paper [18–20]. An increase of 16% in DE was observed by using the enzyme consortium obtained from *Rhizopus microsporus* AH3 by Hasanin and coworkers in 2020 [16].

Analysis of the effect of enzymatic deinking on the recycled fiber characteristics is an essential prerequisite for achieving biotechnological steps to fabricate pulp and paper [21]. Enzyme activity and productivity play a crucial role in their industrial application. *Thermomyces lanuginosus* is a thermophilic fungus and provides thermostable enzymes for industrial applications. Scientists have tried to design enzymes suitable for industrial application by multiple techniques [22–24]. In such efforts, Wei and colleagues 2021 rationally designed lipase from *T. lanuginosus*, which was stable at 80 ℃ and approximately 79% enzyme activity was retained after 12 h [25]. The present study examined the DE of endoglucanase and lipase enzymes produced by *Thermomyces lanuginosus* VAPS25 on the old newspaper. The consequences of repulping parameters like reaction time and enzyme doses on deinking efficacy, pulp brightness, and strength distinctiveness were calculated for the deinked newspaper. X-ray diffraction (XRD), Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR), and Scanning electron microscopy (SEM) were used to determine crystallinity, functional attributes and ultrastructure of the deinked newspaper pulp and paper.

2 Materials and methods

2.1 Enzyme production and assay conditions

Endoglucanase and lipase enzymes were produced from *Thermomyces lanuginosus* VAPS25 (accession no. KU366609.1) as per the conditions reported by Dixit and coworkers in 2022 [26]. Briefly, the fungus was inoculated in the minimal salt medium containing a substrate for enzyme production. Carboxymethyl cellulose was used as a substrate for endoglucanase production, while olive oil was used for lipase production. The flasks were incubated at 60℃ for 3 days, and the enzyme was harvested using a muslin cloth. The crude enzyme, after centrifugation, was subjected to partial purification using Sartorius KrosFlo TFF System KR2i (Sartorius AG, Germany) using a 30 kDa cassette and a 200 ml feed rate of the crude enzyme. The enzymes were then mixed and individually subjected for deinking, and the enzyme consortia ELEC thus obtained was used for further studies.

2.2 Old newspaper source

The old newspaper (ONP) containing 70% newsprints and 30% pamphlets (color-magazine) was utilized for the deinking procedure. The ONP was collected from the office of Avantha Centre for Industrial Research & Development, Patiala, Punjab.

2.3 Hydra-pulping process for ONP deinking

The collected ONP was manually worn to shred into tiny parts (2.5 cm^2 in size), furthermore, 300 g of oven-dried (OD) papers were kept in warm water for 24 h to get desired pulp consistency. The hydra-pulping process was carried out using endoglucanase and lipase enzyme for the deinking process as described by Dixit and coworkers in 2022 [26]. Briefly, the hydra-pulping process was carried out at various enzyme doses and reaction times at fixed 10% pulp consistency. For the enzyme dose optimization, different endoglucanase doses (ranges 0.02–0.1 IU/g OD wt. of pulp) were used with a fixed 0.1 IU/g of lipase enzyme dose at pH 8.0. The process parameters optimization for enzymatic deinking of ONP is given in the Supplementary Table 1. The pH of the pulp slurry during the hydra-pulping process was maintained with H_2SO_4/NaOH. The reaction time (30–120 min) was maintained by interrupting the hydra-pulping process (30 min) to keep the constant mechanical agitation.The tween-80 (0.05%) was used as a surfactant during the deinking process. After hydra-pulping, the pulp was transferred into a boiling water-containing container and kept for 15 min to avoid enzymatic action. The washing process followed by hydra-pulping was done to separate the large and small-sized toner particles from the pulp fibers. Control samples were run in the same conditions to compute the deinking efficiency of the enzyme and ERIC value of the paper sample.

2.4 Determination of physical and mechanical properties of pulp and hand-sheet

Physical and optical properties were analyzed using standard TAPPI methods (see Supplementary information) [26]. British hand-sheet equipment was used to make fifteen hand sheets of 70 gsm. All handmade sheets were accustomed to 27 ± 1 °C and 65 ± 5% humidity for 24 h before testing. The physical potency and optical characteristics of handmade

sheets were observed by the instruments of AB Lorentzen and Wettre Company and Elrepho 070E, respectively.

2.5 Estimation of deinking efficiency (DE)

Based on the ERIC value, the DE for ONP was evaluated using the following procedure:

$$\mathrm{DE}(\%) = \frac{EP - EFW}{EP - EB} \times 100$$

where DE stands for deinking efficiency based on ERIC value (%), EP stands for ERIC value of the pulp following pulping (prior to ink exclusion), EFW stands for ERIC value after washing processes, EB stands for non-detachable ink particles already present in the paper [27].

2.6 Analysis of structural and ultrastructural alterations in the pulp after enzymatic deinking

The ATR-FTIR (PerkinElmer, USA) and SEM were used to study the modification of surface functional groups and morphological changes of deinked pulp samples. The FTIR spectra were verified over a range of 400–4000 cm^{-1} [28].

The SEM analysis helped to scrutinize the morphological and ultrastructural modifications for the deinked pulp and control samples. The deinked pulp was air-dried and pulverized with the help of a pulverization machine. The treated and untreated samples were analyzed using SEM (JEOL, Model JSM-6100).

2.7 Crystallinity evaluation of deinked pulp

The cellulose crystallinity of deinked pulp and control were analyzed by XRD with the help of a diffractometer (XPERT-PRO). The spectra were obtained amid 10 to 70° of 2θ (scattering angle). The following equation evaluated the cellulose crystalline index (Xc) from the XRD patterns [28].

$$\mathrm{Xc} = \frac{I_{002} - I_{am}}{I_{002}} \times 100\%$$

Where I_{002} and I_{am} is the peak intensity of crystalline and amorphous phases from the (002) lattice plane.

3 Results and discussion

3.1 Effect of enzyme dose on old newspaper deinking

The consistency of pulp (10%) and accumulation of enzymes during the hydra-pulping process were constant for all the experimental sets to find the best enzyme dose as per formerly suggested reports [29]. The enzyme doses play an essential role in the degradation of cellulosic fibers and alter the physical properties of the pulp at a high dose [5]. During analysis, 0.05 IU/g of endoglucanase enzyme dose was set up considerably for deinking of ONP. At this concentration, residual ink (957.5 ppm) (Fig. 1b), brightness (41.61% ISO), and deinking efficiency (28.9%) (Fig. 1a) was observed as compared to control. The brightness (41.11% ISO) and DE (27.8%) were slightly decreased with an increase in the enzyme dose. The strength properties also deteriorated with increased enzyme dose (Fig. 2a). The independent enzyme dose such as endoglucanase (0.05 IU/g) and lipase (0.1 IU/g) was used to determine the effect of a single enzyme dose. At this point, DE (26.8% and 23.9%) and residual ink (987 ppm and 976 ppm) from endoglucanase and lipase were obtained, respectively (Fig. 1a and b). Previously, the cellulase enzyme with enzyme activity of 15 FPU/g was produced by *Aspergillus oryzae* MDU-4. This enzyme

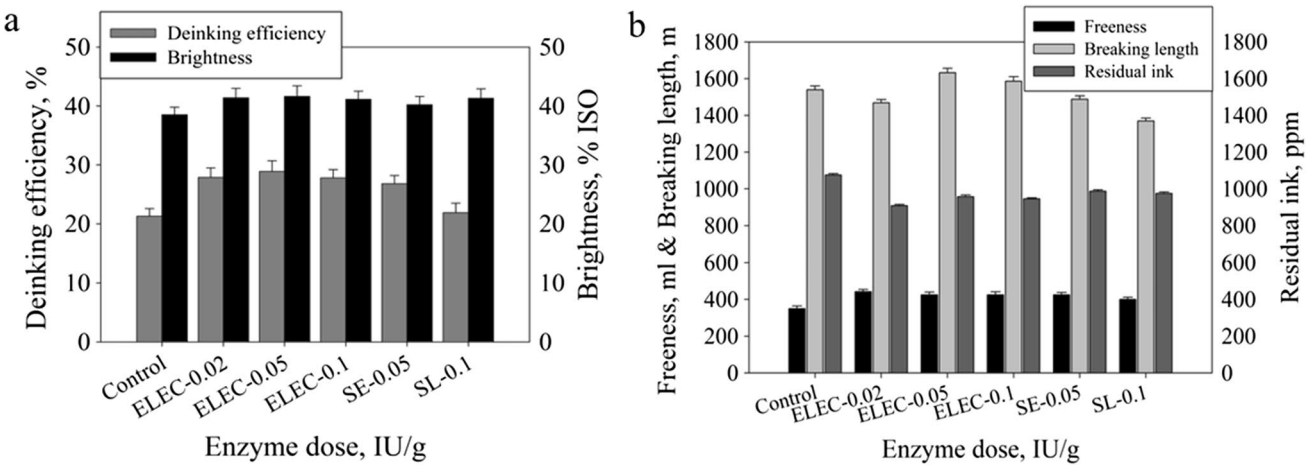

Fig. 1 Outcome of different enzyme dosages on (**a**) brightness (ISO) and deinking efficiency; (**b**) freeness, breaking length, and residual ink of ONP (ELEC refers to enzyme consortium; SE refers to single endoglucanase and SL refers to single lipase)

has been reported for efficient newspaper deinking at a treatment time of 2 h [28]. Xu et al. (2011) demonstrated the use of cellulase enzyme dose (300 U/kg) for deinking of ONP. They added the enzyme at an initial stage, at pH 7, and incubated at 50 °C up to 3 h [30]. It was reported that high enzyme dosages would decrease the strength properties and increase the darkness effect, while a lower dosage of the enzyme would have a less deinking effect [31]. The significant enzyme concentration may differ depending on enzyme production resources, quality of paper, and ink used. Finally, it could be concluded from this analysis that a small range of enzyme doses can enhance paper sheets' physical and optical characteristics.

3.2 Effect of reaction time on old newspaper deinking

To select the optimal time of reaction, all the trials were performed using 0.05 IU/g of endoglucanase enzyme addition all through the hydra-pulping and fixed pulp consistency (10%) at reaction time variations (altering from 30 to 120 min). The maximum DE (38.6%), minimum residual ink (879.3 ppm), and maximum brightness (42.7% ISO) were obtained at 90 min of reaction time (Fig. 2a and b). The reaction time of 30 min was not suggested for the enzyme-based deinking due to deterioration in the measurable paper properties. The DE (25.9% at 30 min, 28.9% at 60 min, 38.6% at 90 min and 21.4% at 120 min) was increased while increasing the reaction time, but it diminished at high reaction time, while brightness was increased on increasing the reaction time (Fig. 2a) at the optimized endoglucanase dose. The residual ink particles 954.0 ppm, 957.5 ppm, 879.3 ppm, and 962.2 ppm were obtained at 30 min, 60 min, 90 min, and 120 min of reaction time, respectively. The longer pulping process is also not suitable because the ink particles redeposit on pulp fibers during elongated reaction time [1]. Kumar and coworkers used bacterial cellulase-xylanase enzyme dosage at 10% for 1 h reaction time and observed a significant deinking process [32]. In the present study, 90 min of reaction time was reported to be the most favorable due to maximum DE and brightness, minimum residual ink, and increased strength properties of the handsheets.

3.3 Deinking efficiency of enzyme consortia (ELEC)

The maximum DE was obtained at an endoglucanase dose of 0.05 IU/g and 90 min of reaction time with 28.9% and 38.6%, respectively. There was no considerable difference in DE in the enzyme dose at 0.02 and 0.1 IU/g of endoglucanase. While using single enzyme doses such as endoglucanase (0.05 IU/g) and lipase (0.1 IU/g), DE was obtained at 26.8% and 23.9%, respectively, which are lower than the consortium enzyme (ELEC) dose of endoglucanase and lipase (Fig. 1a). In the reaction time, maximum DE was attained at 90 min (38.6%) and decreased at lower and higher reaction times (Fig. 2a). At the 90 min reaction time, the remaining ink of control and treated pulp was found at 1076 ppm and 879.3 ppm, respectively (Fig. 2b). About 50% of ink particles were removed by cellulase-treated pulp [7]. Approximately 11.8% brightness of xylanase-treated old newspapers was reported earlier [32]. Pathak et al. (2011) revealed maximum deinking and minimum ink particles in just 30 min of reaction time with the treatment of fungal cellulase. This may occur as the enzymes promote the enhancement of toner hydrophobicity property by the entrapment mechanism of ink particles [33]. Table 1 describes the diverse optimal parameters obtained for maximum deinking of old newspaper.

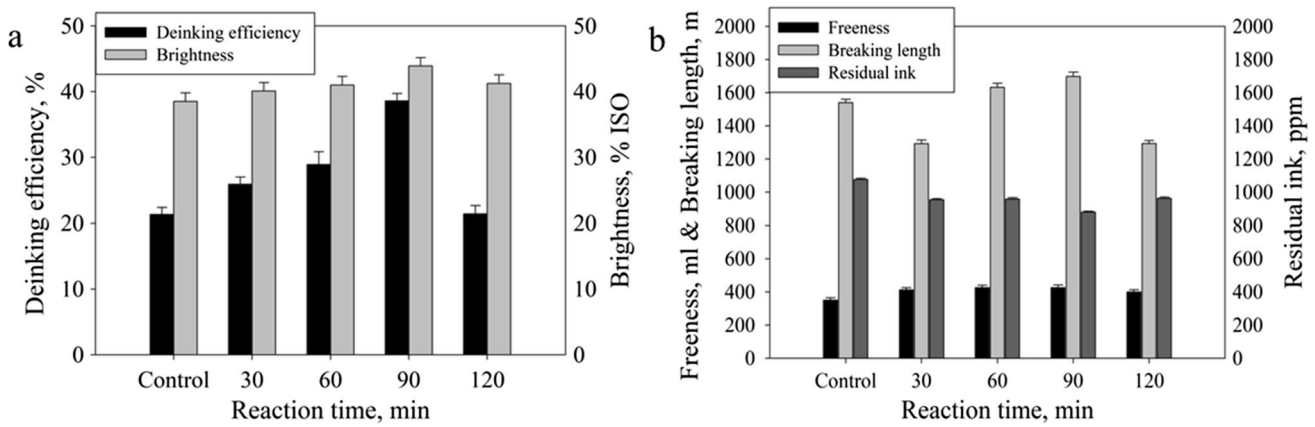

Fig. 2 Optimization of reaction time using favourable enzyme dose on (**a**) brightness and deinking efficiency; (**b**) freeness, breaking length, and residual ink of ONP

Table 1 The analysis of physical and optical features of hand sheets prepared after treatment with individual enzyme and enzyme consortium (ELEC)

Parameters / Particulars	Control	Enzyme dose, IU/g OD wt. of pulp					Endoglucanase (E) 0.05 IU/g with predetermined lipase (L) dose of 0.1 IU/g (ELEC) Treatment time, min			
		Endoglucanase (E) with predetermined lipase (L) dose of 0.1 IU/g (ELEC)			Single enzyme dose					
					Endoglucanase (SE)	Lipase (SL)				
		0.02	0.05	0.1	0.05	0.1	30	60	90	**120**
Initial ink, ppm	1367	1260	1348	1310	1349	1249	1288	1348	1431	1224
Residual ink, ppm	1076.0	908.9	957.5	946.2	987.0	976	954.0	957.5	879.3	962.2
Deinking efficiency, %	21.3	27.9	28.9	27.8	26.8	23.9	25.9	28.9	**38.6**	21.4
Brightness, % ISO	38.5	41.4	41.61	41.11	40.2	41.3	40.1	41.8	**42.7**	41.24
CSF	350	**442**	425	425	425	400	412	425	425	400
Bulk	2.07	2.06	2.25	2.14	1.58	1.67	2.11	**2.24**	2.17	2.06
Breaking length, m	1540	1469	1632	1586	1488	1370	1292	1632	**1698**	1293
Burst factor	20.1	18.5	**19.1**	18.1	17.3	16.2	17.3	19	19	15.5
Tearing index, mNm^2/g	4.57	5.52	5.68	4.99	4.93	4.89	5.10	5.65	**5.83**	4.89
Tensile strength, kN/m	1.11	1.01	1.15	1.18	1.03	0.95	0.92	1.15	**1.20**	0.92
Double fold	**9**	8	7	7	5	6	6	8	7	5

The bold values indicate the highest and efficient optical and physical values of deinked pulp and handmade sheets

3.4 Effect of enzyme dose and reaction time on optical properties of paper

In this work, about 42.7% ISO of brightness was attained in contrast to the control (38.5% ISO) (Fig. 2a). The brightness of sheets was almost identical in either ONP treated with an individual enzyme dose or in consortium (ELEC) (Fig. 1a). Similarly, a slight reduction in brightness was observed with decreasing and increasing the reaction time (Fig. 2a). The decline in brightness may be due to pigment present in the enzymes, which leads to the accumulation of pigments on the fiber surface. The brightness (44.0% ISO) of deinked pulp treated with cellulase-LVS enzymes has been reported. Similarly, deinked pulp treated with hemicellulase-LVS enzymes has 60.4% ISO after H_2O_2 bleaching [7]. The maximum brightness of deinked pulp was reported after 3 h of incubation [34].

3.5 Evaluation of handsheet mechanical strength properties

In the present study, the strength properties of handmade sheets were improved at optimized dose and reaction time. At the enzyme dose, breaking length, tear index, tensile strength was improved by 5.97% (Fig. 1b), 24.28%, 3.60%, respectively (Fig. 3a). In comparison, the bursting factor

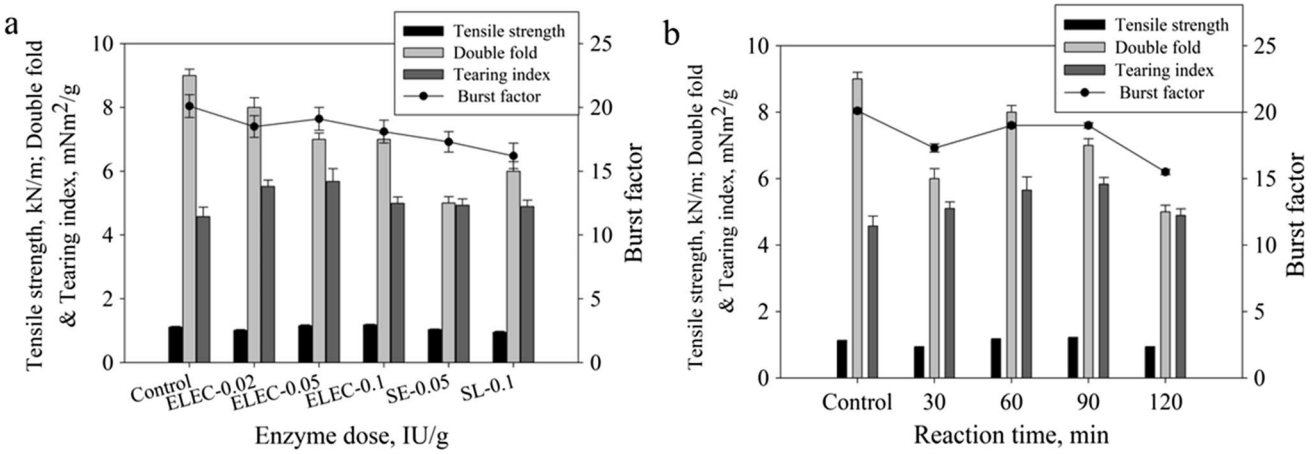

Fig. 3 Effect of (**a**) enzyme dosages (ELEC refers to enzyme consortium; SE refers to single endoglucanase and SL refers to single lipase) and (**b**) reaction time on strength properties of handmade sheets from deinked ONP pulp

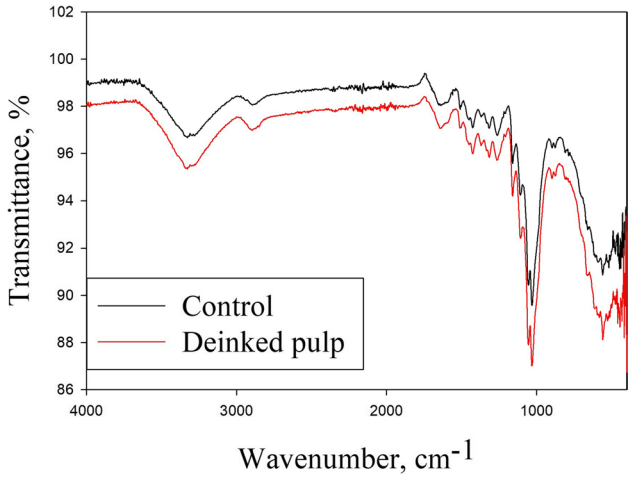

Fig. 4 The functional groups' analysis using ATR-FTIR (**a**) control and (**b**) deinked ONP sample

was decreased by 4.97% as compared to control (Fig. 3a). In the optimized reaction time, breaking length, tear index, tensile strength were improved by 10.25% (Fig. 2b), 27.57%, 8.10%, respectively (Fig. 3a). In comparison, the bursting factor was decreased by 5.47% as compared to the control (Fig. 3b). Freeness was also improved to about 75 ml and 50 ml at optimized enzyme dose and independent lipase dose, respectively, compared to control (Fig. 1b). With increased reaction time, the freeness was also improved (Fig. 2b). The improvement in the pulp freeness can promote paper mill production by enhancing the machine speed rate, thus producing paper with better quality [35]. There was a gradual reduction in the strength properties at the high reaction time. The improvement in the handsheet strength attributes can also show enhanced inner and surface fibrillation of pulp fibers through the enzymatic action of ONP pulp. According to Xu et al. (2011), the breaking length of LVS-deinked pulp was reduced for cellulase deinked pulp. Previously, many researchers reported an improvement in the physical properties of handsheets [7, 24, 35].

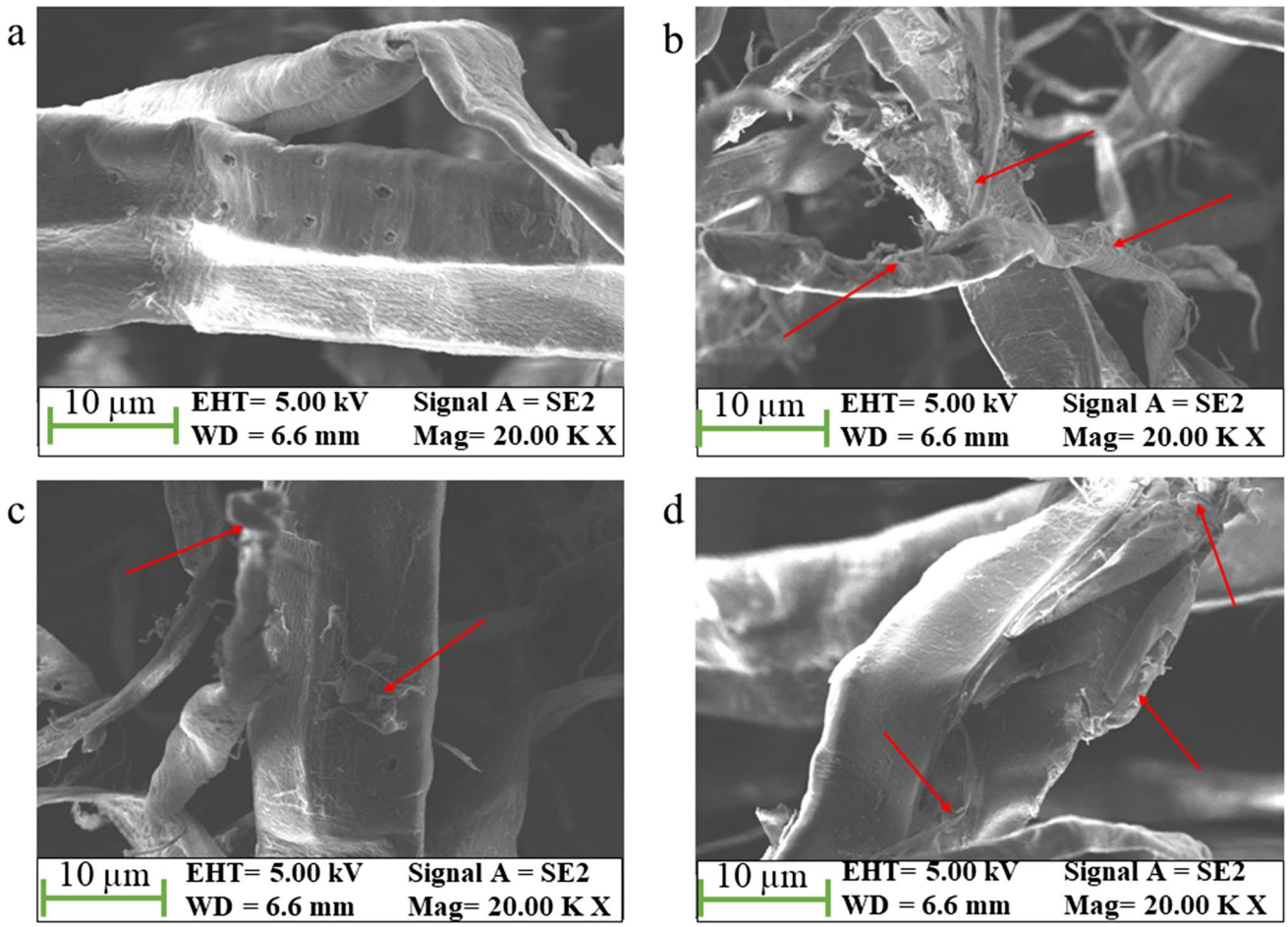

Fig. 5 Scanning electron microscopy photographs: (**a**) Control showing ink particles attached with surface fibers and no fibrillation appeared (20KX); (**b**-10KX; **c**-15KX and **d**-20 K X) Deinked ONP treated with endoglucanase (0.05 IU/g)for 90 min reaction time (Arrow showing disconnection of toner particles along-with some fibrillation and perforation)

3.6 Structural and ultrastructural study of deinked pulp

ATR-FTIR revealed the changes in functional groups of deinked and control samples (Fig. 4). The results obtained were similar to the previous work reported by Xu and coworkers [36, 37]. The peak between 3600 and 3200 cm^{-1} represents the hydroxyl group of cellulose [38]. The ink particles were represented by peak at 3373 cm^{-1}, which was greatly reduced in the enzyme-treated deinked ONP sample compared to the control. The elevation at 2851 and 2920 cm^{-1} represents the oil stuff present in ink particles. These peaks were slightly reduced in the deinked sample due to the mode of action of the lipase enzyme used in combination with endoglucanase. The peaks from 1400 to 1750 cm^{-1} depict the presence of the lignin component.

The peak intensity at 1715 and 1680 cm^{-1} was observed low in deinked pulp, representing the carbonyl group of saturated open-chain and conjugated ketone, respectively [39]. The removal of methoxyl group was suggested by the reduction in peak intensity at 1457 cm^{-1} [40]. Additionally, a decrease in peak intensity at 1270 cm^{-1} in deinked pulp confirms the cellulose degradation [41]. Similar results were observed in another study [28]. Ultimately, with these observations, it can be said that changes in the functional groups established the considerable ONP deinking compared to the control samples.

The scanning electron microscopy provided the changes that occurred in surface fiber properties during deinking (Fig. 5). The results indicated the crack formation and perforation in deinked pulp fibers which facilitated ink disconnection and removal of small fibrils. Fibrillation confirms the breakdown and exclusion of ink particles from the pulp surface [33]. The SEM analysis of the enzyme-treated ONP pulp provided results similar to previous works [28, 42].

3.7 Cellulose crystallinity index (CCI) measurement of deinked pulp

The XRD analysis provided information about the cellulose crystallinity of enzyme-treated deinked ONP and the control sample. It was found that there was only a slight reduction in crystallinity index as compared to the control one. The CCI decreased from 70.84 (control) to 66.27% for the enzyme-treated ONP (Fig. 6). The same study was reported by Virk et al., in 2013 when, laccase and cellulase enzyme were used for the deinking process of ONP [34]. This study was also supported by the report of Efrati et al. (2013) during the biobleaching process using cellulase enzyme [42]. The results indicate the deterioration of fibers as compared to an untreated pulp with minor removal of crystalline cellulose content [43].

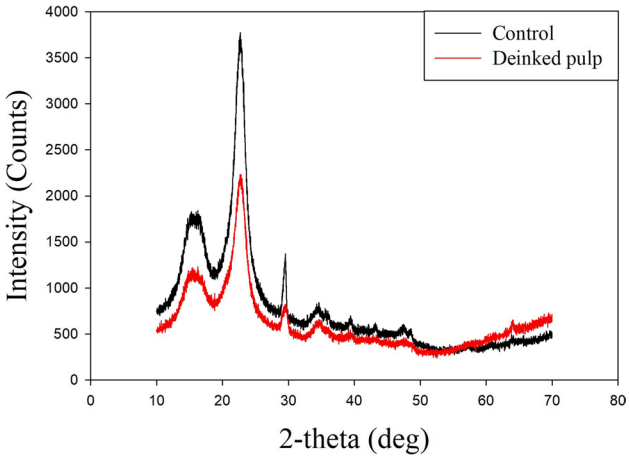

Fig. 6 X-ray diffraction study of enzymatically treated ONPwith respective control

4 Conclusions

The enzyme consortia (ELEC) achieved about 38.6% deinking efficiency with 10% pulp consistency. The optimum time of reaction obtained was 90 min with fixed enzyme dose of lipase, i.e., 0.1 IU/g and 0.05 IU/g endoglucanase in ELEC consortium. This enzyme consortium (ELEC) also concurrently improved the optical and strength characteristics, escalating the pulp's freeness characteristics. Ultimately, it can be considered that the enzyme preparation with endoglucanase and lipase enzyme consortium (ELEC) of *T. lanuginosus* VAPS25 can be an effective environment beneficial substitute to the conservative chemical deinking procedure and can be further tested at a large scale in the paper recycling industry.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13399-022-03310-6.

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Author contribution **Pratyoosh Shukla** contributed to the conceptualisation.

Mandeep Dixit, Guddu Kumar Gupta, Puneet Pathak contributed to analysis, investigation, and writing—original draft preparation;

Pratyoosh Shukla, Nishi K. Bhardwaj contributed to writing—review and editing;

Pratyoosh Shukla, Nishi K. Bhardwaj contributed to funding acquisition and supervision.

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Declarations

Ethics approval This manuscript does not contain any studies with human or animal participants performed by any of the authors.

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