The Enzymatic Deinking of Waste Papers by Engineered Bifunctional Chimeric Neutral Lipase – Endoglucanase

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Endoglucanase and lipase showed good deinking efficiency for waste papers. The performances could be greatly improved further by the combined use of the two enzymes. To reduce the enzyme production cost and enhance synergistic action of endoglucanase and lipase on laser-printed paper and newspaper, a chimeric enzyme with endoglucanase and lipase activity was constructed and expressed in *Pichia pastoris*. The data indicated that the chimera Lip-EG1CD improved the ink removal efficiencies and sheet brightness better than a single enzyme or a mixture of two enzymes. The chimera Lip-EG1CD demonstrated an 89% removal of toner on both papers and 91% ISO and 60% ISO sheet brightness for laser-printed paper and newspaper, respectively. Handsheet strength was also clearly improved. It revealed that the combined deinking of endoglucanase and lipase on waste papers could be strengthened by constructing proper chimera due to intramolecular synergistic action. This would be useful for developing an economical process for waste paper recycling.

Keywords: Endoglucanase; Lipase; Chimeric enzyme; Synergistic action; Laser-printed paper; Newspaper; Flotation deinking

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INTRODUCTION

Waste papers are now the major fiber resources for paper manufacturers in China and other countries (Vyas and Lachke 2003). In the waste paper recycling process, the printed ink in the paper needs to be removed by a deinking process. Deinking is mainly comprised of two general procedures, including the release of ink particles from the fibre surface using chemical or enzymatic methods, and the removal of released ink from the pulp slurry by washing or flotation (Singh *et al*. 2012). During conventional chemical deinking, large amounts of chemicals, such as sodium hydroxide, sodium carbonate, sodium silicate, hydrogen peroxide, surfactants, and chelating agents, are used by manufacturers, which leads to the generation of hazardous effluents (Heise *et al*. 1996; Zollner and Schroeder 1998). Nevertheless, the printed ink on laser-printed paper is still difficult to remove by conventional methods due to differences in the ink formulation (Shrinath *et al*. 1991). Compared with conventional chemical deinking, enzymatic deinking technology provides a more environmentally friendly alternative in waste paper recycling and has attracted a great amount of attention in recent years (Pala *et al*. 2004). Previous reports had demonstrated the successful use of various enzymes for waste paper deinking, like cellulases, xylanases, pectinases, amylases, lipase, and laccases (Bajpai 1999; Zhang *et al*. 2008; Nyman and Hakala 2011; Pathak *et al*. 2011, 2014; Zhang *et al*. 2017). Among the different enzymes assayed, endoglucanase has shown the most promising results for the deinking of different waste papers (Gübitz *et al*. 1998; Marques

et al. 2003; Pelach *et al*. 2003; Vyas and Lachke 2003). It attacks the fiber-ink bonding regions and hydrolyzes cellulose micro-fibrils, which consequently dislodges the inks by peeling off fibres or fines on the paper surfaces. The release of oil-carrier-based inks can be boosted by the simultaneous action of lipases or esterases, due to the enzymatic decomposition of either the oil-based binder or the resins in the ink (Morkbak *et al*. 1999; Mohandass and Raghukumar 2005). In many cases, enzymatic deinking performed more effectively when combined using two or more enzymes due to the synergistic effect of enzymes in polysaccharide and ink digestion. To date, the combined enzymatic deinking using cellulases, lipases, and other enzymes has been widely demonstrated on different secondary fibers (Gübitz *et al*. 1998; Marques *et al*. 2003; Lee *et al*. 2007; Sui *et al*. 2009; Xu *et al*. 2009; Ibarra *et al*. 2012; Virk *et al*. 2013).

Although enzymatic treatment has shown some advantages over chemical deinking, further efforts are still required to prepare more cost effective enzymes for the commercial applications. Genetic engineering has been widely used to artificially fuse two or more different genes into a single open reading fragment for constructing chimeric enzymes. The chimeric enzymes could display bifunctional properties and sometimes pose kinetic advantages over a simple mixture of individual enzymes (Lee *et al*. 2011; Yang *et al*. 2016).

Thus, the use of bifunctional enzymes could reduce the number of enzymes required for deinking in the paper industry and is likely to reduce the enzyme production cost significantly, which may make the deinking process cost effective and environmentally safe. Previously, the endoglucanase and lipase genes were cloned and characterized from *Volvariella volvacea* (Ding *et al*. 2001; Zheng and Ding 2013) and *Thermomyces lanuginosus* (Zheng *et al*. 2011). Both enzymes exhibited the same optimal activities near a pH of 7.5. In this study, the authors reported the construction of a chimeric enzyme consisting of endoglucanase (EG1CD) and lipase (Lip) moieties and evaluated the bifunctional properties and potential of the chimeric enzyme in the deinking of waste laser-printed paper and newspaper.

EXPERIMENTAL

Materials

Strains, culture conditions, vectors, and chemicals

Escherichia coli DH5α (Invitrogen, Carlsbad, CA, USA) was used as the host for the propagation of plasmid. The plasmid pPICZαA (Invitrogen, Carlsbad, CA, USA was used as the expression vector. The *Pichia pastoris* KM71H strain (Invitrogen, Carlsbad, CA, USA) was used as the host for protein expression. For extraction of the total RNA, the *Thermomyces lanuginosus* was grown in an induction medium containing (per liter): 5.0 g of yeast extract, 20.0 g soluble starch, 18.3 g of olive oil, 5.0 g of potassium phosphate (K_2PO_4), 0.15 g of calcium chloride ($CaCl_2$), 1.0 g of magnesium sulfate ($MgSO_4 \cdot 7H_2O$), and 15.0 g of agar for 48 h at 50 °C (Zheng *et al*. 2011). The media used for the culture of *E. coli* and *P. pastoris* KM71H were referred according to standard methods. The *p*-nitrophenyl octanoate and carboxymethylcellulose (CMC), and other chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise.

Construction of EG1CD, Lip, and chimera Lip-EG1CD

The total RNA was extracted from *T. Lanuginosus* using a TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was conducted to synthesize cDNA at 42 °C for 2 h using 3 mg of total RNA and 100 U Superscript II reverse transcriptase according to the manufacturer's manual (Life Technologies Inc., Gaithersburg, MD). The full-length cDNA of Lip (Genbank accession No. AGH70111.1) was amplified by PCR using the pair primers 1 and 2, and subcloned into the pGEM-T vector. The fragment encoding mature Lip was amplified by PCR with the plasmid pGEM-T-Lip (the full length Lip linked to the pGEM-T vector) as template and using pair primers 3 and 4 with restriction enzyme sites of *Eco*RI/*Xba*I (Underlined in Table 1), and Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). After enzymatic digestion, the DNA fragment was inserted into the *Pichia* expression pPICZaA vector at the *Eco*R I and *Xba* I sites and transformed into *Escherichia coli* DH5α, then sequenced for confirming the correct expression frame.

The gene fragment, *EG1CD*, encoding the linker and catalytic domain of EG1 (Genbank accession No. AF329732) from *V. volvacea* was generated by PCR amplification from a plasmid pBluescript-EG1 with the full-length gene of *V. volvacea EG1* using the pair primers 5 and 6 with restriction enzyme sites of *Sac*II/*Xba*I (Underlined in Table 1), and Pfu DNA polymerase (Stratagene). After digestion, the DNA fragment was cloned into the *Pichia* expression pPICZaA vector at the *Sac*II/*Xba*I sites and transformed into *Escherichia coli* DH5α, then confirmed for the correct expression frame by sequencing.

Table 1. Primers for PCR used in the Study

Primer Name	Nucleotide Sequence (5' to 3')
Primer 1	CGGCCTGTTCGACGAGCGGT
Primer 2	ATCACACTCTGAAATGGGAC
Primer 3	AGAGAGGCTGAAGCTGAATTCCGGCCTGTTCGACGAGCGGT
Primer 4	GAGATGAGTTTTTGTTCTAGAAATCAATGATGATGATGATGATGATCACACTCTGAAAT
Primer 5	TCCCCGCGGGGTGTCAACCAGGCTGGTGC
Primer 6	GCTCTAGATCAATGATGATGATGATGATGCACGAATGGTTTCAAAGCCT
Primer 7	AGAGAGGCTGAAGCTGAATTCCGGCCTGTTCGACGAGCGGT
Primer 8	AGGTGGGGTTGGGTGCGCTGCTGGTTGTCGTAGGTCCATCACACTCTGAAAT
Primer 9	ACCCAACCCCACCTCCAGTGGCTGCCCGAATGCCACCAAGTTCAGATTCTTCGGT
Primer 10	GAGATGAGTTTTTGTTCTAGAAATCAATGATGATGATGATGATGCACGAATGGTTTCAA

The chimeric gene was constructed by end-to-end fusion (Khandeparker and Numan 2008). Firstly, the Lip gene was obtained *via* PCR amplification using the forward primer 7 with restriction enzyme sites of *Eco*RI (Underlined), and reverse primer 8. Secondly, the fragment encoding for the linker and catalytic domain of EG1CD was amplified using the forward primer 9 and reverse primer 10 with restriction enzyme sites of *Xba*I (Underlined). Lastly, the gel-purified products were further amplified by PCR using the pair primers 7 and 10 to obtain the synthetic gene Lip-EG1CD, in which the Lip at 5'-end is linked with EG1CD at 3'-end *via* the native linker sequence from EG1CD. Then, the products were digested with *Eco*RI and *Xba*I, and cloned into *Eco*RI/*Xba*I

linearized pPICZaA to construct the recombinant expression plasmid pPICZaA-Lip-EG1CD. The gene insert in the constructed pPICZαA-Lip-EG1CD was confirmed by DNA sequencing.

Methods

Expression and purification of proteins from P. pastoris

After digestion by *Sac*I, all linearized plasmids, pPICZαA-Lip, pPICZαA-EG1CD, and, pPICZαA-Lip-EG1CD, were separately transformed into a KM71H strain by electroporation using a Bio-Rad Genepulser II apparatus (Hercules, CA, USA). The positive colonies were isolated after incubation at the YPD plate with sorbitol (1 M) and Zeocin (100 μg/mL) (Invitrogen, Carlsbad, CA, USA) at 28 °C for 3 to 5 days, according to the manufacturer's instructions. The individual colonies with the highest lipase and/or endoglucanase activity were isolated based on the activity assay and used for recombinant enzyme production. The *P. pastoris* transformants were grown and induced as previously described (Zheng and Ding 2013). The purified recombinant Lip, EG1CD, and Lip-EG1CD were obtained by Ni–NTA Agarose affinity chromatography (Qiagen, Valencia, CA, USA) according to the manufacturer's manual. The purity and molecular mass of the purified proteins were verified by 10% (w/v) sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) analysis.

Enzyme assay

Endoglucanase activity was determined according to the previously described method with 2.0% (w/v) CMC as the substrate in 0.1 M phosphate sodium buffer (pH 7.5) for 30 min at 55 °C. The amount of reducing sugar released was then determined by the Somogyi-Nelson method (Somogyi 1952). One unit of the endoglucanase activity (U) was defined as the amount of enzyme that produced 1 μmol of reducing sugars per min.

The lipase activity was determined according to the previously described method with 2 mM *p*-nitrophenyl octanoate as the substrate in 0.1 M phosphate sodium buffer (pH 7.5) for 10 min at 35 °C (Morkbak *et al*. 1999). One unit of lipase activity (U) was defined as the amount of lipase necessary to liberate 1 μmol of *p*-nitrophenol per min. All of the experiments were performed in triplicate.

Effects of temperature and pH on enzyme activity

The optimal temperatures for endoglucanase and lipase activities were estimated at pH 7.5, with the temperature ranging from 20 °C to 65 °C. The optimum pHs were determined at 55 °C for endoglucanase activity and 35 °C for lipase activity in a universal buffer, with the pH ranging from 5.0 to 11.0. The universal buffer was comprised of 50 mM phosphoric acid (H_3PO_4), acetic acid (CH_3COOH), and orthoboric acid (H_3BO_3), and the pH was adjusted with 0.2 M sodium hydroxide (NaOH) at 25 °C. To estimate thermostability, the enzyme was pre-incubated over a temperature range of 40 °C to 60 °C without a substrate for specific times. The residual activity was then determined at 55 °C for endoglucanase activity and 35 °C for lipase activity. The pH stability assay was conducted in a universal buffer with a pH range of 5.0 to 11.0 at room temperature for 24 h, followed by measurement of the residual activity as above.

Effects of metal ions and chemical reagents on enzyme activity

Metal ions Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Ni^{2+}, Co^{2+}, NH_4^+ (final concentration 1 mM and 5 mM), and EDTA (final concentration 0.1 mM and 0.5 mM) were used to determine their effects on endoglucanase and lipase activities. The enzyme activities were assayed under the standard assay condition in the presence of various metal ions or chemical reagents at the concentrations mentioned above.

Substrate specificity and kinetic constants

The lipase substrate specificities were determined under standard assay conditions by measuring the amount of *p*-nitrophenol liberated in reaction mixtures that contained different *p*-nitrophenyl acyl esters. The endoglucanase substrate specificities were estimated under standard condition by assaying the amount of reducing sugar equivalents released in reaction mixtures containing one of the following substrate solutions/suspensions: CMC, filter paper, phosphoric acid, or swollen cellulose. The specific activities of endoglucanase and lipase were defined as units per micromole protein. The kinetic constants (V_{max} and K_m) were examined at optimum conditions of temperature and pH by incubating the diluted enzymes for 5 min and 30 min, respectively. In the phosphate sodium buffer (pH 7.5) containing *p*-nitrophenyl octanoate, the lipase activity ranged from 0.2 mmol mL^{-1} to 4.0 mmol mL^{-1} and CMC for endoglucanase ranged from 0.2 mg mL^{-1} to 4.0 mg mL^{-1}. The V_{max} and K_m values were calculated by GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) using nonlinear regression.

Deinking of laser-printed paper and newspaper pulp

The laser-printed paper and newspaper used in this study were torn into pieces of approximately 1 cm^2 and immersed into H_2O at a consistency of 5% (w/v) for 24 h at room temperature. The water-soaked papers were macerated with a grinder to obtain a pulp slurry. Thereafter, the pulp slurry was dehydrated, fluffed, and stored at 4 °C as previously described (Chutani and Sharma 2015). The pulp consistency was adjusted to 3% (w/v) with 100 mM phosphate sodium buffer (pH 7.5). The same molecule numbers of Lip, EG1CD, and Lip-EG1CD (5.53 nmol/g dried waste paper, each) and 0.3% (v/v) surfactant (AEO-9) were added into pulp and incubated at 35 °C for Lip, 40 °C for Lip-EG1CD, 40 °C for Lip/EG1CD, and 55 °C for EG1CD, at 150 rpm for 3 h. Controls that used water instead of enzymes were run simultaneously. After treatment, the pulp mixtures were boiled for 5 min to deactivate enzyme activities. The decolorized pulps were diluted to 1% (w/v) with deionized H_2O and the released ink was removed by flotation in a laboratory flotation unit for 10 min. Then, calcium chloride ($CaCl_2$) (0.3 %, w/w dry pulp weight) and 0.3 % (v/v) AEO-9 were added as flotation aids. AEO-9 and $CaCl_2$ contribute to the formation of bubbles for trapping the released ink particles. Lastly, pulps were washed with H_2O in a 60-mesh wire sieve and collected for testing. All of the experiments were performed in triplicate.

The generated reducing sugars and chromophores in the filtrate were determined after 3 h enzymatic deinking. The amount of reducing sugar released was measured by the Somogyi-Nelson method. The amount of chromophores generated was estimated by measuring the absorbance at λ equal to 231 nm (Patel *et al*. 1993). All of the experiments were performed in triplicate and their average values are presented.

The deinked pulps were used for making handsheets (TAPPI T205 sp-02 (2002)) with a grammage of 60 g/m^2. The handsheets were analyzed by measuring the burst index

(TAPPI T403 om-10 (2010)), tensile index (TAPPI T494 om-1 (2001)), and tearing index (TAPPI T414 om-04 (2004)) under the standard procedures. The amounts of effective residual ink on the handsheets were measured by image analysis (TAPPI T213 om-01 (2001)) (SpecScan 2000, Thwing-Albert Instrument Company, West Berlin New Jersey, USA). The handsheet brightness was measured according to the TAPPI T452 om-98 (1998) standard. The ink removal efficiency (%) was calculated according to Eqs. 1 and 2. The Ink Removal Efficiency (IRE) of Control Samples is given by,

$$\mathrm{IRE_c}\ (\%) = 100 \times \frac{N_{\text{No surfactant treated paper}} - N_{\text{surfactant treated paper}}}{N_{\text{No surfactant treated paper}}} \quad (1)$$

whereas the Ink Removal Efficiency of Enzyme Treated Samples is given by

$$\mathrm{IRE_e}\ (\%) = 100 \times \frac{N_{\text{No surfactant treated paper}} - N_{\text{enzyme treated paper}}}{N_{\text{No surfactant treated paper}}} \quad (2)$$

where N is the residual ink number. No surfactant treated paper was made directly from pulp slurry without 3 h treatment.

Scanning electron microscopy (SEM) for fiber morphology

The SEM images of the pulp samples were observed using a FEI Quanta 200 environmental scanning electron microscope (SEM, FEI Company, Hillsboro, USA). The working voltage and distance were 15 kV and 10 mm, respectively. The pulp samples were washed three times with deionized water, air-dried, placed on a short bar, mounted with silver ribbon, and surface sprayed with gold (Hitachi S-4800, Hitachi, Tokyo, Japan). Electron micrographs of the fibers were recorded by the SEM at the desired magnifications.

RESULTS AND DISCUSSION

Design of the Chimera Lip-EG1CD

It has been found to be more cost effective and time saving in the industrial production of chimeric enzymes with two or more enzyme activities in comparison to the separate production of multiple parent enzymes (Fan *et al*. 2009). Because endoglucanase and lipase have shown high deinking efficiency (Gübitz *et al*. 1998; Morkbak *et al*. 1999; Marques *et al*. 2003; Pelach *et al*. 2003; Vyas and Lachke 2003), the authors assumed that the combined use of two enzymes could promote better deinking efficiency. With such assumption, a chimeric enzyme with endoglucanase and lipase activities was constructed in this study by an end-to-end fusion technique, in which the Lip catalytic domain at N-terminus was fused with the EG1 catalytic domain at C-terminus (Khandeparker and Numan 2008). Several studies have suggested that linker peptides between the individual moieties may play an important role in maintaining the proper conformation and function of the moieties (Shan *et al*. 1999; Lu and Feng 2008). Therefore, in this study, the native linker peptide of EG1 was used to link the Lip and EG1CD moieties in the chimeric enzyme.

Expression and Purification of Lip, EG1CD, and Chimera Enzyme

The gene fragments encoding Lip, EG1CD, and Lip-EG1CD were each ligated into the *Pichia* expression pPICZaA vector. By screening, dozens of positive

transformants for each enzyme were isolated, and the transformants with high activity levels were used for enzyme production. The recombinant enzymes were overexpressed as an active form with 6 x His tag at C-terminus under transcriptional control of the AOX1 promoter in *P. pastoris* KM71H after 0.8% methanol induction for 6 days. The purified Lip, EG1CD, and chimeric enzyme Lip-EG1CD were prepared by Ni-NTA agarose affinity chromatography. The purified recombinants Lip, EG1CD, and chimera Lip-EG1CD revealed bands of approximately 34 kDa, 37 kDa, and 71 kDa, respectively, on SDS-PAGE gel. These were identical to their theoretical molecular masses (Fig. 1).

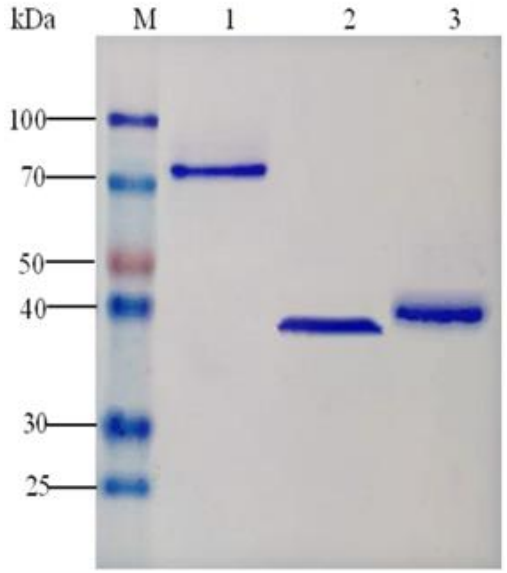

Fig. 1. SDS-PAGE analysis of purified recombinant proteins; Lane M, molecular weight marker; Lane 1, Lip-EG1CD; Lane 2, Lip; and Lane 3, EG1CD

Effects of Temperature and pH on Enzyme Activity

The chimera Lip-EG1CD displayed bifunctional activities with both lipase and endoglucanase activities. The chimera Lip-EG1CD displayed the highest lipase activity at 35 °C, and the highest endoglucanase activity was at 55 °C, which were identical with parent lipase and endoglucanase activities (Figs. 2A and 2B). However, compared to the parent Lip, the Lip moiety in Lip-EG1CD had higher stability at temperature ranges of 35 °C to 45 °C. More than 90% of its initial activity of lipase was retained after 4 h of incubation at 35 °C to 45 °C (Fig. 2C). The EG1CD moiety in chimera Lip-EG1CD and parent EG1CD had similar stability. Both endoglucanase activities changed minimally at 40 °C after 2 h. However, the residual activity declined to 60% and the initial activity declined to 15% after 2 h of incubation at 50 °C and 60 °C, respectively (Fig. 2D).

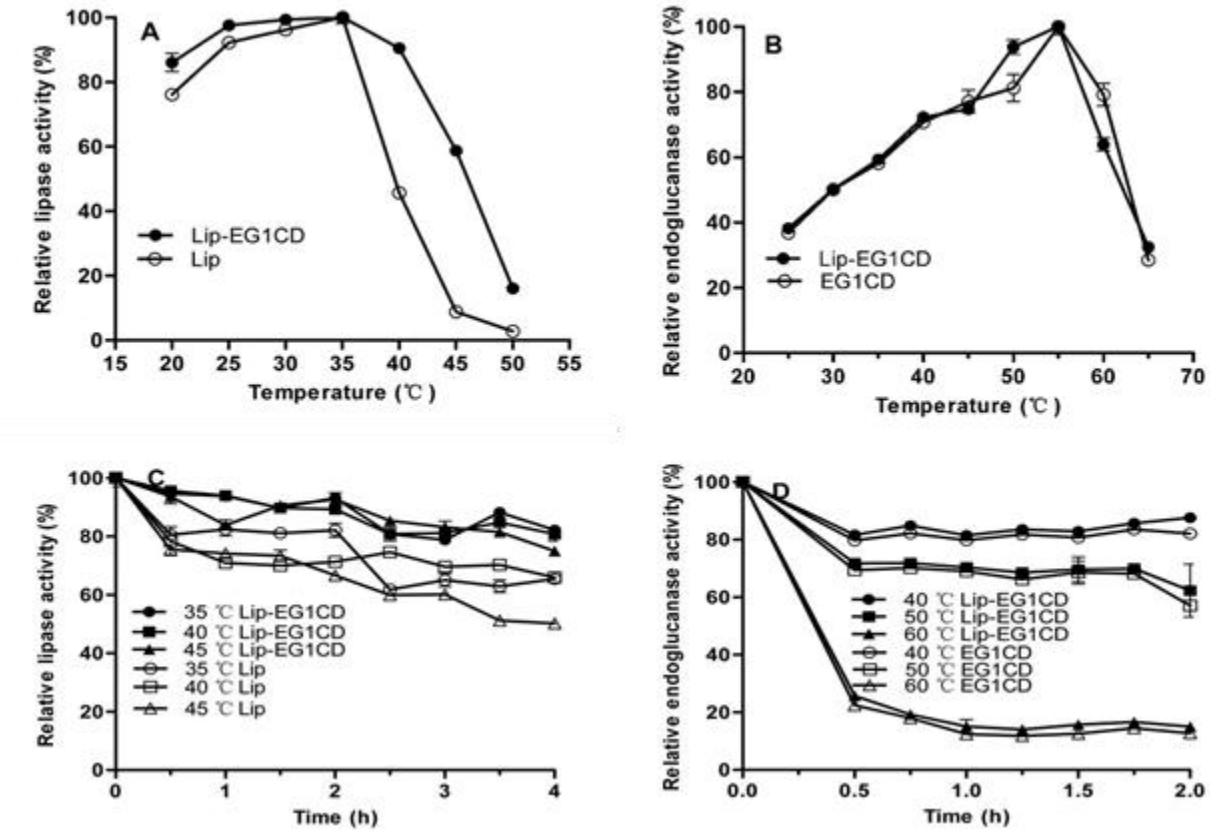

Fig. 2. Effect of temperature on the enzyme activity and stability; A) The optimum temperature on the lipase activity of chimera Lip-EG1CD and parent Lip; B) The optimum temperature on the endoglucanase activity of chimera Lip-EG1CD and parent EG1CD; C) The thermostability on the lipase activity of chimera Lip-EG1CD and parent Lip; and D) The thermostability on the endoglucanase activity of chimera Lip-EG1CD and parent EG1CD; values shown are the means of the results of triplicate experiments ± standard errors of the means (SE).

The chimera Lip-EG1CD displayed the highest endoglucanase and lipase activities at a pH of 7.5, which was identical with the parent Lip and EG1CD (Figs. 3A and 3B). The result showed that moieties in chimera Lip-EG1CD displayed a similar pH stability over the pH range of 5.0 to 11.0 as the parent Lip and EG1CD after treatment at room temperature for 24 h (Figs. 3C and 3D).

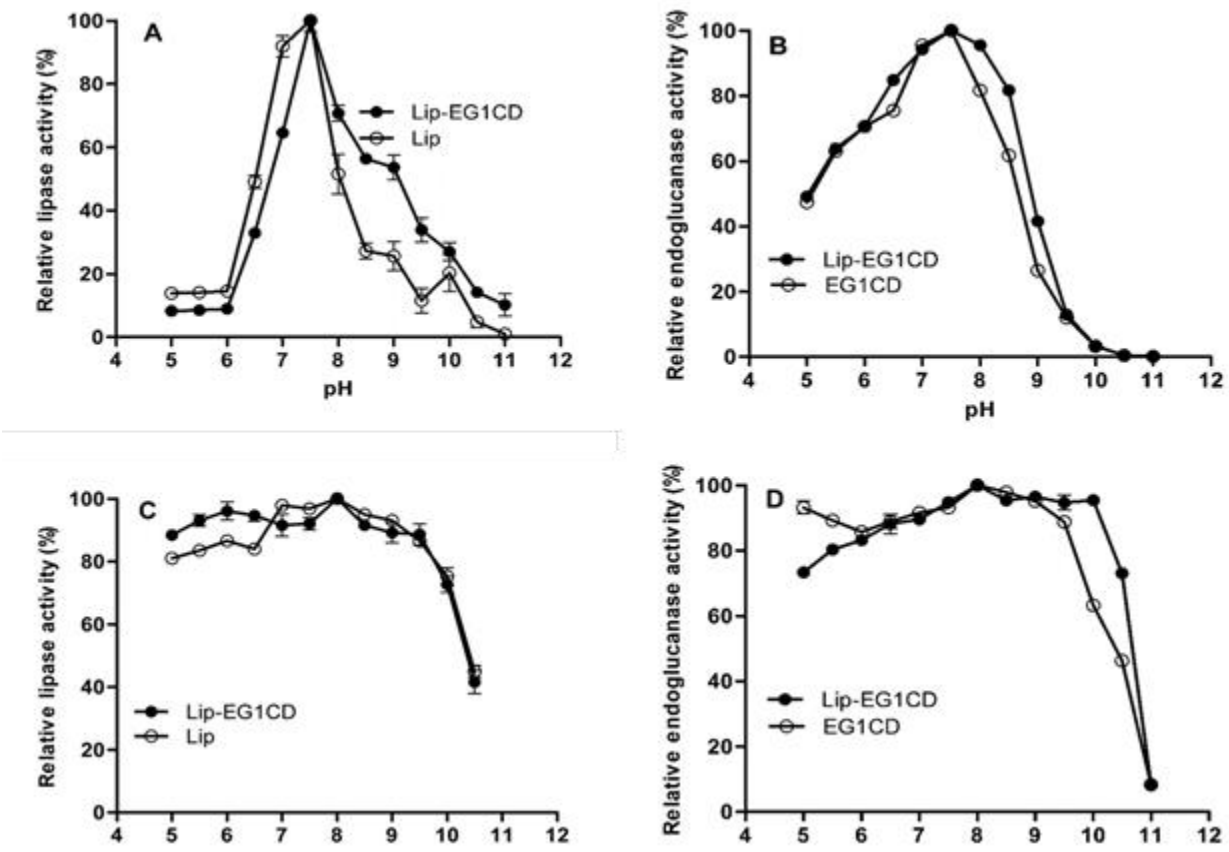

Fig. 3. Effect of pH on the enzyme activity and stability; A) The optimum pH on the lipase activity of chimera Lip-EG1CD and parent Lip; B) The optimum pH on the endoglucanase activity of chimera Lip-EG1CD and parent EG1CD; C) The pH stability on the lipase activity of chimera Lip-EG1CD and parent Lip at room temperature for 24 h; and D) The pH stability on the endoglucanase activity of chimera Lip-EG1CD and parent EG1CD at room temperature for 24 h; values shown are the means of the results of triplicate experiments ± standard errors of the means (SE).

Effects of Metal Ions and Chemical Reagents on Activity

Metal ions may bind to some of the amino acid side chains of protein, therefore influencing the structure and function of enzymes and altering the enzyme activity and stability (Ebrahimpour *et al*. 2011). In general, all metal ions had similar effects on the lipase and endoglucanase activities of parent enzymes and chimera Lip-EG1CD. However, Ni^{2+} gently increased the lipase activity of chimera Lip-EG1CD (Table 2). The endoglucanase activity in chimera Lip-EG1CD was slightly activated by Zn^{2+} at the concentration of 1 mM, but this was not the case for the parent EG1CD. Chimera Lip-EG1CD was more tolerant to EDTA than parent Lip and EG1CD (Table 2).

Table 2. Effects of Metal Ions and Chemical Reagents on Enzyme Activity

Metal-ions and Chemical Agents	Lipase Activity of Lip (%)		Lipase Activity of Lip-EG1CD (%)		Endoglucanase Activity of EG1CD (%)		Endoglucanase Activity of Lip-EG1CD (%)	
	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM
Ca^{2+}	96.58	81.07	89.33	81.25	90.86	89.41	89.19	96.41
Mn^{2+}	86.74	86.44	88.45	90.57	90.00	91.10	87.47	90.41
Mg^{2+}	99.89	98.85	96.23	103.48	93.30	58.52	97.07	75.66
Cu^{2+}	105.93	94.82	92.99	90.91	75.49	72.98	86.19	79.26
Zn^{2+}	99.18	96.31	99.25	99.72	100.29	97.76	112.03	101.14
Fe^{3+}	84.43	83.76	96.16	94.53	88.00	64.59	95.54	72.55
Ni^{2+}	90.35	93.72	108.97	106.50	98.65	68.46	96.57	84.69
Co^{2+}	102.70	101.39	102.44	98.27	99.33	69.90	99.05	84.49
NH_4^+	97.62	96.53	97.22	96.62	97.26	68.57	98.86	85.13
EDTA (0.1 mM)	87.47		98.48		102.14		106.68	
EDTA (0.5 mM)	96.95		99.93		99.06		112.13	

Specific Activities of the Parental and Chimeric Enzyme

The substrate specificity of enzymes was determined by measuring their activities against different substrates (Table 3). The lipase activity of parent Lip and chimera Lip-EG1CD showed the highest activity on *p*-nitrophenyl octanoate, followed by *p*-nitrophenyl decanoate, *p*-nitrophenyl laurate, *p*-nitrophenyl myristate, and so on. The endoglucanase activity of parent EG1CD and chimera Lip-EG1CD exhibited the highest activity on CMC, followed by phosphoric acid swollen cellulose and filter paper.

Interestingly, the lipase activity of the chimeric enzyme Lip-EG1CD was dramatically higher than that of the parental Lip (Table 3). Meanwhile, the endoglucanase activity of Lip-EG1CD on CMC was similar to parent EG1CD.

Table 3. Specific Activities of Lip, Eg1CD, and Chimera Lip-Eg1CD

Substrate	Enzyme Activity （U/μmol）		
	EG1CD	Lip	Lip-EG1CD
CMC	1484.44 ± 37.37	0	1481.11 ± 27.38
Phosphoric acid swollen cellulose	241.24 ± 7.41	0	235.32 ± 4.07
Filter paper	10.73 ± 0.37	0	9.25 ± 0.41
p-Nitrophenyl acetate	0	68.68 ± 10.54	607.76 ± 78.1
p-Nitrophenyl propionate	0	115.60 ± 6.80	412.51 ± 58.22
p-Nitrophenyl butyrate	0	199.92 ± 30.94	738.40 ± 71.71
p-Nitrophenyl valerate	0	299.54 ± 34.68	1838.90 ± 192.41
p-Nitrophenyl octanoate	0	29272.64 ± 768.40	110807.57 ± 4162.02
p-Nitrophenyl decanoate	0	14984.73 ± 578.68	56722.55 ± 2431.75
p-Nitrophenyl laurate	0	5089.80 ± 116.28	34861.71 ± 1101.21
p-Nitrophenyl myristate	0	463.42 ± 109.14	5915.72 ± 213.71
p-Nitrophenyl stearate	0	590.24 ± 112.88	3770.1 ± 144.13

Kinetic Properties of Lip, EG1CD and Chimera Lip-EG1CD

Table 4 shows the K_m and V_{max} values for the purified Lip, EG1CD, and chimera Lip-EG1CD. It was previously reported that fusion can improve the catalytic efficiency of moiety in chimera enzymes, and the chimeric enzyme often had kinetic advantages over the single parent enzyme or a simple mixture of individual enzymes (Seo *et al*. 2000; Wang *et al*. 2007; Yang *et al*. 2016). In this study, the lipase activity (V_{max}) of chimera Lip-EG1CD was greater than parent Lip toward *p*-nitrophenyl octanoate. However, the endoglucanase activity (V_{max}) of chimera Lip-EG1CD was similar to parent EG1CD toward CMC (Table 4), which indicated that the existence of the EG1CD moiety contributed to the activity and thermal stability of Lip.

Table 4. Kinetic Parameters of LIP, EG1CD, and Chimera LIP-EG1CD

Enzymes	Kinetic Parameters	
	K_m (mg/mL)	V_{max} (U/umol)
Lip (*p*-Nitrophenyl octanoate)	0.54	40351
Lip-EG1CD (*p*-Nitrophenyl octanoate)	0.51	152849
EG1CD (CMC)	0.41	2318
Lip-EG1CD (CMC)	0.42	2313

Effect of Lip, EG1CD, and Lip-EG1CD on Ink Removal Efficiency and Brightness

The same amount of molecules of Lip, EG1CD, and Lip-EG1CD (5.53 nmol/g dried waster paper for each enzyme) were used in this study to directly compare the ink removal efficiency and brightness by Lip, EG1CD, Lip/EG1CD mixture, and chimera Lip-EG1CD in flotation deinking. The control obtained an average ink removal efficiency of approximately 71% for both pulp samples with flotation. The Lip slightly enhanced the ink removal efficiency, whereas substantial increases in the ink removal efficiency were observed after EG1CD deinking. Approximately 82% of ink was removed by flotation for both waste papers. The ink removal efficiencies were elevated by the combined treatment of Lip/EG1CD mixture. Maximum ink removal efficiencies, approximately 83% and 87%, were achieved against laser-printed paper and newspaper, respectively. The ink removal efficiency was further improved by using the chimera Lip-EG1CD (Figs. 4A and 4C). Approximately 89% efficiency was obtained for both paper samples when flotation deinking, which was likely a function of the intramolecular synergistic action of Lip-EG1CD during hydrolysis of the surface material (ink particles and cellulose fibres).

All enzymatic deinking also brought obvious improvements in sheet brightness (Figs. 4B and 4D). Compared to the single action of Lip or EG1CD, the mixture of Lip/EG1CD enhanced the brightness, which demonstrated approximately 86 %ISO and 56 %ISO against laser-printed paper and newspaper, respectively. A higher brightness was achieved by the chimera Lip-EG1CD than by the Lip/EG1CD mixture (with 5.53 nmol Lip and EG1CD) (Figs. 4A through 4D). A maximum brightness of 91 %ISO for laser-printed paper and 60 % ISO for newspaper was obtained using the chimera Lip-EG1CD with flotation removal of ink particles. Because the doses used for Lip, EG1CD, Lip/EG1CD and Lip-EG1CD are 5.53, 5.53, 11.06 and 5.53 nmol/g dried waste paper,

respectively. The double dose of enzyme in terms of molecules was used in the Lip/EG1CD mixture as compared to the chimera Lip-EG1CD, the use of the chimera enzyme noticeably reduced the number of enzymes, and therefore the cost of enzyme production. The authors' study clearly demonstrated that the chimera Lip-EG1CD could be used to effectively deink laser-printed paper and newspaper. Figure 5 showed the release of chromophores (λ231 nm) and reducing sugars, which was consistent with the enzymatic deinking efficiencies.

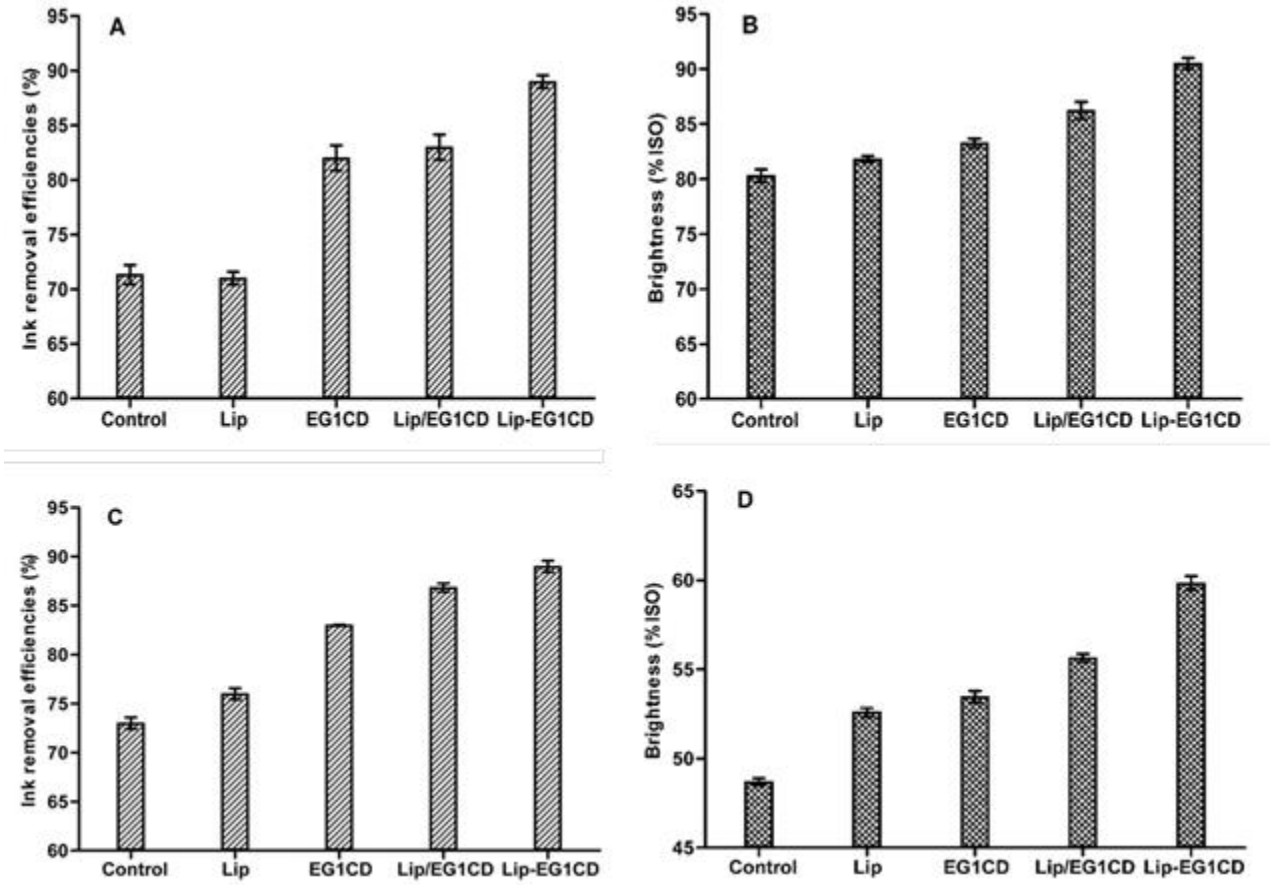

Fig. 4. Ink removal efficiencies of flotation deinking and brightness of deinked laser-printed paper and newspaper after treatment with Lip, EG1CD, Lip/EG1CD mixture, and chimera Lip-EG1CD; A) Ink removal efficiency of flotation deinking for laser-printed paper; B) Brightness of deinked laser-printed paper; C) Ink removal efficiency of flotation deinking for newspaper; and D) Brightness of deinked newspaper; *Control: No enzyme treated pulp

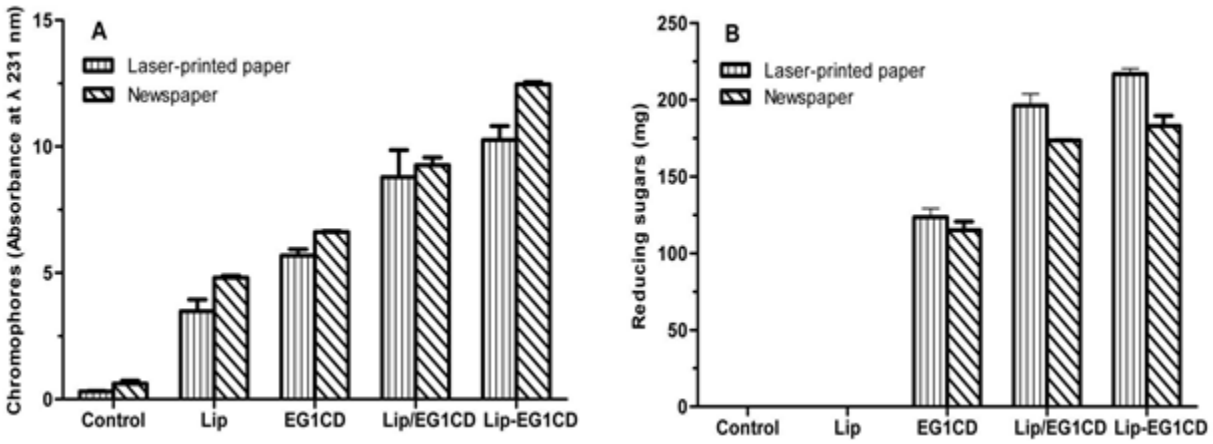

Fig. 5. Analysis of chromophores and reducing sugars in effluent released from laser-printed paper and newspaper after enzyme treatment; A) Release of chromophores in effluent after enzyme treatment; B) Release of total reducing sugars in effluent after enzyme treatment; *Control: No enzyme treated pulp

Effect of Lip, EG1CD, and Lip-EG1CD on the Physical Properties of Deinked Papers

The physical properties of the deinked papers, mainly including burst, tensile, and tearing indices, were compared in this study. The single Lip- and EG1CD-treated laser-printed paper retained similar or marginally decreased burst, tensile, and tearing indices as the control. Compared to a single enzyme, the Lip/EG1CD mixture and chimera Lip-EG1CD enhanced the physical properties to the same extent. The Lip/EG1CD mixture-treated laser-printed paper showed 1.77%, 2.94%, and 3.37% increases in the burst index, tensile index, and tearing index, respectively, in comparison to the control-treated paper (Figs. 6A through 6C). In contrast, the corresponding values for the chimera Lip-EG1CD-treated laser-printed paper were 2.18%, 3.00%, and 5.47%. For enzymatic-deinked newspaper, except for the marginal drop in tearing index *via* single Lip treatment, the single Lip and EG1CD treatment slightly improved the overall newspaper physical properties. The Lip/EG1CD mixture or chimera Lip-EG1CD treatment (Figs. 6D through 6F) could improve the physical properties of deinked newspaper further. The authors observed 11.25%, 12.32%, and 3.12% increases in the burst index, tensile index, and tearing index, respectively, of the Lip/EG1CD mixture-treated newspaper compared to the control-treated newspaper, whereas the corresponding values for the chimera Lip-EG1CD-treated newspaper were 13.44%, 14.14%, and 4.90% increases, respectively (Figs. 6D through 6F).

Previous investigations reported that the enzymatic deinking can result in positive or negative effects on the physical properties of deinked paper (Pala *et al*. 2004; Pathak *et al*. 2015). These differential effects on deinked paper could be related to various factors including types of enzymes and waste papers, and the dosage and treatment duration of the enzyme during enzymatic deinking (Chutani and Sharma 2015). In this study, the handsheets strength was clearly improved after deinking by Lip-EG1CD for both waste papers. Therefore it was concluded that chimera Lip-EG1CD will be useful for developing an economical process for waste paper recycling.

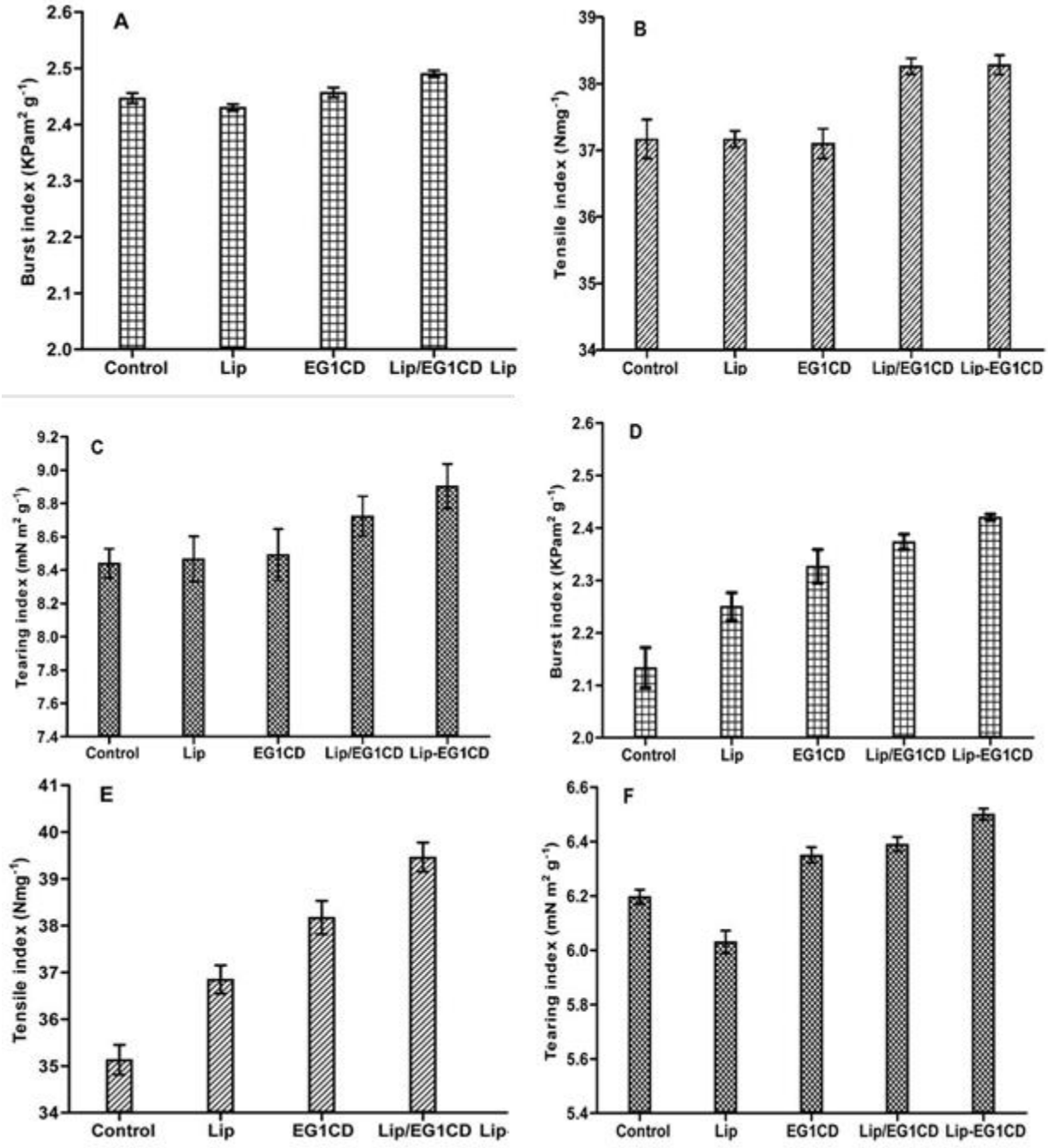

Fig. 6. Physical properties of handsheets after treatment with Lip, EG1CD, Lip/EG1CD mixture, and chimera Lip-EG1CD; A) through C) Physical properties of deinked laser-printed paper; D) through F) Physical properties of deinked newspaper; *Control: No enzyme treated pulp

Scanning Electron Microscopy for Fiber Morphology

The SEM images of the deinked pulp sample are shown in Figs. 7 and 8. As shown in Figs. 7A and 8A, the fiber surfaces of the control pulp were comparatively smooth. The fiber surface was rough after the pulp samples were treated with single Lip or EG1CD (Figs. 7B, 7C, 8B, and 8C), and slight fibrils were visible on the fiber surface. The fiber surface became rougher after the Lip/EG1CD mixture and chimeric Lip-EG1CD treatment and more fibrils were observed (Figs. 7D, 7E, 8D, and 8E), which

indicated that the simultaneous hydrolysis of endoglucanase and lipase on the fiber surface facilitated the release of fibrils. In general, the morphological changes of the fibers, like cracks and peeling, were observed in the enzyme-deinked pulps (Xu *et al*. 2009). These morphological modifications enhanced the contact area between the pulp fiber surface, which may explain the improvement in the paper properties by the Lip/EG1CD mixture and chimera Lip-EG1CD treatment (Virk *et al*. 2013).

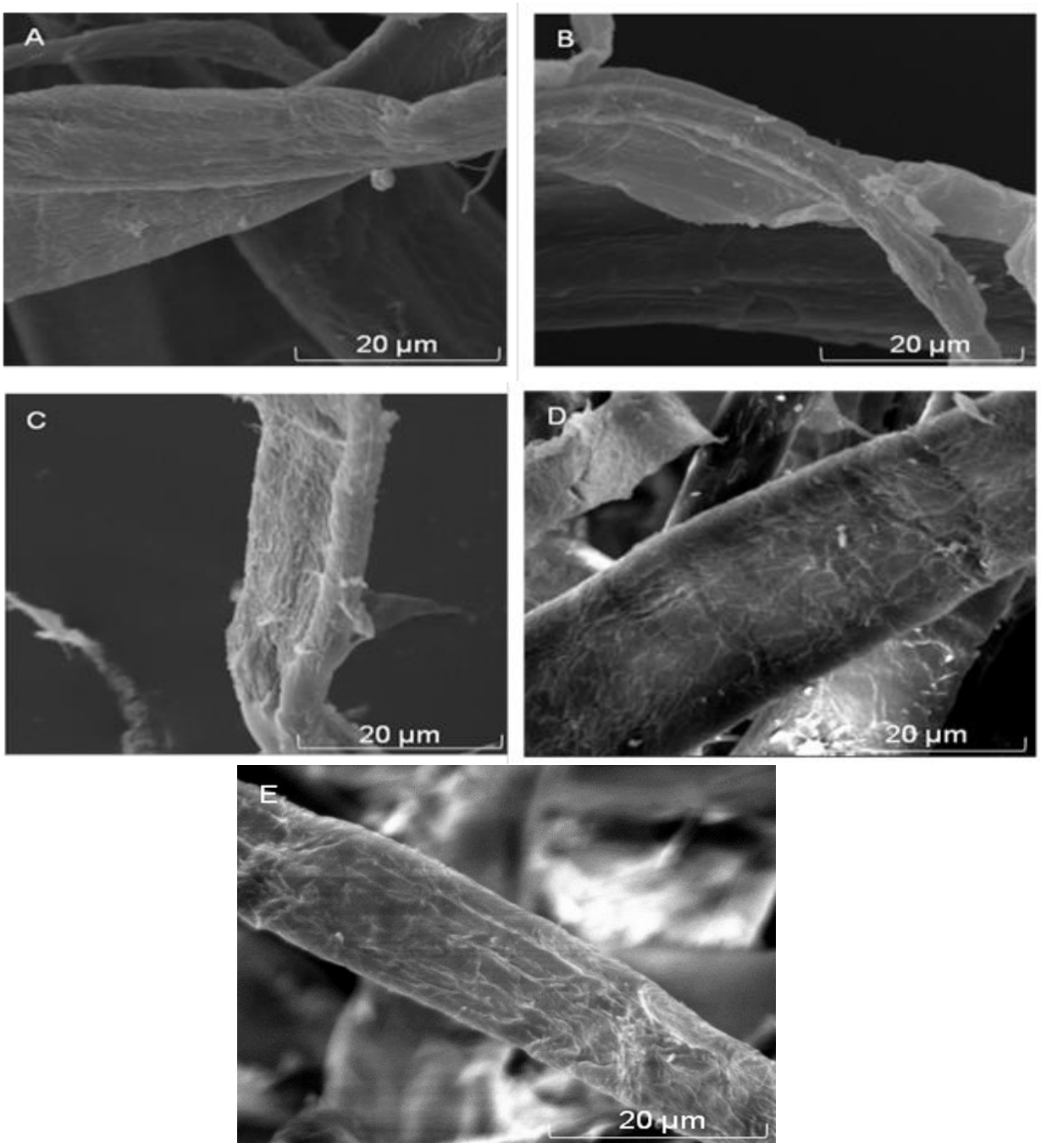

Fig. 7. Scanning electron micrographs of deinked laser-printed paper at magnification 3000x. A) Control laser-printed paper; B) Lip deinked laser-printed paper; C) EG1CD deinked laser-printed paper; D) Lip/EG1CD mixture deinked laser-printed paper; E) Lip-EG1CD deinked laser-printed paper.*Control: no enzyme treated pulp

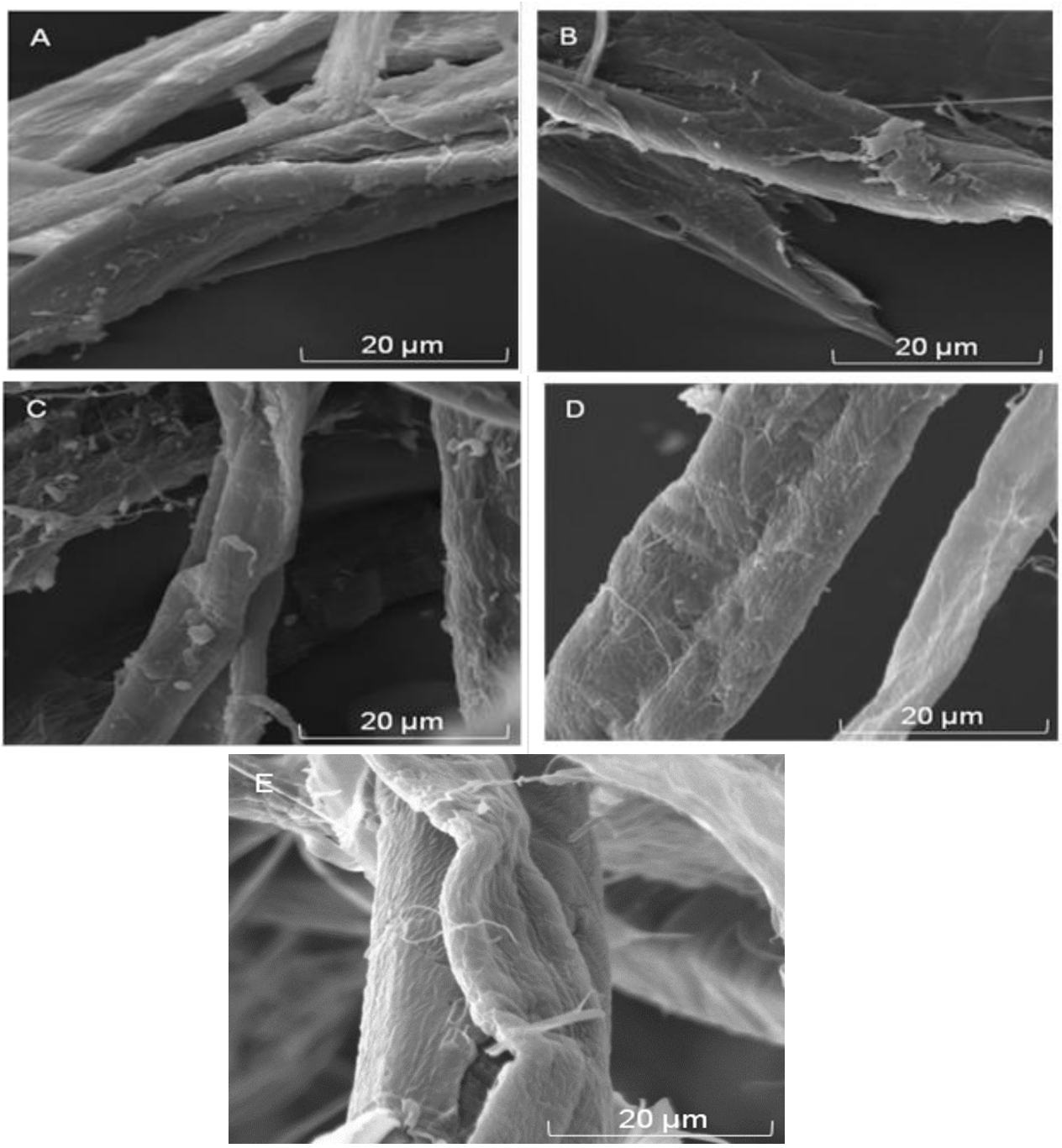

Fig. 8. SEM images of deinked newspaper at magnification 3000x; A) Control newspaper; B) Lip-deinked newspaper; C) EG1CD-deinked newspaper; D) Lip/EG1CD mixture-deinked newspaper; E) Lip-EG1CD-deinked newspaper; *Control: No enzyme-treated pulp

CONCLUSIONS

1. A chimeric enzyme with endoglucanase and lipase activities was constructed and expressed in *Pichia pastoris*.
2. The chimeric enzyme Lip-EG1CD had similar endoglucanase activity, but as much as

four times higher lipase activity on *p*-nitrophenyl octanoate than the parental enzymes.

3. The chimera Lip-EG1CD showed a better deinking performance than the parental enzymes or the parental enzyme mixture. The chimera Lip-EG1CD demonstrated an 89% removal of toner on both papers and 91% ISO and 60% ISO sheet brightness for laser-printed paper and newspaper, respectively. The handsheets strength was also clearly improved using Lip-EG1CD. Because the production of chimeric enzymes were more cost-effective and timesaving in the industry than the separate production of multiple parent enzymes, the engineered chimeric Lip-EG1CD will be useful for developing an economical process for waste paper recycling.

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