Engineering a Chimeric Lipase-cutinase (Lip-Cut) for Efficient Enzymatic Deinking of Waste Paper

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Lipase and cutinase belong to the esterase family and have biological applications in many fields. To develop more efficient biocatalysts that can be used for waste paper deinking, a chimeric lipase-cutinase (Lip-Cut) was constructed and successfully overexpressed in *Pichia pastoris*. The chimeric Lip-Cut exhibited lipase and cutinase activities that were 127% and 210% higher than their parent enzymes, respectively. Cut was superior to Lip in ink removal and improvement of paper brightness than Lip. The Lip-Cut displayed a better ink removal efficiency and paper brightness than that of the Lip, Cut, and Lip/Cut mixture. When the chimeric Lip-Cut was used, the ink removal efficiencies were 25.8% and 16.2% higher than that of the control-treated laser-printed paper and newspaper, which had sheet brightness values of 88% ISO and 59% ISO, respectively. The results demonstrated that the proper construction of bi-functional Lip-Cut could enhance the catalytic properties through the synergistic action of the two moieties because of the complementary advantages in the substrate specificities and catalysis patterns of both enzymes. This may provide an effective way to engineer more efficient bi-functional lipases and cutinases for deinking waste paper.

Keywords: Chimeric enzyme; Waste paper; Lipase; Cutinase; Enzymatic deinking

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INTRODUCTION

Waste paper is now being recycled and acts as a major fiber resource for paper manufacturers around the world (Vyas and Lachke 2003). In the waste paper recycling process, deinking is the most important step. During deinking, ink particles are detached from fibers. Various types of polyesters and synthetic polymers with ester bonds, including polymer vinyl acetate (PVAC), polyacrylate (PA), ethylene vinyl acetate (EVA), and styrene acrylate (SA), are important components that act as adhesives in papermaking and as binders in synthetic toner and ink (Zhang *et al*. 2017a). During conventional chemical deinking, large amounts of chemicals are used to break down the strong adherence of toner ink particles to fibers, which leads to the generation of hazardous effluents (Heise *et al*. 1996; Zollner and Schroeder 1998; Singh *et al*. 2012).

Previous reports have demonstrated the successful use of various enzymes, such as cellulases, xylanases, and lipases, in waste paper deinking (Pathak *et al*. 2011; Das *et al*. 2013; Pathak *et al*. 2014; Saxena and Chauhan 2017; Zhang *et al*. 2017b). Cellulases and hemicellulases can hydrolyze cellulose and hemicellulose, respectively, and hence detach ink from fibers by peeling fibers or fines from the paper surface. The deinking ability of lipases comes from the hydrolysis of triglycerides in vegetable oil-based inks

into di- and monoglycerides and glycerols, which dislodges ink particles from waste paper (Mohandass and Raghukumar 2005).

Cutinase is in the esterase family and can hydrolyze the ester bonds in cutin, which is the insoluble biopolyester matrix in plant surfaces. Cutinases have multiple functions in the degradation of various polyesters, water-insoluble triglycerides, and soluble esters, and have been used in plastic recycling (Feuerhack *et al*. 2008; Yang *et al*. 2013; Khan *et al*. 2017). Lipases and cutinases are differentiated on the basis of the hydrolytic cleavage of acyl glycerols with different acyl chain lengths. Lipases hydrolyze acyl esters with greater than 10 carbon atoms, whereas cutinases catalyze the breakdown of esters with chain lengths of less than 10 carbon atoms (Shah *et al*. 2014). Furthermore, contrary to lipases, whose activity is greatly activated in the presence of a lipid-water interface, cutinases display little interfacial activation.

Although previous reports have demonstrated the successful use of various cellulase and lipase enzymes in waste paper deinking (Mohandass and Raghukumar 2005; Das *et al*. 2013; Saxena and Chauhan 2017), there have been some disadvantages during the bio-deinking. For example, a significant detrimental effect on physical properties of deinked paper was observed because of cellulose degradation caused by cellulase treatment in some cases (Lee *et al*. 2017). Lipases prefer to deink the paper printed with soy bean oil based ink because of their ability to hydrolyze the triglycerides in vegetable-oil based inks into di, monoglycerides and glycerol (Morkbak *et al*. 1999; Mohandass and Raghukumar 2005). It was reasonable to assume that cutinases could be used effectively alone or with lipases in deinking processes because of the multiple functions of cutinase and the complementary nature of the substrate specificities and catalysis patterns of both enzymes. Furthermore, compared to cellulase, cutinase, as well as other esterases, does not affect the properties of fiber because of its strict specificity toward the esters in inks or coating (Carniel *et al*. 2017; Gamerith *et al*. 2017). Enzymes that have been engineered by a genetic technique to be bi- or multi-functional showed some advantages over the parent enzymes and their mixture for various enzymatic parameters and properties required in biotechnological applications (Yu *et al*. 2015). In this study, a chimeric lipase-cutinase (Lip-Cut) was constructed and successfully overexpressed in *Pichia pastoris*. The characteristics and application of chimeric Lip-Cut in degrading polyesters, such as PVAC, and the deinking performance were investigated and compared with that of the parent enzymes and their mixture.

EXPERIMENTAL

Materials

Strains, culture conditions, vectors, and chemicals

Escherichia coli DH5α and *P. pastoris* KM71H were used for the construction of recombinant plasmids and protein expression, respectively. The recombinant plasmid pPICZαA-Lip, containing lipase (Lip) from *Thermomyces lanuginosus*, was constructed in the previous work by the authors (Liu *et al*. 2017). The gene encoding for a characterized cutinase (Cut) from *Thielavia terrestris* NRRL 8126 (accession no. XP_003656017.1) was also synthesized using optimized codons of *P. pastoris* (accession no. MF537431) from Springen Biotech Co. (Nanjing, China), and inserted into pPICZαA (Invitrogen, Carlsbad, USA) at the *Eco*RI/*Xba*I sites to construct the recombinant expression plasmid pPICZαA-Cut. Then, the pPICZαA-Lip and pPICZαA-Cut were used as templates for amplification of the Lip and Cut moieties in the chimeric Lip-Cut. The

culture medium required for the growth of *E. coli* and *P. pastoris* KM71H were prepared according to EasySelect *Pichia* Expression Kit (Invitrogen, Carlsbad, USA). The *p*-nitrophenyl octanoate and *p*-nitrophenyl butyrate were purchased from Sigma-Aldrich (St. Louis, USA). Polycaprolactone (PCL) with a viscosity-average molecular weight of 80000 Daltons was supplied by Suzhou Zhong Zhicheng Plasticizing co., Ltd. (Suzhou, China). The PVAC was provided by Jiangsu Yinyang Gumbase Materials, Co., Ltd. (Jintan, China). All of the other chemicals were of reagent grade or higher and purchased from Sigma-Aldrich (St. Louis, USA) unless otherwise indicated.

Construction of the chimeric lipase-cutinase (Lip-Cut)

All of the primers used for plasmid construction are listed in Table S1. The chimeric gene was constructed by end-to-end fusion. The native linker peptide of EG1 from *Volvariella volvacea* (GPTTTSSAPNPTSSGCPNATK; Genbank accession no. AF329732) was used to link the Lip and Cut moieties in the chimeric enzyme. The Lip and Cut gene fragments used for encoding a partial linker and mature proteins were obtained *via* polymerase chain reaction (PCR) amplification using the primer pairs 1/2 and 3/4, respectively (Table S1). The two PCR products were gel-purified and used as templates for the synthesis of the chimeric gene Lip-Cut by overlap-extension PCR using the pair primers 1 and 4, in which the Lip at the 5'-end was linked with the Cut at the 3'-end *via* the native linker sequence from EG1. Lastly, the PCR products were digested with *Eco*RI and *Xba*I, and cloned into pPICZαA to construct the recombinant expression plasmid pPICZαA-Lip-Cut. The recombinant vector was transformed into competent DH5α cells and sequenced to verify its identity and the absence of mutation.

Methods

Expression and purification of proteins from Pichia pastoris

The three recombinant plasmids were integrated into *P. pastoris* KM71H by electroporation using a Genepulser II apparatus (Bio-Rad, Hercules, USA). The individual colonies with maximum Lip and/or Cut activities were selected for the production of recombinant enzymes. Crude enzymes were purified by affinity chromatography using Ni-NTA Agarose gel (Qiagen, Valencia, USA) according to the manual. The purified proteins were subjected to sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) using 10% (w/v) gel to determine the enzyme homogeneity and molecular weight.

Enzyme activity assay

The Lip activity was measured based on the liberation of *p*-nitrophenol from *p*-nitrophenyl octanoate according to Liu *et al*. (2017). The Cut activity was determined in a similar manner with 10 mM *p*-nitrophenyl butyrate as the substrate in 0.05 M Tris-HCl buffer (pH 9.0) at 50 °C for 10 min (Yang *et al*. 2013). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol per min per mg of protein under the above assay conditions. All of the analytical measurements were performed in triplicate.

Effect of the temperature and pH on the enzyme activity and stability

The effect of the temperature on the enzyme activity was determined at a pH of 7.5 for the Lip activity and 9.0 for the Cut activity over the temperature range of 20 °C to 60 °C using the conditions from the assay. The effect of the temperature on the enzyme

stability was estimated by pre-incubating the enzyme over the temperature range of 35 °C to 55 °C for the time specified and then measuring the residual activity.

The effect of pH on the enzyme activity was determined at the optimum temperature in a universal buffer for the Lip activity and four buffers (sodium citrate (pH 3.0 to 6.0), phosphate sodium (pH 6.0 to 8.0), tris(hydroxymethyl) aminomethane-HCl (Tris-HCl; pH 8.0 to 9.0), and glycine-sodium hydroxide (pH 9.0 to 11.0)) for the Cut activity, over the pH range of 3.0 to 11.0. The universal buffer was comprised of 50 mM each of 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. The pH stability was measured by assessing the residual enzyme activity after the incubation of the enzymes at room temperature for 24 h in a universal buffer.

Effect of metal ions and chemical reagents on the activity

The effect of metal ions and chemical reagents on the Lip and Cut activities was determined by measuring the activities under enzyme activity assay conditions described above in the presence of various metal ions (final concentrations of 1 mM and 5 mM, respectively) and ethylenediamine tetraacetic acid (EDTA) (final concentrations of 0.1 mM and 0.5 mM, respectively) in a reaction mixture.

Substrate specificity and kinetic constants

The substrate specificities of the Lip and Cut were investigated under optimum conditions by measuring the amount of *p*-nitrophenol liberated in the reaction mixtures with different substrate solutions. The specific activities of the Lip and Cut were given in unit/mg of purified protein.

The kinetic constants (V_{max} and K_m) of the Lip and Cut were examined by measuring the enzyme activity at the optimum temperature and pH. A concentration was used of at least 8 substrates of *p*-nitrophenyl octanoate in the range of 0.2 mmol/mL to 4.0 mmol/mL for Lip and *p*-nitrophenyl butyrate from 1.0 mmol/mL to 20.0 mmol/mL for Cut. All of the assays were conducted in duplicate. The V_{max} and K_m were analyzed by fitting the experimental data in the GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) using nonlinear regression.

Effect of the Cut, Lip, and chimeric Lip-Cut on the release of acetic acid from PVAC

The reaction mixture contained 0.05 g of ground PVAC and purified Lip, Cut, and chimeric Lip-Cut (2.174 nmol, each) in a total 1.5 mL of phosphate sodium buffer (pH of 8.0, 100 mM). The reactions were carried out at 200 rpm for 12 h at 35 °C for the Lip and 50 °C for the Cut, respectively, according to their corresponding optimal temperatures, and 40 °C for the Lip-Cut chimera and Lip/Cut mixture, based on the compromise optimal temperature of two moieties. Then, the mixtures were boiled for 10 min. The acetic acid was detected using an Acetic Acid Assay Kit (Megazyme, Bray Business Park, Bray, Co. Wicklow, Ireland) according to the instructions. All of the hydrolysis experiments were conducted in duplicate.

Deinking of the laser-printed paper and newspaper pulp

The laser-printed paper and newspaper used in this study were domestic wastes, which were pretreated as previously described by Liu *et al*. (2017). Afterwards, the pulp was partially disintegrated and stored at 4 °C. The disintegrated paper was diluted to a 2% consistency with 10 mM phosphate sodium buffer (pH 8.0). All of the samples were

treated with 0.3% (v/v) surfactant (AEO-9) and the same molecule numbers of Lip, Cut, and Lip-Cut (5.53 nmol, each). Enzymatic deinking was performed in a shaker (150 rpm) at the optimum temperature for 3 h. The optimum temperature was 35 °C for the Lip, 40 °C for the Lip-Cut chimera and Lip/Cut mixture, and 50 °C for the Cut. Control experiments using a buffer instead of enzymes were also performed simultaneously. After treatment, the enzymes were deactivated by boiling the pulp for 5 min. Flotation was performed for 10 min at a 1% (w/v) consistency with a laboratory flotation unit. Finally, the pulp was washed with tap water in an 80-mesh wire sieve and recovered for testing. All of the experiments were performed in triplicate.

Analysis methods

The released chromophores were measured in the filtrate by absorbance at a λ=231 nm (Patel *et al*. 1993). After deinking, the handsheets (TAPPI T205 sp-02 (2002)) were made with a standard basis weight of 60 g/m. The optical properties of the handsheets were measured (effective residual ink amounts and brightness), and the burst index (TAPPI T403 om-10 (2010)), tensile index (TAPPI T494 om-13 (2013)), and tear index (TAPPI T414 om-12 (2004)) were also measured to analyze the strength properties of the handsheets. Image analysis of the handsheets was performed using a Hewlett Packard Scan Jet 3c scanner (Palo Alto, CA, USA) and Spec Scan 2000 software (Thwing-Albert Instrument Company, West Berlin, USA). The analyzed area of the handsheets was 314 cm^2 for each duplicate. The effective residual ink amounts on the handsheets were determined by image analysis (TAPPI T213 om-15 (2001)). The brightness of the handsheets was measured according to the standard TAPPI T 452 om-08 (2008). The ink removal efficiency (%) was calculated according to Eqs. 1 and 2,

$$\text{Ink removal efficiency of control paper (\%)} = 100 \times (N_{\text{No surfactant-treated paper}} - N_{\text{surfactant added-treated paper}}) / N_{\text{No surfactant-treated paper}} \quad (1)$$

$$\text{Ink removal efficiency of enzyme-treated paper (\%)} = 100 \times (N_{\text{No surfactant-treated paper}} - N_{\text{enzyme-treated paper}}) / N_{\text{No surfactant-treated paper}} \quad (2)$$

where N is the residual ink number. The paper not treated by surfactants was made directly from the pulp slurry without a 3-h treatment.

RESULTS AND DISCUSSION

Design of the Chimeric Lip-Cut

Artificial chimeric enzymes have been constructed for various purposes, including targeted delivery, improved stability, novel specificity, and superior detection (Fan *et al*. 2009). A key goal in the construction of chimeric enzymes that must be considered is to maintain or improve the enzymatic characteristics of the individual components. Therefore, the addition of peptide linkers between the two proteins often confers the necessary flexibility for the individual proteins to maintain or improve their functionalities (Fan *et al*. 2009). In this work, two well characterized enzymes, Lip from *Thermomyces lanuginosus* (Zheng *et al*. 2011) and Cut from *Thielavia terrestris* (Yang *et al*. 2013), were used to construct the chimeric Lip-Cut to lower the production costs and increase the enzymatic catalytic efficiency for biological applications. A native 21-aa residue linker (GPTTTSSAPNPTSSGCPNATK), derived from endoglucanase EG1 from

V. volvacea, was used to join the two functional domains to avoid structural and functional interferences.

Expression and Purification of the Lip, Cut, and Chimeric Enzyme

The chimeric Lip-Cut and its parent enzymes, Lip and Cut, were successfully expressed in *P. pastoris* KM71H. The high homogeneity of the enzymes was visualized by 10% SDS-PAGE (Fig. 1). The molecular weights of the final purified recombinants Lip, Cut, and chimeric Lip-Cut were approximately 34 kDa, 23 kDa, and 53 kDa, respectively, as shown in the SDS-PAGE profile (Fig. 1), which were identical to their theoretical molecular weights.

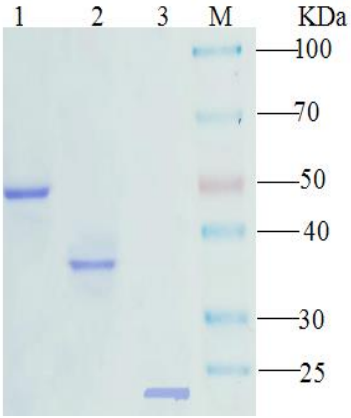

Fig. 1. SDS-PAGE analysis of the purified recombinant proteins: Lane M, molecular weight marker; Lane 1, Lip-Cut; Lane 2, Lip; Lane 3, Cut

Effect of the Temperature and pH on the Enzyme Activity and Stability

When assayed using *p*-nitrophenyl octanoate as a substrate, the Lip had an optimum temperature of 35 °C, and the Lip moiety in the chimeric Lip-Cut had a shifted optimum temperature of 30 °C (Fig. 2A1). The Cut and Cut moiety in the chimeric Lip-Cut had the same optimum temperature of 50 °C (Fig. 2A2). The Lip moiety in the chimeric Lip-Cut had a higher thermal stability than Lip at 35 °C to 45 °C. However, the Cut moiety in the chimeric Lip-Cut had a lower thermal stability than the Cut after 1.5 h of incubation at 45 °C to 55 °C (Figs. 2B1 to 2B2). The Lip, Cut, and Lip and Cut moieties in the chimeric Lip-Cut had optimum pH values of 7.5, 8.0, 8.0, and 9.0, respectively (Figs. 2C1 and 2C2). The moieties in the chimeric Lip-Cut displayed pH stabilities similar to that of the parent Lip and Cut (Figs. 2D1 and 2D2).

Effect of Metal Ions and Chemical Reagents on the Activity

The influence of diverse metal ions and chemical reagents on the Lip and Cut activities was investigated using *p*-nitrophenyl octanoate and *p*-nitrophenyl butyrate as substrates, respectively. The results showed that the Lip was almost unaffected by the cations Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, and NH_4^+, while a slight inhibition was detected in the presence of Mn^{2+} and Fe^{3+} at the concentration of 1 mM. All of the tested metal ions showed some inhibition effects on the Lip when the concentration was increased to 5 mM. The Lip moiety in the chimeric Lip-Cut was found to be activated by 1 mM of Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+}, and Co^{2+}, but certain cations, such as Cu^{2+} and Zn^{2+}, resulted in the reduction of the Lip activity of the Lip-Cut (72.1% and 74.1%, respectively; see Table 1).

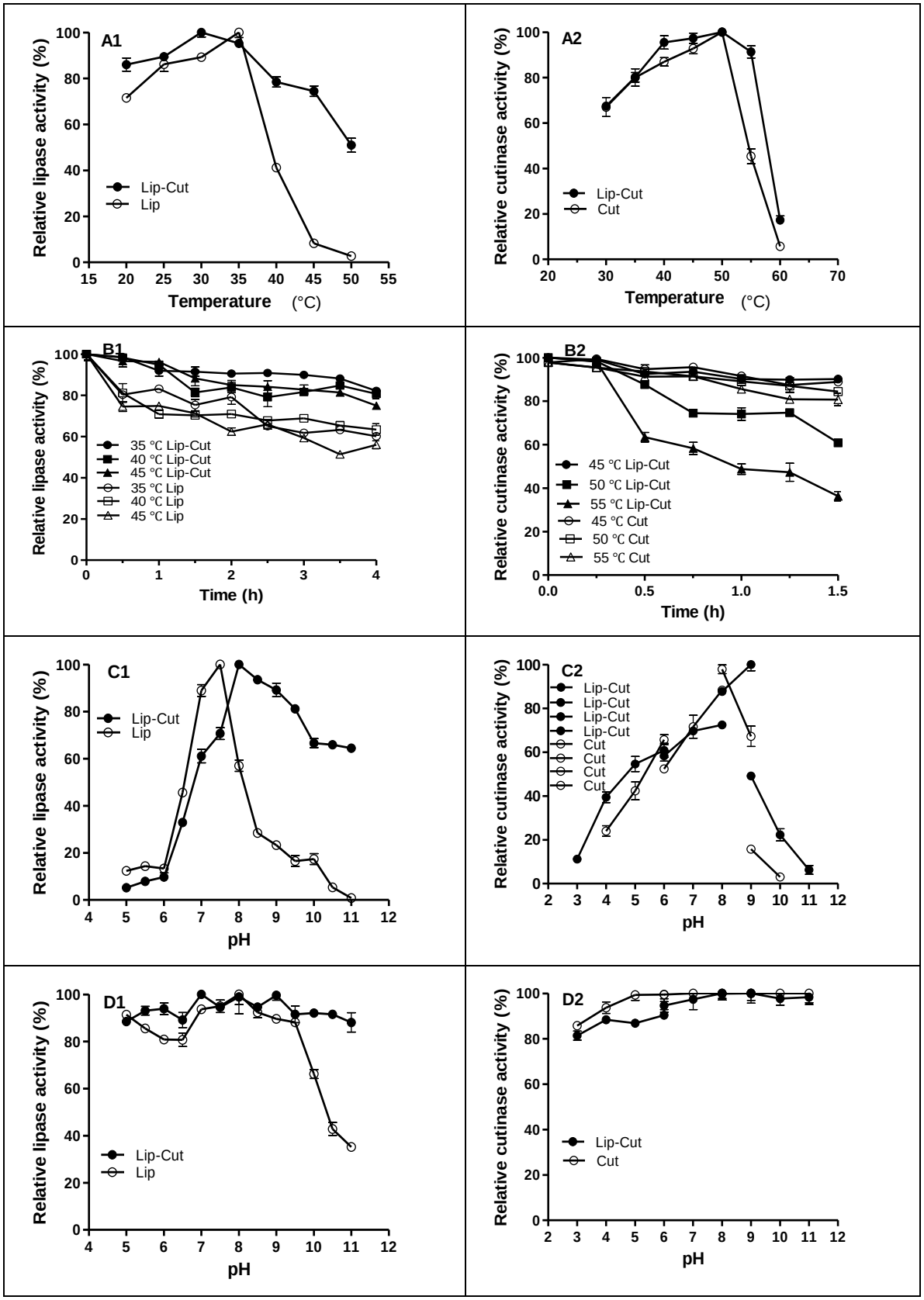

Fig. 2. Effect of the optimum temperature (A), thermostability (B), optimum pH (C), and pH stability (D) on the Lip (1) and Cut (2) activities of the chimeric Lip-Cut and parent enzymes; values shown are the means of the results of the triplicate experiments ± the standard error (SE)

When the metal concentration was increased to 5 mM, all of the metal ions exhibited a stimulation effect on the Lip activity of the chimeric Lip-Cut. The Cut was slightly stimulated in the presence of 1 mM cations, but no such activation was observed in the chimeric Lip-Cut. Inhibition was detected when the concentration of metal ions, such as Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+}, was increased to 5 mM. The chelating agent EDTA did not remarkably influence the Lip, Cut, and Lip-Cut activities (Table 1).

Table 1. Effect of Different Metal Ions and Chemical Reagents on the Enzyme Activity

Metal Ion or Chemical Agent	Lipase Activity of Lip (%)		Lipase Activity of Lip-Cut (%)		Cutinase Activity of Cut (%)		Cutinase Activity of Lip-Cut (%)	
	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM
Ca^{2+}	94.2	84.0	115.9	126.0	112.5	94.5	97.0	71.1
Mn^{2+}	81.5	87.9	106.1	114.8	112.8	91.5	95.8	72.4
Mg^{2+}	97.9	95.6	100.1	143.3	112.3	90.5	94.8	68.1
Cu^{2+}	101.4	96.8	72.1	114.2	113.2	86.8	99.4	87.8
Zn^{2+}	100.1	93.1	74.1	114.8	114.9	93.6	91.0	83.2
Fe^{3+}	83.5	85.1	123.8	109.5	112.5	96.7	98.0	147.1
Ni^{2+}	90.9	94.7	90.7	159.7	113.6	100.9	98.4	102.8
Co^{2+}	100.7	99.4	133.1	128.7	100.4	90.4	95.9	118.6
NH_4^+	93.2	95.1	92.1	125.8	104.2	97.2	102.2	140.2
EDTA (0.1 mM)	89.2		89.8		98.0		82.5	
EDTA (0.5 mM)	91.6		91.6		101.8		81.1	

Specific Activities and Kinetic Properties of the Parent and Chimeric Enzymes

The specific activities of the Lip, Cut, and Lip-Cut for various substrates are given in Table 2. The Lip and Lip moiety in the chimeric Lip-Cut had similar substrate specificities, *i.e*. the highest activity was observed against *p*-nitrophenyl octanoate. The enzymes also possessed notable activities toward *p*-nitrophenyl decanoate and *p*-nitrophenyl laurate, but they were one to five times lower than the *p*-nitrophenyl octanoate activity. The Cut and Cut moiety in the chimeric Lip-Cut showed the highest activities for *p*-nitrophenyl butyrate, followed by *p*-nitrophenyl valerate, *p*-nitrophenyl octanoate, and *p*-nitrophenyl propionate.

Table 3 lists the K_m and V_{max} values of the Lip and Cut moieties in the chimeric Lip-Cut compared with their parent enzymes. The Lip activity of the chimeric Lip-Cut was 2.4-fold higher that of the parent Lip (V_{max}) toward *p*-nitrophenyl octanoate. For the Cut moiety, the Cut activity of the chimeric Lip-Cut was 3.4-fold higher that of the parent Cut (V_{max}) toward *p*-nitrophenyl butyrate (Table 3). The obtained results clearly indicated that the co-existence of Lip and Cut moieties in the chimeric Lip-Cut promoted the activity of each other.

Table 2. Specific Activities of the Lip, Cut, and Chimeric Lip-Cut

Substrate	Enzyme Activity (U/μmol)			
	Cut	Cutinase Activity of Lip-Cut	Lip	Lipase Activity of Lip-Cut
p-Nitrophenyl acetate	2839.1	7250.9	102.7	6083.5
p-Nitrophenyl propionate	8990.5	25257.0	100.0	16462.5
p-Nitrophenyl butyrate	18650.2	63741.3	206.4	45880.1
p-Nitrophenyl valerate	17454.2	55601.3	292.1	47183.5
p-Nitrophenyl octanoate	9786.0	34081.0	29628.0	72363.5
p-Nitrophenyl decanoate	5775.1	13944.5	15166.4	55587.0
p-Nitrophenyl laurate	4983.2	12032.4	5486.9	49483.1
p-Nitrophenyl myristate	1731.2	2519.6	595.3	15094.5
p-Nitrophenyl stearate	1083.1	4377.2	349.2	14272.1

Table 3. Kinetic Parameters of the Lip, Cut, and Chimeric Lip-Cut

Enzyme	Kinetic Parameter	
	K_m (mg/mL)	V_{max} (U/μmol)
Lip (*p*-Nitrophenyl octanoate)	0.61	40843
Lip-Cut (*p*-Nitrophenyl octanoate)	0.83	92807
Cut (*p*-Nitrophenyl butyrate)	0.92	31878
Lip-Cut (*p*-Nitrophenyl butyrate)	1.71	98821

Effect of the Cut, Lip, and Chimeric Lip-Cut on the Release of Acetic Acid from PVAC

The release of acetic acid gradually increased with the reaction time (Fig. 3), except for the single Lip treatment.

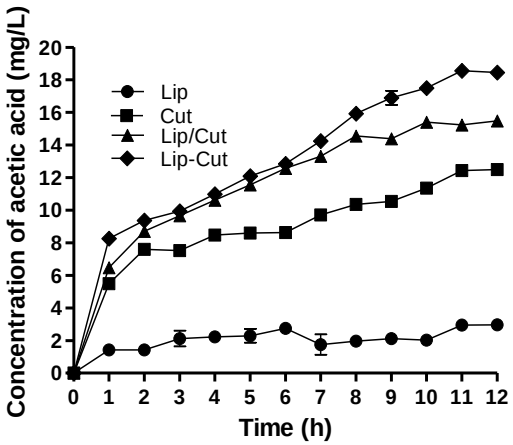

Fig. 3. Release of acetic acid from the PVAC after degradation by the Lip, Cut, Lip/Cut mixture, and chimeric Lip-Cut; values shown are the means of the results of the triplicate experiments ± the SE

After hydrolysis for 12 h, the concentration of the released acetic acid by the chimeric Lip-Cut was the highest (18.5 mg/L), followed by the Lip/Cut mixture (15.5 mg/L), Cut (12.5 mg/L), and Lip (3.0 mg/L). This demonstrated that the Cut degraded the PVAC and the effect was improved by the synergistic action of the moieties in the chimeric Lip-Cut.

Effect of the Lip, Cut, and Lip-Cut on the Ink Removal Efficiency and Paper Brightness

The same amount of molecules in the Lip, Cut, and Lip-Cut (5.53 nmol/g dried waster paper for each enzyme) was used in the deinking trials. Figure 4 shows the results obtained in terms of the effective ink removal efficiency and brightness. It was observed that a considerable ink removal efficiency was attained from mechanical treatment. In fact, the control (no enzyme action present) had an average ink removal efficiency of approximately 71% for both pulp samples (laser-printed paper and newspaper). This was probably the result of environmental factors, such as the pH, temperature, and washing, which enabled a partial release of ink particles from the fibers due to the breakdown of physical and chemical bonds (Marques *et al*. 2003).

The present work revealed that the Lip showed only a 1.3% to 4.1% higher ink removal efficiency compared with the control. When the Cut was added to the deinking trials, an ink removal efficiency was obtained that was 8.9% and 16.2% higher compared with the control-treated laser-printed paper and newspaper, respectively (Figs. 4A1 and 4A2). The Lip/Cut mixture showed 22.1% and 10.2% higher ink removal efficiencies for the laser-printed paper and newspaper, respectively, while the values for the chimeric Lip-Cut were 25.8% and 16.2% higher than the control-treated paper, respectively (Figs. 4A1 and 4A2). The higher ink removal efficiency of the chimeric Lip-Cut was likely due to the inner-molecular synergistic action of the Lip-Cut during the removal of surface material (ink particles and binder). Moreover, the Lip-Cut displayed 2.4- and 3.4-fold higher Lip and Cut activities than the parent enzymes, respectively (Table 3). The higher Lip and Cut activities might have been an important contributing factor to the higher ink removal efficiency observed during waste paper deinking.

Brightness is one of the main appearance indicators used to determine the paper quality (Fillat *et al*. 2015). In this study, the effect of proper enzymatic treatment on the brightness of laser-printed paper and newspaper was investigated. The experimental results are shown in Figs. 4B1 and 4B2. The enzymatic deinking resulted in improvements to the sheet brightness (Figs. 4B1 and 4B2). However, the effects of the Lip and Cut on the improvement of the sheet brightness were not as good as that of the Lip/Cut mixture and chimeric Lip-Cut. The highest brightness was achieved with the chimeric Lip-Cut, which displayed a brightness of approximately 88% ISO and 59% ISO for the laser-printed paper and newspaper, respectively.

Figures 4C1 and 4C2 revealed that the enzymatic treatment caused the release of chromophores (λ = 231 nm) from the pulp. Chromophores are groups that can cause the absorption of compounds in the ultraviolet and visible light spectrum (Chutani and Sharma 2015). The deinking solution exhibited the highest absorbance at a λ=231 nm, which meant that some aromatic compounds were released into the pulp slurry. Figures 4C1 and 4C2 showed that the release of chromophores was gradually enhanced by the enzymatic treatment in the order (from low to high) of Lip, Cut, Lip/Cut mixture, and chimeric Lip-Cut, which was consistent with the enzymatic ink removal efficiencies. The higher release of chromophores by the chimeric Lip-Cut was because its ability to

degrade plastic adhesives was better than that of the parent enzymes and their mixture (Fig. 3). However, the chromophores were not all from the ink binder; some chromophores in newspaper fibers could be also released during deinking process.

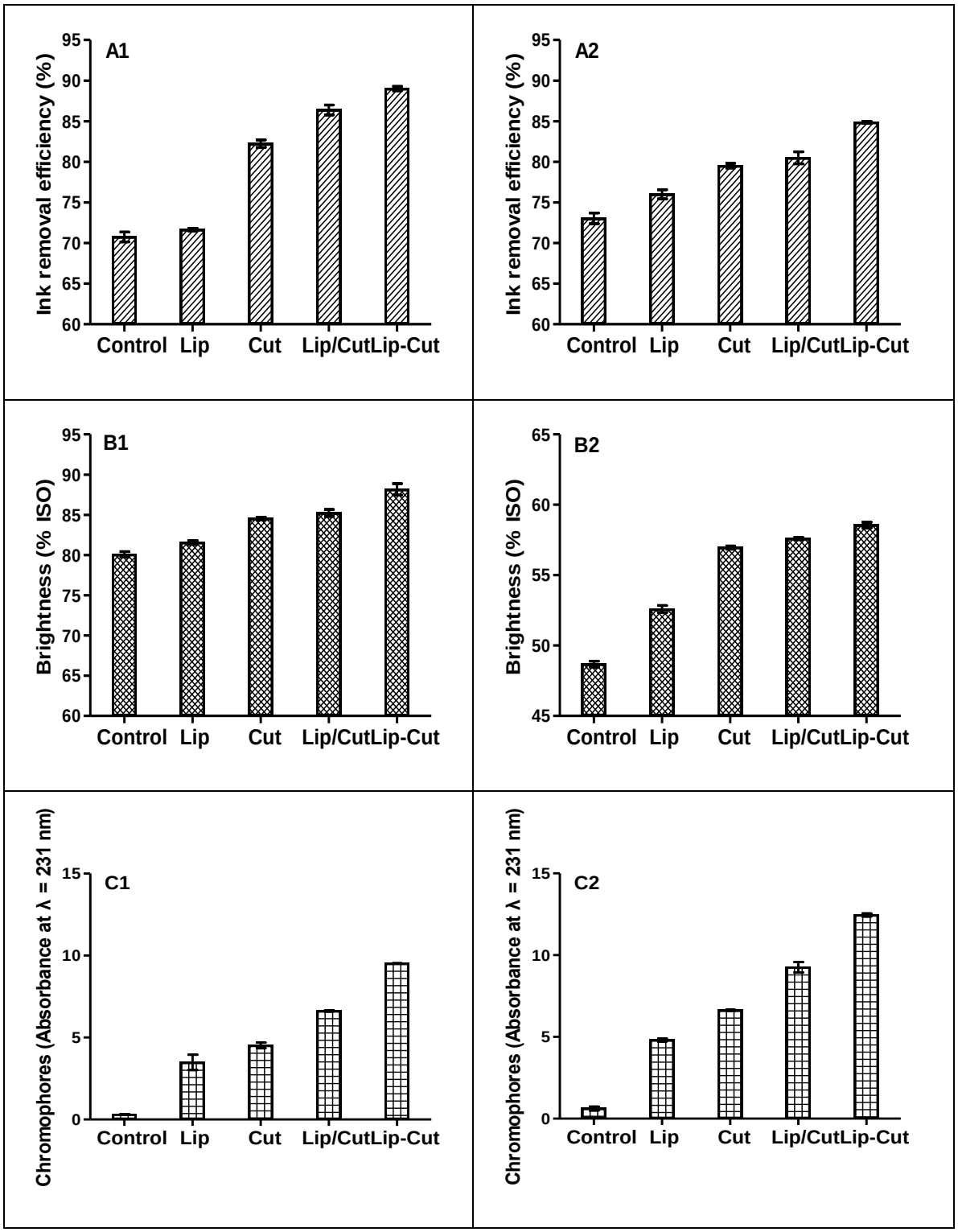

Fig. 4. Ink removal efficiencies of the laser-printed paper (A1) and newspaper (A2); brightness of the laser-printed paper (B1) and newspaper (B2); released chromophores in the effluent of the laser-printed paper (C1) and newspaper (C2)

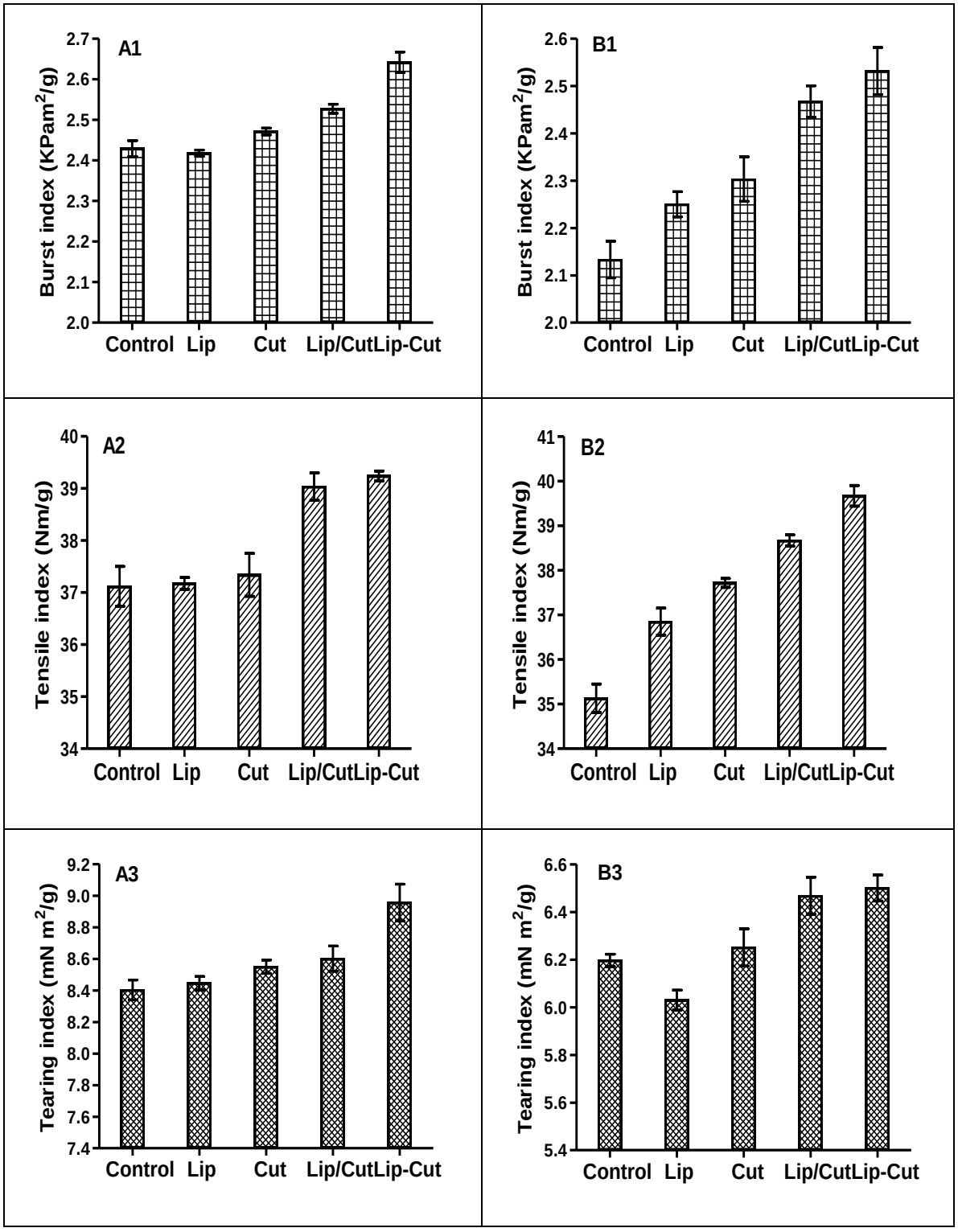

Fig. 5. Strength properties of the deinked laser-printed paper (A) and newspaper (B) after enzyme treatment; A1 and B1: burst index; A2 and B2: tensile index; and A3 and B3: tear index

Effect of Lip, Cut, and Lip-Cut on Strength Properties of the Deinked Paper

Figure 5 shows the effect of enzymatic treatment on the pulp properties, including the burst, tensile, and tear indices. All of the enzymes contributed to the improvement of the strength properties, with the exception of the tear index for the Lip deinked newspaper, which was marginally decreased. These results supported previous investigations by other authors concerning the occurrence of enhanced, decreased, or unchanged strength properties (Heise *et al*. 1996; Marques *et al*. 2003; Pala *et al*. 2004; Pathak *et al*. 2015). The chimeric Lip-Cut increased the strength properties of both pulp samples compared with the Lip, Cut, and Lip/Cut mixture. The chimeric Lip-Cut-treated laser-printed paper showed 8.8%, 5.7%, and 6.6% higher burst, tensile, and tear indices than that of the control-treated paper, respectively (Figs. 5A1 to 5A3). Figures 5B1, 5B2, and 5B3 showed that the burst, tensile, and tear indices for the Lip-Cut treated newspaper were 18.7%, 12.9%, and 4.9% higher than that of the control-treated newspaper, respectively (Figs. 5B1 to 5B3). The strength properties of paper is not only determined by the physical properties of the fiber (fiber length, cellulose viscosity, etc.), but is also determined by the effective bond between fibers. The enzymes used in the present work did not affect the properties of fiber, but improved the effective bond between fibers due to the removal of dirt on fibers during enzymatic treatment, thereby increasing the strength properties of the deinked papers.

The doses used for the Lip, Cut, chimeric Lip/Cut, and Lip-Cut mixture were 5.53 nmol/g, 5.53 nmol/g, 11.06 nmol/g, and 5.53 nmol/g dried waste paper, respectively. Compared with the Lip/Cut mixture, the chimeric Lip-Cut not only noticeably reduced the amount of enzyme used, but also majorly improved the paper strength for both waste papers; therefore, it was concluded that the chimeric Lip-Cut is useful for the deinking of laser-printed paper and newspaper (Zhang *et al*. 2017a). Compared with the chimeric Lip-EG1CD constructed in this laboratory, the chimeric Lip-Cut achieved better strength properties, especially for the laser-printed paper. The Lip-Cut-treated laser-printed paper had 5.7%, 2.5%, and 0.6% higher burst, tensile, and tear indices than that of the Lip-EG1CD-treated paper, respectively. The Lip-Cut-treated newspaper had marginally decreased or maintained similar tensile and tear indices as that of the Lip-EG1CD-treated newspaper, whereas the burst index of the Lip-Cut treated newspaper was 4.6% higher than that of the Lip-EG1CD-treated newspaper (Liu *et al*. 2017). Since the burst index was mainly influenced by the bonding between fibers, this phenomenon indicated the Lip-Cut treatment was more effective in improvement of bonding ability of the recycled newspaper than Lip-EG1CD was.

CONCLUSIONS

1. A chimeric enzyme with Lip and Cut activities was constructed and successfully produced in *Pichia pastoris*. The Lip-Cut displayed Lip and Cut activities that were 127% and 210% higher than that of the parent enzymes, respectively.
2. The Lip-Cut showed a higher ink removal efficiency and sheet brightness than that of the parent enzymes and mixture of enzymes. The strength of the handsheets was also improved using the Lip-Cut.

3. The results suggested that the proper construction of bi-functional Lip-Cut could enhance the catalytic properties through the synergistic action of the two moieties because of the complementary nature of the substrate specificities and catalysis patterns. This chimeric enzyme can be used for the efficient deinking of waste paper.

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APPENDIX

Table S1. PCR Primers Used in this Study

Primer	Nucleotide Sequence (5'-3')
Primer 1	AGAGAGGCTGAAGCTGAATTCCGGCCTGTTCGACGAGCGGT
Primer 2	AGGTGGGGTTGGGTGCGCTGCTGGTTGTCGTAGGTCCATCACACTCTGAAAT
Primer 3	ACCCAACCCCACCTCCAGTGGCTGCCCGAATGCCACCAAGGCCCCAACACAGCCA
Primer 4	GAGATGAGTTTTTGTTCTAGAAATCAATGATGATGATGATGATGAGCATCACCAATCTT