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Characterization of cellulase, hemicellulase and lipase and its use in deinking of laser printed paper

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ABSTRACT

Aims: It is recognized that laser printed paper are difficult to deink using conventional method. This had lead to the suggestion of enzymatic approach to overcome the problem encountered by commonly employed deinking techniques. The present study aimed to investigate 7 commercially available enzymes for their suitability use in deinking of laser printed paper.
Methodology and results: 3 cellulases, hemicellulases, xylanase and 2 lipases were used in enzymatic deinking of laser-printed wastepaper. Cellulase A "Amano"3 (C), Hemicellulase (H) and lipase (L) were selected for used in deinking because they possess either highest activity or broad pH stability compared to others enzymes. Different combination of enzymes was carried out to evaluate their effectiveness in deinking process. CH enzymes sequence was determined to be the most effective sequence in toner removal with 1.90% of brightness increment. However, only 0.95% of brightness increment was gained by enzyme sequence L. Highest deinking efficiency obtained was not proportional to the highest total reducing sugar produced.
Conclusion, significance and impact of study: Enzyme (cellulase and hemicellulase) can be used to de-ink laser-printed wastepaper, which are difficult to be deinked by conventional chemical deinking process. Thus, enzyme deinking has high possibility as alternative method to current chemical deinking process which is not environmental friendly.

Keywords: Laser-printed paper; cellulase; hemicellulase; lipases; enzymatic deinking

INTRODUCTION

Recycled waste paper has become an important and environmentally benign source for new fiber for papermaking. Office waste papers that mainly consist of laser-printed and xerographic papers are a fast growing source for recycling due to increase utilization of office photocopiers and computers print out. However, the reuses of these waste papers are limited because the ink formulations used in these papers are difficult to remove by conventional techniques such as washing and flotation. The toners used in xerographic and laser printers consist of thermoplastic resin pellets such as copolymers of acrylate and styrene along with pigments such as carbon black. Toners are fixed by fusion with combination of pressure and heat (Carr, 1991). The flat, plate like particles of toners is difficult to disperse and remove by conventional process. Moreover most of the conventional deinking techniques require usage of large amount of chemical agents such as sodium carbonate, sodium hydroxide, sodium silicate, hydrogen peroxide and surfactants (Prasad *et al.,* 1993; Woodward *et al.,* 1994, cited: Marques *et al.,* 2003) which resulted in a costly wastewater treatment (Jeffries *et al.,* 1994). Furthermore, enzymatic deinking can avoid the alkaline environments required in traditional deinking process, which consequently reduce chemical and waste treatment cost.

Alternatively, enzyme approaches have been assessed and proven successful using a number of different enzymes types. Lipases can degrade vegetable oil based ink while cellulases and hemicellulase are believed to alter fiber surface or bonds in the vicinity of ink particles thereby facilitating subsequent ink removal (Zeyer *et al.,* 1994; Ow *et at.,* 1995; Welt & Dinus, 1995, cited: Marques *et al.,* 2003, Anne *et al.,* 1999). Since enzymatic deinking have been proven and reported to be a potentially efficient. Therefore, several commercial available enzymes such as cellulase, hemicellulase and lipase were investigated in present study for their efficiently used in deinking process.

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MATERIALS AND METHODS

Enzymes

7 commercially available enzymes were study for their suitability used in deinking of laser-printed paper. Five enzymes obtained from Amano Pharmaceutical Co., LTD (Nagoya, Japan) were consisting of cellulase A "Amano"3 (C), cellulase T "Amano"4 (T), hemicellulase "Amano"90 (H), lipase F-AP 15 (L) and lipase AY "Amano"30 (AY). Two other enzymes designated cellulase (S) and xylanase (X) were purchased from Sigma (USA) and Fluka, (USA), respectively. All the enzymes were either light yellowish or light brownish powder and soluble in water.

Cellulases assay

The cellulase activity was determined based on the carboxymethyl cellulase (CMC)-activity. For optimum temperature, CMC was dissolved in 50 mM citric-NaOH buffer, pH 4.5 as substrate. To 1 mL of CMC solutions, 0.5 mL of appropriately diluted enzyme was added and the solutions were incubated at 25 °C to 75 °C (5 unit intervals) for 20 min, respectively. The reactions were terminated by adding 3 mL of dinitrosalicylic acid (DNS) reagent followed by 5 min of boiling. The released sugar was measured spectrophotometrically at 575 nm using glucose as the standard (Gessesse & Gashaw,1999). The control experiment was performed as described above using heat-inactivated enzyme by boiling for 10 min. For optimum pH, the experiments were performed as described in optimum temperature experiments excepted CMC was dissolved in 50 mM of different buffer solution at pH ranging from 1.5 to 12.5 with 1 unit intervals as substrate and the solutions were incubated under the determined optimum temperature for 20 min. One unit of cellulase activity was defined as the amount of enzyme that released 1 µmol of reducing sugar equivalent to glucose per minute under the above assayed condition.

Xylanases assay

Hemicellulase, H and xylanase, X activities were assay as described by Gessesse & Gashe (1997). The experiment was performed as described in cellulase assay except 1% (w/v) of oat spelt xylan was used as substrate for xylanase and hemicellulase. One unit of enzyme activity was defined as the amount of enzymes, which catalyzes the liberation of 1µmol of reducing sugar equivalent to xylose per minute under specified assayed condition. Xylose was used as the standard.

Lipase assay

Lipases activities were assayed by the olive oil emulsion method as described by Mustranta *et al.* (1993) with some modification. For optimum temperature, the substrate was prepared by emulsifying 30 mL of olive oil and 70 mL of emulsification reagent by homogenizing for 3 min. 5 mL of emulsion was mixtures with 4 mL of 0.2 M phosphate buffer, pH 7.0 at 200 rpm at 30 °C to 60 °C (5 interval) for 10 min, respectively before 1.0 mL of appropriately diluted enzyme was added. Thereafter, the solutions were incubated for 30 min and the reactions were stopped by addition of 20 mL of 95% ethanol (Sugihara *et al*., 1995). Control was performed as described above using heat-inactivated enzyme. Fatty acids released during the incubation were immediately titrated with 50 mM NaOH. One unit of lipase represented the release of 1 µmol of fatty acid per min under the above assay condition. For optimum pH, the experiments were carried out as described above for optimum temperature excepted the emulsions were mixed with different buffer at pH ranging from 2.5 to 12.5 with 1 unit interval. The solutions were incubated under determined optimum temperature for 30 min.

Selection of paper and enzymes

A4 size paper with a basis weight of 70g/m^2 produced by Asia pulp & paper (Malaysia) Sdn Bhd was printed with toner on a canon laser shot LBP-1120 laser printer on 2.5 cm x 2.5 cm square area for 40 squares per sheet. Only one side of each sheet of the paper was printed. The printed areas were cut and used in all the experiments. The printed papers were pre-treated with 0.5N HCI for 30 min before applied to enzyme treatment. One enzyme from each group was selected for used in deinking of laser printed-paper. Cellulase C, hemicellulase H and lipase L were selected for used in deinking of laser-printed waste paper. The selection was based on the high activity, broad pH stability and high thermostability.

Effect of different enzymes sequences on ink removal

Enzymatic hydrolysis was performed at pH 5.5 and temperature 40 °C. 16 pieces (±0.8g) of papers were dipped into 0.2 M citric-NaOH buffer for 10 min and shake at 60 rpm prior to enzyme addition. Each enzyme (C, H and L) was previously diluted to 10 U/mL to ensure an even enzymes distribution on the papers. The dosage chosen was based on preliminary experiments makes to determine the approximate amount of enzymes necessary to produce a reasonable deinking level. Enzymes of different sequences were added at 10 min interval. The mixtures were incubated for 60 min. Control was run simultaneously using thermally inactivated enzyme (Gubitz *et al*, 1998). The reactions were stopped by boiling for 10 min. The solutions were used to assay for reducing sugar. The papers were rinsed three times with distilled water before applied to the flotation process. The initial conditions for the flotation process were set at pH 6.0, 0.5% (w/w) of Tween 20, 2 L/min of air flow rate, room temperature and 30 min of flotation time. After flotation process, the papers were taken for brightness analysis. The brightness increase (%) was determined by subtracting blank brightness from sample brightness. The most efficient enzymes sequence on toner removal was selected for further study.

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Observation of enzymatic treated papers under scanning electron microscope

After the flotation process, the de-inked papers were rinsed three times with distilled water and air-dried under room temperature. The de-inked paper was cut and mounted onto a scanning electron microscope specimen stud with a double-sided sticky tape. The papers were then coated with a layer of approximately 20 nm thick gold using sputter coater (POLARON SC515). The coated papers were examined under SEM (Gemini Supra Puls) with 500 times magnification.

RESULTS AND DISCUSSIONS

7 commercially available enzymes were used in this study. Five enzymes were obtained from Amano Pharmaceutical Co., LTD (Nagoya, Japan). Another two enzymes were purchased from Sigma (USA) and Fluka, (USA), each. Different manufacturer used different assay methods to determine the enzyme activity. Thus, there is a need to use one assay method to quantify and standardized the enzymes activity obtained from different manufacturer prior to apply in this study.

Effect of temperature and pH on cellulases activities

The temperature profiles of cellulases are shown in Figure 1. All enzymes distinctively exhibited different optimum temperatures. The cellulases produced displayed optimum activities for temperatures range between 55^{o}C to 65^{o}C. The result was in agreement with Marques *et al*., (2003) who reported that the cellulase produced showed maximal activity at temperature range between 55^{o}C to 60^{o}C. However, Maijala *et al*., (2012) reported endoglucanase produced by *Myceliophthora fergusii* MTCC 9293 and *Malbranchea cinnamomea* MTCC 9294 showed optimum temperatures at 70 and 80^{o}C, respectively. Cellulase C showed an optimum temperature of 55^{o}C compared to S at 65^{o}C and T at 60^{o}C. The optimum activities at their respective optimum temperature and pH 4.5 were 816 U/g, 408 U/g, 862 U/g, respectively. Although cellulase C and S were produced by similar fungus of *Aspergillus niger*, but did not displayed identical optimum temperature. Thus, the result indicated that cellulase C and S might be from different sources or different strain of *A. niger*.

Figure 1b shows the pH profiles of cellulases from different sources with optimum pH ranging from 2.5 to 4.5. The optimum pH for C was at pH 2.5 while S and T were at pH 3.5 and 4.5, respectively. These findings were similar to the cellulase produced by *T. viride* CCMI 84 strain that exhibited optimum pH of 4.5 (Marques *et al*., 2003). On the other hand, Howard *et al*., (2003) reported that cellulase produced by *A. niger* showed an optimum pH of 5.0. While, cellulase produced by *Bacillus pumilus* EB3 showed at optimum pH of 6.0 (Ariffin *et al*., 2006). Under the respective optimum conditions, C (55^{o}C, pH 2.5) showed the highest activities of 1018 U/g, followed by T (60^{o}C, pH 4.5) of 860 U/g and S (65^{o}C, pH 3.5) of only 490 U/g of CMC activities. Findings reported that most cellulases have an optimum pH value of 3.5 or above (Ghosh & Ghosh, 1992). Nevertheless, it was observed that the cellulase used in this work has an optimum pH of 2.5 (cellulase C). However, it must be noted that cellulase activity is very dependent on the type of buffer used since for lower pH value (2.0-3.0), the activity can be higher or ever double depending on the buffer solution (Marques *et al*., 2003).

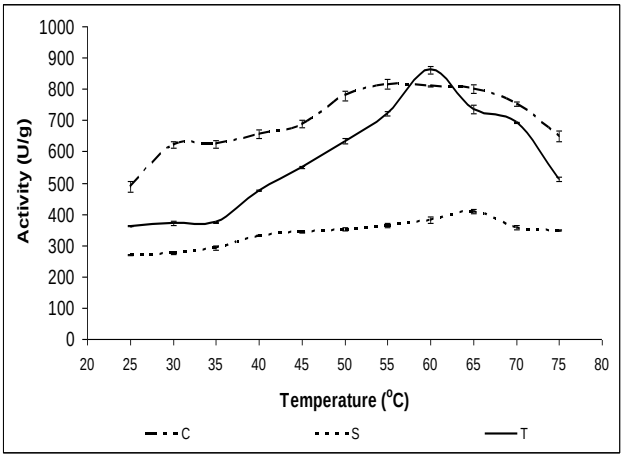

Figure 1a: Effect of temperature on cellulases activities.

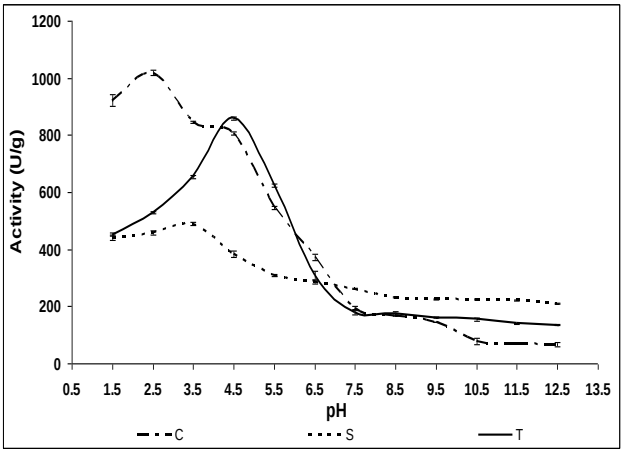

Figure 1b: Effect of pH on cellulases activities.

The assay was performed at 55^{o}C, 65^{o}C and 60^{o}C, for cellulase C, S and T, respectively for 20 min. The ionic strength for all buffers was 50 mM; Glycine-HCl, pH 1.5 and 2.5; citric-NaOH, pH 3.5 to 5.5; sodium phosphate, pH 6.5 and 7.5; Tris-HCl, pH 8.5; glycine-NaOH, pH 9.5; carbonate, pH 10.5; phosphate, pH 11.5 and hydroxide-chloride, pH 12.5. Note: Arrow bars indicate means with standard error of three replicates.

Effect of temperature and pH on hemicellulase and xylanases activities

The temperature profiles of hemicellulase H and xylanase X were shown in Figure 2a. The result demonstrated that

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both enzymes have similar optimum temperature of 50°C. The result was in agreement with Marques *et al*., (2003) and Rajashri & Anandrao (2012), who reported that the xylanase produced showed optimal activity at 50°C. Compared to H, X exhibited a broader optimum temperature profile. The maximal activities for H and X at the optimum temperatures and pH 4.5 were 9434 U/g and 11255 U/g, respectively. These findings were not similar to the result obtained by Howard *et al*., (2003) and Maijala *et al*., (2012) in which the xylanase produced displayed an optimum temperature at 45°C and 70°C, respectively. In conclusion, the results indicated that xylanases produced by different strains may have different characteristics with respect to their temperatures optimum.

Result presented in Figure 2b shows the effect of pH on the activities of H and X. The optimum pH of H and X differs for different enzyme producer strain. H and X displayed maximum activity at pH 4.5 and 5.5, which were produced by *A. niger* and *T. viride*, respectively. Under the optimum condition, the highest activity of 9372 U/g was obtained by H compared to X of 11486 U/g. However, both activities dropped drastically after pH 6.5 and extremely low activity was detected at pH 8.5 and above. Marques *et al*., (2003) reported that the more acidic xylanase of pH 4.5 was produced by *T. viride* compared to xylanase of pH 5.5 that produced by *A. terreus*. In addition, Rajashri & Anandrao (2012) reported that, xylanase produced by *Bacillus* sp. showed at optimum pH of 8.0. It can be concluded that, xylanase produced by *Aspergillus* and *Trichoderma* sp. have an optimum pH at acidic condition.

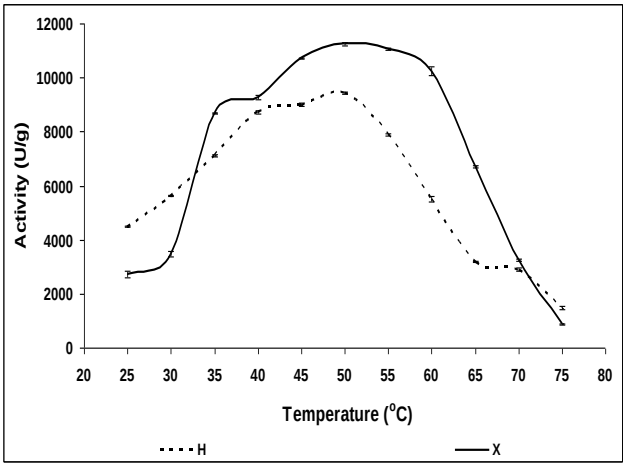

Figure 2a: Effect of temperature on hemicellulase H and xylanase X activities.

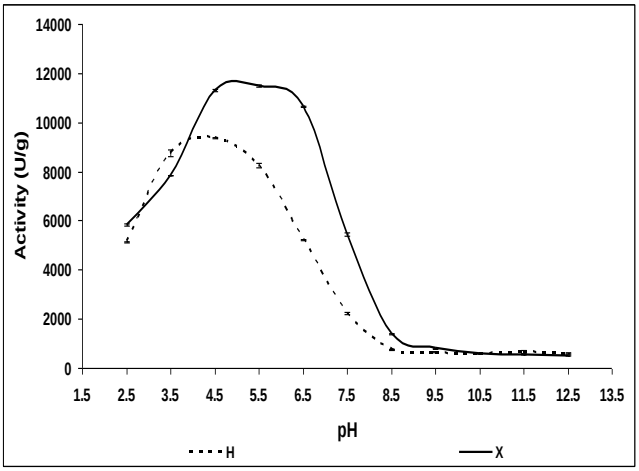

Figure 2b: Effect of pH on hemicellulase H and xylanase X activities.

The assay was performed at 50°C for hemicellulase H and xylanase X for 20 min. The ionic strength for all buffers was 50 mM; Glycine-HCl, pH 2.5; citric-NaOH, pH 3.5 to 5.5; sodium phosphate, pH 6.5 and 7.5; Tris-HCl, pH 8.5; glycine-NaOH, pH 9.5; carbonate, pH 10.5; phosphate, pH 11.5 and hydroxide-chloride, pH 12.5.**Note**: Arrow bars indicate means with standard error of three replicates.

Effect of temperature and pH on lipases activities

The temperature profiles of lipases L and AY were shown in Figure 3a. As shown in the figure, L and AY exhibited an optimum temperature of 40 °C and 45 °C, respectively. The lipase produced by *Penicillium wortmanii* has a maximum activity at 45 °C (Costa & Peralta, 1999). While, lipase produced by *Bacillus stearothermophilus* showed an optimum temperature at 70 °C (Muhannad & Fatima, 2011). At the optimum temperature and pH 7.0, L and AY displayed maximal activities of 23750 U/g and 7917 U/g, respectively. Lipase produced by *A. niger* strain MTCC 2594 through solid state fermentation using gingerly oil cake as substrate exhibited an optimum temperature of 37 °C (Kamini *et al*., 1998). Wang *et al*., (1995) reported the production of a highly thermostable lipase by *Bacillus* strain A 30-1 had an optimum temperature of 60 °C. Both L and AY activities dropped drastically at the temperature above 50 °C. L and AY only showed about 41% and 26% of its activity at 60 °C, respectively when compared to their respective optimum temperature. Lipase was deactivated at 50 °C and above. The result was in disagreement with Essamri *et al*., (1998), who reported that the intracellular lipase produced by *R. oryzae* showed an optimum temperature at 30 °C and was totally deactivated at 45 °C. In conclusion, lipase produced by *R. oryzae* showed an optimum temperature in the range between 30 °C and 40 °C.

Figure 3b shows the effect of pH on the activity of L and AY. L and AY exhibited optimum pH of 7.5 and 6.5. Under the respective optimum temperature and pH, L and AY showed the highest activities of 25833 U/g and 8750 U/g, respectively. At pH 8.5, the relative activity of L drastically dropped to 14.0% compared to its optimum pH. Similar result was reported by Hiol *et al*., (2000), which observed that the optimum pH for *R. oryzae* was at pH 7.5. Nevertheless, the lipase produced by *Penicillium wortmanii* and *Bacillus stearothermophilus showed* an

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optimum pH of 7.0 and 6.0, respectively (Costa & Peralta, 1999; Muhannad & Fatima, 2011). Unlike L, AY showed about 72.5% of its relative activity at pH 8.5 when compared to its optimum pH of 6.5. The results suggested that lipase produced by *R. oryzae* was very pH dependent and the enzyme only exhibited high activity within a narrow pH range.

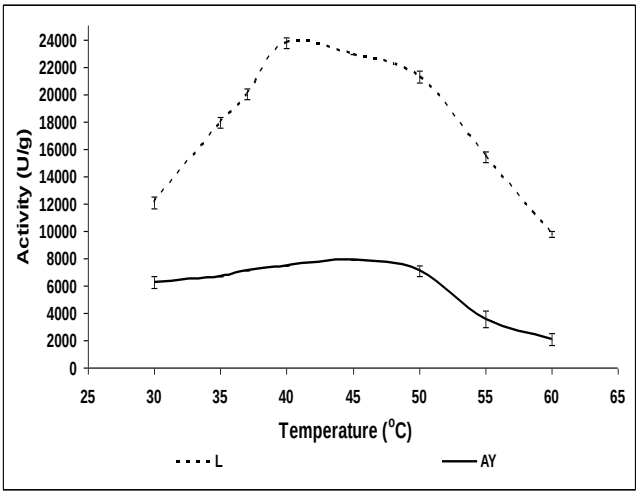

Figure 3a: Effect of temperature on lipase L and lipase AY activities.

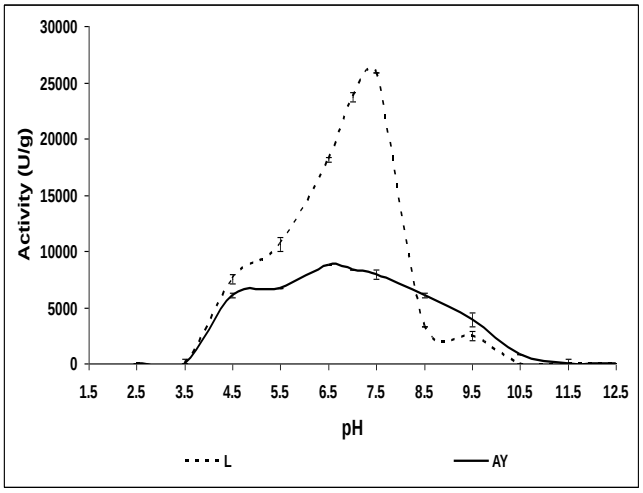

Figure 3b: Effect of pH on lipase L and lipase AY activities.

The assay was performed at 40°C and 45°C, for lipase L and AY, respectively for 30 min). The ionic strength for all buffers was 0.20 M; citric-NaOH, pH 3.5 to 5.5; sodium phosphate, pH 6.5 and 7.5; Tris-HCI, pH 8.5; glycine-NaOH, pH 9.5; carbonate, pH 10.5; phosphate, pH 11.5 and hydroxide-chloride, pH 12.5. **Note**: Arrow bars indicate means with standard error of three replicates.

Selection of enzymes for use in deinking of laser printed waste paper

The effectiveness of using these enzymes in the deinking process of waste papers was evaluated. The selection of enzymes in the hydrolysis of papers was based on the ability of the enzymes to show maximum hydrolysis onto the waste papers. The hydrolysis was performed using the optimum conditions of the enzymes with respect to the pHs and temperatures. Therefore, the enzymes to be selected for used in the hydrolysis must possess high activity, broad pH stability and higher thermostability. Based on these characteristics, the enzymes cellulase C, hemicellulase H and lipase L were selected. Cellulase C and lipase L were selected because they possess high activity and broad pH stability compared to cellulase T, cellulase S and lipase AY, respectively. Although xylanase has higher activity compared to hemicellulase, but hemicellulase possess broader pH stability and higher thermostability. Therefore instead of xylanase, hemicellulase was selected for used in deinking process.

The laser printed-paper was the alkaline-sized paper and the pH of the paper slurry was between 8 to 9.5. This was not compatible with C, H and L having optimum activities at pH 2.5, 4.5 and 7.5, respectively. Therefore pre-treatment of the papers with HCI was necessary in order to neutralize the papers. Enzymatic hydrolysis of laser-printed papers was performed at pH 5.5, 40°C. The pH of 5.5 was selected because all the enzymes used remained active at this pH although pH 5.5 was not the optimum pH for all the enzymes. The temperature of 40°C was selected because 40°C was the lowest temperature that gave the maximum activity as indicated by the lipase L. Besides the activities, thermostabilities of the enzymes were maximum at 40°C.

Effect of different enzymes sequences on ink removal

The result indicated that more than one enzyme were required in deinking of laser printed-paper (Figure 4). Highest brightness increased was obtained by enzyme sequence CH, which was 1.90% followed by sequence HC of 1.85%. This slightly difference in the brightness increment probably due to the 10 min interval time before each of the enzymes added was too short to show the differences. The increased in brightness or toner removal was due to action of cellulase (endoglucanase), which released fibers from the surface of hairy toners, which in turn enhanced the flotation efficiencies by increasing hydrophobicity of ink particles (Vyas & Lachke, 2003). Similar increased in brightness was observed for the enzyme sequence of HLC and LCH (1.40%). However, the lowest brightness increment was gained using the enzyme sequence L, with the increment of about 0.95%. Enzymatic deinking efficiency obtained from three enzyme combination sequences was lower when compared to two enzyme combination sequence of LC and CL with exception for enzyme sequence of CHL and LHC. The results suggested that cellulase and lipase showed antagonistic effect in toner removal in the presence of hemicellulase. On the other hand, the combination of three enzymes resulted in slightly higher in deinking

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efficiency compared to the addition of only lipase, indicating a synergistic effect in the hydrolysis process. Morkbak & Zimmermann, (1998) reported that the combined enzyme hydrolysis of vegetable oil based ink resulted in a 2-3% increase in deinking efficiency compared to the addition of only lipase.

Total reducing sugar produced was used to quantify the fiber degradation by using DNS method (Figure 5). Total reducing sugar produced from hydrolyzed waste papers by H of 15.39 µmol was lower than the total reducing sugar produced by C of 21.44 µmol. Moreover, total reducing sugar obtained by enzyme sequences CL and LC were higher relative to the enzyme sequence of HL and LH. The lower total reducing sugar produced by H, HL, and LH compared to C, CL and LC, respectively was due to the low xylan content in the waste papers compared to the fiber (cellulose). At the same time, total reducing sugar produced by C and H alone was lower compared to the enzyme sequence CH (22.74 µmol) and HC (23.86 µmol). These results were expected because the two enzymes (C and H) were able to hydrolyze the papers faster than using only one enzyme (C or H). Nevertheless, cellulase and hemicellulase do not show synergistic effect on the hydrolysis of waste papers. The highest total reducing sugar was obtained by enzyme sequence CHL, which was 35.91 µmol followed by 35.86 µmol by enzyme sequence HCL. On the other hand, H alone gave the lowest total reducing sugar produced of 15.39 µmol. Moreover, the result demonstrated that highest brightness increment (CH) was not proportional to the highest total reducing sugar (CHL) produced. This was in agreement with the result reported by Jeffries *et al*., (1996), who observed that the efficiency of enzymatic deinking did not follow a consistent pattern with respect to enzymatic activities as measured using CMC as substrate.

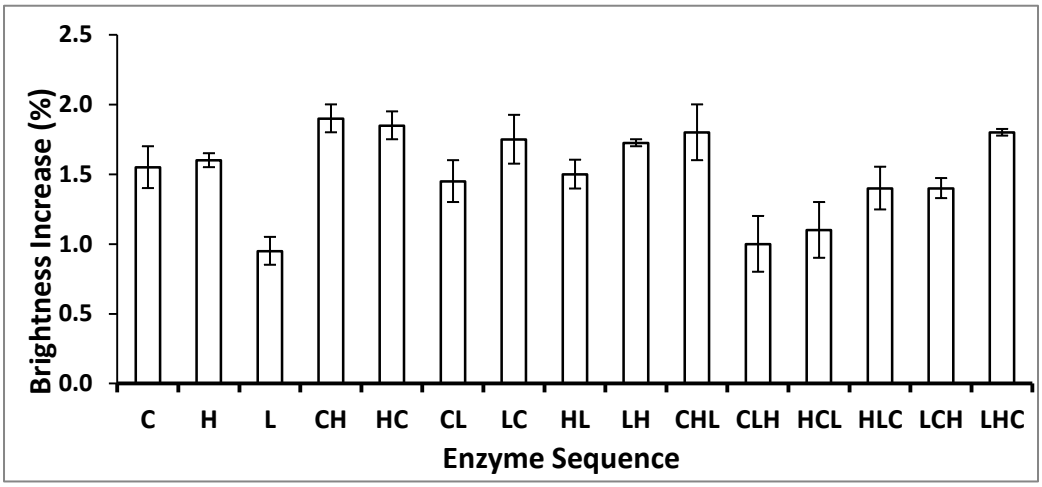

Figure 4: Effect of enzymes sequences on ink removal of laser printed paper

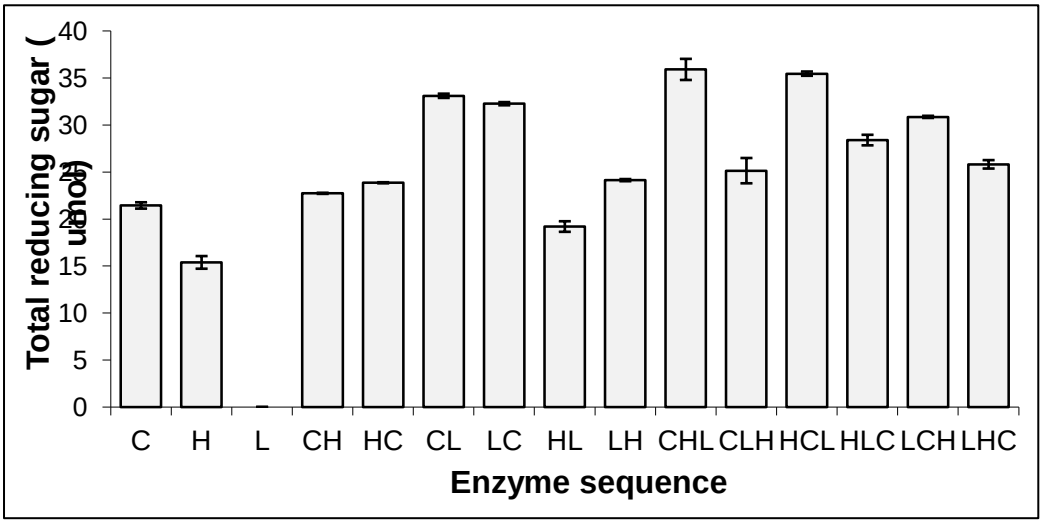

Figure 5: Total reducing sugar produced from hydrolyzed laser-printed paper by different enzyme sequence. **Note**: Arrow bars indicate means with standard error of three replicates

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Although lipase was also involved in the hydrolysis of waste papers, however it decreased the deinking efficiency. Lipase partially degraded acrylic and hydrocarbon resins of the toner (Borchardt, 1995), which resulted in the accumulation of toner particles on the surface of the paper, making removal by flotation process difficult. Thus, the brightness decreased. The efficiency of flotation process relied strongly on toner particle size, suggesting that flotation process play an important role in toner.

Observation of enzymatic treated papers under scanning electron microscope

The toners used in laser printer are consisting of thermoplastic resin pellets such as copolymer of acrylate and styrene or polyester along with pigment such as carbon black (Carr, 1991). The toner particles were heated to about 200°C to fuse the styrene-acrylate and to bind the toner particles to the paper (Shrinath *et al.,* 1991; Birkenshaw, 1993). Figure 6 showed that the toner was in flat, plate like particle which was homogeny dispersed onto a paper fiber. After enzymatic hydrolysis, some of the toner will be ripped off from the paper, which was mainly carried out by the enzymes cellulase and hemicellulase as indicated in Figure 7. However, these toners released from fiber was difficult to disperse and removal by conventional deinking process. This was because flotation process is size dependent and most favorable remove ink particle in the range of 10 to 150um (Viesturs, *et al*., 1999). As a conclusion the optimization of the enzymic reaction process with continuous removal of hydrolyzed ink must be carried out to enhance brightness of the paper.

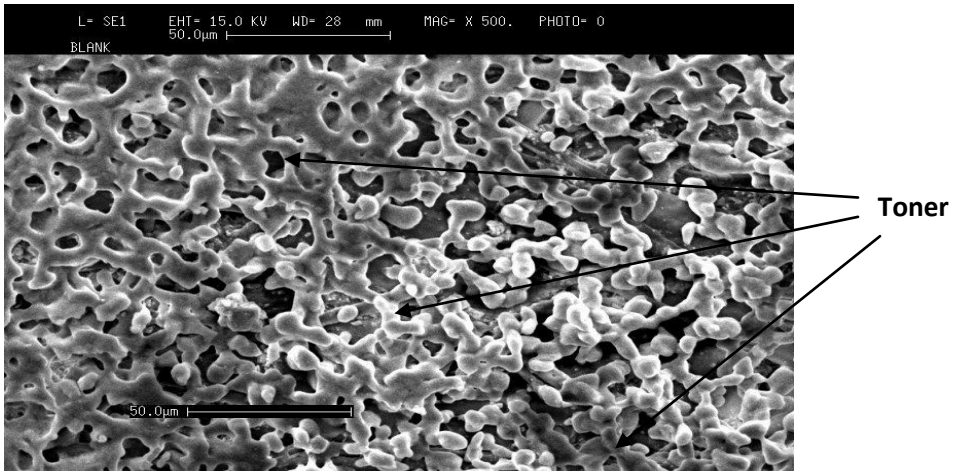

Figure 6: Scanning electron micrograph showing the particles of toner on the laser printed-paper.

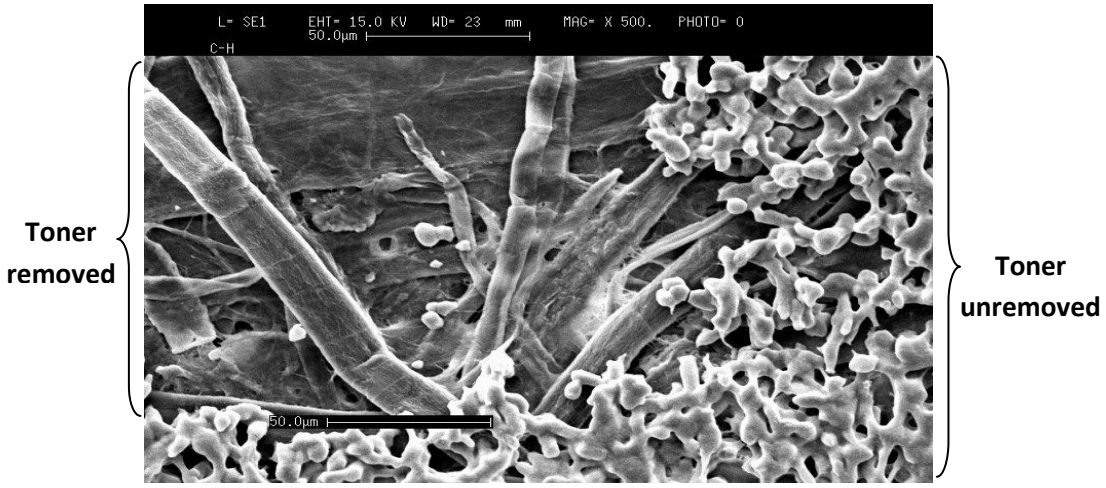

Figure 7: Scanning electron micrograph showing the removal of the toner from laser printed-paper after enzymatic hydrolysis using cellulase and hemicellulase. The hydrolysis was performed at pH 5.5; 40°C for 60 min.

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CONCLUSIONS

Enzyme (cellulase and hemicellulase) can be used to deinked laser-printed paper, which are difficult to be deinked by conventional chemical deinking process. However, lipases are not suitable for used in deinking of laser-printed paper because it showed antagonistic effect with cellulase and hemicellulase actions. Brightness increments obtained in present study are still low. Thus, factors that affect the deinking process such as pulping process, enzymatic hydrolysis process and flotation process will be carried out in order to further increase the deinking efficiency. Enzyme deinking has high possibility as alternative method to current chemical deinking process which is not environmental friendly.

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