Efficacy of *Aspergillus fumigatus* R6 Pectinase in Enzymatic Retting of Kenaf

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Enzyme retting can be a viable alternative to water retting, which is the currently utilised method for extracting fibres from kenaf. The advantages of enzyme retting are its greater environmental friendliness, shorter retting time, and more controllable fibre quality. The objective of this study was to determine the efficacy of pectinase produced from locally isolated *Aspergillus fumigatus* R6 in kenaf retting. *A. fumigatus* R6 pectinase effectively separated the fibres from non-fibre components. Scanning electron micrographs showed that the surface of pectinase-treated kenaf bast fibres appeared to be smoother and finer. The degree of retting increased with incubation time. A retting time of 32 h produced good-quality kenaf bast fibres with high tensile strength (459 MPa). No significant differences were found between the tensile properties of kenaf bast fibres treated with *A. fumigatus* R6 pectinase-containing culture filtrate and other sources of commercial pectinase enzyme. Hence, it was concluded that *A. fumigatus* R6 pectinase was capable of retting kenaf effectively.

Keywords: Kenaf; Enzyme retting; Pectinase; High tensile strength

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INTRODUCTION

Kenaf (*Hibiscus cannabinus* L.), a member of the hibiscus family, is one of the most versatile bast fibres in western Africa. Kenaf is being cultivated for use as animal feed, cordages, and paper products (Dempsey 1975; Warnock *et al*. 2002). Natural fibres are emerging as a substitute for carbon and glass fibres in the automobile industry and in building materials, as well as in orthopaedic medicine and the prosthetic industry (Fowler *et al*. 2006; Baltina *et al*. 2012; Me *et al.* 2012). Kenaf fibre is sustainable and environmentally safe because it is biodegradable and renewable. Furthermore, the rapid growth of kenaf allows two harvest times annually, which increases production yields and makes it more cost effective (Song and Obendorf 2007; Tahir *et al*. 2011).

Kenaf fibres are obtained through retting, which is a biochemical process that splits biopolymers such as pectin, hemicelluloses, and other mucilaginous substances found in the cuticle and epidermis. These substances hold the adjacent fibre bundles together, so the removal of non-fibrous substances exposes the fibre bundles. Ultimately, fibres can be produced (Van Sumere 1992; Othman *et al*. 2014). Non-cellulosic substances have a negative effect on the fibre's properties, which will affect the downstream process, especially in the textile industry (Othman *et al*. 2014). Hence, the removal of non-cellulosic materials is necessary to obtain high-quality kenaf bast fibres.

Water retting is one of the traditional methods used to produce high-quality fibres; however, the major drawbacks of this method are water pollution and a strong odour that is produced during the retting process (Van Sumere 1992; Akin *et al*. 2007). Another retting method, dew-retting, produces fibres of inconsistent quality, as this method is geographically limited and is dependent on the weather, which makes the retting conditions uncontrollable (Van Sumere 1992). Efforts have thus been made to find alternative retting methods, and the focus has been on enzymatic retting.

Enzyme technology is gaining global recognition because it is environmentally friendly and has a specific and focused performance (Bledzki *et al*. 2010; Hanana *et al*. 2015). Enzyme retting is also more controllable and the production of effluents can be reduced (Kozlowski *et al*. 2006). Biodegradation of pectin occurs as a result of the synergistic action of different extracellular enzymes that are used in this process (Brühlmann *et al*. 1994). Polygalacturonase and pectin lyase, two types of pectinase, are the main enzymes involved in the retting process (Othman *et al*. 2014). High-strength fibres with consistent quality and varying fineness can be produced *via* enzyme retting using pectinase. These high-quality fibres can be used in novel resins or developed for natural fibre agricultural feedstock composites (Foulk *et al*. 2011; Bernava *et al*. 2015). Van Sumere (1992) suggested that the presence of hemicellulases and cellulases is required to assist pectinase to obtain good retting results. The efficacy of pectinase in enzyme retting has varied with the type of pectinase and the source of the microorganism (Henriksson *et al*. 1999).

Although there have been achievements in enzyme retting research that led to a semi-industrial scale trial, no commercial system has been developed (Tian *et al*. 2013). Today, many commercial enzyme preparations are available for fibre modification, but the main hurdles are moderate-to-low shelf life, high cost, low activity, and low renewability (Bera *et al*. 2014). Parameters such as fibre strength, softness, and tenacity largely depend on the quality of the enzyme treatment.

Enzyme retting seems to be a promising method for producing high-quality fibres in easily controllable conditions. Most studies to date have focused on retting natural fibre such as flax and ramie (Foulk *et al*. 2011; Bera *et al*. 2014; Bernava *et al*. 2015) using commercial enzymes. However, dependence on commercial enzymes can increase the kenaf fibre production costs. Hence, the objective of this study is to determine the efficacy of the locally isolated *A. fumigatus* R6 that produces a high yield of pectinase enzymes in kenaf retting. This work can serve as a baseline for future work in this field.

EXPERIMENTAL

Materials

The kenaf plants used in this work were obtained from the National Board of Kenaf and Tobacco, Malaysia. Kenaf variety V36 was grown in Rompin, Pahang. Age at harvest was 4 months. Kenaf stem was harvested mechanically and brought to the laboratory for processing. Decorticated kenaf bast was cut into 10-cm pieces from the middle of the stem, followed by immersion in tap water at 30 °C for 4 h to remove pigmentation, and air-dried for 24 h prior to retting.

Enzymes

The commercial enzymes used in this study were products of Sigma-Aldrich (USA). The culture filtrate containing pectinase activity from *Bacillus subtilis* ADI1 was provided by the Microbiology Department, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. All of the enzymes were prepared in 0.2 M sodium acetate buffer with a pH of 5.

Methods

Cultivation and harvesting of Aspergillus fumigatus R6 pectinase

A. fumigatus R6 used in this study was isolated from the kenaf-water-retting tank (Institute of Tropical Forestry and Forest Products, Malaysia). Previous study has shown that *A. fumigatus* R6 produced high pectinase enzyme and hence, could be potentially used in kenaf retting process. Spore suspension of *A. fumigatus* R6 was prepared by suspending a loopful of spores in 0.01 % Tween 80® solution and using it as inoculum. The inoculum was inoculated on rice bran supplemented with a mineral solution and incubated at 33 °C for 129 h. Upon incubation, 0.2 M of sodium acetate buffer, pH 5, was added to the culture. Pectinase was harvested by centrifugation at 10,000 × *g*, 4 °C for 15 min using Avanti® J-25I Centrifuge (Beckman Coulter, USA). The clear supernatant was used as crude enzyme throughout the study. A solution of 0.05% sodium azide (NaN_3) was added to the supernatant to prevent microbial growth.

Enzyme assays

To determine polygalacturonase activity, suitably diluted crude enzyme was incubated with 1% polygalacturonic acid in 0.2 M sodium acetate buffer, pH 5, for 10 min at 40 °C. The reaction was terminated by adding 400 µL of 3, 5-dinitrosalicylic acid (DNS) and boiled for 15 min. Then, 4.4 mL of distilled water was added and measured at 530 nm wavelength (Kashyap *et al*. 2000). The concentration of reducing sugar released was determined using galacturonic acid as a standard. One unit of enzyme activity was defined as the amount of enzyme required to produce one µmol of the product in standard conditions.

Pectin lyase activity was determined spectrometrically according to the method of Nedjma *et al*. (2001). One unit was defined as changes in 0.01 absorbance of 550 nm wavelength in standard assay conditions.

Carboxymethylcellulase (CMCase) and xylanase activities were determined by estimating the release of reducing sugar using the 3, 5-dinitrosalicyclic acid (DNS) method (Miller 1959), where 1% carboxymethylcellulose and xylan were used as a substrate in the reaction mixture, respectively.

In vitro retting

Kenaf bast was treated with suitably diluted pectinase enzyme (*A. fumigatus* R6), placed in a polyethene box, and incubated at 30 °C for 40 h. Samples were collected in 8-h intervals and washed under running tap water. Kenaf bast was cleaned and combed upon drying. The quality and morphology of the retted fibres were evaluated.

Morphological characterisation of the retted kenaf fibres

Kenaf bast was cut to a matchstick size of 2 cm × 0.5 cm × 0.5 cm and heated in distilled water to remove oxygen. The sticks were soaked in a glacial acetic acid and hydrogen peroxide solution according to Franklin's (1945) method. After the sticks became

colourless, they were washed with distilled water several times to remove chemicals. Kenaf bast sticks were transferred to new test tubes, shaken in distilled water, and stained with safranin. The fibres were then observed under a microscope.

The brightness and yellowness index was measured according to ASTM E313 (2015). All measurements were taken under the conditions of the CIE standard illuminant D65 (average daylight) and 10° observation angle. The surface topography of kenaf fibres was qualitatively investigated to evaluate the efficacy of *A. fumigatus* R6 pectinase in kenaf retting using a scanning electron microscope (JOEL, Japan).

Fourier Transform Infrared Spectroscopy (FT-IR) analysis

An FT-IR spectrophotometer (Perkin Elmer, USA) was used to evaluate the chemical composition of the treated kenaf bast fibres. The spectra were recorded in the frequency range of 4000 to 280 cm^{-1}.

Tensile characterisation

The tensile properties were determined using a Universal Testing Machine (Instron®, USA). Tests were performed according to ASTM International D3379 (1975). Thirty samples were tested to determine the average single fibre strength. The tests were conducted in a standard laboratory atmosphere of 25 °C and 50% relative humidity. The crosshead rate in this study was 3 mm/min, and the gauge length was constantly measured to 10 mm.

Statistical analysis

All statistical tests were performed by an analysis of variance (one-way ANOVA) and post hoc Turkey's test using a probability level below 5% ($P < 0.05$).

RESULTS AND DISCUSSION

Fibre Morphology

Pectinase-treated kenaf fibres are shown in Fig. 1.

Fig. 1. Pectinase-treated kenaf fibres; from left to right: untreated, 8 h, 16 h, 24 h, 32 h, and 40 h treatment with *A. fumigatus* R6 pectinase

The fibres produced were finer and smoother, compared with the untreated fibres. The colour of the fibres was also satisfactory, where the brightness index increased with the retting period, indicating the removal of lignin. Natural bast fibres are classified and graded according to their colour, lustre, strength cleanliness, and freedom from the retting defects (Rowell and Stout 2006). No grading standard has been developed to date to specify kenaf fibre's quality; however, according to the grading of jute in India, jute fibres having the colour of light cream to white are considered good in terms of colour specification (Bhattacharya 2012). Hence, kenaf fibres treated with *A. fumigatus* R6 pectinase enzyme are considered to have good colour specification.

Fibre Dimensions

It is known that the morphology and properties of fibres affect their physio-mechanical characteristics, which eventually determines their final usage in the downstream process. Table 1 shows the kenaf fibre dimensions after being retted by *A. fumigatus* R6 pectinase for different lengths of time. The length of a single unit fibre increased with retting time up to 24 h, while the lumen width and fibre diameter increased up to 32 h. Fibres with longer unit cells, bigger lumen width, and fibre diameter have improved strength properties (Narendra and Yiqi 2005; Benazir *et al*. 2010). The derived values from fibre dimensions are slenderness ratio, flexibility coefficient, and Runkel ratio. The slenderness ratio, which measures the fibre stiffness, plays a vital role in determining how the fibre will be used. Fibre that is too stiff has difficulty adapting to movements such as rolling, revolving, and twisting in textile processing. However, fibres that are not stiff enough have too little springiness. They do not retain their shape after deformation and have insufficient longitudinal resistance. The slenderness ratio increased with retting time, and the highest was observed in kenaf retted for 24 h (197.88). As the slenderness ratio increases, the fibre will show higher resistance to the force of tearing. For textile processing, the preferred slenderness ratio is between 200 and 300 (Maiti 1980), while for the pulp and paper industry, a slenderness ratio greater than 70 is valuable for making high-quality paper (Ververis *et al*. 2004).

Table 1. Dimensions of *A. fumigatus* R6 Pectinase-treated Kenaf Fibre

Time (h)	0	8	16	24	32	40
Length (mm)	2.22 ± 0.63	2.21 ± 0.64	2.48 ± 0.52	2.75 ± 0.65	2.31 ± 0.79	1.58 ± 0.68
Diameter (µm)	16.83 ± 4.66	16.91 ± 4.54	16.70 ± 2.79	14.29 ± 2.57	17.40 ± 3.77	13.93 ± 3.72
Lumen Size (µm)	8.38 ± 2.99	11.77 ± 3.92	10.03 ± 2.42	9.40 ± 2.64	10.84 ± 2.99	7.81 ± 3.01
Cell wall Diameter (µm)	4.23 ± 2.41	2.57 ± 1.09	3.33 ± 1.43	2.44 ± 0.77	3.28 ± 1.07	3.06 ± 0.97
Slenderness Ratio	141.90 ± 59.78	141.48 ± 59.86	154.84 ± 50.46	197.88 ± 57.36	135.48 ± 47.72	124.23 ± 73.24
Flexibility Coefficient	51.28 ± 14.89	69.14 ± 12.42	60.66 ± 14.11	65.42 ± 11.09	62.25 ± 9.71	55.33 ± 12.24
Runkel Ratio	1.18 ± 0.90	0.50 ± 0.30	0.76 ± 0.50	0.57 ± 0.26	0.64 ± 0.26	0.90 ± 0.44

The flexibility coefficient of *A. fumigatus* R6 pectinase enzyme-retted fibres ranged from 55 to 70. According to Istas *et al.* (1954), fibres with a flexibility ratio between 50 and 75 are classified as elastic. Fibres with a high flexibility coefficient are flexible, crumple readily, and provide good surface contact as well as fibre-to-fibre bonding, yielding low bulk paper that is suitable for the pulp and paper industry.

The Runkel ratio measures the suppleness of the fibre by determining the lumen thickness and cell wall thickness. A Runkel ratio between 1 and 2 implies that the fibres would be suitable for use in textiles, while 1 or lower is favourable for papermaking (Tamolong *et al*. 1980). In this work, the Runkel ratio of the pectinase-treated fibre ranged from 0.5 to 0.9, which is better suited to the pulp and paper industry. Fibre with a Runkel ratio below 1 is normally associated with good mechanical strength properties (Istek 2006).

Fibre Surface Topology

The *A. fumigatus* R6 pectinase-treated fibres were examined by scanning electron microscopy (SEM) to qualitatively evaluate the structural modification of the surface. Table 2 shows the evolution of kenaf fibres' surface at different stages of treatment. The pectinase enzyme treatment cleaned the fibre's surface effectively without showing any indication of fibre damage.

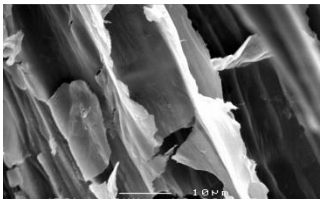
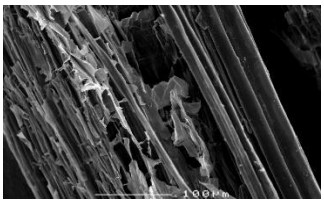
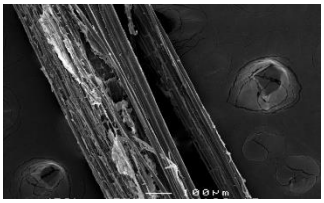
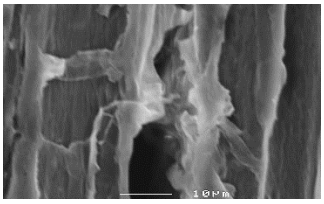
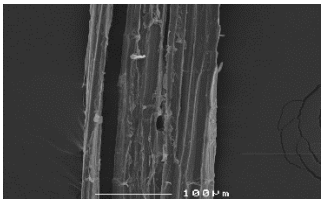
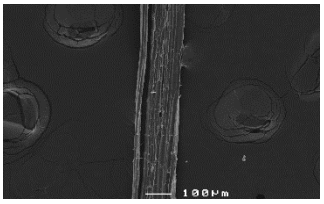
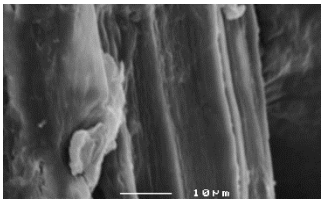
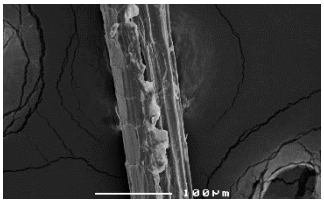
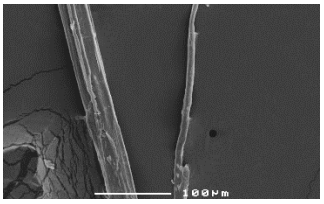
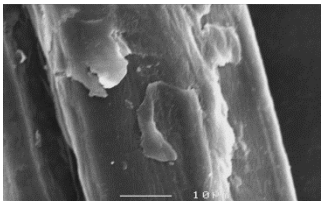
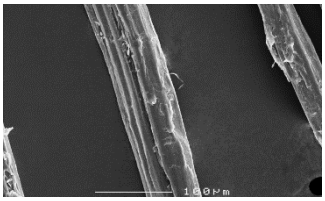
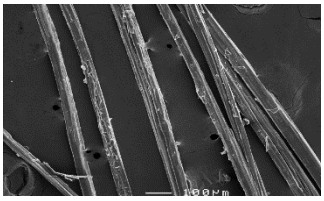
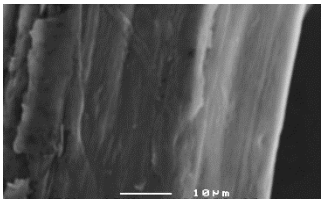
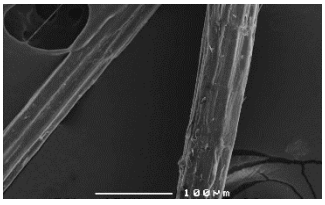
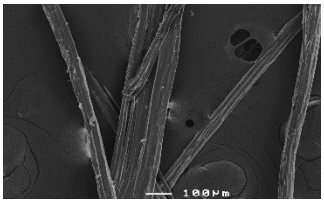
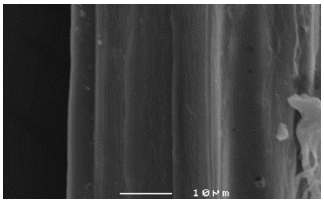
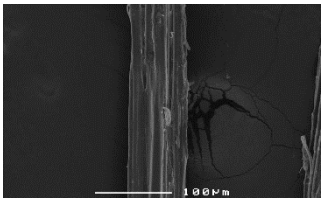
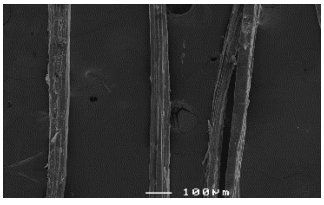
The surface of the untreated fibre was covered with waxes, fats, and cementing materials, such as pectin and lignin. The non-fibrous materials were removed substantially by *A. fumigatus* R6 pectinase enzyme treatment. A trace amount of these materials could still be observed during the course of treatment; however, the surface became smoother and fibrillation was obvious by increasing the retting time. Hanana *et al*. (2015) reported that pectinase treatment was the most effective enzyme treatment for separating alfa fibres. Pectinase allowed better separation of the fibres due to the removal of pectin that holds the fibre bundles together. As expected, the fibre fineness also decreased with increasing enzyme exposure.

FT-IR Analysis

The results of FT-IR analysis are shown in Fig. 2. The region from 3000 to 3600 wavelengths indicates the O-H stretching vibration. A shift from 3285 to 3335 cm^{-1} was observed for untreated and treated kenaf. The peak at 3285 cm^{-1} represents the intermolecular hydrogen bonding, while the one at 3335 cm^{-1} represents intramolecular hydrogen bonding. This indicates the hydrolysis of the pectin chain to form monomers. There was a decrease in absorbance at the peak around 2920 cm^{-1}, which was associated with the asymmetrical stretching vibration of CH_2 in lignin and the presence of waxy substances (Abidi *et al*. 2008). A new sharp peak found at 1730 cm^{-1} indicates the formation of aliphatic ketones.

Pectic substances have distinctive bands at the wavelengths around 1615, 1445, and 1425 cm^{-1}, which are associated with COO- and CH_2 bonds. There was a decrease in these wavelengths, which implies that the removal of pectin by pectinase in kenaf fibre occurred. The wavelength at 1022 cm^{-1} represents -C-O-C pyranose skeletal vibration from the polysaccharide component in cellulose, which increased with treatment. Enzymatic treatment on bast kenaf successfully removed the non-cellulosic materials. A 24-h treatment successfully removed the most pectin, lignin, and hemicellulose.

Table 2. Scanning Electron Micrographs of Kenaf Fibres

Time (h)	2000× magnification	300× magnification	100× magnification
0			
8			
16			
24			
32			
40			

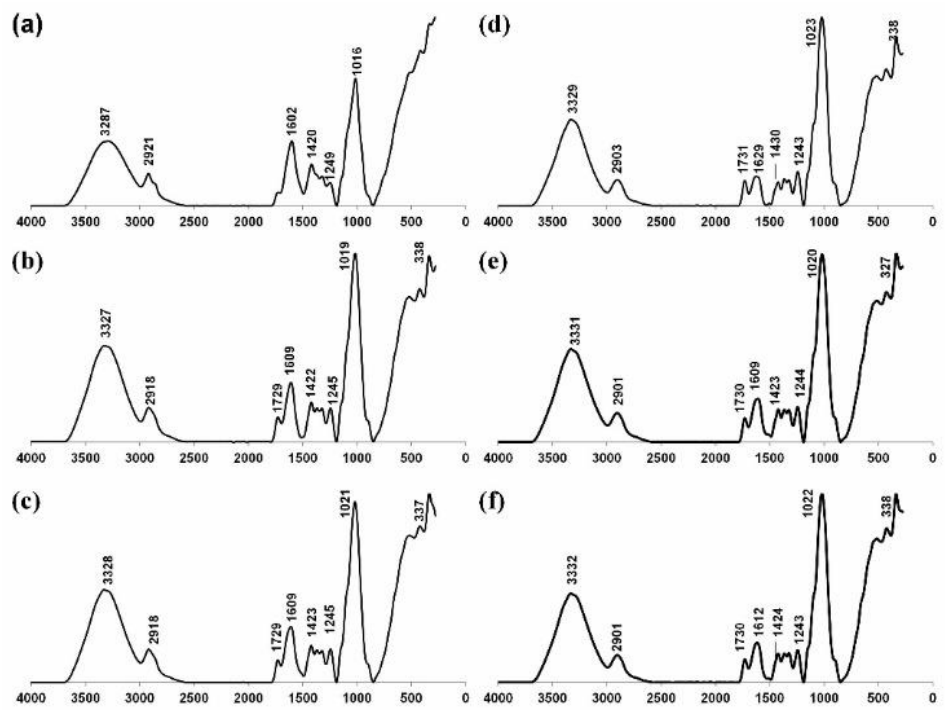


Fig. 2. FT-IR spectra of kenaf fibre in the frequency range 280 to 4000 cm^{-1}: (a) untreated, (b) 8 h, (c) 16 h, (d) 24 h, (e) 32 h, and (f) 40 h treatment with pectinase enzymes

Tensile Properties

According to the tensile test, the highest tensile strength was observed after 32 h of retting time (Table 3). Any further increment in retting adversely affected the tensile strength. Tensile properties are affected by the cellulose content in the fibre. The sudden increment in 8-h treatment might be due to uneven distribution of enzymes during the retting process. The kenaf single fibres were obtained manually using a tweezer. The kenaf bast fibres enzyme-treated for 8 h were under retted where there was a difficulty in getting single fibres. The high tensile strength in fibre treated for 8 h could be attributed to the strength of the fibre bundle rather than a single fibre.

The Young's modulus indicates the elasticity of the fibre. A high Young's modulus was also obtained from the 32-h treatment, although there was no significant difference in tensile properties between 24-h and 32-h treatments. Brühlmann *et al*. (1994) suggested that prolongation of the degumming time or application of concentrated supernatant results in fibre that contains less gum using pectate lyase in ramie retting. Hence, the quality of the fibre can be improved by optimising the enzyme concentration and other factors.

Table 3. Tensile Properties of *A. fumigatus* R6 Pectinase-Retted Kenaf Fibre

Time (h)	Young's Modulus (MPa)	Tensile Strength (MPa)
0	7330 ± 1289^{c}	121 ± 69^{c}
8	11189 ± 3052^{a,b}	426 ± 136^{a}
16	8026 ± 2055^{c}	305 ± 173^{b}
24	10213 ± 2786^{a,b}	396 ± 119^{a,b}
32	11893 ± 3143^{a}	459 ± 166^{a}
40	9408 ± 2062^{b,c}	342 ± 155^{a,b}

$^{a-c}$ Mean values followed by different superscript letters within the same column are significantly different at $P < 0.05$.

Comparison with Commercial Enzymes

Four different sources of pectinase enzymes were used to compare the effectiveness of pectinase in kenaf retting. The highest tensile strength was obtained when kenaf bast was treated with *B. subtilis* ADI1 culture filtrate containing pectinase enzymes. Kenaf bast treated with *A. fumigatus* R6 pectinase had similar tensile strength to kenaf bast treated with commercial pectinase from *A. niger* (Table 4). However, bast treated with culture filtrate of *B. subtilis* ADI1 had lower Young's modulus compared with bast treated with *A. fumigatus* R6 pectinase and *A. niger* pectinase. No significant difference in tensile properties was found between all treatments, except pectinase from *Rhizopus* sp. Since the pectinase culture filtrate used in this work had comparable tensile properties to the commercial pectinase, it can be concluded that *A. fumigatus* R6 pectinase offers the potential for efficient retting and can be used to further establish an optimal formulation for maximum efficiency in enzymatic retting of kenaf.

Enzyme profiling of the commercial enzymes and culture filtrates used in this study showed that the main components are pectin-degrading enzymes with some xylanase activity and a low concentration of cellulase (Table 5). Van Sumere (1992) suggested that in order to obtain good retting results, hemicellulase and cellulase are required in addition to pectinase.

Table 4. Comparison of Tensile Properties of Kenaf Bast Fibre Treated with Different Sources of Pectinase

Enzymes	Young's Modulus (MPa)	Tensile Strength (MPa)	Extension at Break (mm)
B. subtilis ADI1 Pectinase	9264 ± 2447[a]	315 ± 85[a]	0.38 ± 0.10[a]
A. fumigatus R6 Pectinase	10529 ± 3365[a]	283 ± 106[a,b]	0.29 ± 0.06[b]
A. japonicus Pectin Lyase	9437 ± 4812[a]	220 ± 148[a,b]	0.26 ± 0.07[b]
A. niger Pectinase	11414 ± 2810[a]	283 ± 113[a,b]	0.27 ± 0.11[b]
Rhizopus sp. Pectinase	8502 ± 3496[a]	196 ± 140[b]	0.24 ± 0.11[b]

[a-b] Mean values followed by different superscript letters within the same column are significantly different at P <0.05.

Table 5. Enzyme Composition of Different Sources of Pectinase Enzymes

Enzymes	Polygalacturonase Activity (U/mL)	Pectin Lyase Activity (U/mL)	Xylanase Activity (U/mL)	CMCase Activity (U/mL)
B. subtilis ADI1 Pectinase	10.70	N.D.	3.12	0.26
A. fumigatus R6 Pectinase	73.84	17.40	17.15	0.64
A. japonicus Pectin Lyase	113.80	60.13	26.51	1.01
A. niger Pectinase	100.26	26.27	2.46	0.35
Rhizopus sp. Pectinase	26.67	46.67	6.75	0.38

N.D.- not detected

Additionally, other enzymes might have the ability to modify the surface characteristics and improve the properties of yarns or composites (Sharma 1987). Specific interactions between enzymes and plant tissues involved in enzymatic retting, particularly those related to the efficiency of the type of pectinase, are not completely understood, and further research into these interactions could help optimise the process.

CONCLUSIONS

1. Pectinase enzyme from *Aspergillus fumigatus* R6 was effective enough to be used in the kenaf enzyme retting process.
2. A 32-h treatment using pectinase produced high-quality fibres that are suitable for use in the pulp and paper industry.
3. Optimisation of the retting process is required to further improve the quality of pectinase-retted kenaf fibre so it can be used in the textile industry.

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