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Bamboo fibre processing: insights into hemicellulase and cellulase substrate accessibility

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Abstract

A biological approach for degumming bamboo substrates has been assessed. The ability of various commercially available enzymes, including cellulase, xylanase, pectinase and mannanase, to hydrolyze bamboo powders was investigated. In addition, a commercial cellulase preparation was applied onto bamboo fibre bundles obtained by natural retting. It was found that almost all enzymes applied can use bamboo material as a substrate. Mild autoclave pre-treatment can enhance reducing sugar yield from different enzyme treatments. A most pronounced effect was observed with cellulase treatment in which the hydrolysis degree increased 1.7 fold as measured by reducing sugars for autoclave pre-treated bamboo powders versus non-treated powders after only a short period of incubation. The combined treatment of hemicellulase preparations showed no effect on the hydrolysis of bamboo substrates. The effect of autoclave pre-treatment on cellulase-treated samples was confirmed by the increase of sugar yield, protein absorption as well as by the enhancement of surface modification and enzyme penetration observed by CLSM (confocal laser scanning microscopy). This work establishes a base for future studies to develop enzymatic hydrolysis of bamboo materials, making them suitable for textile processing.

Keywords: *bamboo, cellulase, xylanase, pectinase, mannanase, autoclave, CLSM*

Introduction

Bamboo is a giant woody, tree-like, perennial evergreen plant belonging to a primitive subfamily of grasses (Sathitsuksanoh et al. 2010). With more than 87 genera and 1,500 species worldwide (Diver 1976), bamboo constitutes an economically important group of plants, especially in Asia (Parameswaran & Liese 1976). Currently, bamboo is emerging as a natural, eco-friendly raw material in the textile industry due to its many attractive properties such as fastest growth rate of any known plant, anti-microbial properties, etc. Structurally, bamboo fibres are distributed in the internodes of vascular bundles as fibre caps or sheaths surrounding the conducting elements (including vessels, sieve tubes and companion cells) (Liese 1998), which contributes to the superior strength and toughness of bamboo culm. A single bamboo fibre (monofilament) varies between 1.5 and 4.5 mm in length (Liese 1987). Caustic soda degumming is most widely applied on bamboo processing, and considerable research has been focused on this method (Shi et al. 2005; Wang et al. 2005; Xu & Tang 2006; Wang et al. 2007; Das & Chakraborty 2008). Such a process requires high chemical consumption and energy input, produces hazardous waste resulting in serious environmental pollution, damages the fibre quality (Brülmann et al. 2007; Zheng et al. 2001; Fu et al. 2008) and cannot be tailored to selectively produce fibre bundles. A semi-controlled degumming technique is required to process the bamboo culms, in which a certain amount of gummy material or non-cellulosic substances is maintained to aggregate single fibres and form the fibre bundles necessary for spinning requirements.

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Biotechnology has attracted wide attention for its role in the development of environment-friendly production technologies in textile processing and in strategies to improve the final product quality and functionality (Guebitz et al. 2006). The degumming effect is easier to control by biotechnological approaches, which offers an array of enzymes and reaction conditions for selective targeting to a particular polymer in the complex lignocellulosic structure. Although studies are available on the application of biotechnology for bast fibre extraction (Guebitz & Cavaco-Paulo 2001), its application to bamboo degumming is still limited. Little work has been reported on the isolation of microbes (Li et al. 2006) or application of commercial enzymes (Fu et al. 2007; Fu et al. 2008; Deng et al. 2009) for bamboo fibre processing for textile applications.

The objective of this work was to investigate the ability of commercially available enzymes (cellulase, xylanse, pectinase and mannanase) to hydrolyze bamboo polymers. A biological degumming using enzymes was assessed, making bamboo fibres suitable for textile processing. An attempt was also made to study the influence of autoclave pre-treatment of bamboo on enzyme penetration and release of hydrolyzed products. In addition, the effect of combined enzyme treatment on bamboo powders was investigated.

Materials and methods

Substrates

One-year-old moso bamboo (*Phyllostachys*) was harvested from Jiangxi Province, P.R. China. The epidermis of bamboos was removed and the remaining culm was chopped into small strips. The air-dried strips were crushed in a hammer mill. The fractions passing through a No. 40 mesh sieve but retained on a No. 60 mesh sieve were collected. The resulting powders were subsequently extracted with an organic solvent to remove the fat, wax and impurities which might interfere with enzyme accessibility. The extraction was carried out according to Li (2004) with minor modifications. Two grams of air-dried bamboo powder was extracted with a 2:1 (v:v) mixture of alcohol (99.8% ethanol with 1% MEK, ROTH) and distilled toluene for 3 h. Afterwards, the bamboo powders were placed in a ventilating hood overnight to evaporate the organic solvents and then boiled and rinsed with distilled water to remove the residual solvent and water-soluble substances. The extracted bamboo powder was then dried in an oven at 40°C for 48 h and stored in a glass jar for subsequent use. Bamboo fibre bundles were obtained by natural retting of the culms mentioned previously. Fibre bundles were rinsed with distilled water to remove the impurities from retting and then dried in an oven at 40°C for 48 h.

Enzymes

Various enzymes were used in this work. Commercial Celluclast 1.5 L (a cellulase preparation mixture from *Trichoderma reesei*), Pulpzyme HC (Xylanase endo-1,4-pulpzyme HC novozyme) and mannanase (Novozyme 51054) were kindly provided by Novozymes (Denmark). Mutifect 720 (alkaline xylanase) was a generous gift from Genencor International (USA). Pectinase from *Aspergillus niger* (Fluka 17389), Pectinase from *A. aculeatus* aqueous solution, Pectinex Ultra SPL (Sigma P2611) and Pectinase from *A. niger* aqueous solution, Pectinex 3XL (Sigma P2736) were purchased from Sigma-Aldrich (St. Louis, MO). Xyl-lab (endo-1,4-xylanase) was obtained in the laboratory.

All other chemicals were purchased from Sigma and ROTH.

Enzymatic processing of bamboo powder

Three series of enzymatic treatments were conducted on bamboo materials. The enzyme dosage was 10 nkat/ml for all the enzymes tested, unless otherwise specified. The first series of experiments was carried out to study the influence of autoclave pre-treatment (125°C, 1 h) on the effect of different enzymes. In the second investigation, the combined enzyme treatment was performed on autoclaved bamboo powders to assess multiple effects of different enzymes. In the final series, the celluclast preparation was further applied on bamboo fibre bundles with and without autoclave pre-treatment (125°C, 1 h) to investigate modification of the structure and surface of the bundles.

Single-enzyme bamboo powder treatment studies

Bamboo powders (with and without autoclave pre-treatment) weighing 200 mg were immersed in a 10 ml buffer (50 mM) containing 0.05 nkat/$mg_{substrate}$ of specific enzyme. The pH was adjusted depending on the enzyme under investigation according to the producer's recommendations, that is, pH 4.8 acetate buffer for commercial cellulase (Celluclast), pH 4 citrate buffer for pectinase (17389 Fluka), pH 5 citrate buffer for pectinase (P2611) and pectinase (2736), pH 6 phosphate buffer for mannanase, as well as pH 7.5 phosphate buffer for xylanases (Pulpzyme HC, Mutifect 720 and Xyl-lab). Before adding the enzyme, samples and buffer were pre-heated in a boiling water bath for 10 min to pasteurize the samples to avoid

microbial action. Xylanase, pectinase and mannanase treatments were carried out in test tubes by incubating in a shaking water bath for 48 h at the recommended temperature (37°C for pectinases, 50°C for xylanases and mannanase). Cellulase treatment was conducted in a 50 ml Erlenmeyer flask, incubating in a shaker at 50°C, 140 rpm for 1.5 h.

Combined enzyme treatment of autoclaved bamboo powder

Autoclaved bamboo powders weighing 200 mg were treated with xylanase in combination with pectinase and mannanase. Reactions were performed in test tubes containing a total volume of 10 ml buffer (50 mM, pH 7.5) with an enzyme dosage of 0.05 nkat/$mg_{substrate}$. The tubes were placed in a shaking water bath at 50°C for 48 h. Prior to the addition of enzyme, the bamboo powders and the buffer were pre-heated as described earlier to eliminate microorganisms. A blank and control (without adding enzyme) was incubated at the same time and under the same assay conditions. After incubation, the samples were cooled immediately on ice and the same amount of enzyme was added to the control and mixed well before measuring the sugars released.

Cellulase treatment of bamboo fibre bundles

Bamboo fibre bundles [with and without autoclave (125°C, 1 h) pre-treatment] were treated with 0.025 nkat/$mg_{substrate}$ of commercial cellulase preparation. Fibre bundles were suspended in a 50 mM sodium acetate buffer (pH 4.8). After the addition of the enzyme, the reaction mixtures (supplemented with 0.01% sodium azide) were incubated at 50°C, 120 rpm for 1.5 h and 72 h. Reaction liquor (2.00 ml) was sampled at specified times and centrifuged at 12,000 rpm for 15 min. The supernatant was kept at -20°C for further analysis. The reaction was stopped by boiling for 10°min. Treated bamboo fibre bundles were rinsed with distilled water and dried in an oven at 40°C to constant weight. All of the experiments in the series were run in duplicate.

Determination of the protein concentration and reducing sugars

To investigate the correlation between the adsorbed protein and the incubation time, the quantity of protein in the solution was measured using the Bradford (1976) method and protein concentration estimated using bovine serum albumin as standard. Aliquots were taken at different periods of time for 72 h of incubation. The experiments were done in triplicate. The concentration of reducing sugars was determined by the DNS (2-hydroxy-3,5-dinitrobenzoic acid reagent) method mentioned previously (Fu et al. 2008) and expressed as the glucose equivalent for cellulase treatment and xylose equivalent for xylanase treatments, pectinase treatments, mannanase treatments as well as combined enzyme treatments. The DNS reagent was prepared according to IUPAC (Ghose 1987) using analytical grade chemicals. The reaction liquor was centrifuged at 12,000 rpm for 20 min. An aliquot of 0.5 ml of supernatant was dispensed into 1.5 ml of distilled water. Afterwards, 1.5 ml DNS solution was added and the mixture immersed in a boiling bath for 5 min. After cooling, 21.5 ml distilled water was added, mixed well and the optical density at 540 nm measured. The data shown is the absolute value minus the control value.

Effect of moisture on bamboo fibre bundle structure

The deformation behaviour of a single elementary bamboo fibre bundle was investigated by environmental scanning electron microscopy (ESEM, Philips XL30). Temperature adjustments for the sample were made with the Peltier stage. This was set to 0°C before observation. Samples were cut from the cross-section and placed onto the stage vertically with an electrically conductive adhesive transfer tape. The pressure inside the specimen chamber was varied from 6.1 to 0 torr in steps of 0.5 torr every 5 min to achieve a relative humidity varying from 100% to 0%. Observations of the behaviour of the sample (cross-section) were made at different time points with images acquired at 5°C.

Determination of enzymatic activities

Xylanase activity. Xylanase activity was measured according to an improved dinitrosalicylic acid (DNS) method, using xylose solution as a reference (Grous et al. 1986). An aliquot of 0.5 mL of enzyme solution was incubated with 1.5 mL of 0.1% (w/v) oat spelt xylan solution (0.05 M K_2HPO_4/NaOH buffer, optimum pH) at optimum temperature for 30 min. Afterwards, 1.5 mL DNS solution was added and the mixture immersed in a boiling bath for 5 min. After cooling, 21.5 mL distilled water was added, mixed well and the optical density at 520 nm (OD_{520}) measured with a spectrophotometer. Xylanase was tested under various conditions to determine the optimum temperature and pH for enzyme activity. The temperature was varied from 45 to 70°C at pH 7.6 and the pH was varied from 6.4 to 8.0 at 60°C. Enzyme assays were all performed in triplicate and the mean values were reported.

Cellulase activity. Cellulase activity was measured using the same method described for xylanase but with CMC as substrate and glucose as standard reference (Grous et al. 1986; Bhat 2000).

Pectinase activity. Pectinase activity was measured using the same method described for xylanase but with polygalacturonic acid as a substrate, and the standard curve for calibration was performed with D-galacturonic acid (Grous et al. 1986).

Mannanase activity. Mannanase activity was determined using the same method described for xylanase but with locust bean gum as substrate. Reducing sugar in the samples were determined by using the dinitrosalycylic method with mannose as a standard (Grous et al. 1986).

For all the enzymes used, the activity is expressed as U mL^{-1}, where one unit (U) of enzyme activity is defined as the amount of enzyme required to release 1 mmol of reducing sugar equivalent per minute from substrate, under optimum assay conditions.

Weight loss evaluation

At the end of the enzyme treatment, the fibre bundles were removed from the liquor, boiled to inactivate the enzyme, washed thoroughly and dried at 40°C for 72 h, followed by weight loss determination. The weight loss of the samples was calculated as follows:

$$\text{Weight loss } (\%) = [(W_0 - W_1)/W_0] \times 100,$$

where W_0 was the original weight of bamboo before enzymatic treatment and W_1 was the residual weight of the bamboo after treatment.

Labelling enzyme with Fluorescein isothiocyanate (FITC) dye

Cellulase was labelled with FITC dye according to the protocol provided by Sigma-Aldrich, Inc with minor modifications. A solution of at least 10 mg/ml of protein was prepared by diluting cellulase in 0.1 M fresh sodium carbonate buffer (pH 9). For each 1 ml of enzyme solution, 50 µl of FITC solution (2 mg/ml, dissolved in anhydrous DMSO) was added, very slowly in 5 µl aliquots while stirring the protein solution. After the addition of FITC solution, the reaction was incubated in the dark for at least 24 h at 4°C with gentle and continuous stirring.

Monitoring and separation of the unbound FITC from labelled enzyme was carried out with an Äkta-Purifier (version 10) chromatographic system from Amersham–Pharmacia Biotech (Sweden), using a 5 ml HiTrap Desalting column. The injected sample volume was 0.2 ml for each time point, with PBS buffer (pH 7.4, 10 mM) at a flow rate of 3 ml/min. Fractions with absorbance at both 495 nm and 280 nm were collected as labelled cellulase.

Microscopic observation of surface modification and enzyme penetration

The bamboo samples were analyzed with a confocal laser scanning microscope Leica SPE (Leica Microsystems, Wetzlar, Germany). Bamboo tissues were excited with a 532 nm laser beam and the autofluorescence emitted in the range 600–695 nm recorded. The FITC-labelled enzyme was excited with a 488 nm laser beam and the detection window was optimized for every field of view to obtain a better discrimination of the signals. Z-stacks were acquired with a Z-step of 0.17 µm for the objective 63X (used for observing the enzyme-treated transversal sections) and 2.4 µm for the 10X objective (used without a coverslip for observing the uncut samples). Voxel size in the x-y plane was 0.074 µm (obj. 63X) and 0.72 µm (obj. 10X). Volume renderings and three-dimensional reconstructions were produced with the software Imaris 6.4.2 (Bitplane, Zurich, Switzerland).

Twisting testing

Twisting tests were performed based on the NP EN ISO 2061 procedure using James H. Heal equipment. Samples of 6 cm of bamboo were submitted to twisting until breakage. The number of twists needed to break were counted and registered. The values recorded represent the mean of ten assays for each sample.

Results and discussion

Influence of autoclave pre-treatment on bamboo powders

The autoclave pre-treatment increased the sugars released after 48 h of xylanse, pectinase and mannanase treatments, compared to non-autoclaved samples (Figure 1). The reducing sugars released from autoclaved bamboo powders by using xylanase (Pulpzyme HC), xylanase (Multifect 720) and xylanase (Xyl-lab) were 52.82%, 26.19% and 38.27% higher, respectively. A corresponding increase in the reducing sugar yield was observed with pectinase (Fluka 17389, Sigma P2611 and Sigma P2736) pre-autoclaved bamboo powders, resulting in 15.38%, 38.98% and 54.33% higher yields, respectively.

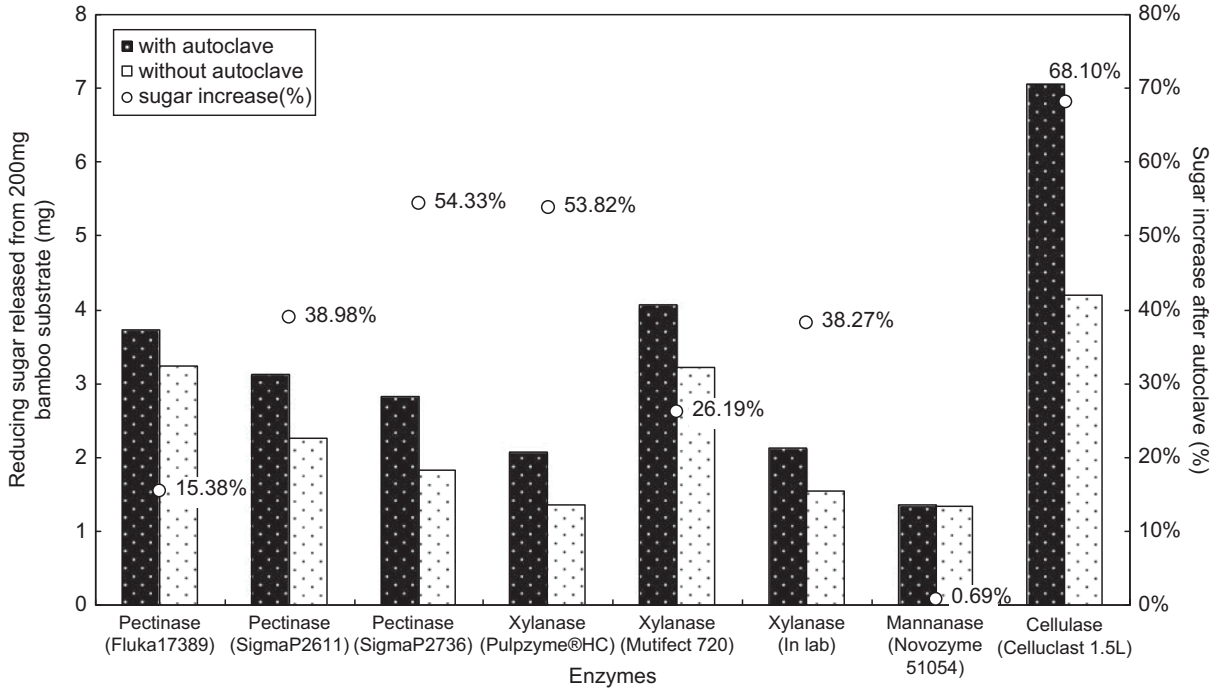

Figure 1. The effect of autoclave pre-treatment on the reducing sugars produced by enzymatic treatment with pectinase, xylanase, mannanase and cellulase preparation. The conditions used were bamboo powders weighing 200 mg (with and without autoclave pre-treatment) were immersed in a 10 ml buffer (50 mM) containing 0.05 nkat/$mg_{substrate}$ specific enzyme. Conditions were as described in methods. Cellulase treatment was conducted at 50°C, 140 rpm for 1.5 h.

The limited action of mannanase on most pulp has been already reported (Tenkanen et al. 1997) and our finding suggested that the difference between autoclaved and non-autoclaved powders was not significant. Only a marginal increase (0.69%) in sugar yield with autoclaved bamboo powders was found. Interestingly, a considerable increase in reducing sugar yield of samples incubated with cellulase preparation was observed after autoclave pre-treatment. After 1.5 h incubation, the sugar yield was almost 1.7 times higher than that obtained for un-pre-treated powders. Autoclaving bamboo powder enhanced the penetration of moisture, leading to sedimentation, while in non-autoclaved samples, the bamboo powder remained floating. Compared with steam explosion, which is frequently applied as a pre-treatment (Bhat 2000), autoclaving is a milder and gentler process. Moreover, after autoclaving, the bamboo powder's general structure is still compact for enzyme penetration. The results from cellulase treatment revealed that cellulose was more accessible in bamboo powders. It is clear that the autoclave treatment causes swelling of the bamboo structure, leading to an increase of enzymatic susceptibility. Additionally, bamboo fibre materials pre-treated by autoclaving showed higher enzymatic digestibility by all enzymes (cellulase preparation, pectinases and xylanases) tested. Although the results of conversion were not so pronounced in terms of overall saccharification, they can be considered sufficiently significant for textile purposes, because the goal was the surface modification, and higher processability can be achieved after bamboo hydrolysis. The fibres became suitable for twisting and their resistance increased.

Performance of combined enzyme treatment

As a lignocellulosic material, bamboo has cellulose, hemicellulose and lignin as its main constituents (over 90% of the total mass). Therefore, hemicellulose and lignin are the main target polymers for removal during the bamboo degumming process. Approximately 20–30% of the total mass in bamboo is hemicellulose, mainly consisting of β-D-xylan (Aoyama 1999), meaning that xylanases are expected to play a central role during bamboo processing. In combined enzyme treatment, pectinase and mannanase were therefore added to the xylanase treatment system. As shown in Table I, the reducing sugar yield increased considerably compared with single xylanase treatments under the same incubation conditions. However, when comparing the reducing sugar yield of the combined enzyme treatment to the sum of reducing sugar yield from individual enzyme treatment under the same assay conditions, the level of synergy was very low (Table I). This may be attributed to the fact that the incubation pH (alkaline) was not optimal for pectinase and mannanase (require

Table I. The level of synergism for different combinations of enzymes.

Enzymes	Sugar increase (%) compared with xylanase treatment	Level of synergism[a]
Xylanase (Mutifect 720) + Mannanase (Novozyme 51054)	15.74	0.87
Xylanase (Mutifect 720) + Pectinase (Sigma P2611)	30.68	0.74
Xylanase (Pulpzyme®HC) + Mannanase 51054)	25.57	0.76
Xylanase (Pulpzyme®HC) + Pectinase (Sigma P2611)	152.06	1.00
Xylanase (In lab) + Mannanase (51054)	32.69	0.81

[a]The level of synergism = Reducing sugar yield from mixture enzyme treatment/Sum of reducing sugar yield from individual enzyme treatment contributing in the mixture.

acidic conditions) because they were optimal for xylanase. Therefore, the effect of pectinase and mannanase was less than with the single enzyme treatment under the assay conditions.

Treatment of bamboo fibre bundles with cellulase preparation

Based on the study on bamboo powders, the ability of *T. reesei* cellulase preparation to penetrate the bamboo fibre bundles and hydrolyze them was investigated. The used crude fibre bundles, obtained by natural retting, were subjected to moisture, facilitating enzyme penetration. As shown by environmental scanning electron microscopy (Figure 2D), the oven-dried bamboo bundle was too compact due to shrinkage caused by evaporation, and this was accompanied by a decrease in enzymatic susceptibility (Grous et al. 1986). Therefore, during enzyme treatment of bamboo bundles, enzyme penetration and accessibility to its respective substrate constitute the limiting step.

Considering these factors, pre-treatment of presoaked bamboo by autoclaving was investigated. It was reasoned that the steam would help to open the bamboo structure, thereby enhancing enzymatic penetration and the removal of simple sugars. The first step in cellulase reaction is its adsorption onto the surface of the cellulosic substrate (Andreaus et al. 1999; Azevedo et al. 2002), regarded as a prerequisite step for subsequent hydrolysis (Kim et al. 1997; van Wyk 1997; Kyriacou et al. 1988; Boussaid & Saddler 1999). The ability of the cellulase preparation to adsorb and/or penetrate bamboo bundles was investigated by using FITC-labelled cellulase. As shown in CLSM (Figure 3), the majority of the enzyme remained on the outmost layer of the fibre bundles regardless of the autoclave pre-treatment. Nevertheless, a small amount of enzyme managed to penetrate the autoclaved bamboo bundles mainly in

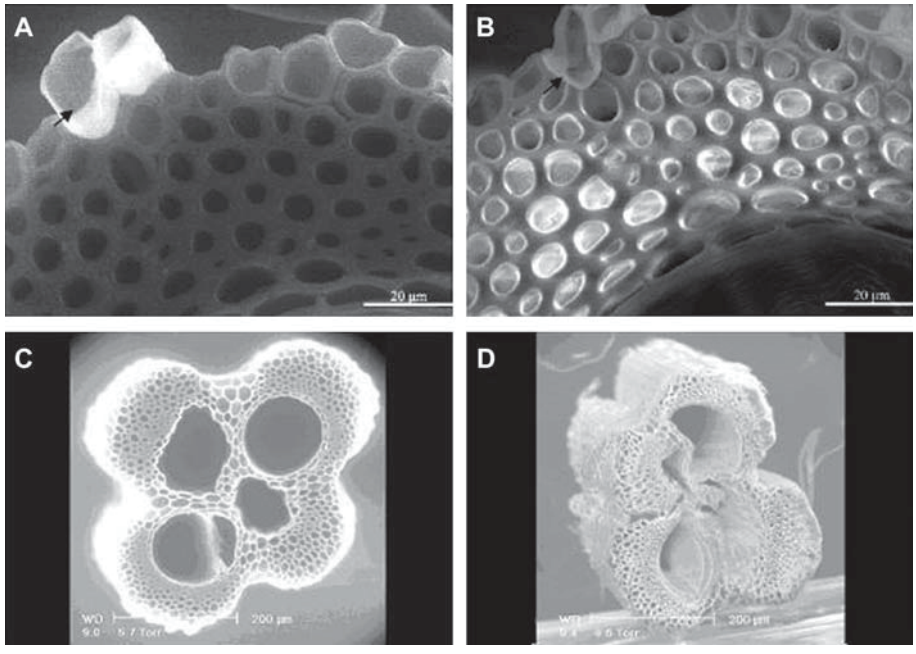

Figure 2. ESEM photos of morphology changes of bamboo fibre bundles under different moisture conditions: A. wet fibre bundles under 100% RH, B. wet fibre bundles under 0% RH, C. wet fibre bundles and D. fibre bundles after drying in an oven for 5 h at 80°C.

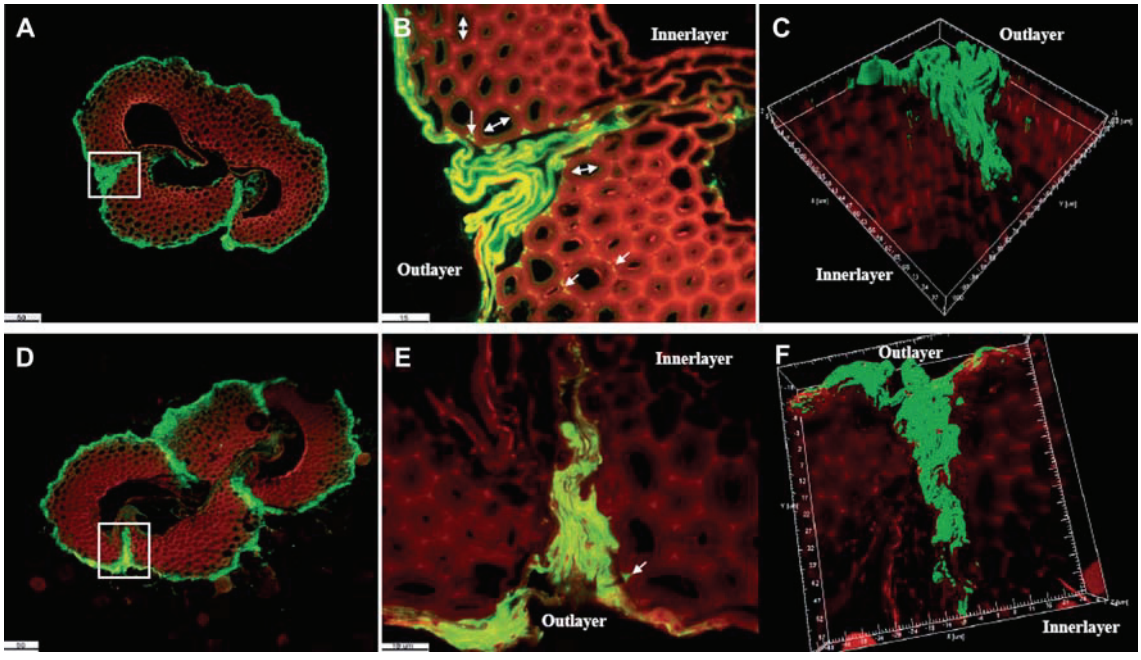

Figure 3. CLSM photos of bamboo bundles with/without autoclaving, treated with labelled cellulase at 0.025 nkat/$mg_{substrate}$ of enzyme, 50°C, pH 4.8, 120 rpm, 2 h: A. bamboo bundle with autoclave pre-treatment, B. bamboo bundle with autoclave pre-treatment with high magnification of a part in A marked by white frame, C. 3D image of the white frame highlighted area in A, D. bamboo bundle without autoclave pre-treatment, E. bamboo bundle with autoclave pre-treatment with high magnification of a part in *e* marked by white frame and f. 3D image of the white frame highlighted area in D.

the space between single fibres (single-arrow in Figure 3B) after autoclaving, while no enzyme was detected in the control (Figure 3E and F). Thus, autoclave pre-treatment led to the opening of bamboo bundles or of the three-dimensional structure of the network of lignin, hemicellulose and cellulose, enhancing the accessibility of the enzyme molecules to the surface, benefiting further penetration. Similar studies with other lignocellulosic polymers showed that cellulase treatments were restricted to the fibre surface, with minimal degradation (Azevedo et al. 2002; Cavaco-Paulo 1998; Morgado et al. 2000). This is an important observation as it allows the controlled modification of lignocellulosic polymers.

The degree of hydrolysis increased as shown by the increase in reducing sugars after autoclaving when compared to non-pre-treated bamboo bundles (Figure 4). The efficiency of cellulase action on solid substrates is known to be related to the specific surface area (Grethlein 1985). Autoclave pre-treatment

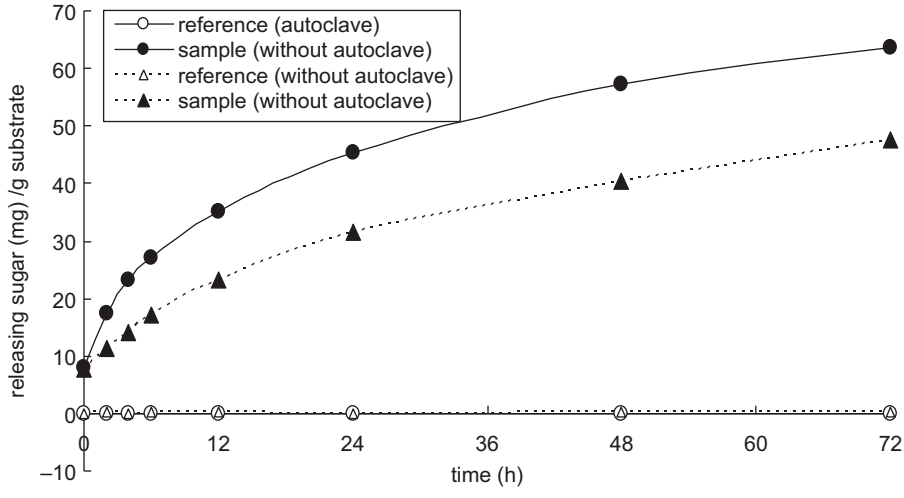

Figure 4. Dependence of the sugar concentration on the reaction time for bamboo bundles with/without autoclave pre-treatment under the treatment condition of 50°C, pH 4.8, 120 rpm and 0.025 nkat/$mg_{substrate}$ of cellulase preparation. Points are the averages of two experiments. Reference refers to samples without enzyme and sample refers to samples with enzyme.

Table II. The weight loss of bamboo fibre bundles.

Bamboo material	Reference	Cellulase treated[a]
Without autoclave	2.54%	2.64%
Autoclave	2.14%	4.95%

[a]Cellulase treatment with a dosage of 100 mg/g substrate, 50°C, pH 4.8, 120 rpm, 72 h.

of bamboo bundles increased the surface area by opening more pores for enzyme penetration. This was also confirmed by measuring the dry weight of treated samples, as shown in Table II. The weight loss of bamboo bundles with and without autoclave pre-treatment is 4.95% and 2.64%, respectively, with a corresponding reference weight loss of 2.14% and 2.54%. Therefore, the weight loss of pre-treated bundles is 25 times higher than that of un-pre-treated bundles. However, this huge difference is not evident when comparing released sugars (Figure 4). One possible explanation is due to underestimation in the DNS method, which cannot properly detect the cello-oligosaccharides of DP 3 or higher released by EG I in the cellulase mixture on the autoclave pre-treated samples (Pere et al. 2001). As the mode of action of EGs is based on the cellulose chain cutting at the most accessible points, it is expected that bigger fragments of cellulose would be released into the solution (Azevedo et al. 2002).

The protein content in the liquor treatment was also quantified (Figure 5). As can be seen, protein adsorption was higher for the previously autoclaved samples. Autoclave pre-treatment causes swelling of the fibres promoting their ability to adsorb the protein. In the first period of incubation, the highest level of adsorption was achieved (near 37%) for the samples previously autoclaved. After the first 2–3 h of incubation, the level of protein is constant, meaning that saturation of the substrate has been reached (Figure 5).

The surface change of bamboo fibre bundles after cellulase preparation treatment was also recorded by CLSM. Figure 6 shows that the enzyme-treated fibre bundles with and without autoclave pre-treatment had larger spaces or cracks on the surface (Figure 6B, D, arrow) compared to bundles without any treatment (Figure 6A, arrow). Particularly, for the bundles with autoclave treatment, cellulase preparation had a relatively pronounced effect. One bundle separated into several pieces from the vertical section, as can be seen in Figure 6C. Separated thinner bundles could sometimes overlap with one another (Figure 6E, marked with a frame). In the meantime, the exposed small fibres might wrap together and form a big ball shape on the surface (Figure 6E, marked with a circle). This change in morphology could be attributed to the synergistic action of different components of the cellulase mixture (Cavaco-Paulo 1998).

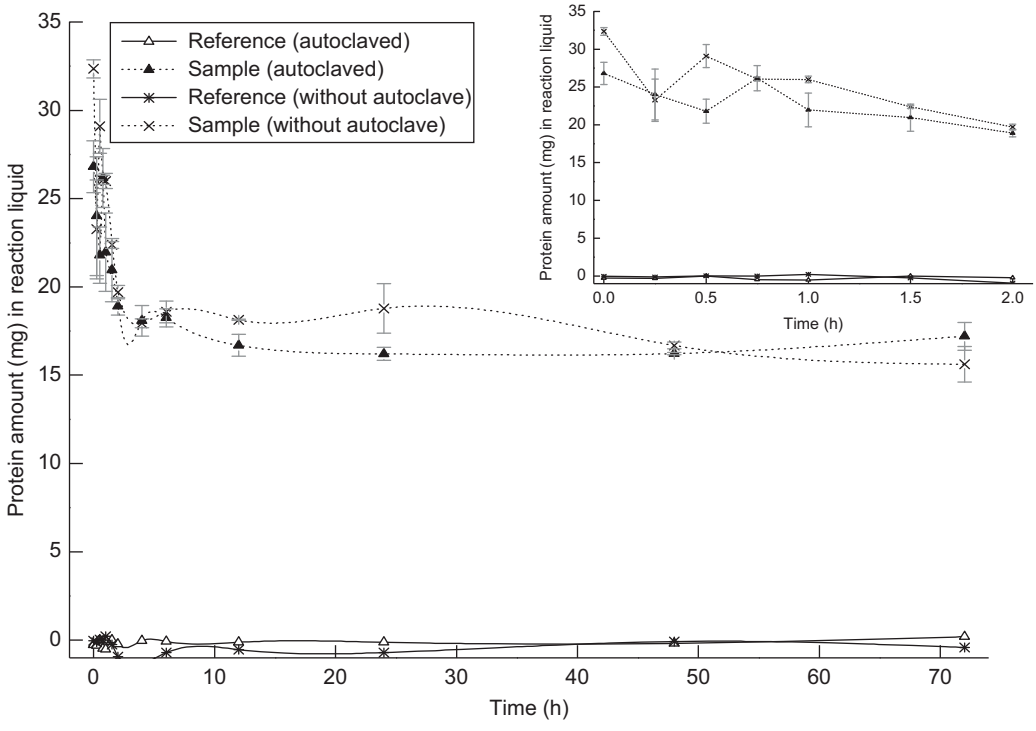

Figure 5. Monitoring of protein content (%) in the liquor treatment containing cellulase preparation (0.025 nkat/$mg_{substrate}$). The experiments were conducted at 50°C, pH 4.8, 120 rpm with 72 h of incubation. The small figure at the top right is an amplification of the first 2 h of incubation.

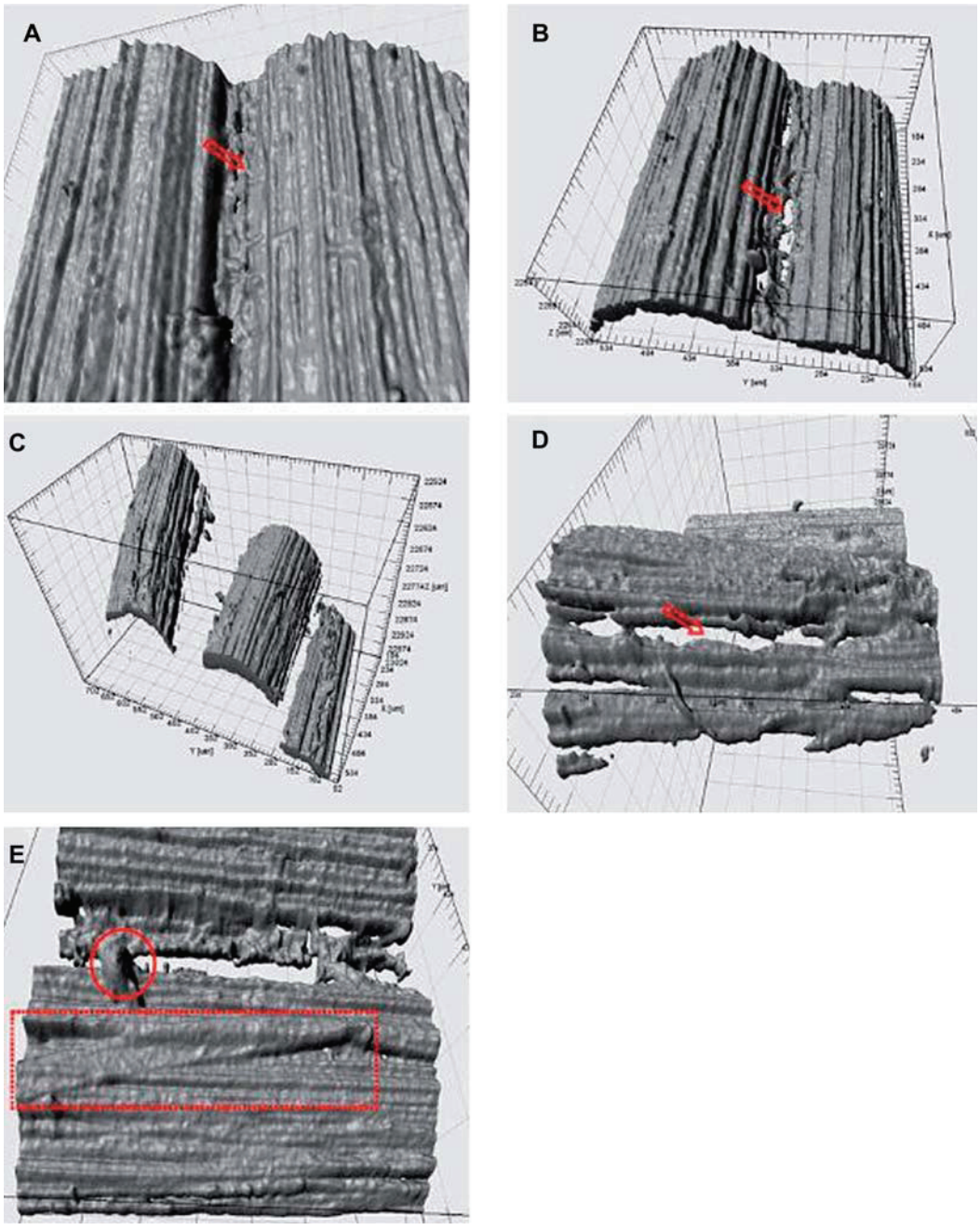

Figure 6. CLSM photos of surface change of bamboo bundles under the enzymatic treatment condition: 0.025 nkat/$mg_{substrate}$ of cellulase preparation, 50°C, pH 4.8, 120 rpm, 72 h: A. original crude bamboo bundle without any treatment, B. cellulase-treated bamboo bundle (without autoclave pre-treatment), C. cellulase-treated bamboo bundle (with autoclave pre-treatment), D. cellulase treated bamboo bundle (with autoclave pre-treatment), higher magnification of bundle piece in C and E. cellulase-treated bamboo bundle (with autoclave pre-treatment), higher magnification of bundle piece.

Cellobiohydrolases (CBHs) attack chain ends in a stepwise fashion, releasing cellobiose units, but they can act on crystalline regions of cellulose, whereas endoglucanases (EGs) cause random hydrolytic chain scission at the most accessible points of the cellulose chain, producing new chain ends on the cellulose surface for exoglucanase attack and resulting in a rapid decrease in cellulose chain length (Azevedo et al. 2002; Heikinheimo et al. 2003; Cavaco-Paulo 1997). The action of CBH I resulted in a clearly fibrillated bundle surface (Figure 6E, circle). Meanwhile, EG I is more likely to produce erosion, cracks and defects in the bundles, causing separation of the bundles into small pieces (Figure 6C). These results were confirmed in a previous report on cotton fibre and yarn treatment by a cellulase preparation (Pere 2001).

Enzymatic treatment can lead to structural and surface modification of fibre bundles (Figure 6), resulting in a change in bundle resistance. In this study, we applied twisting as a parameter to evaluate enzyme performance. The breakage of fibre bundles is mainly due to limited twisting ability. The more twists that can be added in the fixed length of a bundle, the more resistant the bundle is. The results of twist testing on different bundles are listed in Table III. The air-dried untreated fibre bundles gained the lowest number of twists before breaking, presumably resulting from their relatively low moisture content. With long incubation times, water could penetrate in the treated samples as well as in the reference ones. It is unavoidable that a certain amount of water might remain inside the bundle structure. This also explained why autoclaved and non-autoclaved samples references could withstand more twists before breaking. Surprisingly, the twists added by sample autoclaving were maintained after cellulase treatment. One possible explanation is that the autoclave pre-treatment enhanced the effect of cellulase on samples and resulted in bundle separation in a certain particle area which became the weak links in the bundle. The existence of weak links finally led to an uneven stress on the bundles and made them easier to break. In contrast, the effect of cellulase treatment on un-autoclaved samples was limited. Therefore, only minor modification of the surface was observed. However, the softness of the bundle was improved, because it was necessary to apply a higher force to twist and break the sample after enzyme treatment. The sample was less rigid and its malleability had increased (Table III).

Conclusions

We have investigated the substrate accessibility of pectinases, hemicellulases and cellulase preparation during bamboo fibre processing. The results have clearly demonstrated that all tested enzymes were able to attack bamboo with reducing sugar release. Autoclave pre-treatment was effective in enhancing all enzyme activity on bamboo powders as shown by an increase in reducing sugar release, although mannanase treatment activity was minimal. The accessibility of bamboo bundles to cellulase hydrolysis can be increased by autoclave pre-treatment as confirmed by protein absorption, enzyme penetration (observed by CLSM) and greater weight loss.

Based on this work, it is suggested that the establishment of an enzyme-bamboo reaction system requires effective pre-treatment which increases the surface area of penetration of the enzyme into the bamboo structure.

Table III. Twisting of bamboo fibre bundles.

Samples		Number of twists add in fibre bundles
Untreated samples (air-dried)		4
Without autoclave pre-treatment	Reference	6
	Cellulase treated sample	7
With autoclave pre-treatment	Reference	6
	Cellulase treated sample	6

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