Biotechnology Reports 23 (2019) e00358

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Flax treatment with strategic enzyme combinations: Effect on chemical fiber composition and ease of fiber extraction

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ARTICLE INFO

Article history:
Received 26 February 2019
Received in revised form 28 May 2019
Accepted 25 June 2019

Keywords:
Natural fibers
Enzymatic treatment
Polygalacturonase
Xylanase
Extraction efficiency

ABSTRACT

The effect of treatment of flax with strategic enzyme combinations on the ease of fiber extraction and the chemical fiber composition is reported in this study. To contribute to the increasing demand for bio-based and sustainable materials, it is of great importance to develop optimal enzyme formulations which can replace the yet poorly controlled traditional dew retting process. Regarding the chemical composition of the fiber, enzymatic treatments all resulted in similar improvements, with an enhanced cellulose content of 81 ± 1% after polygalacturonase + xylanase treatment (vs. 64 ± 2% for green fibers). Evaluation of extraction efficiency (EE) showed that several enzyme combinations significantly increased EE in comparison with green fibers. An EE of 23 ± 6% was found for fibers extracted after polygalacturonase + pectinmethylesterase treatment, in comparison with an EE of 11 ± 1% for green fibers. Combinations with three enzymes resulted in a higher reduction of the pectin content of the fibers. The combination of enzymes shows hence promising potential but further evaluation of mechanical performance of fiber reinforced composites is needed.
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1. Introduction

The future of natural fiber reinforced composites (NFRC), emerging as a promising alternative for glass fiber reinforced composites (GFRC), should be assured by producing NFRC's of a consistent quality [1–3]. Substitution of dew retting by enzymatic retting will contribute to this future in order to provide consistent and high quality flax fibers. The drawbacks of traditional dew retting, like dependence on weather, region and climate changes and the long duration of the process, can be overcome by the introduction of enzymatic retting thanks to the enzyme specificity and high controllability of the process [4–6]. Moreover, due to the low environmental impact of biocatalysts, an important contribution can be made towards a more sustainable bio-economy.

Major chemical components of flax fibers are cellulose, hemicellulose, lignin and pectin. Hereby, cellulose is most strongly present with concentrations ranging from 43 to 65% w/w as reported in literature for untreated flax fibers [7–9]. During retting, fibers need to be loosened from the stem by interaction of enzymes with hemicellulose and pectin in the surrounding network, implying that pectinases and hemicellulases may play a major role in the retting process [10].

Previous studies on enzymatic treatment of flax stems within our research group have shown that polygalacturonase, pectate lyase and xylanase showed most potential for the extraction of flax fibers [11,12]. Hereby, chemical characteristics as well as mechanical properties, e.g. unidirectional longitudinal and transversal composite strengths, were evaluated. Purified fibers with a cellulose content of 79 w/w % were obtained after enzymatic treatment with polygalacturonase while exhibiting improved mechanical properties [11,12]. The improvement of mechanical properties of composites impregnated with fibers extracted after enzymatic treatment of flax can be attributed to the increase in cellulose content of the fibers combined with a good orientation of the micro fibrils and the reduction in pectin content [11,12]. Zhang et al. [13] also reported the key role of polygalacturonase in the

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https://doi.org/10.1016/j.btre.2019.e00358
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retting process by degradation of non-methylated polygalacturonic acid in the middle lamella pectin which was later on confirmed by Evans et al. [14]. Polygalacturonase treatment also resulted in the highest improvements concerning moisture sensitivity while results concerning extraction efficiencies (EE) illustrated a significant enhancement in ease of extraction after treatments with pectate lyase, polygalacturonase and xylanase, compared to extraction of green fibers which will lead to less fiber damage and a higher fiber yield [11,12]. Therefore, flax treatments with polygalacturonase, pectate lyase and xylanase seem to be the most promising for enzymatic retting of flax.

Based on these insights, strategic combinations of enzymes can be made and evaluated to study possible synergism and improved retting performance resulting in fibers of a high consistent quality. Some research groups studied enzyme combinations or commercial enzyme preparations containing different enzyme activities. Research of [9,15] showed that the enzyme combination xylanase + cellulase increased cellulose content of the flax fiber and decreased hemicellulose and pectin content, but not as successfully as polygalacturonase treatment. Stuart et al. [16] tested the commercial enzyme preparation 'Pectinex AR', containing xylanase, cellulase and pectinase activity but the effect on chemical composition of the fiber was not investigated. Akin, Morrison, Gamble, & Rigsby (1997) performed tests with the commercial Flaxzyme, Ultrazym and an enriched pectinase mixture (EPM) which all showed improvements based on glucose content. Endopolygalacturonase (EPG) was studied by Akin, Slomczynski, Rigsby, & Eriksson (2002) separately and in combination with other enzymes, i.e. pectin lyase, pectinmethylesterase, xylanase and endoglucanase. Enzymatic treatments on flax stems were evaluated with Fried Test scores, fineness and fiber strength determination. Addition of supplemental enzymes to the EPG preparation produced fibers with similar properties as after EPG treatment alone [17]. Foulk et al. [18] investigated the effect of combinations with enzymes from Inotex on flax and these were evaluated with the Fried Test. Characterization of the chemical composition of fibers is hence not very often included in the evaluation of enzymatic treatments but is however an important factor to consider. Moreover, enzymatic treatments are often performed on flax fibers immediately, while this research aims at treatment of flax stems to facilitate the extraction of the fibers.

Therefore, this research deals with the application of strategic enzyme combinations and evaluation of possible synergy for the extraction of flax fibers from the stems by determining the resulting chemical composition of fibers and their ease of extraction. Characterization of chemical properties of the fiber illustrates the purity of the fiber after treatment. Extraction efficiency (EE) on the other hand is an important factor that gives more information on the ease of fiber extraction after flax treatment. A high EE value results from a better loosening of the fibers from the bast stem, implying a less severe mechanical post-treatment is necessary, leading to less fiber damage and a higher fiber yield.

2. Materials and methods

2.1. Materials

Verhalle supplied green flax samples (Amina cultivar) harvested in Belgium in 2015. Green flax was dried during 24 h at 105 °C and used as a reference and starting material to perform the enzymatic treatments. Green flax of the same cultivar was also traditionally dew retted and supplied by Verhalle as second reference. In addition, dew retted flax stems were submitted to a manual extraction procedure in order to extract the fibers (DRm). Dew retted flax of another cultivar (from France, harvested in 2017) was traditionally mechanically processed. These scutched fibers were provided by Vanacker Rumbeke (DRs). Finally, FlaxTape (200 g/m^2) from Lineo, a commercially available hackled flax fiber product was included as reference material as well.

Combinations of different pectinases and hemicellulases were tested on flax in this study. Scourzyme L (Sc, a pectate lyase), NS59049 (NS, a pectin lyase) and Pulpzyme (Pz, an endoxylanase) were provided by Novozymes (Switzerland). Rohapect MPE (MPE, a pectinmethylesterase) was supplied by AB Enzymes. Polygalacturonase from *Aspergillus niger* (PAn) and xylanase from *Thermomyces lanuginosus* (XTl) were purchased from Sigma-Aldrich.

2.2. Enzymatic treatment

Enzymatic treatments on green flax were performed as described in De Prez et al. [19]. Prior to enzymatic treatment, flax stems of 25 cm were dried at 105 °C during 24 h. 50 g of dried flax stems were immersed in a 1 l enzyme formulation of pH 6.5 containing 25 mM ethylenediaminetetraacetic acid (EDTA disodium salt dihydrate, VWR) and 0.30 v/v % enzyme, with exception of PAn (0.60 v/v %) due to its lower solubility. Enzymatic treatment was effectuated by incubating flax stems at 40 °C during 24 h. After washing, flax stems were dried again at 105 °C during 24 h.

Two additional treatments were performed as a reference, i.e. water treatment and EDTA treatment. Flax stems were treated with tap water at 40 °C during 24 h. EDTA treatment consisted of incubating the flax stems in an EDTA solution of 25 mM at pH 6.5 and 40 °C during 24 h. Subsequently, fibers were manually extracted as described in De Prez et al. [19]. Each enzymatic treatment was performed in duplicate.

2.3. Determination of extraction efficiency

Fibers were manually extracted from the flax stems from top to bottom as stated in De Prez et al. [19]. Manual extraction enables us to assign any changes in fiber characteristics to enzymatic treatment and minimizes fiber damage introduced by the mechanical action. The time necessary for the extraction of fibers and the yield of long fibers are measured. Time efficiency (E_t), fiber efficiency (E_f) and the overall extraction efficiency (EE) or separation efficiency (E_s) can be determined according to the adjusted equations as described in De Prez et al. [19]:

$$Ef = \frac{Amount\ of\ long\ fibers\ extracted}{Total\ amount\ of\ flax\ stems} \quad (1)$$

$$Et = \frac{Amount\ of\ long\ fibers\ extracted}{Time\ needed\ for\ extraction} * 2 \quad (2)$$

$$EE = E_s = E_f{}^{*}E_t \quad (3)$$

Where, "*amount of long fibers extracted*" and "*total amount of flax stems*" are expressed in weight (g), and "*time needed for extraction*" in (min). The factor 2 in Eq. (2) is expressed in min. g^{-1} and represents 100% time efficiency as reference, with 10 g long fibers extracted during 20 min. The amount of long fibers extracted is defined as the amount of fibers exhibiting a length greater than 15 cm after separation [11]. The abovementioned efficiencies permit to evaluate fiber yield and extraction duration, and thus ease of extraction.

2.4. Determination of chemical composition of fiber

Chemical composition of the fiber was determined with a gravimetric method as described in De Prez et al. [11] and is based on procedures described by Bledzki et al. [20] and Ramadevi et al.

[21]. The gravimetric method enables us to determine cellulose, hemicellulose and lignin content of the fiber. Pectin content was determined spectrophotometrically by using 3-phenylphenol (90%, Acros Organics) as described in De Prez et al. [11] and is based on methods described by [22–24]. Samples were analyzed in triplicate and assays were repeated twice.

2.5. Statistical analysis

Statistical analysis was performed by running one-way Analysis of variance (ANOVA) in SPSS with a confidence level of 95% and a Tukey post hoc test.

3. Results and discussion

3.1. Enzyme combinations

Previous research of the effect of individual enzyme treatments on flax revealed promising results for polygalacturonase, along with pectate lyase and endoxylanase based on chemical fiber composition and extraction efficiency [11]. Further evaluation of the enzyme treatments was done by characterization of fiber fineness, moisture absorption properties and determination of mechanical performance of flax fiber reinforced epoxy composites [12]. Enzymatically treated flax yielded fibers and resulting composites with promising high mechanical properties. Technical fibers with an E-modulus of 84GPa and strengths of 800 MPa were obtained. Within the current study, several combinations were investigated. The enzymes used as a base for the combinations are shown in Table 1, along with their corresponding abbreviation and enzyme activity.

Combinations of two enzymes as well as three enzymes were studied. In view of earlier published results [11,12], first strategic combinations were made with the promising polygalacturonase (PAn). PAn was respectively combined with Sc, NS, MPE, Pz and XTl. An overview of the strategic combinations is illustrated in Fig. 1. Furthermore, the other important pectinase activities, i.e. pectate lyase (Sc) and pectin lyase (NS), were tested in combination with the promising xylanase (XTl). Combination of PAn + MPE is expected to improve the retting behavior. Pectinmethylesterase (MPE) cleaves methyl groups from the pectin backbone which should give more access to the depolymerizing enzymes like polygalacturonase and pectate lyase [10]. Based on this hypothesis, combinations with three enzymes were also tested with complementary activities to PAn and MPE, namely by addition of Sc, NS, Pz and XTl. The combination of PAn + MPE + NS could exhibit some competition since MPE and NS are favoring the same substrate. However, it is also possible that pectin polymers with methyl groups as well as pectate polymers can be degraded more efficiently. Finally, combinations of PAn + Sc + XTl and PAn + NS + Pz were investigated for their retting effect as well.

Table 1
Overview of enzymes and their activity.

Enzyme	Abbreviation	Activity
Pectinases		
Scourzyme L	Sc	Pectate lyase
NS59049	NS	Pectin lyase
Rohapect MPE	MPE	Pectinmethylesterase
Polygalacturonase from *Aspergillus niger*	PAn	Polygalacturonase
Hemicellulases		
Pulpzyme	Pz	Endoxylanase
Xylanase from *Thermomyces lanuginosus*	XTl	Endo-β-(1,4)-xylanase

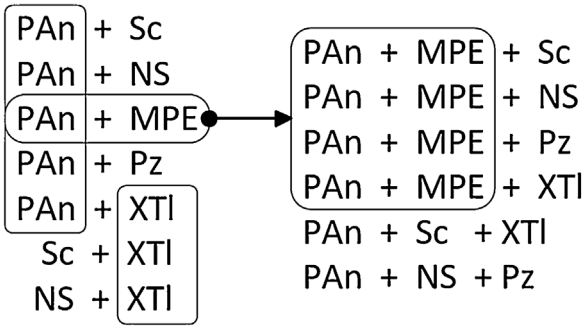

Fig. 1. Overview of strategic enzyme combinations.

Enzymatic treatments were effectuated as described in the Materials and methods section. Each enzyme was added with the concentration of 0.30 v/v %, except for PAn (0.60 v/v %). Fibers were manually extracted according to De Prez et al. [19] which enabled us to characterize the fiber extraction efficiency. Fibers were then chemically characterized by the gravimetric method.

3.2. Characterization of chemical fiber composition

Determination of the chemical composition of the fiber gives more insight on the effect of enzymatic treatments in view of degradation of surrounding polymers and removal of unimportant matter (e.g. waxes). Cellulose, hemicellulose, lignin and pectin content of extracted fibers were analyzed and results are illustrated in Table 2. The included reference materials are green fibers (GR), fibers extracted after water treatment (WATER) and EDTA treatment (EDTA), manually extracted dew retted fibers (DRm), scutched dew retted fibers (DRs) and FlaxTape fibers (FT).

Green flax as starting material resulted in fibers with a cellulose content of 64 ± 2% [11]. A significant increase of the cellulose content of fibers extracted after enzymatic treatment was definitely observed for the individual enzymes (78 to 80%), as well as for FlaxTape fibers. Additional reference materials also exhibited a significantly higher cellulose content compared to green fibers, but to a lesser extent in comparison with enzymatic treatments and FlaxTape. Results from the individual enzymatic treatments were extensively discussed in De Prez et al. [11]. Hemicellulose content of fibers only decreased significantly after NS (9.2 ± 1.6%) and XTl (9.4 ± 0.0%) treatment of flax compared with GR (13.3 ± 1.0%), WATER, EDTA and DRs fibers, while pectin content was reduced significantly after all individual enzymatic treatments compared with green fibers.

Furthermore, results of enzymatic treatments with the combination of two enzymes are presented in Table 2. PAn and Sc are enzymes specifically acting on the same substrate, i.e. pectate or homogalacturonic acid, but were nevertheless also combined. The combination of PAn + NS should be able to affect more of the pectic network around the fiber. With polygalacturonase (PAn) and pectin lyase (NS), both non-methylesterified and methylesterified pectins can be degraded. Next to non-esterified pectins, PAn + MPE treatment should be able to degrade methyl-esterified pectins by cleaving of methyl groups by MPE and further action of PAn. Combinations of pectinases and hemicellulases like PAn + Pz, PAn + XTl, Sc + XTl and NS + XTl should disconnect the surrounding network of the fiber more thoroughly due to the impact on pectins as well as on hemicelluloses.

Treatment combinations with two enzymes seemed to result in fibers with similar cellulose contents as fibers after treatments with individual enzymes and significantly higher cellulose contents compared with reference materials (with exception of FT). Next to NS and XTl, combinations PAn + Sc (9.8 ± 0.6%)

Table 2
Chemical characterization of extracted fibers after enzymatic treatment in comparison with various reference materials.

Treatment	Cellulose (w/w %)	Hemicellulose (w/w %)	Lignin (w/w %)	Pectin (w/w %)	Rest fraction (w/w %)
References					
GR*	64 ± 2^{a}	13.3 ± 1.0^{a}	4.9 ± 1.2^{abc}	6.1 ± 0.4^{a}	12.0 ± 0.9^{a}
WATER*	71 ± 3^{b}	12.4 ± 1.2^{ab}	2.4 ± 1.1^{cd}	5.5 ± 0.2^{a}	8.8 ± 2.0^{ab}
EDTA*	70 ± 2^{b}	12.4 ± 0.3^{ab}	6.1 ± 2.3^{a}	2.5 ± 0.3^{cdef}	9.0 ± 3.4^{bcd}
DRm*	72 ± 2^{bcd}	9.7 ± 0.4^{bcd}	3.8 ± 0.1^{abcd}	4.0 ± 0.1^{b}	10.0 ± 2.8^{bc}
DRs	71 ± 1^{bc}	13.4 ± 0.3^{a}	5.2 ± 0.7^{ab}	4.0 ± 0.1^{b}	6.1 ± 0.1^{cde}
FT*	76 ± 0^{cdef}	11.7 ± 0.2^{abcd}	3.3 ± 0.5^{bcd}	2.9 ± 0.1^{bcdef}	6.3 ± 0.1^{de}
Pectinases					
Sc*	78 ± 1^{efg}	10.7 ± 0.4^{abcd}	2.9 ± 0.3^{bcd}	3.3 ± 0.6^{bcde}	5.4 ± 1.1^{de}
NS*	79 ± 1^{efg}	9.2 ± 1.6^{d}	3.0 ± 0.8^{bcd}	2.9 ± 0.4^{bcdef}	5.9 ± 1.5^{de}
MPE*	78 ± 2^{efg}	11.0 ± 0.7^{abcd}	2.4 ± 0.5^{cd}	3.0 ± 0.0^{bcdef}	5.9 ± 2.3^{de}
PAn*	79 ± 2^{efg}	11.5 ± 0.8^{abcd}	3.5 ± 1.1^{abcd}	2.8 ± 0.2^{bcdef}	3.6 ± 1.3^{e}
Hemicellulases					
Pz*	80 ± 1^{fg}	10.8 ± 0.6^{abcd}	2.8 ± 0.8^{bcd}	3.2 ± 0.2^{bcdef}	3.2 ± 1.9^{e}
XTl*	80 ± 1^{fg}	9.4 ± 0.0^{cd}	3.3 ± 1.1^{bcd}	3.0 ± 0.3^{bcdef}	4.5 ± 0.0^{e}
Combinations with two enzymes					
PAn + Sc	80 ± 1^{fg}	9.8 ± 0.6^{bcd}	2.0 ± 0.8^{d}	2.4 ± 0.6^{cdef}	6.2 ± 0.8^{de}
PAn + NS	79 ± 2^{efg}	11.3 ± 1.8^{abcd}	2.9 ± 0.9^{bcd}	2.9 ± 0.1^{bcdef}	4.4 ± 1.1^{e}
PAn + MPE	79 ± 1^{fg}	11.1 ± 0.2^{abcd}	2.4 ± 0.7^{cd}	3.2 ± 0.3^{bcdef}	3.9 ± 0.4^{e}
PAn + Pz	79 ± 2^{efg}	10.7 ± 0.9^{abcd}	3.2 ± 1.3^{bcd}	3.2 ± 0.4^{bcdef}	3.9 ± 0.8^{e}
PAn + XTl	81 ± 1^{g}	9.0 ± 0.6^{d}	3.0 ± 0.3^{bcd}	2.9 ± 0.5^{bcdef}	4.1 ± 0.6^{e}
Sc + XTl	77 ± 0^{defg}	11.1 ± 0.9^{abcd}	3.4 ± 0.3^{bcd}	3.4 ± 0.1^{bcd}	5.3 ± 0.5^{de}
NS + XTl	78 ± 2^{efg}	9.8 ± 0.8^{bcd}	2.7 ± 1.0^{bcd}	3.6 ± 0.9^{bc}	5.6 ± 0.7^{de}
Combinations with three enzymes					
PAn + MPE + Sc	77 ± 0^{defg}	10.8 ± 0.4^{abcd}	4.7 ± 0.8^{abc}	2.3 ± 0.1^{cdef}	5.6 ± 0.6^{de}
PAn + MPE + NS	76 ± 1^{cdef}	12.5 ± 1.1^{ab}	3.4 ± 0.8^{bcd}	2.9 ± 0.3^{bcdef}	5.6 ± 1.0^{de}
PAn + MPE + Pz	79 ± 1^{fg}	10.6 ± 0.5^{abcd}	2.4 ± 0.1^{cd}	2.3 ± 0.1^{def}	5.4 ± 1.6^{de}
PAn + MPE + XTl	78 ± 3^{efg}	10.3 ± 3.2^{bcd}	4.9 ± 0.5^{abc}	2.0 ± 0.1^{f}	5.1 ± 0.4^{e}
PAn + Sc + XTl	75 ± 1^{bcde}	12.2 ± 0.4^{abc}	4.4 ± 1.1^{abcd}	2.1 ± 0.2^{ef}	6.8 ± 0.3^{de}
PAn + NS + Pz	78 ± 2^{efg}	10.6 ± 1.8^{abcd}	3.5 ± 1.1^{abcd}	2.3 ± 0.3^{def}	5.9 ± 1.1^{de}

a,b,c,d,e,f,g: values within columns with different letters differ at $P < 0.05$.
* Data adapted from De Prez et al. [11].

PAn + XTl (9.0 ± 0.6%) and NS + XTl (9.8 ± 0.8%) also realized a significant drop in hemicellulose content compared to green fibers (13.3 ± 1.0%). Besides this, the PAn + Sc combination was the only enzymatic treatment that resulted in a significantly lower lignin content (2.0 ± 0.8%) compared to green fibers (4.9 ± 1.2%). For the pectin content and rest fraction on the other hand, all enzymatic treatments resulted in a significant reduction compared to the starting material. In contrast to the hypothesis, the combination PAn + MPE did not realize an increased reduction of the pectin content, nor was a big decrease observed for the hemicellulose content. Combining all parameters, PAn + Sc showed the best results taking into account the reductions in hemicellulose, lignin and pectin content, while PAn + XTl and NS + XTl resulted in a decline in hemicellulose and pectin.

Compared with the single enzyme treatments, the addition of an extra enzyme to the enzyme formulation did not lead to drastic enhancements on fiber chemical composition. However, also the extraction efficiency should be thoroughly examined in view of the necessity of a second or third enzyme in the formulation.

Combinations with three enzymes tended to produce fibers with similar chemical properties and no significant differences in cellulose content compared with FlaxTape fibers and other enzymatic treatments; this with exception of PAn + MPE + NS (76 ± 1%) and PAn + Sc + XTl (75 ± 1%) treatments, which resulted in fibers with a significantly lower cellulose content than PAn + XTl (81 ± 1%). This lower cellulose content could be explained by the limited reduction of the other components. Only pectin contents of NS + XTl (3.6 ± 0.9%) and Sc + XTl (3.4 ± 0.1%) were significantly different from PAn + MPE + XTl (2.0 ± 0.1%) and PAn + Sc + XTl (2.1 ± 0.2%) among enzymatic treatments. A possible explanation for the minimal differences after treatment with a combination of two or three enzymes could be the arising competition between different enzyme activities or possible inhibition.

In research of George et al. [9], flax fibers were reported with a cellulose content of 68% after xylanase + cellulase treatment and up to 80% after polygalacturonase treatment. The enzymatic treatments were performed on fibers, not on stems. Furthermore, the polygalacturonase treatment resulted in fiber contents of 3.34% hemicellulose, 1.36% pectin and 1.87% lignin [9]. Lignin and pectin content in this study were in line with these results. However, hemicellulose contents were minimally 9.0% in this study and thus much higher than the hemicellulose content obtained in the research of George et al. [9]. Hemicellulose content of the fiber after xylanase + cellulase on the other hand amounted to 10.85% [9], which can be compared with our hemicellulose results. Remarkably, a higher hemicellulose reduction was observed after polygalacturonase treatment than after xylanase + cellulase treatment. A possible explanation for this observation is that the degraded pectin cohered with hemicellulose in the network, resulting in the degradation of both substances. Akin et al. [25] characterized chemical fiber properties after treatment with Flaxzyme, Ultrazym and EPM by performing gas-liquid chromatography. Glucose contents increased from 43.4% for unretted fibers to 65.0% for dew retted fibers and 69.9% after Flaxzyme (4%) treatment [25]. The chromatography characterization reported rhamnose, arabinose, xylose, mannose and galactose contents instead of hemicellulose and pectin contents, which makes a direct comparison more difficult.

3.3. Extraction efficiency

Next to the chemical composition of the extracted fiber, and even more important concerning feasibility and implementation in industry is the ease of fiber extraction, along with fiber yield.

After enzymatic treatment, flax stems were dried and fibers were manually extracted from top to bottom. Mechanical extraction was intentionally not chosen in order to be able to assign the changes in properties to the enzymatic treatments and not to severe mechanical extraction effects. Fig. 2 illustrates the fiber efficiency (E_f), time efficiency (E_t) and extraction efficiency (EE) for all enzymatic treatments, in comparison with the reference materials and reference treatments, i.e. green fibers, fibers extracted after water treatment and after EDTA treatment and manually extracted dew retted fibers.

Fig. 2A illustrates that E_f, i.e. long fiber yield after extraction, was ranging from 34 to 38%, except for DRm. These values are close to a maximal long fiber yield, since the amount of fibers present in flax stems is limited to circa 39% for the Amina cultivar [19]. Stephens [26] reported fiber percentages ranging between 20 to 35% in flax stems. Hence, further improvement concerning E_f is not possible since the E_f value cannot exceed the total fiber percentage of the flax stem. The higher E_f value of DRm fibers could be caused by the loss of flax stem fragments during handling and transportation. Since shives are lost, the total amount of flax stems referring to the long fiber yield may deviate from the original amount.

Since fiber efficiencies were in general closely in line with each other, with exception of DRm as stated earlier, the decisive factor influencing the overall extraction efficiency is the time efficiency. As seen in Fig. 2B, green fibers were extracted with a E_t of $25 \pm 4\%$. Water and EDTA treatment resulted in fibers that were faster and more easily extracted from the stem, with time efficiencies of $33 \pm 9\%$ and $47 \pm 12\%$, respectively. EDTA clearly had an important effect in the retting process, thanks to its complexation characteristics with calcium [10]. The presence of EDTA results in the chelate

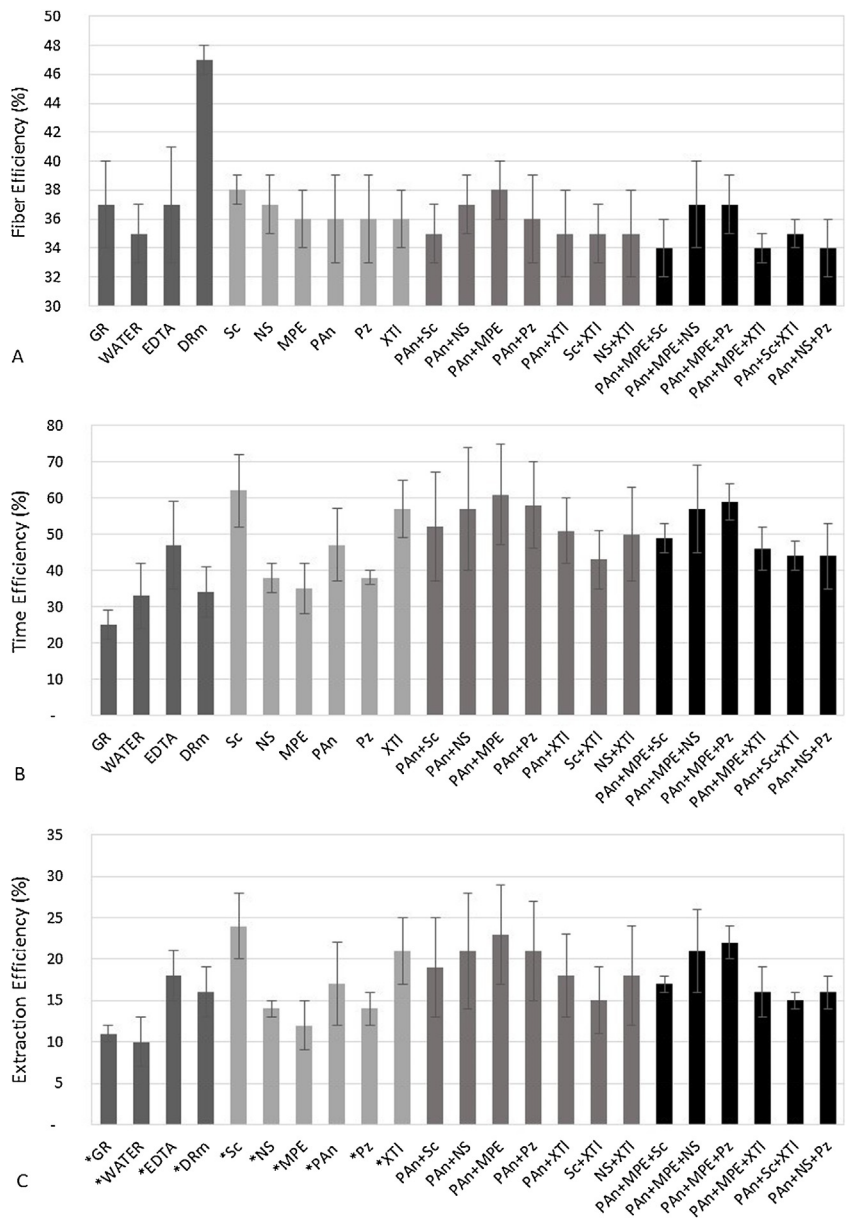

Fig. 2. Overview of (A) fiber efficiency (E_f), (B) time efficiency (E_t) and (C) overall extraction efficiency (EE) of references and fibers after enzymatic treatment. * Data adapted from [11].

formation with calcium, which is extracted from the epidermis. Degradation of the epidermis will lead to an easier degradation of the fiber surroundings and will improve retting efficiency [27–29]. Manual extraction of dew retted fibers only showed a E_t of 34 ± 7%. Even though fibers were already very loosened due to the extraction process, fibers were misaligned and chaotically ordered. This led to an increase in time necessary for the extraction. In contrast to this, mechanical treatment usually improves this alignment issue when present but at the same time, fiber yield is brought down as well due to fiber losses.

Among single enzymatic treatments, pectate lyase (Sc), polygalacturonase (PAn) and xylanase (XTl) treatment led to fibers that were extracted with the highest time efficiency of 62 ± 10%, 47 ± 10% and 57 ± 8%, respectively. Pectin lyase (NS), pectinmethylesterase (MPE) and endoxylanase (Pz) treatments produced fibers with a similar E_t as water and DRm fibers. The high time efficiencies of Sc, PAn and XTl correspond with extraction efficiencies of 24 ± 4%, 17 ± 5% and 21 ± 4%, respectively [11].

Concerning the time and extraction efficiencies, two enzyme systems all seemed to exhibit a better performance, with exception of Sc + XTl treatment, which only resulted in an EE of 15 ± 4%. In contrast, PAn + MPE treatment resulted as expected in a promising EE of 23 ± 6%, while PAn + NS and PAn + Pz obtained extraction efficiencies of 21 ± 7% and 21 ± 6%, respectively. Therefore, it was a well estimated decision to use PAn + MPE as a base for combinations with a third enzyme.

Combinations with three enzymes were successful as well since PAn + MPE + NS and PAn + MPE + Pz treatments obtained fibers with an extraction efficiency of 21 ± 5% and 22 ± 2%, respectively. The possible competition that could arise between the PAn + MPE + NS combination did hence not occur, but chemical properties were somewhat less promising. Other combinations with three enzymes did not achieve an EE higher than 17%.

Compared to green fibers, significant improvements in EE have been realized after combined treatments with PAn + NS, PAn + MPE, PAn + PZ, PAn + MPE + NS and PAn + MPE + Pz. Remarkably, the enzymes which performed best on an individual basis could not be retrieved in the optimal enzyme combinations.

It is crucial however to investigate also the mechanical performance of the fibers after these enzymatic treatments, more specifically when used as reinforcement in composite materials. Based on these additional insights, substantiated decisions can be made regarding the most optimal enzyme combination(s) to perform retting.

4. Conclusions

This study investigated the effect of combined enzymatic treatments on chemical composition of extracted fibers and extraction efficiency. The enzyme combinations were selected based on the promising results for single enzymatic treatments, which were realized with pectate lyase (Sc), polygalacturonase (PAn) and xylanase (XTl). Combinations with two and three enzymes tended to produce fibers with similar chemical properties as fibers after single enzymatic treatment. Some combinations with three enzymes did seem to realize a higher reduction of the pectin content of the fibers. Furthermore, study of the ease of fiber extraction showed similar long fiber yields for all enzymatic treatments and reference materials, with exception of DRm. Time efficiency was hence the decisive factor to determine the overall extraction efficiencies. Evaluation of extraction efficiencies illustrated that, next to the single treatments with pectate lyase (Sc) and xylanase (XTl), combined enzymatic treatments with polygalacturonase + pectin lyase (PAn + NS), polygalacturonase + pectinmethylesterase (PAn + MPE), polygalacturonase + endoxylanase (PAn + PZ), polygalacturonase + pectinmethylesterase + pectin lyase (PAn + MPE + NS) and polygalacturonase + pectinmethylesterase + endoxylanase (PAn + MPE + Pz) improved ease of fiber extraction significantly compared to green fibers and reached values better than these for dew retted fibers. Since treatments with two enzymes resulted in fibers with similar properties as after treatments with three enzymes, additional insights are needed regarding fineness of fibers and mechanical performance of the composite materials reinforced with enzymatically extracted fibers which forms the basis of future work.

Acknowledgements

The researchers would like to thank the Flemish Agency for Innovation and Entrepreneurship (VLAIO) for funding of this project [grant number IWT.141387] and are very grateful for the support from flax company Verhalle and Elmar Janser from Novozymes.

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