Effect of cell wall modifying enzymes on the rheological behaviour of citrus fibre dispersions

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Nowadays there are no doubts about the positive impact that fruits and vegetables have on human health. Plant fibres (i.e. non-digestible carbohydrates) are widely present in daily food, such as soups, dressings and sauces. On the other hand, agricultural (non-wood) by-products have a low economical value. However, those residues are frequently important sources of fibres so an important research is being done in order to obtain new high-value applications for such materials. Within Unilever, a technology is being developed based on plant fibre material for product structuring purpose. Structural changes on plant fibres dispersions due to enzymatic treatments were therefore studied along this project, based on the rheological behavior. In this project, the effect of several cell wall modifying enzymes (purified, industrial, and mix of enzymes) on the microstructure of plant fibres dispersions has been studied. Besides rheological tests, other techniques – CSLM and density tests – have been applied in order to fully characterize the aforementioned microstructure.

Introduction

The plants primary cell walls comprise mostly on a network of the so-called cellulose microfibrils, hemicellulose and pectin.

Cellulose is one of the most abundant biopolymers on earth occurring in wood, cotton, hemp and other plant-based materials (including fruits and vegetables) and serving as the dominant reinforcing phase in plant structures [1]. It is a polysaccharide made of β-1,4-D-glucopyranose units [2], in which every unit is rotated 180^{o} comparing with the next one. Single cellulose polymers form microfibrils via hydrogen bonds in a so-called crystalline structure [3].

Hemicellulose is a branched network of several types of polysaccharides. It refers to those that are extracted from cell walls by dilute alkali, which is believed to dissociate hydrogen bonds between cellulose and hemicellulose [4]. It is possible to separate them in four different types, depending on the main sugar (or sugars) unit, being linked mostly by β-1,4-glycosidic bonds [5]: xyloglucan, xylan, mixed linkage glucans, and mannan.

Pectin has three major components [6]: homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II. These polysaccharides give wall structure due to its interactions with cellulose and hemicellulose, but pectin has also a role in the development and defense of the plant, cell-cell adhesion, cell signaling, pH control and ionic strength [4]. Several models have been proposed to the pectin structure. The most accepted places homogalacturonan as the smooth region and rhamnogalacturonan as the hairy region [7].

Each cellulose microfibril can be partially coated by hemicellulosic polysaccharides,

which chain can have the sufficient length to span another microfibril linking them together [8]. A polysaccharide network is therefore formed, via hydrogen bonds. It's also widely accepted that the cellulose-hemicellulose network is embedded on pectin network, by covalent or non-covalent links [9].

As to the enzymatic modification of plant fibres, glycoside hydrolases catalyze the glycosidic bond break between two carbohydrates or between a carbohydrate and a non-carbohydrate, via general acid/base catalysis [10]; polysaccharides lyases catalyze an elimination reaction, by β elimination mechanism [11]; carbohydrate esterases hydrolyze ester linkages also through acid/base catalysis [12].

In this project, enzymatic modification of PF dispersions (considered as viscoelastic material) was studied in terms of rheology as it provides a good insight on the PF microstructure changes. Macroscopic mechanical behavior of dispersions (including PF dispersions) is a key property, which often determines the usability of such materials for a given industrial application. In terms of rheology, there are some characteristics that influence its behavior: volume concentration of the dispersed phase (fibres in this case); viscosity of the continuum phase; shape, size and size distribution of the dispersed phase; surface chemistry of the dispersed phase, which affects the repulsion/attraction of the cellulose microfibrils and thus its aggregation [13]. Viscoelastic response can thus be quantified by two material measures, namely the elastic storage modulus (G´) and the viscous loss modulus (G˝).

Plant cell wall fibres present some interesting properties from the product structurant point of view: water-holding and swelling capacity; viscosity or gel formation; etc. The porous matrix which is formed by the cellulose, hemicellulose and pectin network can hold a significant amount of water due to the formation of hydrogen bonds. These properties are related with the particle size, extraction conditions, ratio between insoluble and soluble dietary fibre, and plant source.

The goal of this project was then to obtain knowledge about the effect of different enzymatic treatments on the microstructure of plant fibres dispersions.

Materials and Methods

Pure enzymes as well as industrial enzymes were tested; for confidentiality reasons their names are given as letters, from A to L. Plant Fibres (from a commercial supplier) dispersions were prepared using demineralised water, by two different methods. 1L disposable jars were used (34.5cm of diameter, 12.3 cm of height).

Incubation

Method A: 1%PF[1] samples (600g or 750g) with and without addition of 0.2%[2] of Enzyme H were incubated for 3h at 45°C and 150 rpm using a thermostatic shaker (from New Brunswick, Innova 40 model). Method B (Dilution method): 2%PF samples (300g) were prepared containing 2% of PF. Depending on the experiment, a certain concentration (regarding the initial sample mass) of a certain

[1] PF concentration are all in terms of %(w/w)
[2] Enzyme concentration are all in terms of %(v/v) assuming a dispersion density equal to 1kg.m^{-3}

enzyme was added and the incubation was performed as described for Method A (excluding the shaking speed, which was 125rpm instead of 150rpm). After enzymatic inactivation, a dilution was made in order to achieve a 1%PF concentration. Some experiments were performed taking in account Method B with a small variation. For these cases pH was adjusted before incubation in the shaker, using a NaOH solution (0.1 and 1M from VWR Chemical) which was added drop by drop in order to avoid an abrupt pH increasing.

Inactivation of enzymatic activity was done by subjecting the samples to 1000W on a microwave in order to achieve a minimum temperature of 80^{o}C during at least 10 minutes. Samples obtained by Method B were cooled in water + ice for 15min to avoid high temperatures during processing.

Processing

PF dispersions were homogenized using an overhead mixer (Silverson L4RT-A mixer, standard emulsor screen workhead) at the following conditions: 6000 or 7000rpm for 10 minutes.

Rheology

A stress controlled Rheometer (TA-instruments AR-2000ex) with plate-plate geometry was used whereas the top plate is 40mm steel roughened plate and the gap was 1000µm. Following protocol was used, on a temperature of 20^{o}C: 1) Equilibrate Step (eq.) - the sample is subjected to a time sweep test for 5min, at 1Hz and 0.1% strain which enables the standardization between samples; 2) Continuous ramp – shear rate varies from 0.1s^{-1} to 500s^{-1} (for 2 minutes) and then to 0.1s^{-1} again (for 2 minutes); Strain Sweep (str. sw.) - %strain varies from 0.1% to 200%, with a fixed frequency of 1Hz.
Samples were tested at least two times.

Density Assay

To study the effect of air bubbles on rheology, caused by Silverson processing, a density test was performed on some samples. Identical small jars were filled with samples and its mass was determined. Differences on mass values between samples and a reference (expected mass of 1%PF without air, referred to the same type of small jar) were assumed as being caused by air bubbles.

Confocal Scanning Laser Microscopy

CSLM images were made by Caroline Remijn at Unilever R&D Vlaardingen. Congo Red was used to visualize fibre dispersions because of its strong affinity with cellulose. 1 ml of sample was stained with 1 droplet Congo Red and gently stirred through the sample. Imaging was done using the Leica TCS-SP5 confocal microscope with the DMI6000 inverted microscope. A solid state laser emitting at 561 nm was used for excitation. The emission bandwidth used was 565 nm to 677 nm.

Results and Discussion

The effect of several enzymes on the microstructure of commercially available plant fibres dispersions has been studied. Rheology tests, CSLM images, and density tests have been used to properly characterize the aforementioned microstructure. Besides, the effect of pH has been also studied based on rheology tests.

Enzymatic and pH effect on PF microstructure

Method B was used to prepare all samples which have been tested from the rheological point of view. Besides pure enzymes, it was also experimented the combination of enzyme H with pure enzymes and also other industrial enzymes. Results for a Silverson processing of 10minutes at 7000rpm (for all samples) are presented in Figure 1.

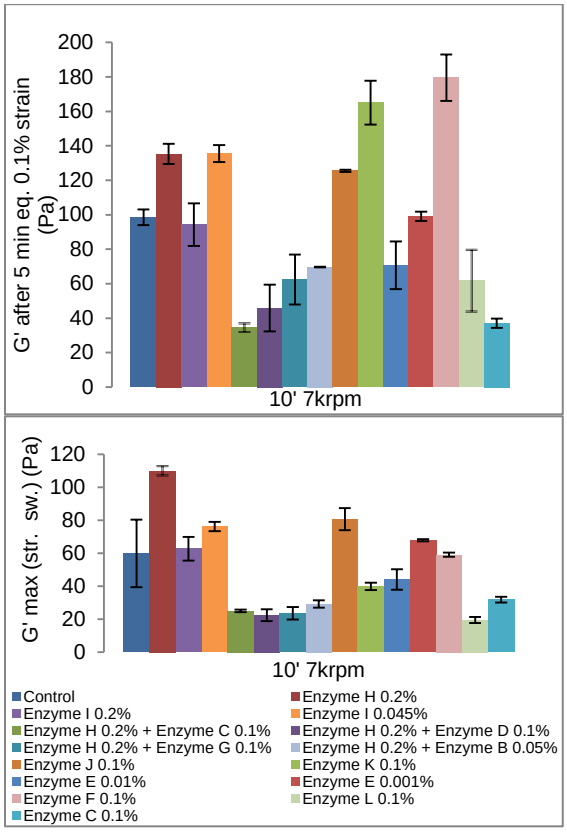

Figure 1 - G' of Plant Fibres, untreated and treated for 3h with different enzymes. Samples have been processed on a Silverson mixer for 10minutes at 7000rpm

When comparing with the control, sample treated with enzyme H presents the most interesting result. A significant increase on G' on the Equilibrate step (around 37%) and the higher increase on the Strain Sweep (around 83%) have been achieved. For that reason, it was decided to study more thoroughly the effect of this specific enzyme on PF dispersion microstructure.

On the Equilibrate step, enzyme K presents an increasing of 67%. However the same enzyme has led to a decrease of 33% on the Strain Sweep step. Although the sample presents higher elasticity on the Equilibrate, the microstructure is more sensible to the strain amplitude test.

All other treatments in this experiment didn't present a positive effect when comparing with the untreated sample.

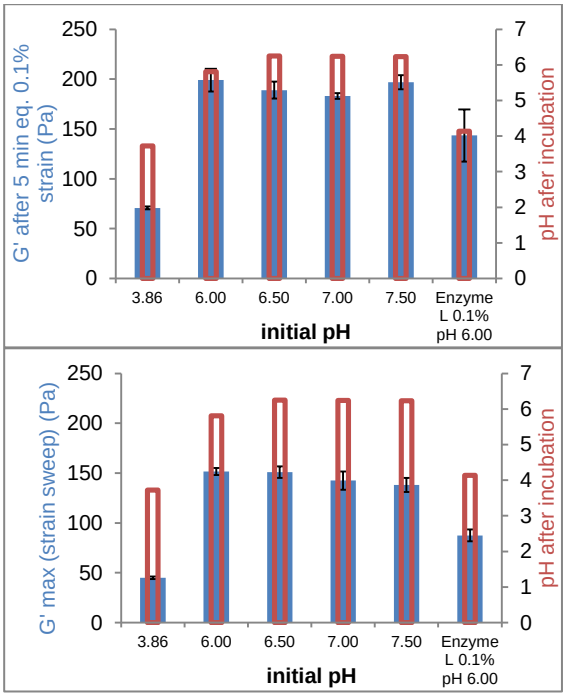

Figure 2 – pH effect on G' of Plant Fibres, incubated for 3h, untreated and treated with Enzyme L. Samples have been processed on a Silverson mixer for 10minutes at 7000rpm.

G' values are quite stable for higher pH values and they almost triplicate when compared with the sample without pH adjustment. At lower pH protonation of the acidic groups of the fibres reduces electrostatic repulsion. Thus the G' value also decreases since the fibres present more aggregation. At higher pH the opposite phenomenon occurs as electrostatic repulsion

increases and consequently the G' also increases.

Enzyme L activity was studied at pH 6 and G' decreases when compared with untreated sample with the same pH.

Effect of Enzyme H on PF microstructure

The influence of enzyme H on the PF dispersion microstructure was studied more thoroughly using rheology, CSLM and density tests.

Table 1 –Density results of PF with and without Enzyme H incubation

Sample N^o	Visual Air Bubble	Density (g/cm^3)		Air (%)		G' after 5min Equilibrate (Pa)		Max G' (Pa) Strain Sweep	
	Control / Enzyme H	Cont.	Enz. H	Cont.	Enz. H	Cont.	Enz. H	Cont.	Enz. H
1	No / Yes	1.00	0.97	1.02	3.57	76	101	66	90
2	No / Yes	1.01	1.00	0.12	1.22	115	137	98	133

Concerns about air intake after Silverson processing were taken in account. In particular, enzyme H treatment seemed to result in samples with visible air bubbles. Samples obtained from Method A were weighted and density was estimated as described in the Methods chapter. Results are summarized on Table 1. Differences on air percentage do exist but are very low, reaching a maximum difference of 2.6%.

Concerns about temperature effect on the microstructure were also taken in account. Two samples (PF processed by Silverson with and without enzyme H treatment) were reheated in the microwave in order to test the temperature effect but also to remove the air bubbles by stirring for 10minutes at a high temperature. During Silverson processing, these two samples achieved a temperature around 65^{o}C. The reheating was performed to reach a temperature around 85oC. Results from Table 2 show that G' values have remained constant.

Table 2 – Effect of temperature on the rheology of 1%PF

Sample N^o	G' after 5min Equilibrate (Pa)		Max G' (Pa) Strain Sweep	
	Control	Enz. H	Control	Enz. H
3	199	242	184	207
3 Reheated	236	233	187	207

At last, CSLM pictures of Figure 3 didn't reveal any significant differences between the two samples with and without enzyme H treatment presented on (table 2), which has led to increases of 21.6% (Equilibrate) and 12.2% (Strain Sweep) on G' in these samples. Both present compact cell wall material. Reheating on the microwave has led to a little bit less space filled.

Magnification: 40*1

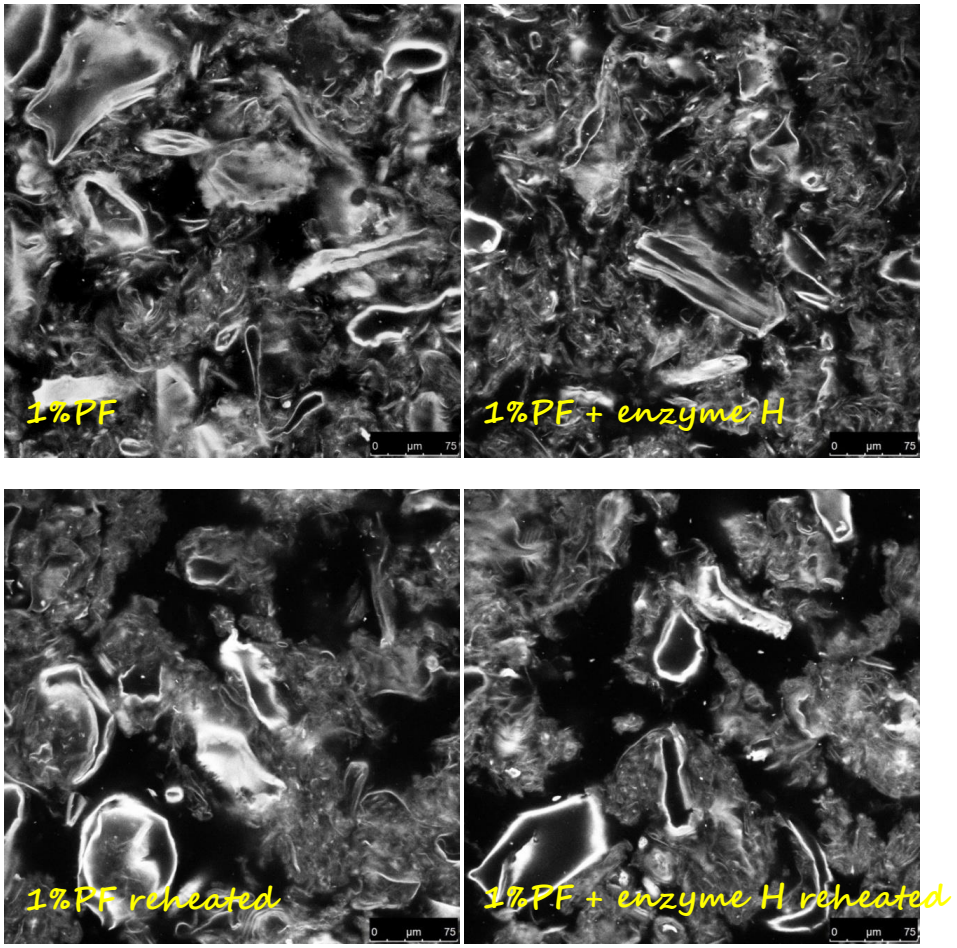

Figure 3 – CSLM images of 1%PF treated with and without enzyme H

Conclusions

Treatment of 1%PF dispersions with enzyme H had a positive effect on the G'. CSLM images didn't present any significant differences between control and sample treated with enzyme H, meaning the microstructure (visible at CSLM) wasn't deeply affected by enzymatic activity.

Enzymes C, D, E, F, G, and L have led to a decrease on G' both on Equilibrate and Strain Sweep tests.

Some enzymes had mixed results but none of them led to a positive effect as relevant as enzyme H.

It was observed that there is some air intake when processing samples treated with enzyme H. However the differences are considered to be small (about 3% is air). Regarding the temperature, heating up to 85^{o}C didn't affect the rheology results on tested samples.

pH adjustments do have an effect on PF dispersions microstructure. Adjusting pH of 1% PF dispersions, from 4 to 6.5, has led to higher electrostatic repulsion and thus to an increase of G'.

Future work

Prior to this project, it is recommended to study the effect of other enzymes and enzymes combinations on PF dispersions. Regarding enzyme H, different incubation conditions, including a range of enzyme concentration,

should be considered to test in order to optimize its effect.

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