



Autohydrolysis of *Annona cherimola* Mill. seeds: Optimization, modeling and products characterization



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ABSTRACT

Annona cherimola Mill. seeds are a residue of the industrial processing of this fruit, for which, presently, there is no industrial application. They have a considerable amount of oil, which can be converted into biodiesel, but the remaining lignocellulosic fraction still needs relevant added-value valorization routes.

In this work, the selective hemicelluloses removal by autohydrolysis was optimized aiming to maximize the yield of oligosaccharides with potential applications in food, pharmaceutical and cosmetic industries. A maximum of 10.4 g L⁻¹ of oligosaccharides was obtained, for a severity factor of 3.6, where 74.5% of the original hemicellulose was solubilized.

The process kinetics is presented, modeled (based on the Arrhenius equation) and its scale-up is discussed. The hydrolyzate shelf-life was evaluated and the produced oligosaccharides are stable at room temperature for, at least, 3 weeks. Furthermore, all oligosaccharides are also stable at 100 °C for 1 h, in pH values between 1 and 11, enabling their industrial processing, and at 37 °C for 3 h, in pH values between 1 and 3, thus indicating its potential classification as non-digestible oligosaccharides. The remaining cellulose enriched solids presented an increased enzymatic digestibility (as a function of the autohydrolysis severity) that assures its efficient use in subsequent processes (e.g., bioethanol production).

The upgrade route developed in this work in combination to the previously reported use of *A. cherimola* seed oil for biodiesel production can lead to an integrated zero-waste valorization strategy within the biorefinery framework.

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1. Introduction

Annona cherimola Mill. seeds are the main residue of the industrial processing of cherimoya. Currently it has no significant use, being considered a waste disposable problem.

The seeds have a significant oil content (up to 30%) that can be used for biodiesel production [1]. Cherimoya is produced mainly in Spain (3000 ha) and Chile (1000 ha), being common in the Andean region and in the Iberian countries. Moreover, Annonaceae are well spread all over the globe and cherimoya, being one of the most important species of this family, can maybe be considered a model for other Annonaceae.

Oil extraction yields a lignocellulosic fraction, the seed cake, which must be further upgraded. The presence of xyloglucans in

the seeds of another *Annona* species, namely *A. muricata*, has been reported [2], and hence the presence of these compounds is also expected in the lignocellulosic fraction of cherimoya seeds. This is a strong indication that this residue can be used to produce novel xylo-oligosaccharides (XOS), thus, assuring a potential valorization of cherimoya seeds within the biorefinery. In fact, oligosaccharides' (OS) demand has increased significantly and, thus, the search for new OS, and specifically of novel XOS sources is currently a relevant endeavor, as it can lead to novel products with applications in food, pharmaceutical and cosmetic industries [3,4].

The autohydrolysis process is considered to be the most appropriate choice [3,4] for the selective separation of hemicelluloses and the maximization of the OS production. This process has many significant advantages e.g., reduced production of monosaccharides [5], but the optimal operation conditions depend significantly from the composition of the feedstock.

There are several tools to help to better understand and control the behavior of the autohydrolysis process [6], namely the use of the severity factor ($\log R_0$ – Eq. (1)) and formal kinetic models. The severity factor, R_0 , was developed to compare steam explosion and

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autohydrolysis treatments of lignocellulosic materials between different conditions and equipment [7,8], and is given by the following equation, for non-isothermal conditions:

$$R_O = \int_0^t \exp\left(\frac{T(t) - T_{\text{ref}}}{\omega}\right) \times dt \quad (1)$$

where $T(t)$ is the temperature as a function of time, T_{ref} is the temperature up to which the hydrolysis process is considered to be negligible (usually 100 °C) and ω is a term that can be related to a conventional energy of activation, typically 14.75 [7,9].

Nevertheless, the application of the severity factor can be subjected to criticism, as it is observed that dissimilar responses may be obtained for similar severity factors, even if the operational conditions (e.g., heating/cooling profiles, liquid to solid ratio, target – maximum attained – temperature), are not that diverse. Therefore, formal kinetic models are usually preferable, as the severity factor does not give any mechanistic insight [7].

The determination of the kinetic parameters is a powerful tool for scale-up procedures, either to the pilot and demonstration levels or to a later commercial phase [10], but the rigorous kinetic modeling of acid catalyzed hemicelluloses' degradation is very complex. Several constraints do exist, like the uneven production and diffusion of the hydronium ions through the heterogeneous and complex polymeric matrix, the occurrence of side reactions with other components of the matrix (e.g., lignin), the limited access of the H_3O^+ ions to the glycosidic bonds due to steric hindrance, or the surface area availability and accessibility of the substrate during the reaction [6,11], that make the system difficult to model in detail. Therefore, simplifications have to be made to overcome these constraints. The main assumption is that polysaccharide degradation (and also the subsequent reactions) is irreversible and follow a first order kinetic. It is also considered that the system is pseudo-homogenous. Furthermore, the kinetics are assumed to follow the Arrhenius equation,

$$K = Ae^{\frac{E_a}{RT}} \quad (2)$$

where A is the pre-exponential factor, E_a the activation energy, R the ideal gas constant and T the temperature. Therefore, the determination of the Arrhenius parameters requires extensive experimental data to fit Eq. (2), namely, the study of the process kinetics at diverse temperatures, what is typically laborious, expensive and time consuming, and clearly an alternative approach is required.

In order to maximize OS yield a series of non-isothermal autohydrolysis was carried out, varying the maximum temperature of the treatment. The obtained results were successfully fitted to a kinetic model, aiming to predict the behavior of the process. Also, bearing in mind a zero waste goal, it is important to characterize both the liquid and solid fractions resulting from the autohydrolysis process. Therefore, expedite methods for evaluating OS stability were applied and the enzymatic digestibility of the cellulose-enriched solid fractions were evaluated.

2. Materials and methods

2.1. Materials

2.1.1. Feedstock

A. cherimola seeds were provided frozen by J. Faria & Filhos Lda. (Funchal, Portugal). Upon collection seeds were washed and dried to constant weight. After drying, the seeds were stored at room temperature until further processing. The seeds were ground using a simple blender and its oil extracted with commercial hexane. Seeds were then dried in an oven, further milled with an IKA® Werke (Staufen, Germany) MF10 basic mill and screened to retain the fraction under 1.5 mm.

2.1.2. Reagents

Sulfuric acid (95–97%), D-(+)-xylose ($\geq 99\%$), levulinic acid (98%), furfural (99%) and 5-hydroxymethylfurfural (99%) were purchased from Sigma–Aldrich (Steinheim, Germany), hydrochloric acid (37%), citric acid monohydrate (99.5–100.5%), L-(+)-arabinose ($\geq 99\%$), acetic acid ($>99.8\%$) and potassium chloride ($>99.5\%$) were purchased from Merck (Darmstadt, Germany), D-(+)-glucose ($>99.5\%$) was purchased from Duchefa Biochemie (Haarlem, The Netherlands), formic acid (98%) was purchased from PANREAC (Barcelona, Spain), sodium hydrogen phosphate heptahydrate (99.5–100.5%) was purchased from Riedel-de Haën (Seelze, Germany), Celluclast® 1.5 L and Novozyme 188 were purchased from Novozymes (Bagsvaerd, Denmark) and sodium hydroxide was purchased from eka (Bohus, Sweden).

2.2. Autohydrolysis treatments

Experiments were carried out in batch mode using a Parr Instruments Company (Moline, IL, USA) two liter T316SS steel reactor heated externally by an electric mantle. Two turbines, of four blades each, agitated the mixture and cold water cooled the system through an internal serpentine, when necessary. Temperature and agitation were monitored and controlled with a Parr Instruments Company model 4842 PID controller. Agitation was set to 150 rpm. Pressure was measured by the same controller. In each treatment milled seeds and water were mixed in a liquid-to-solid ratio (LSR) of 5 (w/w) to a total mass of 1.2 kg. The experiments were conducted under non-isothermal conditions to reach target temperatures ranging from 150 to 230 °C. Heating profiles were obtained for all treatments. After a rapid cooling, (typically less than two minutes to reach temperatures below 100 °C), liquid and solid phases were separated by pressing (up to 200 bar) with a Sotel (Portugal) manual oil press. The solid residues were washed with 500 mL of distilled water and pressed once again.

For comparison purposes, the severity of the treatments was estimated by calculating the log R_O (Eq. (1), see Section 1) based on the measured temperature profiles data.

2.3. Scanning electron microscopy

Samples were sputter-coated with gold/palladium and observed using different magnifications with a Philips XL30 FEG Scanning Electron Microscope (Eindhoven, The Netherlands) at 10 kV.

Both the cherimoya seed cake and the solid residue obtained after autohydrolysis at 190 °C were observed.

2.4. Mathematical modeling

Numerical integration was carried out by implementing Euler's method in MS Excel 2010. The MS Excel Solver tool was used to estimate the kinetic model parameters that fit the experimental data, by minimizing the sum of square deviations [12,13].

2.5. Analytical procedures

Both the raw material and the solid residues obtained after autohydrolysis were characterized according to standard NREL protocols [14–17]. The monosaccharides (glucose, xylose and arabinose), aliphatic acids (formic, acetic and levulinic), and furan derivatives (HMF and furfural) present in the liquid fraction were quantified by HPLC using a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) (Hercules, CA) in an Agilent 1100 series HPLC system (Santa Clara, CA, USA) equipped with a refractive index detector (G1362A) controlled at 35 °C and a diode array detector (G1315B). The mobile phase was H_2SO_4 5 mM, the column temperature 50 °C, and the flow rate 0.6 mL min^{−1}. The system

Table 1

Buffers solutions used for the evaluation of the stability to pH and temperature of the produced OS.

pH	Solution A	Solution B	V _{Sol A} (mL)	V _{Sol B} (mL)	V _{Total} (mL)
1 ^{a,c}	0.2 M KCl	0.2 M HCl	50.0	97.0	200.0
2 ^{a,c}	0.2 M KCl	0.2 M HCl	50.0	10.6	200.0
3 ^a	0.1 M citric acid	0.2 M Na ₂ HPO ₄	39.8	10.2	100.0
7 ^a	0.1 M citric acid	0.2 M Na ₂ HPO ₄	6.5	43.6	100.0
11 ^{b,c}	0.05 M Na ₂ HPO ₄	0.1 M NaOH	100.0	8.2	108.2

^a Gomori.¹⁷

^b Robinson and Stokes.¹⁸

^c These are not buffer solutions but will be considered as so, similarly to what is described in the literature.

was equipped with a Micro-Guard Cation-H Refill Cartridge from Bio-Rad before the HPX-87H column. The same HPLC system was equipped with a Bio-Rad Aminex HPX-87P column (300 × 7.8 mm) (Hercules, CA) for monosaccharide quantitation, as indicated by standard NREL protocols [14–17]. The mobile phase was water, the column temperature 80 °C and the flow rate 0.6 mL min⁻¹. Injection volume was, for both cases, 5 µL.

All samples were filtered with Millipore® (Cork, Ireland) 0.45 µm cellulose acetate membrane filters prior to analysis.

The liquid fractions were also characterized using standard NREL protocols [17]. Sulfuric acid was added to 100 mL of the liquid fraction to a final concentration of 4% (w/w). The samples were hydrolyzed in an autoclave for 1 h at 121 °C. After cooling, the hydrolyzates were filtered with 0.45 µm membrane filters before HPLC analysis, as previously described. This procedure was performed at least in duplicate. Oligosaccharides' concentration was calculated from the increase in sugar monomers, before and after acid post-hydrolysis.

2.5.1. Stability of the liquid fraction and oligosaccharides

To evaluate the shelf life of the hydrolysate obtained in optimal conditions, 500 mL were filtered through 0.45 µm membrane filters to Schott flasks (under non-sterile conditions) and kept in a dry place at room temperature away from light. At defined intervals a sample was taken (also under non-sterile conditions), hydrolyzed and analyzed according to NREL standard protocol, as previously described [17].

To evaluate the OS' stability to pH and temperature the hydrolysate was filtered and evaporated to dryness in a rotary evaporator (Büchi, Switzerland) at 35 °C (down to 0.015 bar) to recover OS. The evaporated sample was dissolved to obtain concentrations of 30 g L⁻¹, using diverse buffer solutions (Table 1). The solutions were thermostatically kept for 3 h at 37 °C (at pH 1–3, away from sunlight, simulating human digestion) and 1 h at 100 °C (simulating cooking procedures) in a Memmert (Schwabach, Germany) WNB 22 oil bath and then analyzed as previously described [17].

2.5.2. Enzymatic digestibility of the remaining solid

Enzymatic digestibility was evaluated according to NREL standard protocol [18].

The reaction mixture contained 0.15 g of frozen biomass (dry weight basis) 5 mL of sodium citrate buffer (0.1 M, pH 4.8), 100 µL of sodium azide solution (2% w/v), as an anti-microbial agent, and Celluclast® 1.5 L and Novozyme 188 enzymes in prescribed amounts to obtain 60 FPU/g and 64 pNPGU/g of dry biomass, respectively. The total volume was adjusted to 10 mL with water.

Each biomass sample was hydrolyzed in duplicate. A biomass blank (without enzymes) and enzyme blank (without biomass) were also carried out. The blanks were used to correct the results to any free saccharides present in the biomass and eventual products

Table 2

Initial seed cake composition, solid yield (%) and solid residues composition (g/100 g dry matter) obtained in the autohydrolysis assays of cherimoya seeds as a function of the severity factor.

Seed cake	Target temperature (°C)					
	log R ₀	150	170	190	210	230
	Solid yield	2.37	2.83	3.60	4.23	4.70
		96.01	83.12	78.04	58.61	59.17
Glucan	22.05	23.56	24.01	33.78	39.00	38.65
Xylan	14.17	12.56	12.34	5.03	0.00	0.00
Arabinan	3.61	3.34	3.33	0.00	0.00	0.00
Acetyl groups	2.15	2.13	2.15	1.49	0.00	0.00
Klason lignin	25.63	38.41	42.92	58.50	60.98	61.81
Ash	2.84	0.92	0.84	0.45	0.39	0.38
Protein/others	29.53	19.08	14.41	0.75	0.00	0.00

inherent to the reaction conditions or to determine the amount of glucose present in the enzymes.

The hydrolysis was carried out in a Comecta (Barcelona, Spain) 200D orbital shaker at 50 °C and 250 rpm for 72 h. After this period, the samples were immersed in a water bath at 90 °C for 5 min in order to inactivate the enzymes. The samples were then filtered through 0.45 µm membrane filters and analyzed by HPLC (Aminex HPX-87H column) as previously described [17].

3. Results and discussion

3.1. Autohydrolysis liquors and solid residues' composition

In order to understand the behavior of the autohydrolysis of cherimoya seed cake, the process was carried out under non-isothermal conditions and the composition of liquid and solid phases was evaluated for all experiments.

On the liquid phase, OS concentration reached 10.4 g L⁻¹ at the optimal experimental condition, 190 °C, log R₀ = 3.60 (Fig. 1). Monosaccharides formation follows a similar pattern and their maximum yield is achieved at the same severity. For more severe conditions sugars degradation becomes predominant over sugar production from oligosaccharides. In fact, degradation products (DP) – namely furans and formic acid – formation is clearly the main reaction for high severity conditions. Acetic acid formation is a consequence of acetyl groups' removal from the hemicellulose moiety and, therefore, it is correlated with the data presented in Table 2 regarding residual solids composition.

The severity condition leading to the highest production of oligosaccharides (log R₀ = 3.60) is lower than for other lignocellulosic materials [10,13,19], that typically present values close or above 4, thus, indicating that this material is less recalcitrant to hydrolysis and it will require milder/economical operational conditions at industrial level.

The concentrations of soluble compounds, namely monosaccharides and degradation compounds, such as 5-hydroxymethylfurfural (HMF) and furfural, do not impose excessive purification problems. So it will be possible to easily purify OS e.g., using a membrane technology based process as formerly described [20], or the simpler evaporative concentration process (see below).

Table 2 contains the data obtained for the solid yield and the composition of the solid residues after autohydrolysis. As expected, the solid yield diminished with the severity. This trend is justified by hemicellulose removal and also by the removal of extractives, namely inorganics and other soluble compounds that decreased significantly already at mild severities, what is an advantageous trait if their recovery is required. Regarding the most noteworthy fraction, hemicellulose, its removal reaches 74.5% for the optimal condition for XOS production and comparing to the seed cake composition, is completely removed for the higher temperature

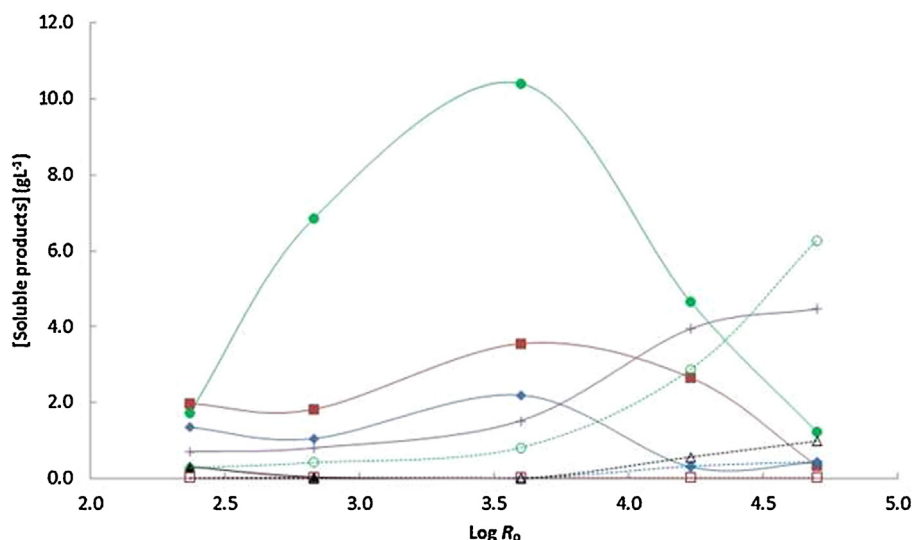


Fig. 1. Soluble products' concentrations (g L^{-1}) in the hydrolysates as a function of $\log R_0$ (●) Oligosaccharides, (◆) glucose, (■) xylose, (▲) arabinose, (+) acetic acid, (○) formic acid, (□) levulinic acid, (◇) HMF and (△) furfural, lines are used for eye guidance only for the non-isothermal autohydrolysis of cherimoya seed cake. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

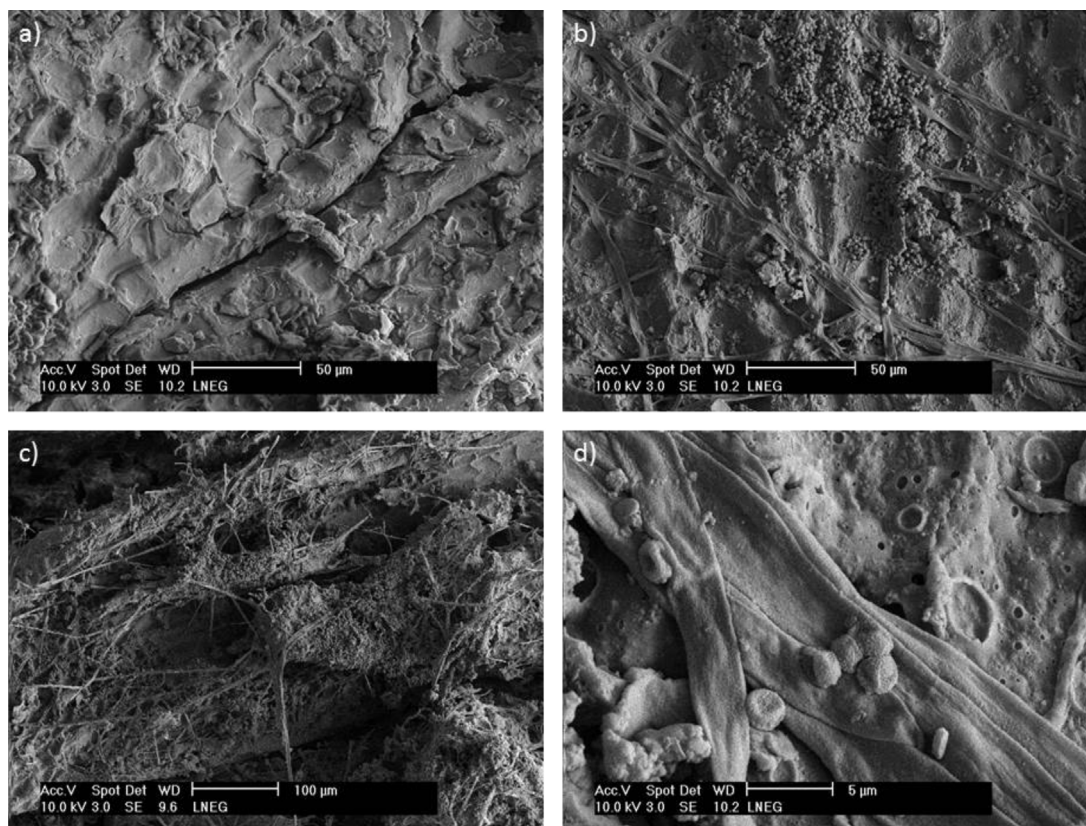


Fig. 2. SEM photomicrographs (a) before and (b), (c) and (d) after autohydrolysis at 190 °C.

treatments. Conversely, and as a consequence of these removals, the relative amounts of glucan and lignin in the solid residues increased with the severity of the treatment. A complete recovery of glucan was observed, but lignin recovery typically exceeds 100%. This behavior has already been reported for several other lignocellulosic materials e.g. [21] and may be related to binding reactions, for instance between protein and lignin [22,23]. These results showed that after autohydrolysis, the solid phases were

enriched in glucan and, putatively, lignin (and protein), clearly indicating the process' high selectivity for hemicellulose removal and the solids' possible suitability for further processing e.g., lactic acid or ethanol production through simultaneous saccharification and fermentation. In fact, this is advantageous for subsequent cellulose hydrolysis, as it limits cellulase adsorption [22]. Furthermore, the presence of protein in the cellulose enriched solids can also be advantageous for fermentation (as a substitute/complement nitro-

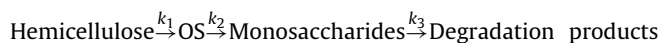
gen source). Nevertheless, if deemed relevant, it is also possible to recover protein from hydrolyzed materials [24]. The assumptions above are confirmed by SEM imaging (Fig. 2). The lignocellulosic matrix, intact in Fig. 2a, is partly removed after autohydrolysis (Fig. 2b) and some cellulose fibers can be distinguished along with re-condensed lignin droplets. The removal of hemicellulose, revealing the cellulose fibers, is evident in less detailed photomicrographs (Fig. 2c). The appearance of lignin droplets on the surface of plant cell walls has been attributed to lignin melting at high temperature and pressure, migration to the surface and subsequent condensation [25–28]. These lignin droplets are more clearly observed in higher magnifications (Fig. 2d).

3.2. Mathematical modeling of the autohydrolysis process

Comparisons based on $\log R_0$ are subject to some criticisms and do not give extra insights on the process kinetics. As such, kinetic models may be a better tool to understand the process. In fact, the optimization of process conditions using kinetic models is a key resource for a scale-up to both the level of proof and a subsequent commercial phase [10]. Unfortunately, the development of such models using isothermal conditions implies the use of many experimental data points that are time- and resource-consuming, especially if the effect of temperature is to be taken into account.

The hydrolysis of lignocellulosic materials can be described by an interdependent sequence of chemical reactions. Based on these reactions it is possible to establish a model which describes the kinetics of the autohydrolysis process, using a system of differential equations based on a mass balance among the species involved.

Several specific kinetic models have been reported for the hydrolysis of the hemicellulosic fraction of raw materials [6,13,29]. In this work it is assumed that the OS are produced from the hemicellulose fraction, further hydrolyzed into monomers that are then degraded into diverse products, according to the following path:



Nevertheless, the rigorous kinetic modeling to study hemicellulose autohydrolysis is a complex problem, especially if the model has to be adapted to describe hemicellulose hydrolysis under non-isothermal conditions and simplifications are assumed, as described in Section 1. Specifically, in this work we assume that the kinetic constants (k_1 – k_3) dependence on temperature do follows Arrhenius' equation, all hemicellulose is hydrolysable and for OS, only one fraction was considered, independently of their molecular weight.

As such, the hemicellulose degradation can be mathematically translated into the following equation:

$$\frac{d[\text{Hemicel}]}{dt} = -k_1 \times [\text{Hemicel}] \quad (3)$$

Taking in consideration the Arrhenius equation (Eq. (2)), Eq. (3) can then be rewritten as:

$$\frac{d[\text{Hemicel}]}{dt} = -A_1 \times e^{\left(-\frac{E_{a1}}{RT(t)}\right)} \times [\text{Hemicel}] \quad (4)$$

As the reaction takes place under non-isothermal conditions, and the cooling time is considered to be negligible as compared to the heating time, the temperature profile as a function of time was empirically described by a linear trend, fitting the experimental data for the heating period. Hence, the $T(t)$ term can be substituted by the expression $T = \alpha t + \beta$, where α represents the heating rate and β the temperature at the beginning of the treatment:

$$\frac{d[\text{Hemicel}]}{dt} = -A_1 \times e^{\left(-\frac{E_{a1}}{RT(\alpha t + \beta)}\right)} \times [\text{Hemicel}] \quad (5)$$

Table 3

Parameters determined for the proposed model for the autohydrolysis of cherimoya seeds – pre-exponential factor ($\ln A_n$) [A_n , h^{-1}] and activation energies (E_{an}) [kJ mol^{-1}].

E_{a1}	$\ln A_1$	E_{a2}	$\ln A_2$	E_{a3}	$\ln A_3$
58.83	17.05	25.53	8.39	56.36	16.48

In the same way, the following reaction step, can be described by:

$$\frac{d[\text{OS}]}{dt} = -A_1 \times e^{\left(-\frac{E_{a1}}{RT(\alpha t + \beta)}\right)} \times [\text{Hemicel}] - A_2 \times e^{\left(-\frac{E_{a2}}{RT(\alpha t + \beta)}\right)} \times [\text{OS}] \quad (6)$$

In a similar way, it is possible to develop the equations for monosaccharides and degradation products (DP) formation, Eqs. (7) and (8), respectively:

$$\frac{d[\text{Monos}]}{dt} = A_2 \times e^{\left(-\frac{E_{a2}}{RT(\alpha t + \beta)}\right)} \times [\text{OS}] - A_3 \times e^{\left(-\frac{E_{a3}}{RT(\alpha t + \beta)}\right)} \times [\text{Monos}] \quad (7)$$

$$\frac{d[\text{DP}]}{dt} = A_3 \times e^{\left(-\frac{E_{a3}}{RT(\alpha t + \beta)}\right)} \times [\text{Monos}] \quad (8)$$

Due to the non-isothermal nature of the system, the system's analytical integration is not trivial, contrary to what happens in models for isothermal conditions [21]. Therefore, the system of non-linear equations that contains only six parameters (the Arrhenius parameters for each of the three reactions) has to be solved numerically and fitted to the full set of experimental data points.

Table 3 shows the values determined for the pre-exponential factor ($\ln A_n$) and activation energies (E_{an}). The reliability of the proposed model is assured by the good agreement between the experimental data points and model predictions (Fig. 3). Furthermore, these results are in the order of magnitude as compared to data obtained for other residues [11,29,30], but present lower levels than the reported values for more recalcitrant lignocellulosic materials, that justify the milder conditions found for optimal cherimoya seeds hydrolysis. In fact, the comparison based on the kinetic modeling, due to taking into account the composition of the materials and temperature influence more effectively, is a more useful tool than severity parameters for the comparison between different biomass feedstock. It is important to stress that autohydrolysis modeling based on data obtained from experiments carried out under isothermal conditions would require far more extensive datasets than the ones presented in this work. These results confirm that the use of non-isothermal conditions is an effective experimental approach to support the kinetic modeling of the autohydrolysis process.

3.3. Hydrolysate's shelf life evaluation

Considering a possible industrial application, the capability of the oligosaccharide-rich hydrolyzates to be stored for long times at room temperature was evaluated.

During the 3 weeks of the test, gluco-oligosaccharides' (GlcOS) and xylo-oligosaccharides' (XOS) concentrations presented no changes (data not shown). Also, no microbial growth was observed, even when, deliberately, no sterilization was made to avoid microbial contamination. The low pH value of the hydrolysate and the significant amounts of furfural and acetic acid, reported inhibitory for microbial growth [31–33], may explain the microbial stability of the hydrolysate. Furthermore, the mild conditions during storage (as compared to the production conditions) may also justify

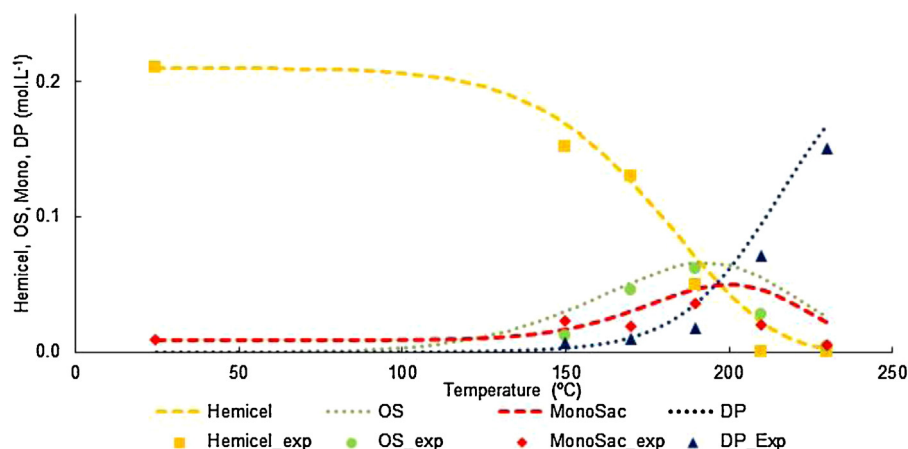


Fig. 3. Experimental and calculated time courses for (■) hemicellulose, (●) oligosaccharides, (▲) monosaccharides, (◆) degradation products for the non-isothermal autohydrolysis of cherimoya seed cake. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

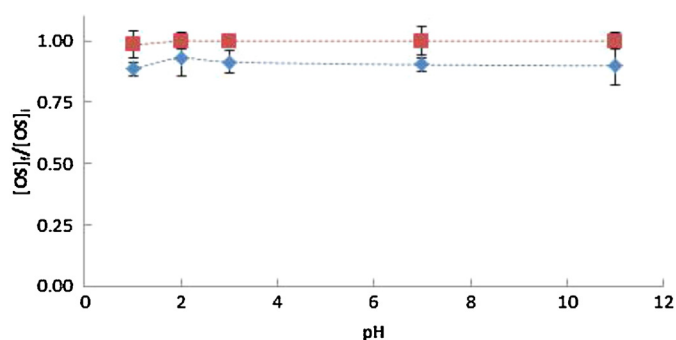


Fig. 4. Stability profiles of the produced oligosaccharides at 100 °C. (◆) GlcOS, (■) XOS (lines are used for eye guidance only). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

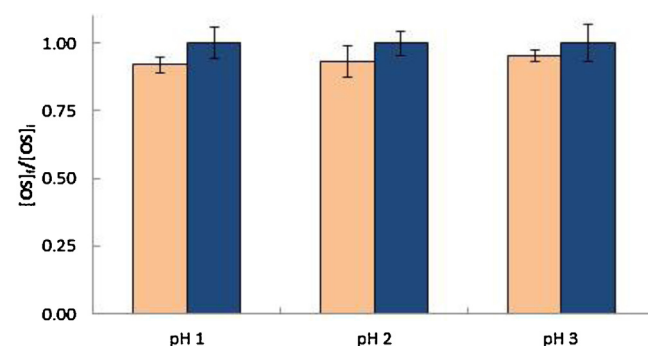


Fig. 5. Stability of the produced oligosaccharides in conditions similar to the human digestion process. (■) GlcOS, (■) XOS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the hydrolysate's chemical stability. As to the best of our knowledge this is the first report regarding the evaluation of the storage stability, and no data is available for comparison.

3.4. OS' stability

OS are mainly used in the food sector as prebiotic agents. For that, their source must be as free as possible from microbial growth inhibitors, such as furans and aliphatic acids. In this work this was easily achieved by using a simple evaporative concentration step that allowed a significant removal of furfural and acetic acid, without OS degradation.

Among the several OS with high dietary and economic value, the non-digestible oligosaccharides (NDOS) are of great importance as they can reach the intestine and be used to feed the bacteria in the gut flora, acting like prebiotic agents [3,4]. In this work two preliminary evaluative simple screening tests are used to assess if the produced and partially purified OS are potential NDOS. These tests evaluate the stability at 100 °C to appraise the possibility of the OS to be processed *per se* or in combination with foodstuffs [34] and the stability at digestive pH and temperature conditions as a first indication that they will be able to pass the gastrointestinal tract to reach the large bowel.

The stability profiles of both GlcOS and XOS at 100 °C for 1 h as a function of the pH is shown on Fig. 4. Both GlcOS and XOS are quite stable in the whole tested pH range (1.5% of XOS and 11.5% of GlcOS were degraded at pH 1). Moreover, XOS are highly stable at pH values between 2 and 11, as expected, since XOS are

described to be more stable at 100 °C than GlcOS [34]. This is a particularly interesting result as OS obtained from wheat bran or chicory have been reported not to be this stable at pH values of 3 and 11, mainly those derived from chicory [34,35]. The produced OS are thus deemed suitable for applications in the food industries and likely to be processed.

Consequently, these OS need to be evaluated according to the pH conditions of the human stomach. The digestive process in the stomach occurs at 37 °C for about 3 h and pH values between 1 and 3. The produced XOS are highly resistant to this simulation of the digestive process (Fig. 5), as no degradation was observed, and only 8% degradation was observed for GlcOS. This is an expected result, according to the reported stability for other similar compounds at 25, 37 and 50 °C at pH values of 1 and 2 [35,36] and a first assurance that their potential nutritional properties may be retained during digestion.

In fact, the ability of these OS to be processed and the possibly to pass the stomach undigested makes them potential NDOS, but this must be study further, as this screening test is only a preliminary step before more complex (and expensive) *in vivo* testing to definitely classify these OS as prebiotic agents.

3.5. Enzymatic digestibility of the solid residues

After the autohydrolysis treatment the remaining biomass must also be valorized. In this sense, the lignin-(protein) and cellulose-rich solids were treated enzymatically to saccharify cellulose.

The enzymatic digestibility is clearly higher for the more severe treatments. Glucose concentration increased 2.3 times (Fig. 6a)

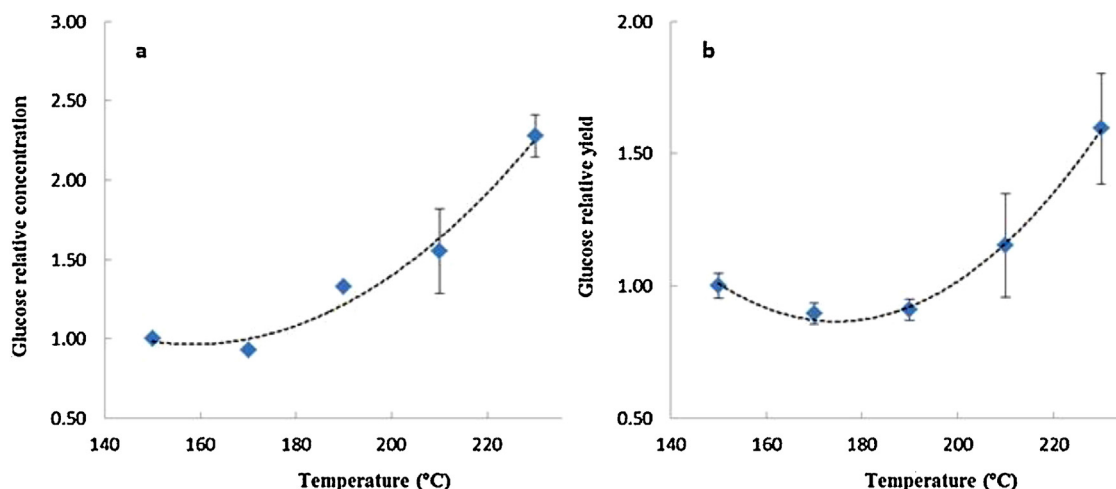


Fig. 6. Enzymatic digestibility of treated cherimoya seeds in terms of (a) glucose relative concentration and (b) glucose relative yield (lines are used for eye guidance only).

from the least severe treatment to the higher concentration obtained (4.77 g L^{-1}) and the highest yield (Fig. 6b) was 83% ($1.6\times$ higher than the yield obtained for the least severe treatment). Here it is important to stress that, as the amount of enzyme was kept constant, due to the glucose enrichment of the solids, less enzyme per glucan is present, which indicates that the digestibility is even higher, and that this value will translate into a significant glucose concentration when use under typical solid loadings reported in literature, e.g., 20%. In fact, the reported digestibility is in line with values reported for similar pretreatment and enzymatic hydrolysis conditions for other materials, e.g., it is significantly higher than the glucose yield reported for eucalyptus residues (54%), and close to 82% for olive tree pruning, and only slightly lower for straws (88, 90 and 91%, respectively for rice, corn straw and wheat straw) [37–39], what may be explained by the higher lignin content of the cherimola seed cake as compared to straws. Although, it is reported that the highest glucose yield can only be achieved after the complete removal of hemicellulose, the lignin also plays a role on preventing cellulose hydrolysis that must not be neglected [22].

This behavior can be related to the extent of the autohydrolysis treatment. Increased severity of the autohydrolysis results in hemicellulose removal, a factor known to increase cellulose digestibility [40]. Nevertheless, this removal only partially explains the high increase in cellulose digestibility. As such, it is suggested that the further increase in cellulose digestibility after hemicellulose removal may be related to the decrease of cellulose crystallinity (observed by SEM imaging – data not shown) or to an increase of pore sizes obtained for more severe conditions that enhance cellulase penetration into biomass [40].

4. Conclusions

The lignocellulosic fraction of cherimoya seeds after oil extraction, seed cake, is a possible source of OS, yielding XOS under mild operational conditions that could be modeled by mathematical kinetic models based on the parameters of the Arrhenius equation. The developed model will be a useful tool to predict the behavior of the autohydrolysis process, namely on scale-up procedures, as the produced OS have been demonstrated to be chemically stable under relevant process and digestive conditions. This enables to maintain their physicochemical properties, and hence their inherent potential nutritional/bioactive properties. In fact, the ability of these OS to be processed and the possibility to pass the stomach

undigested, deem them as potential NDOS, a trait that must be further characterized and explored.

Moreover, in a technological point of view, the autohydrolysis process enabled the recovery of hemicellulose as potentially marketable products, and to obtain cellulose-enriched pretreated solids presenting higher enzymatic saccharification yields, thus increasing the value of these solids as substrates for fermentative processes. Furthermore, it also does not threaten the upgrade potential of the lignin and protein fractions, thus, contributing to a zero waste approach on the valorization of the cherimoya seeds.

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References

- [1] P.C. Branco, P.C. Castilho, M.F. Rosa, J. Ferreira, Characterization of *Annona cherimola* Mill. seed oil from Madeira Island: a possible biodiesel feedstock, *J. Am. Oil Chem. Soc.* 87 (2009) 429–436.
- [2] T. Watanabe, M. Shida, T. Murayama, Y. Furuyama, T. Nakajima, K. Matsuda, K. Kainuma, Cell-wall polysaccharides of rice hull. 2. Xyloglucan in cell-walls of rice hull, *Carbohydr. Res.* 129 (1984) 229–242.
- [3] S.I. Mussatto, I.M. Mancilha, Non-digestible oligosaccharides: a review, *Carbohydr. Polym.* 68 (2007) 587–597.
- [4] A.A. Achary, S.G. Prapulla, Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications, *Compr. Rev. Food Sci. F* 10 (2011) 2–16.
- [5] F.M. Gírio, C. Fonseca, F. Carvalheiro, L.C. Duarte, S. Marques, R. Bogel-Lukasik, Hemicelluloses for fuel ethanol: a review, *Bioresour. Technol.* 101 (2010) 4775–4800.
- [6] G. Garrote, H. Domínguez, J.C. Parajó, Hydrothermal processing of lignocellulosic materials, *Holz Roh Werkst.* 57 (1999) 191–202.
- [7] R.P. Overend, E. Chornet, Fractionation of lignocellulosics by steam-aqueous pretreatments, *Philos. Trans. R. Soc. A* 321 (1987) 523–536.
- [8] E. Chornet, R.P. Overend, Phenomenological kinetics and reaction engineering aspects of steam/aqueous treatments, *Proceedings of the International Workshop on Steam Explosion Techniques: Fundamentals and Industrial Applications* (1988) 21–58.
- [9] R.P. Overend, E. Chornet, Heavy-oil cracking – the case for nonhomogenous kinetics, *Can. J. Phys.* 68 (1990) 1105–1111.

- [10] D. Nabarlatz, X. Farriol, D. Montané, Kinetic modeling of the autohydrolysis of lignocellulosic biomass for the production of hemicellulose-derived oligosaccharides, *Ind. Eng. Chem. Res.* 43 (2004) 4124–4131.
- [11] G. Garrote, H. Domínguez, J.C. Parajó, Mild autohydrolysis: an environmentally friendly technology for xylooligosaccharide production from wood, *J. Chem. Technol. Biotechnol.* 74 (1999) 1101–1109.
- [12] B.A. Ogunnaike, W.H. Ray, *Process Dynamics, Modeling, and Control*, Oxford University Press, New York, NY, USA, 1994.
- [13] G. Garrote, J.C. Parajó, Non-isothermal autohydrolysis of *Eucalyptus* wood, *Wood Sci. Technol.* 36 (2002) 111–123.
- [14] B. Hames, R. Ruiz, C. Scarlata, A. Sluiter, J. Templeton, Preparation of Samples for Compositional Analysis, NREL, Golden, CO, 2005.
- [15] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Determination of Structural Carbohydrates and Lignin in Biomass, NREL, Golden, CO, 2005.
- [16] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Determination of Ash in Biomass, NREL, Golden, CO, 2008.
- [17] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples, NREL, Golden, CO, 2006.
- [18] M. Selig, N. Weiss, Y. Ji, Enzymatic Saccharification of Lignocellulosic Biomass, NREL, Golden, CO, 2008.
- [19] F. Carvalheiro, T. Silva-Fernandes, L.C. Duarte, F.M. Gírio, Wheat straw autohydrolysis: process optimization and products characterization, *Appl. Biochem. Biotechnol.* 153 (2009) 84–93.
- [20] J.L.C. dos Santos, M.C. Fernandes, P.M.L. Lourenço, L.C. Duarte, F. Carvalheiro, J.G. Crespo, Removal of inhibitory compounds from olive stone auto-hydrolysis liquors by nanofiltration, *Desalin. Water Treat.* 27 (2011) 90–96.
- [21] F. Carvalheiro, M.P. Esteves, J.C. Parajó, H. Pereira, F.M. Gírio, Production of oligosaccharides by autohydrolysis of brewery's spent grain, *Bioresour. Technol.* 91 (2004) 93–100.
- [22] X. Pan, D. Xie, N. Gilkes, D.J. Gregg, J.N. Saddler, Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content, *Appl. Biochem. Biotechnol.* 121–124 (2005) 1069–1079.
- [23] W.J.J. Huijgen, J.H. Reith, H. den Uil, Pretreatment and fractionation of wheat straw by an acetone-based organosolv process, *Ind. Eng. Chem. Res.* 49 (2010) 10132–10140.
- [24] B. Bals, L. Teachworth, B. Dale, V. Balan, Extraction of proteins from switchgrass using aqueous ammonia within an integrated biorefinery, *Appl. Biochem. Biotechnol.* 143 (2007) 187–198.
- [25] B.S. Donohoe, S.R. Decker, M.P. Tucker, M.E. Himmel, T.B. Vinzant, Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment, *Biotechnol. Bioeng.* 101 (2008) 913–925.
- [26] J.B. Kristensen, L.G. Thygesen, C. Felby, H. Jorgensen, T. Elder, Cell-wall structural changes in wheat straw pretreated for bioethanol production, *Biotechnol. Biofuels* 1 (2008) 5.
- [27] L.P. Xiao, Z.J. Sun, Z.J. Shi, F. Xu, R.C. Sun, Impact of hot compressed water pretreatment on the structural changes of woody biomass for bioethanol production, *Bioresources* 6 (2011) 1576–1598.
- [28] T.S. Sun, K. Wang, G.H. Yang, H.Y. Yang, F. Xu, Hydrothermal treatment and enzymatic saccharification of corncobs, *Bioresources* 9 (2014) 3000–3013.
- [29] F. Carvalheiro, G. Garrote, J.C. Parajó, H. Pereira, F.M. Gírio, Kinetic modeling of brewery's spent grain autohydrolysis, *Biotechnol. Prog.* 21 (2005) 233–243.
- [30] A.H. Conner, Kinetic modeling of hardwood prehydrolysis. Part I. Xylan removal by water prehydrolysis, *Wood Fiber Sci.* 16 (1984) 268–277.
- [31] G. Garrote, H. Domínguez, J.C. Parajó, Manufacture of xylose-based fermentation media from corncobs by posthydrolysis of autohydrolysis liquors, *Appl. Biochem. Biotechnol.* 95 (2001) 195–207.
- [32] E. Palmqvist, B. Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition, *Bioresour. Technol.* 74 (2000) 25–33.
- [33] L.C. Duarte, F. Carvalheiro, J. Tadeu, F.M. Gírio, The combined effects of acetic acid, formic acid, and hydroquinone on *Debaryomyces hansenii* physiology, *Appl. Biochem. Biotechnol.* 129–132 (2006) 461–475.
- [34] C.M. Courtin, K. Swennen, P. Verjans, J.A. Delcour, Heat and pH stability of prebiotic arabinoxyloligosaccharides, xylooligosaccharides and fructooligosaccharides, *Food Chem.* 112 (2009) 831–837.
- [35] P. Rumpagaporn, A. Kaur, O.H. Campanella, J.A. Patterson, B.R. Hamaker, Heat and pH stability of alkali-extractable corn arabinoxylan and its xylanase-hydrolyzate and their viscosity behavior, *J. Food Sci.* 71 (2011) H23–H30.
- [36] Q. Wang, P.R. Ellis, S.B. Ross-Murphy, The stability of guar gum in an aqueous system under acidic conditions, *Food Hydrocolloids* 14 (2000) 129–134.
- [37] T. Silva-Fernandes, L.C. Duarte, F. Carvalheiro, S. Marques, M.C. Loureiro-Dias, C. Fonseca, F. Gírio, Biorefining strategy for maximal monosaccharide recovery from three different feedstocks: eucalyptus residues, wheat straw and olive tree pruning, *Bioresour. Technol.* 183 (2015) 203–212.
- [38] P. Moniz, L. João, L.C. Duarte, L.B. Roseiro, C.G. Boeriu, H. Pereira, F. Carvalheiro, Fractionation of hemicelluloses and lignin from rice straw by combining autohydrolysis and optimised mild organosolv delignification, *Bioresources* 10 (2015).
- [39] P. Moniz, H. Pereira, T. Quilho, F. Carvalheiro, Characterisation and hydrothermal processing of corn straw towards the selective fractionation of hemicelluloses, *Ind. Crops Prod.* 50 (2013) 145–153.
- [40] B. Yang, Z. Dai, S.-Y. Ding, C.E. Wyman, Enzymatic hydrolysis of cellulosic biomass, *Biofuels* 2 (2011) 421–450.