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Maximizing Bioethanol Production from *Eucalyptus globulus* Using Steam Explosion Pretreatment: A Multifactorial Design and Fermenter Development for High Solid Loads

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Abstract: Steam explosion pretreatment is suitable for bioethanol production from *Eucalyptus globulus* wood. Multifactorial experiment designs were used to find the optimal temperature and residence time required to obtain the best glucose yield from the enzymatic hydrolysis of pretreated materials. The chemical composition, crystallinity index, morphology and polymerization degree of the pretreated materials were correlated with enzymatic accessibility. Simultaneous saccharification and fermentation (SSF) using a fed-batch strategy was applied to three different laboratory-scale fermenters. The optimization of the pretreatment was obtained at 208 °C and 11 min. However, the enzymatic hydrolysis performance did not show significant differences from the material obtained at 196 °C and 9.5 min, which was determined to be the real optimum, owing to its lower energy requirement. The vertical fermenter with type "G" blades and the horizontal fermenter with helical blades were both highly efficient for reaching ethanol yields close to 90% based on dry wood, and ethanol concentrations close to 9.0% *v*/*v*.

Keywords: high-concentration bioethanol; response surface; simultaneous saccharification and fermentation; fed batch; fermenters design; steam explosion pretreatment

Citation: Troncoso-Ortega, E.; Valenzuela, R.; Reyes-Contreras, P.; Castaño-Rivera, P.; Schiappacasse, L.-N.; Parra, C. Maximizing Bioethanol Production from *Eucalyptus globulus* Using Steam Explosion Pretreatment: A Multifactorial Design and Fermenter Development for High Solid Loads. *Fermentation* **2023**, *9*, 965. https://doi.org/10.3390/fermentation9110965

Academic Editors: Miguel Ladero and Victoria E. Santos

Received: 16 September 2023
Revised: 20 October 2023
Accepted: 20 October 2023
Published: 10 November 2023

1. Introduction

Lignocellulosic biomass (LCB) is considered to play an important role in the building of a sustainable society, as it has the potential to replace fossil fuels and chemicals. For the conversion and use of biomass in value-added products, the "biorefinery" concept has emerged, which can contribute to the development of the circular economy by recovering and recycling bio-based products [1]. Currently, one of the main products of biomass is biofuels, which are estimated at a world production of 137 billion liters in 2016 [2]. Currently, bioethanol is the most ideal bio-based fuel or fuel additive for use in motor vehicles as a partial substitute for fossil fuels. Compared to gasoline, bioethanol contains 34.7% oxygen, which enables a 15% higher combustion efficiency, thereby resulting in fewer emissions of particulate nitrogen oxides, which are harmful to the environment [3]. The production of second-generation bioethanol (from lignocellulosic materials) requires several stages: pretreatment, hydrolysis, fermentation and distillation, where each stage will have consequences for the quality and cost of bioethanol [4]. The objective of pretreatments is to

increase the susceptibility of the material to obtain a reactive lignocellulosic substrate that is accessible to the enzyme. The efficiency of pretreatment is measured by the intensity at which hydrolysis becomes easier with minimal degradation and/or loss of carbohydrates, avoiding the formation of inhibitory compounds for the fermentative process. Among the different kinds of pretreatment methods, steam explosion (SE) is one of the most successful and widely used for fractionating lignocellulosic biomass [5,6]. This pretreatment has its origin in the Masonite process to produce fiber boards, described in 1930, where the high-pressure vapor penetrates the cell structure for a short time, and is then released, expanding rapidly when the reactor is depressurized, thereby causing the cell system to deconstruct (as a result of mechanical force by pressure drop). Before depressurization, a hydrolytic mechanism occurs that promotes the breakdown of hemicelluloses (autohydrolysis of the acetyl group), lignin modifications and cellulose exposure, promoting the formation of pores in the biomass and improving the efficiency of hydrolysis and saccharification during the bioethanol production process. It is known that the biomass depressurizes quickly in the expansion chamber. It is also known that the rapid depressurization of the biomass in the expansion chamber, causes a subito stress, which disrupts the glycosidic linkages and hydrogen bonds, facilitating enzymatic hydrolysis of the sugars (pentoses and hexoses) released from the hemicellulose and cellulose [7–9].

Additionally, another challenge for the viability of the bioethanol process and for bioethanol production is obtainment of highly concentrated ethanol during the fermentation or saccharification and simultaneous fermentation (SSF) stage, which would allow for an optimization of the energy consumed during distillation. Approximately 37% of the energy required by the bioethanol production plant is consumed during distillation [10].

To achieve a higher ethanol concentration, high pretreated substrate loading is crucial for the economy of the SSF process [11–13]. Working with the highest solid load has a significant effect on the capital and production cost of the process due to the reduction in size of the required equipment, such as tanks and distillation columns, as well as the reduced amount of energy utilized during distillation and the reduced production of wastewater [14]. For bioethanol produced from lignocellulosic material to be economically feasible on an industrial scale, the ethanol produced must be above 4% (w/v) [15]. High substrate loadings mean that the process is taking place at pretreated solid levels where there are not significant initial amounts of free water [16]. Less water causes an increase in the viscosity of the substrate matrix and creates rheological problems when the mass transfer rate in the substrate matrix is obstructed, thereby increasing the time required to liquefy the matrix and to perform efficient hydrolysis by the enzymes using classical batch-mode SSF [12], [17]. Thus, it has been demonstrated that the glucose and ethanol yields in enzymatic hydrolysis and SSF, respectively, decreased linearly with increasing solid concentration [18,19]. However, this statement has been subject to debate taking into consideration the efficiency and amount of enzyme used during mixing [20].

It is well known that the enzymatic activity of the cellulases complex decreases the viscosity of the lignocellulosic substrate during enzymatic hydrolysis. Some authors have proposed a gradual increment in the substrate concentration, in the fed-batch mode, instead of adding all of the substrate initially in the batch mode [21]. This strategy helps to maintain the viscosity at relatively low levels, thereby avoiding mixing and heat and mass transfer problems [13,22]. Several works using the fed-batch SSF approach have been performed using different raw materials and pretreatments, demonstrating the feasibility of this strategy, not only due to the improvement of the ethanol concentration obtained, but also due to the product yields achieved [17,23]. Guigou et al. [24] published a comparison of ethanol yields and concentrations achieved using different species of Eucalyptus, with initial solid percentages ranging from 4%wt to 27%wt and concentrations between 5.4 g/L to 30.7 g/L. The highest ethanol concentration was achieved using the presaccharification and simultaneous fermentation (PSSF) strategy for *Eucalyptus grandis* pretreated by steam explosion and *S. cerevisiae* PE-2 as the fermentative microorganism.

This work was focused on the optimization of the SE pretreatment for *Eucalyptus globulus*, a most important commercial hardwood species in the world and the second most important in Chile [25,26]. A multifactorial experimental design was used. The optimal parameters of pretreatment were obtained by using a response surface methodology, followed by a physicochemical characterization of pretreated materials to different severities. The physicochemical characteristics of these materials were correlated with the efficiency of enzymatic hydrolysis. The best-pretreated materials, with high enzymatic accessibility, were evaluated in bioethanol production, showing high yields and high concentrations when using fermenters designed for a SSF process with high solid loads.

2. Materials and Methods

2.1. Raw Material

Woodchips from 10- to 12-year-old *E. globulus* trees were donated by CMPC Cellulose S.A., Chile. The woodchips were selected to be a specific size of 2.5 cm × 1.5 cm × 0.3 cm to obtain a homogeneous sample. They were air-dried to a moisture content of 10% and stored under dry conditions until they were used.

2.2. Chemical Characterization of Samples

The woodchips were milled using a knife mill (Maestranza Proinco S.A., Concepción, Chile) and then classified using an ASTM sieve (45–60 mesh). The milled wood was extracted with a 90% acetone solution for 16 h in a Soxhlet extractor to measure the amount of extractives present. The steam-exploded samples were dried until they reached a humidity level of 10% and then they were milled and sieved. However, the sample from pretreatment process not were extracted with acetone. To hydrolyze the milled samples, 3 mL of 72% sulfuric acid was added to 300 mg of each sample and incubated at 30 °C for 1 h. The acid was then diluted to 4% by the addition of 84 mL of water, and the mixture was heated at 121 °C (1 atm) for 1 h. Finally, the resulting residual material was cooled and filtered through a number 4 porous glass filter. The solids fractions were dried at 105 °C until a constant weight was achieved, and then determined to be insoluble lignin. The concentration of soluble lignin in the filtrate was determined by measuring the absorbance at 205 nm, using an absorption coefficient of 110 L/g cm. Glucose, xylose, and acetyl groups were analyzed using a HPLC (Merck Hitachi, Tokyo, Japan) equipped with a refractive index detector and an Aminex HPX-87H (Bio-Rad, CA, USA) column at 45 °C, eluted at a flow rate of 0.6 mL/min with 5 mM H_2SO_4 [27]. To convert sugar monomers to anhydromonomers, 0.90 for glucose (reported as glucan) and 0.88 for xylose (reported as xylans) were used. These factors were calculated based on the water added to polysaccharides during acid hydrolysis, taking into account the molar mass of each original anhydromonomer in the polysaccharide. This resulted in a 10% increase for glucose (from 162 g/mol in the anhydromonomer to 180 g/mol in glucose) and a 12% increase for xylose (from 132 g/mol in the anhydromonomer to 150 g/mol in xylose). The acetyl content was calculated by multiplying the acetic acid content by 0.7. The percentages of reaction products (glucan, xylan, and lignin) in the solid and liquid fractions were calculated based on the initial amount of each component loaded into the SER (*w*/*w* dry basis). The moisture content of the solid fraction was determined using a moisture analyzer (Sartorius MA35).

2.3. Experimental Design to Optimize Steam Explosion Pretreatment

The range of variables for this study was defined based on previous work and the safety restrictions of the reactor and its boiler. Response surface methodology (RSM) was used to determine the optimal pretreatment condition that yields the highest glucose (Y_{EH}) in enzymatic hydrolysis. The influence and optimization of the variables, temperature and residence time were determined using Central Composite Circumscribed design (CCC) with Modde 7.0 software (Umetrics, Sweden). A two-level factorial design was used for each parameter, with levels of −1 and +1, star points with levels of $-\sqrt{2}$ and $+\sqrt{2}$ and a central point (0, 0) which is performed in triplicate to obtain the experimental standard

deviation, as shown in Table 1. The temperature range evaluated was 180–213 °C while the residence time range was 4–15 min. The response variable, Y_{EH}, was obtained through enzymatic hydrolysis for 72 h. The data were analyzed using Modde 7.0 software to obtain the polynomials related to the reaction system and the response surface plot. The model was statistically validated using analysis of variance (ANOVA) with a confidence level of 95%, using the same software. Variables such as wood particle size (3 × 3 cm^2), and impregnation reagent (2 L of water for 200 g of dry base wood) were kept constant.

Table 1. Experimental design results and optimal conditions for steam explosion pretreatment of *E. globulus*. Solid yield, raw material and pretreated materials chemical composition, glucose yields obtained in enzymatic hydrolysis.

	Pretreatment Conditions			**Raw and Pretreated Material Composition (% dwb)** [a]				**%Y_{EH}** [e] **(dwb) (72 h)**
Exp N°	**Temperature (°C)**	**Time (min)**	**Log S_0**	**Solid Yield**	**Glucans**	**Xylans**	**Lignin**	**Glucose Yield**
E.g. [b]	--	--	--	--	45.5	15.3	23.5	--
1	180 (−1)	4 (−1)	2.96	92.0	47.0	16.9	28.0	6.4
2	213 (+1)	4 (−1)	3.93	71.9	64.9	3.2	35.3	71.2
3	180 (−1)	15 (+1)	3.53	85.1	53.1	12.1	31.4	28.5
4	213 (+1)	15 (+1)	4.50	68.1	68.4	1.6	41.3	73.1
5	173 (−1.4)	9.5 (0)	3.13	93.0	48.2	18.3	26.0	8.1
6	220 (+1.4)	9.5 (0)	4.51	66.6	66.8	1.5	40.3	76.9
7	196 (0)	1.7 (−1.4)	3.06	74.3	50.6	15.3	29.3	19.8
8	196 (0)	17 (−1.4)	4.06	68.6	65.0	3.2	36.6	75.9
9 [c]	196 (0)	9.5 (0)	3.80	70.9 ± 3.0	63.2 ± 1.3	4.4 ± 0.4	35.1 ± 1.0	73.3 ± 4.8
10 [d]	208	11	4.22	67.5 ± 0.9	58.3 ± 1.4	2.7 ± 0.1	31.8 ± 0.7	74.5 ± 2.3

[a] Dry wood basis. [b] *Eucalyptus globulus* chips. [c] Exp N° 9 represents the average of triplicate experiments at the central point. [d] Average values obtained from triplicate pretreated material under optimized conditions. [e] Yield enzymatic hydrolysis.

2.4. Steam Explosion Pretreatment

Wood chips were pretreated in a steam explosion reactor (SER) (Maestranza Proinco S.A., Concepción, Chile) consisting of a steam generator, 5 L vessel reactor and an expansion chamber. The steam generator has a volumetric capacity of 150 L and a 25 kW electric heater, allowing it to generate saturated steam between 220 °C and 230 °C. The expansion chamber with a capacity of 120 L is equipped with an automated fast open valve. The reaction vessel is equipped with temperature and pressure sensors. The reaction time and temperature are controlled using a console (control keypad), as shown in Figure 1. For each experiment, the reactor was loaded with 200 g of *E. globulus* woodchips, based on dry wood basis (dwb). Saturated steam at various temperatures (ranging from 173 to 220 °C) was then introduced for different residence times (ranging from 1.7 to 17 min), following the multivariate experimental design outlined in Table 1. Finally, the reactor was depressurized to atmospheric pressure. The slurry was collected and filtered, and the liquid was then stored a temperature of 4 °C for further determine of glucose, xylose and arabinose contents. In order to determine the solids yield (%), the pretreated wood chips were left to dry in air for approximately 24 h. After this period, the wood chips were weighed, and the moisture content was measure. These two data were utilized to calculate amount of dry mass obtained after the pretreatment process. The severity factor (S_0) was calculated using Equation (1):

$$(S_0) = \log(t \times \exp\left[\frac{T_H - T_R}{14.75}\right]) \quad (1)$$

where t is the reaction time in minutes, T_H is the hydrolysis temperature in °C and T_R is a reference temperature of 100 °C. The value of 14.75 represents a constant (ω), which is an empirical parameter related to the activation energy and temperature of the reaction.

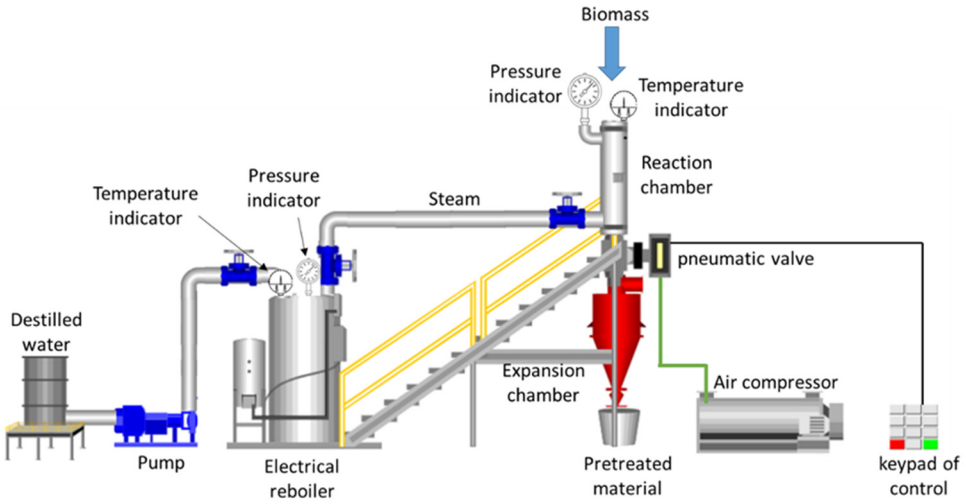


Figure 1. This is a schematic representation of the steam explosion reactor located at the Renewable Resources Laboratory of the University of Concepción.

2.5. Xylose Recovery from Liquid Fraction Post Steam Explosion Pretreatment

The liquid fraction was processed according the method outlined by Castro et al. [28] to measure the amount of xylose present in the liquid fraction. In this method, a sample of containing primarily xylose and xylooligomers is subjected to hydrolysis using 0.6% H_2SO_4 at 121 °C for 60 min in an autoclave. After hydrolysis, the sample is filtered through 0.22 µm porosity membranes. The total xylose is determined using HPLC described above for the quantification of carbohydrates. The proportion of xylooligomers in the liquor is calculated by subtracting the xylose content in hydrolyzed liquor from the xylose content in the non-hydrolyzed liquor.

2.6. Characterization of Cellulose

2.6.1. Cellulose Extraction

The cellulose isolation of pretreated pulps with a wood-like texture at low severity (2.96; 3.53; 3.13 and 3.06) was conducted using the following procedure [29]: 2 g of the sample were treated with an aqueous solution consisting of 80 mL of water, 2.50 g $NaClO_3$ and 2.0 mL of acetic acid at 80 °C for 90 min. The resulting insoluble residue (holocellulose = cellulose + hemicellulose) was cooled, filtered through a glass filter number 2 and washed with distilled water and acetone. The holocellulose was then dried at 40 °C, weighed, and 0.5 g it was treated with 12.5 mL of 0.5% KOH at 20 °C for 5 min. The obtained cellulose was filtered through a glass filter number 4, washed with distilled water, a 1 M solution of acetic acid and water again. Finally, it was dried at 40 °C. The cellulose isolation of high severity pretreated materials with a pulp-like texture (3.93; 4.50; 4.51; 4.06 and 3.80) was performed as follows: 1 g of the sample was treated with an aqueous solution consisting of 40 mL of water, 1.25 g of $NaClO_2$ and 0.5 mL of acetic acid at 80 °C for 25 min. The resulting insoluble residue (holocellulose = cellulose + hemicellulose) was cooled, filtered through a sintered glass filter number 2 and washed with distilled water and acetone. The holocellulose was then dried at 40 °C, weighed, and 0.5 g it was treated with 12.5 mL of 0.5% KOH at 20 °C for 5 min. Subsequently, 12.5 mL of water was added, and the mixture was treated for additional 5 min at 20 °C. The obtained cellulose was filtered through a glass filter number 4, washed with distilled water, a 1 M solution of acetic acid and water again. Finally, it was dried at 40 °C.

2.6.2. Average Degree of Polymerization

The average degree of polymerization (DP) of isolated cellulose samples was calculated from its intrinsic viscosity [η] using the equation: $DP^{0.90} = 1.65\ [\eta]$, as described by Monrroy et al. [29]. Specifically, the viscosity of a 0.5% cellulose solution in 0.5 M cupper ethylenediamine was measured using a capillary viscometer (Cannon Fenske Viscometer, Cannon Instrument Co., State College, PA, USA).

2.6.3. The Cellulose Crystallinity Index by Spectroscopic FT-IR

The cellulose crystallinity index (CrI) was determined using FT-IR. FTIR spectra of isolated cellulose samples were measured by directly transmitting light through a KBr pellet. To prepare the pellet, 1.5 mg of dried sample was mixed with 200 mg of KBr (spectroscopy grade, Merck, Germany) in an agate mortar. The resulting mixture was then pressed at 5000 psi for 2 min. Spectra were recorded between 4000 and 400 cm^{-1}, using a Perkin Elmer System 2000 FT-IR (Perkin Elmer, Inc., Waltham, MA, USA) equipped with a DTGS detector. The background used for FT-IR measurements was a KBr pellet that did not contain any sample. All spectra were measured at a spectral resolution of 8 cm^{-1} and 64 scans were taken per sample. These spectra were normalized to the intensity of the band at 2900 cm^{-1} (C–H stretching vibration), which is a relatively constant band that does not change with the temperature of pretreatment (ranging from 30 to 250 °C). This band was used as a reference to compare the spectra from different samples and determine the cellulose crystallinity. The CrI was evaluated by calculating the ratio of the absorption bands H1372/H2900, which was determined from the averages of four measurements for each sample. The band at 1372 cm^{-1} is related to cellulose crystallinity. The ratio of peak heights at 1372 and 2900 cm^{-1} (H1372/H2900), which represents the ratio of C–H bending to C–H stretching, has been used in various studies to predict cellulose crystallinity [30].

2.7. Surface Morphology by Scanning Electronic Microscopy

Images were captured of the fiber surfaces both before and after pretreatment using a JSM- 6380LV SEM instrument (Jeol, MA, USA) under high vacuum conditions. The SEM is equipped with a secondary electron detector. The samples were dried at room temperature and then coated with conductive gold paint (particle size 500 Å) using an S150 Edwards Sputter Coater (Atlas Copco Group, Stockholm, Sweden). The imaging process was conducted at a beam-accelerating voltage of 20 kV with a tungsten filament serving as the electron source.

2.8. Enzymatic Hydrolysis

Enzymatic hydrolysis (EH) experiments were conducted in 125 mL Erlenmeyer flasks at 50 °C using an orbital shaking incubator (Labtech LSI-4018A, Sorisole, BG, Italy) set at 150 rpm for 72 h. Each experiment was performed in triplicate with a total volume of 50 mL containing 10% solid pretreated material (w/v). A commercial cellulase enzyme complex (NS-22128 CCN3128; 71 FPU mL/mL) supplemented with β-glucosidase (NS-22128 DCN00216; 370 CB mL/mL) and a pH 4.8 buffer of 0.05 M sodium citrate were used. The enzyme dosages employed were 20 FPU and 20 CBU of cellulase and β-glucosidase, respectively, per gram of dry material. The glucose content released during the enzymatic treatment was analyzed by HPLC [8]. Enzymatic digestibility was expressed as enzymatic hydrolysis yield ($\%Y_{EH}$) and was calculated using Equation (2):

$$\%Y_{EH} = \frac{\left(\frac{Gs}{\text{dry substrate loading (g)}}\right) \times \%SR}{Gi} \times 100 \quad (2)$$

where Gs represents the amount of glucose released in grams, the dry substrate loading of pretreated biomass in grams, the percentage of solid recovered after the pretreatment (%SR) and Gi denotes the initial amount of glucose in wood expressed as grams of monomer.

2.9. Description of the Horizontal Fermenter with Helical Blades

The horizontal fermenter (HF) is a stainless-steel container with a semi-cylindrical shape and an internal volume of 500 mL. To regulate the temperature, it was designed with a double jacket. The helical blade, made of 316 stainless-steel are responsible for agitating the pretreated material. The fermenter is equipped with an acrylic cover, allowing for observation of the agitation. To facilitate gas exchange during fermentation, a rubber stopper with a syringe needle is placed on the acrylic cap. Rubber seals are used to ensure a hermetic seal for the fermenter. The stirring speed is externally controlled at 50 rpm using motor.

2.10. Description of the Vertical Fermenter with Type "G" Blades

The vertical fermenter (VF) consisted of a glass container with an internal volume of 420 mL. It was provided by a plastic cover with a rubber stopper to which a syringe needle was connected to allow gaseous exchange during fermentation. The cover and blade assembly were designed using Teflon seals to remain airtight throughout the fermentation stage. The stirring speed was maintained at 50 rpm by using an external motor. The fermenter was immersed in thermostatic water bath to maintain a constant fermentation temperature. The blades are made of 316 stainless steels.

2.11. Simultaneous Saccharification and Fermentation

Thermotolerant *Saccharomyces cereviseae* IR2-9a [31] was used as the fermentative microorganism. *S. cereviseae* IR2-9a are maintained in agar plates with a medium composed of 20 g/L of glucose, 20 g/L of agar, 20 g/L of peptone and 10 g /L of yeast extract; in a grow oven at 40 °C. Prior to the simultaneous saccharification and fermentation (SSF) and fed-batch SSF (FB-SSF) process, an inoculum of yeast was prepared in a growth liquid medium; contained 100 g/L of glucose, 10 g/L of yeast extract, 10 g/L of peptone, 4.14 g/L NH_4Cl, 1.17 g/L of KH_2PO_4 and 0.36 g/L of $MgSO_4 \times 7H_2O$. They were then inoculated with colonies isolated from an agar plate. The mixture was incubated at 40 °C with shaking at 150 rpm for 24 h. Subsequently the cells were harvested by centrifugation at 2500× *g* rpm, washed four times with sterile 0.9% *w*/*v* NaCl and suspended in the same solution. The cell concentration was determined gravimetrically. SSF experiments were performed at two dry-substrate loadings, 10 and 20% *w*/*v*. The enzyme dosages employed were 20 FPU and 20 CBU per gram of pretreated material and initial inoculums of 12 g/L of *S. cereviseae* IR2-9a. Samples were collected after 24, 48 and 72 h, unless otherwise stated. FB-SSF experiments were performed a final dry-substrate loading of 20% *w*/*v*. The initial substrate loading was 12%, and every two hours, the substrate loading was increased by 2% until the final concentration was reached; this process took a period of 8 h. Samples were collected at 24, 48 and 72 h, unless otherwise. The enzyme dosages and initial inoculum of S. cerevisiae IR2-9a were the same as those used in the SSF. All SSF and FB-SSF experiences were performed in 50 mM citrate buffer (pH 4.8) supplemented with 10 g/L of yeast extract, 10 g/L of peptone, 4.14 g/L NH_4Cl, 1.17 g/L of KH_2PO_4 and 0.36 g/L of $MgSO_4 \times 7H_2O$.

The ethanol yield of the reactions was calculated on a dry wood basis ($\%Y_E$) according to Equation (3), considering the ethanol in grams produced, dry substrate loading in grams of pretreated biomass, percentage of solid recovered after the pretreatments (%SR) and potential ethanol from the stoichiometric conversion of potential glucose in the feedstock.

$$\%Y_E = \frac{\left(\frac{\text{ethanol (g)}}{\text{dry substrate loading (g)}}\right) \times \%SR}{\text{potential glucose in wood } \times 0.51} \times 100 \quad (3)$$

2.12. Ethanol Analysis

The ethanol concentration was determined using a Merck Hitachi (Tokyo, Japan) HPLC system equipped with a refractive index detector, a Aminex HPX 87H column (Bio-Rad, Hercules, CA, USA) at 45 °C and H_2SO_4 5.0 mM as mobile phase at a flow rate of 0.6

mL/min was using to determination of ethanol concentration. The ethanol standards were prepared using commercial grade ethanol.

3. Results and Discussion

The chemical composition of *E. globulus* woodchips and pretreated materials by SE is summarized in Table 1. The solid yields ranged from 66 to 93% (dry matter), depending on the severity of pretreatment, expressed as S_0.

3.1. Steam Explosion Pretreatment Optimizaztion by CCC Design

A set of 11 experiments were performed for the different pretreatment conditions, defined by the CCC design. The effects of the pretreatment condition on the pretreated materials and experimental $\%Y_{EH}$ are shown in Table 1. The responses were fitted to a second-order polynomial equation using Modde 7.0 software (Unimetrics), as shown in Equation (4). The model equation was validated through ANOVA with 95% confidence level. The predicted polynomial responses were close to the experimental responses with a correlation coefficient $r^2 = 0.95$.

$$\%Y_{EH} = 74.31 + 25.74\ T + 12.52\ t - 15.82\ T^2 - 13.42\ t^2 - 45.25\ Tt \quad (4)$$

where $\%Y_{EH}$ is the enzymatic hydrolysis yield predicted, (in Wood dry weight basis); T = temperature, °C and t = time, minutes.

The contour diagram describing the estimated response surface for $\%Y_{EH}$ on a wood dry weight basis of *E. globulus* pretreated with SE is shown in Figure 2. Through the response surface and applying least squares regression, the optimal pretreatment values for the temperature and reaction time predicted by mathematical model were 208 °C and 11 min. These parameters were used to validate the optimal condition predicted by polynomial. The chemical composition of the materials pretreated under optimal conditions is shown in Table 1, codified as Exp. N° 10 and represents the average of three experiments. The $\%Y_{EH}$ of the pretreated material under optimal predicted conditions reached a value of 74.5 ± 2.3% glucose on a dry wood basis with a S_0 of 4.22. Similar values were obtained for the materials obtained from experiments 2, 4, 6, 8 and 9, with $\%Y_{EH}$ ranging from 71 to 77% of glucose. Considering the energy involved during pretreatments (S_0), it was evident that the center point experiments ($S_0 = 3.80$) were more competitive than those obtained under the optimal predicted condition. To verify this, a statistical test (Student's *t*-test) allowed asseveration that there is no significant difference between $\%Y_{EH}$ reached by the material obtained at optimal conditions (208 °C for 11 min) compared to those obtained from the center point conditions (196 °C for 9.5 min). For the statistical test, a population of nine replicates per material was considered, resulting in t-calculated = 0.79, which was less than the t-critical (1.74); therefore, there was no significant difference at $p = 0.05$.

3.2. Analysis of Chemical Composition

The chemical composition of *E. globulus* used in this research is detailed in Table 1. The main component found were glucans with 45.5% followed by hemicellulose with 15.3% and lignin with 23.5%. In relation to the pretreated materials, it was observed that the chemical composition of each biomass samples pretreated at low severity, showed no greater removal of wood components towards the liquid fraction during the pretreat-ments. As S_0 increased above 3.8, the increase in dissolved materials became evident, and mainly hemicellulose and lignin were removed. At higher severity values, the degradation of xylan to xylose, which degrades products such a furfural and carboxylic acids, is favored. The curves of xylose and xylooligomer content in the liquid phase versus severity are shown in Figure 3a. It was possible to observe the depolymerization of hemicellulose to xylose up to a severity factor of 4.3. Above this value, xylose concentrations begin to decrease, probably due to its conversion into other compounds such as those mentioned above.

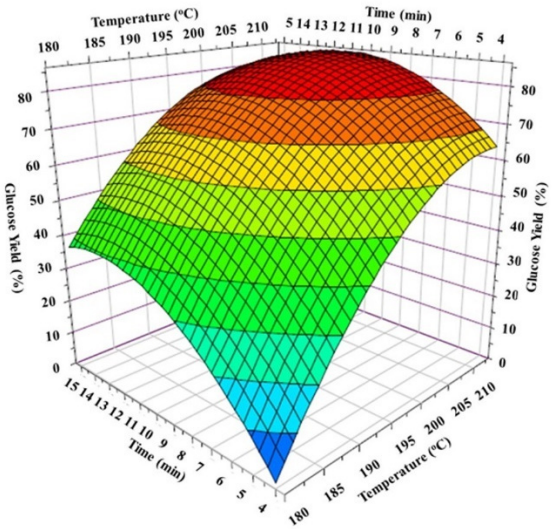
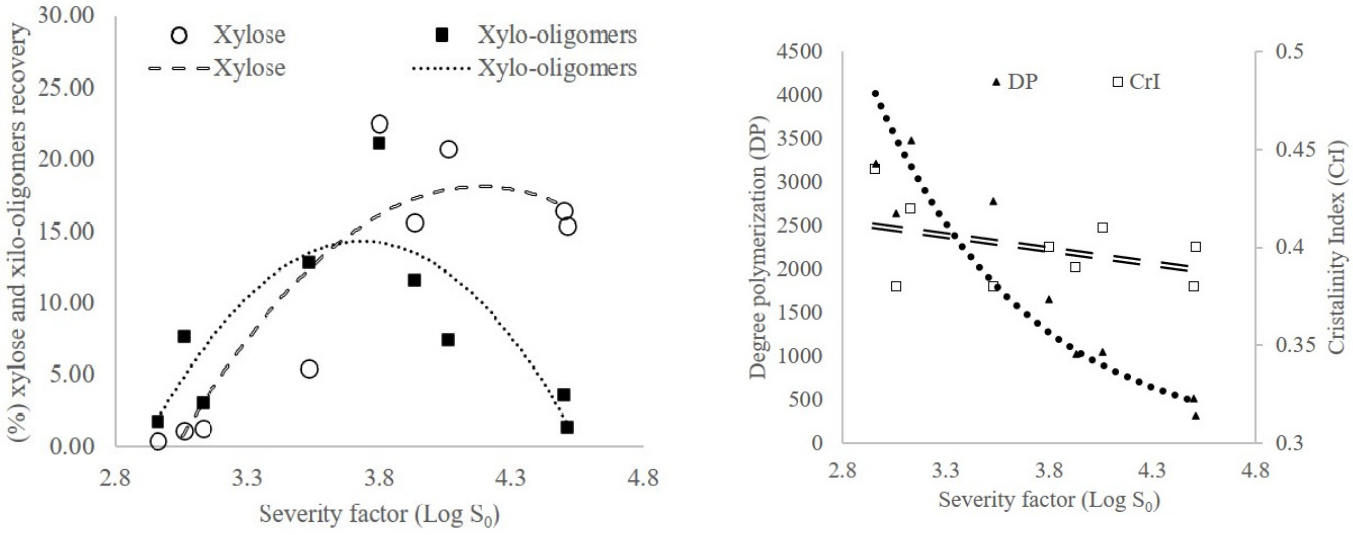


Figure 2. Estimated second-order polynomial response surface to enzymatic hydrolysis yield (%Y_{EH}) expressed in glucose released, in dry wood basis of the *Eucalyptus globulus* pretreated by steam explosion. Blue: lower glucose yields. Red: higher glucose yields.

In contrast, the amount of residual xylans obtained in the materials subjected to pretreatment between S_0 2.96 and 4.51 ranged from 18.9% to 1.5%, approximately 100% and 1.0%, respectively, of the original amount in the wood. The solubility of xylans depends on their molecular weight and the presence of side-chain substituents. Chen et al. [32] mention that acetyl groups, arabinose and uronic acid increase the solubility of xylans. Other authors have described SE pretreatment as an effective method for reducing LCB recalcitrance by removing hemicellulose, disrupting the lignin-hemicellulose matrix, and redistributing lignin in the cell wall layers [8,33] and thus allowing the preservation of glucans under controlled conditions. The increases in S_0 in the pretreatments, also a glucan preservation varied between 100% and 82.6%, while the lignin content in the same pretreated materials varied between 21.5% and 28.1% (Table 1), slightly higher than the lignin content in the raw material. As mentioned above, the reason for this increase could be the generation of pseudo-lignin during the SE pretreatment. Araya et al. [34] indicated that the increase in the lignin content of autohydrolysis pretreated materials at higher severity conditions is partly due to the concomitant loss of polysaccharides and the formation of condensed lignin products. This increase can also be attributed to the formation of lignin-like compounds from lignin and carbohydrate degradation.

3.3. Structural Changes in Cellulose in Pretreated Materials

The DP and crystallinity of cellulose are considered important factors in the recalcitrance of LCB [35]. The DP of cellulose in the raw material and pretreated materials was determined by the intrinsic viscosity method, while its crystallinity index (CrI) was determined by the ratio of bands of the infrared spectrum. *E. globulus* wood showed an initial DP of 3493, decreasing rapidly as the severity of the pretreatment increased, until reaching a value of 317 with the maximum severity (Figure 3b). The FTIR crystallinity index was calculated using the absorbance ratio of the 1372 and 2900 cm^{-1} bands. The CrI is between 0.45 and 0.38, observing a slight decrease as the severity increases (Figure 3b). Therefore, it is possible to attribute a good performance in enzymatic hydrolysis to materials that present a high removal of hemicellulose (xylan in *E. globulus*) and a lower DP of cellulose; however, it cannot be established that CrI plays an important role in the performance value of EH.



(**a**) (**b**)

Figure 3. (**a**) Correlation between the recovery of xylose and xylo-oligomers in the liquid fraction with the severity of pretreatment and (**b**) relationship between the severity factor of the process with the degree polymerization and the crystallinity index of the cellulose isolated from pretreated material by steam explosion.

3.4. Scanning Electron Microscopy

Figure 4 shows images and scanning electron microscopy images for the most representative materials of the present study, that is, the pretreated material with the least severity (Exp. 1: 2.96), with the highest severity (Exp. 6: 4.51), central point of the design (Exp 9: 3.80) and material optimized by response surface (Exp. 10: 4.22). The first row shows a photograph of the materials pretreated by steam explosion, the second row shows scanning electron microscopy (SEM) images at a magnification of 130× to obtain a general idea of the fiber arrangement, and the third row shows SEM images at 1400× magnification to further characterize the surface of the fibers. A low severity does not manage to deconstruct the fibrillar structure of the wood, probably due to the high content of hemicellulose still present in the pretreated material. By applying a high severity of pretreatment, it is possible to completely deconstruct the structure of the wood, and even shorter fibers are observed, which is probably related to the drastic decrease in the DP of cellulose. At intermediate severities (Exp. 9 and Exp. 10), a mixture is observed between structures that have not yet been deconstructed and detached fibers, although they are longer than those present in Exp. 6. This can be explained for the sudden depressurization led to an "explosion" of the steam inside the lignocellulosic matrix, which promotes breakdown and defibrillation of its structure generating a solid fraction which a more open structure. The good-results were observed in the enzymatic hydrolysis of Exp. 9 and Exp. 12 indicate that no further deconstruction is necessary to achieve good glucose release if a significant amount of xylan is removed.

3.5. Simultaneous Sacharification and Fermentation

In Section 3.1 it was mentioned that the pretreated material obtained under the conditions of the central point of the experimental design (Exp. 9) presented a similar performance to the material obtained under the optimization conditions (Exp. 10), but with a lower energy expenditure from the pretreatment point of view (less severity). Severity it was used in subsequent studies. The fermentability of the Exp. 9 pretreated material were studied by SSF at two different substrate loads, 10% and 20% w/v. With a load of 20% solids, the challenge of achieving good agitation that allows the diffusion of the enzymes, liberating the glucose from the cellulose and then its fermentation to ethanol was verified. Agitation is an important

parameter for overcome mass transfer related limitations. To overcome the agitation barrier, a fed-batch strategy was implemented (Figure 5), which allows the feeding of pretreated material to the SSF reactor as it is liquefied by the action of enzymes. This strategy was successfully implemented in three types of laboratory-scale fermenters.

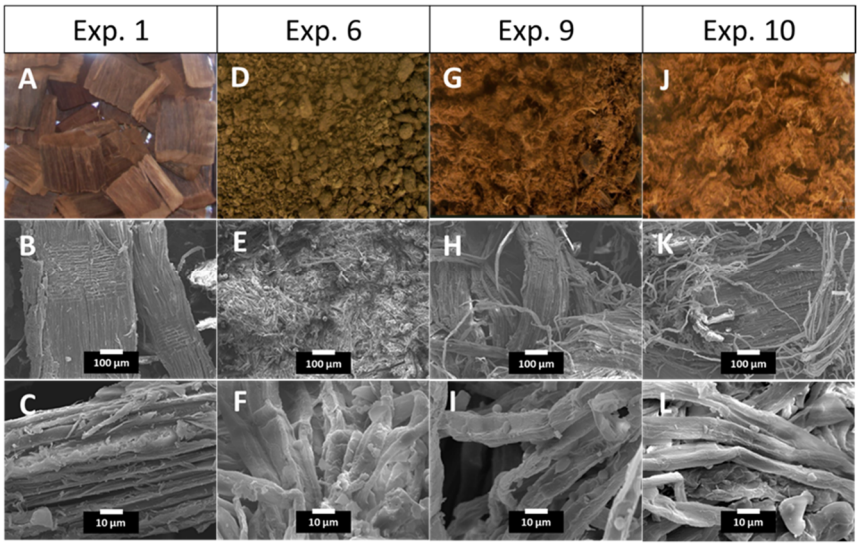


Figure 4. Macroscopic and microscopic images of pretreated materials selected according to severity. (**A**,**D**,**G**,**J**) correspond to the original texture of samples Exp. 1, Exp. 6, Exp. 9 and Exp. 10, respectively. (**B**,**E**,**H**,**K**) correspond to images by scanning electron microscopy with 130× magnification of samples Exp. 1, Exp. 6, Exp. 9 and Exp. 10, respectively. (**C**,**F**,**I**,**L**) correspond to images by scanning electron microscopy with 1400× magnification of samples Exp. 1, Exp. 6, Exp. 9 and Exp. 10, respectively.

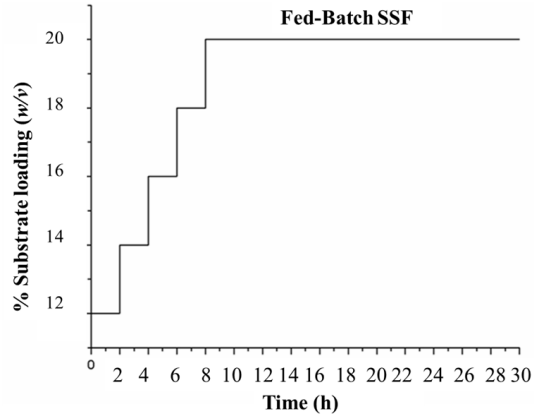


Figure 5. Fed-batch SSF strategy with 20 FPU/20 CBU and 12 g/L of *S. cereviseae* IR2-9a. Every two hours, the substrate loading was increased in 2% until to reach the final concentration, this process took a period of 8 h.

The first laboratory-scale fermenter was a traditional orbitally stirred Erlenmeyer flask. The second and third SSF fermenters are scalable prototypes of the original design with blade systems for high solid loads. The second fermenter corresponds to a horizontal design with helical-type blades, where its design can be seen in Figure 6A and the third fermenter is of a vertical design with "G-type" blades shown in Figure 6B.

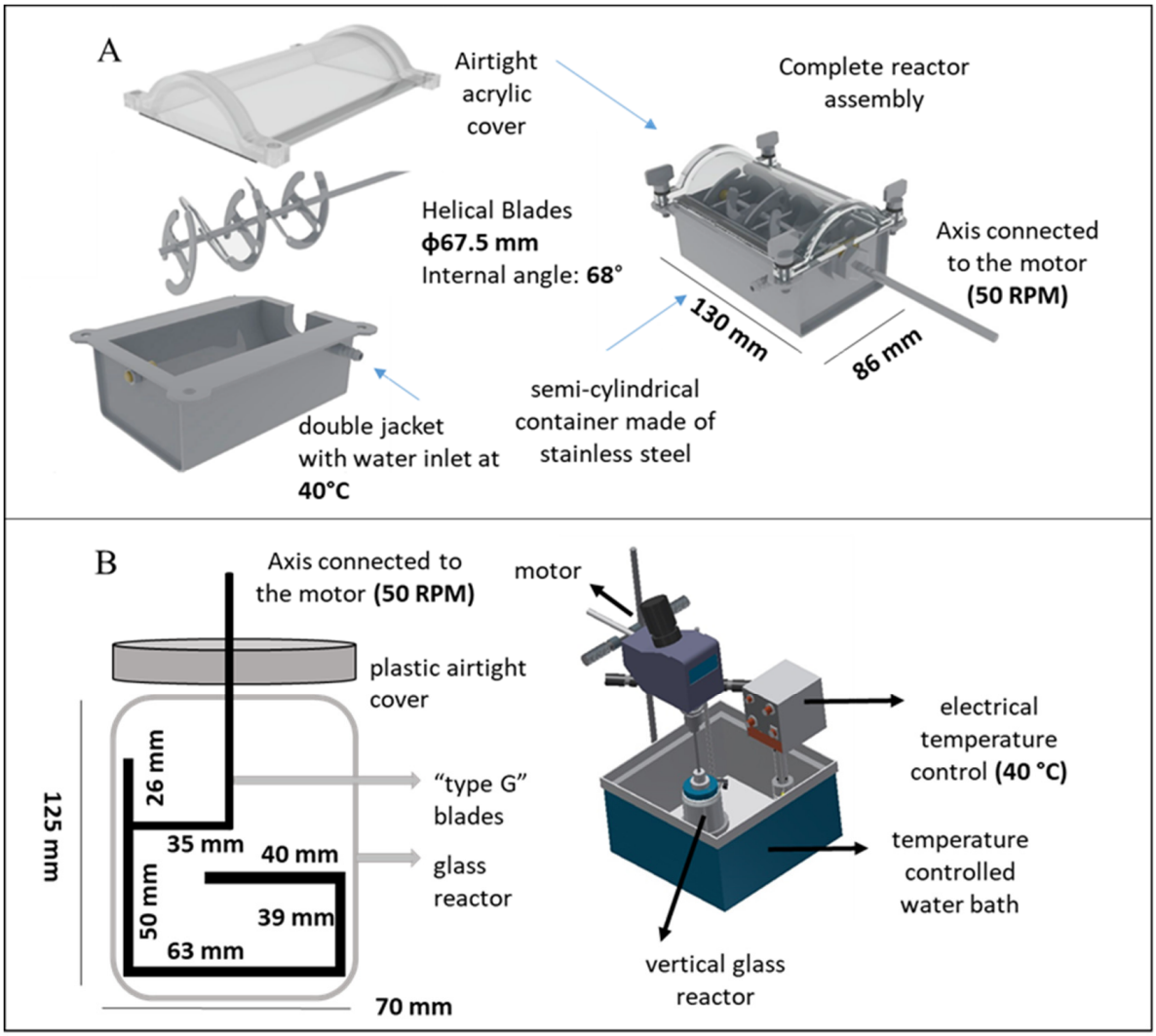


Figure 6. (**A**) Schematic representation of the horizontal fermenter (HF) with helical blades and (**B**) schematic representation of the vertical fermenter (VF) with "type G" blades.

In a traditional fermenter (Erlenmeyer flask), when the solid pretreated biomass was increased from 10% w/v to 20% w/v, the yield decreased from 61.9% to 55.0%. At high substrate loading, a slower rate of mass transfer of enzyme as well also soluble products. Diffusional limitation of enzymes increases with elevated substrate concentration and the same is responsible for slower mass transfer of enzymes [36]. However, when the fed-batch strategy was implemented, the glucose yield increased to 70.6%. SSF increases the yields because the glucose released by the enzymes is rapidly consumed by the yeast, reducing the inhibition per product. Table 2 shows a significant increase in the yield of FB-SSF with 20% w/v of pretreated material, which reached 78.0% at 72 h. However, the profitability and productivity of future bioethanol production plants remain very low. Very promising results were achieved with fermenter two and three, where we achieved a yield of 87.7% equivalent to 8.3% v/v of ethanol with the horizontal fermenter, and 92.5% equivalent to 9.1% v/v of ethanol with the vertical fermenter, both yields reached at 48 h of FB-SSF. Cost and energy studies are required to determine whether a vertical or horizontal fermenter is more viable for scaling up the bioethanol production.

Table 2. Glucose and ethanol yields (wood dry weight basis) obtained by EH, SSF and FB-SSF. Comparison between different fermenter designs.

Solids	SSF Erlenmeyer Flasks (Orbital Agitation)			SSF in Horizontal Fermenter (Helical Blades)			SSF in Vertical Fermenter (Type "G" Blades)		
	Ethanol Yields	Ethanol		Ethanol Yields	Ethanol		Ethanol Yields	Ethanol	
%	%	g/L	%*v*/*v*	%	g/L	%*v*/*v*	%	g/L	%*v*/*v*
10	63.8 ± 0.7 (72) [a]	24.7 ± 0.3	3.2 ± 0.0						
20	61.0 ± 0.1 (96)	44.6 ± 0.1	5.7± 0.0	78.6 ± 1.2 (72)	60.0 ± 1.0	7.7 ± 0.8	77.7 ± 2.3 (72)	57.0 ± 2.1	7.3 ± 2.0
20 [b]	78.0 ± 0.6 (72)	58.0 ± 0.4	7.4 ± 0.1	87.7 ± 0.9 (48)	65.0 ± 0.8	8.3 ± 0.6	92.5 ± 1.9 (48)	71.0 ± 1.5	9.1 ± 1.4

[a] Time in hours of the process at which the yields were reached. [b] Fed-batch strategy in first 8 h.

4. Conclusions

SE pretreatment of *E. globulus* was optimized using RSM. The response polynomial allowed us to obtain the optimal parameter combination of 208 °C and 11 min to maximize glucose release by EH. The study of samples generated during optimization allowed us to determine the physicochemical changes in a wide range of severities, where the removal of xylans and decrease in the DP of cellulose were the main effects caused by SE pretreatment. These effects correlated with the SEM images and Y_{EH}. Finally, a high Y_E was achieved, which is necessary to lower distillation costs through novel SSF fermenter designs and fed-batch strategies.

The combination of fermenters with blades designed for a high concentration of pretreated solids and their incorporation in the fed-batch mode allowed us to achieve a high ethanol concentration without sacrificing the potential yield. Furthermore, this strategy enabled us to achieve the highest ethanol concentration in a shorter time for SSF compared with the conventional orbital agitation method.

Author Contributions: E.T.-O., conceptualization, methodology, software, formal analysis, investigation, and writing—original draft. R.V., conceptualization, software, and formal analysis. P.R.-C., methodology, formal analysis, and writing—original draft. P.C.-R., methodology, investigation, and writing—original draft. L.-N.S., investigation, writing—review and editing, and funding acquisition. C.P., conceptualization, methodology, software, investigation, resources, writing—original draft, supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Innova Chile CORFO grant 208-7302 and the Innovation Found for Competitiveness of the Chilean Economic Development Agency (CORFO) under Grant no. 13CEI2-21839, FONDECYT-ANID 1231086 and ANID BASAL FB210015 CENAMAD.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Acknowledgments: The authors are grateful to ANID—Millennium Science Initiative Program—NCN17_040. Eduardo Troncoso-Ortega thanks the ANID/2020-21202445 National Doctoral Scholarship.

Conflicts of Interest: Author Roberto Valenzuela is employed by the company Innocon S.A. But for purposes of this investigation, there was no financing relationship with the company; therefore, there are no conflicts of interest. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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