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Digestate-Based Liquid Growth Medium for Production of Microbial Chitosan

Silvia Crognale [1], Cristina Russo [1], Eleonora Carota [1], Ilaria Armentano [2], Federico Di Gregorio [1], Alessandro D'Annibale [1,*], Alessio Cimini [1] and Maurizio Petruccioli [1]

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Abstract

This study investigated the feasibility of using both the solid and the liquid fractions of the digestate, a waste from the anaerobic digestion, as a possible liquid growth medium for fungal production of chitosan. An enriched liquid phase (ELP) derived from mild acid hydrolysis treatment combining both fractions was screened for its ability to support biomass and chitosan production by 17 fungal strains. The best results were obtained with *Absidia blakesleeana* NRRL 2696 and *Rhizopus oryzae* NRRL 1510 cultures, which yielded chitosan volumetric productions of 444 and 324 mg L^{-1}, respectively. The chitosan preparations of the former and the latter strain, characterized by infrared spectroscopy, elemental analysis, viscosimetry and thermogravimetric analysis, showed deacetylation degrees of 79% and 84.2%, respectively, and average viscosimetric molecular weights of around 20 and 5.4 kDA, respectively. Moreover, both fungal chitosan samples exerted significant antibacterial activity towards Gram-negative (*i.e., Pseudomonas syringae* and *Escherichia coli)* and Gram-positive *(i.e., Bacillus subtilis)* species.

Keywords: chitosan; production; Zygomycetes; antibacterial activity; deacetylation; waste upgrading; circular economy

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1. Introduction

Chitin is a major constituent of crustacean shells and an abundant component of the exoskeletons of insects and mollusks. For several decades, the production of chitin and its deacetylated counterpart, chitosan, relied on the extraction of crab and shrimp, and shell wastes, where chitin amounts ranged from 14 to 27% and 13 to 15%, respectively. However, the extraction process from these sources involved harsh chemical treatments because of the presence of proteins, lipids, and inorganic salts in the crustacean shell wastes [1].

However, chitin and chitosan are also found in significant amounts in the cell walls of various fungal classes [2]. This discovery has led to extensive research on producing these polymers through fungal bioprocesses. Several technical factors make the fungal production route an attractive alternative to conventional extraction from shellfish residues [2,3]. Firstly, the demineralization step is unnecessary during processing, as the crude chitin from fungal sources has significantly lower ash levels than crustacean shells [4]. In addition to providing a year-round source of chitin and chitosan, fungal bioprocesses can be accurately controlled, potentially ensuring consistent product characteristics [2,3,5]. Moreover, unlike that obtained from marine organisms, chitosan of fungal origin does not have associated protein residues that can trigger allergic reactions in allergic individuals with crustaceans [2,5].

Hence, recent research is now concentrating on fungal biotechnology, relying on strains capable of accumulating chitosan directly in their cell walls. This approach is due to the superior quality of chitin and chitosan found in the fungal cell wall and the lower environmental impact of extracting them from fungal sources compared to crustacean waste [4,6]. The strains that meet this requirement belong to the Zygomycota division and have a high percentage of chitin and chitosan in their cell walls. For this reason, they are very promising sources for producing chitosan [3,6,7].

The economics of chitosan production using inexpensive raw materials can make industrial microbial processes competitive with chemical ones. For this reason, studies on using alternative, low-cost carbon sources to make the fungal chitosan production process highly sustainable are particularly relevant in this area. Several studies have reported the successful production of chitosan in liquid cultures using various waste materials. Examples include apple pomace [8], corn steep liquor [9], cassava wastewater [10] and paper-mill wastewater [11].

The digestate, the waste derived from anaerobic conversion processes, represents a promising source of nutrients to derive a fungal growth medium [12]. The composition and quality of this by-product depend strongly on the raw materials used and the treatment conditions applied [13]. The digestate derived from the anaerobic conversion of wastes from the agricultural or agro-industrial sector can represent an excellent source of carbohydrates due to the significant content of residual fibers [12].

The present study aimed to derive a digestate-based liquid growth medium and to assess its adequacy for microbial chitosan production. To this end, a digestate from an anaerobic process, fed with a mixture of olive pomace and silage as the input flow, underwent various chemical pretreatments to yield a sugar-enriched liquid phase. The medium thus obtained was tested for its ability to support both growth and chitosan production of 17 fungal strains, most of which belong to well-known chitosan-producing species. To the best of our knowledge, this is the first study claiming the use of a digestate-based liquid medium for fungal chitosan production.

2. Materials and Methods

2.1. Materials

The digestate was taken from a biogas plant located in the province of Perugia and derived from an anaerobic process fed 1/3 by silage (of which 50% corn, 25% barley, 25% sorghum) and 2/3 by olive pomace. At the end of the chain, the waste was also subjected to a liquid / solid separation leading to a solid fraction, from here onwards referred to as digestate solid fraction (DSF) and a liquid one, referred to as digestate liquid fraction (DLF). Both fractions underwent a preliminary characterization to determine the possibility of using DLF as the extractant for DSF, thus obtaining an extract called enriched liquid phase (ELP). Before use, the DSF was dried at 50 °C in an oven up to constant weight and underwent grinding on a Cyclotec 1093 mill (PBI Instruments) endowed with a 1-mm sieve to make its granulometry uniform. DLF, instead, was centrifuged (6000×*g*, 15 min), filtered under vacuum through Whatman paper n. 1 (porosity, 11 μm), and, finally, stored at -20 °C until use.

Chemical analyses of DSF were performed as described elsewhere [14] and the most relevant parameters, expressed in g kg^{-1}, were: total sugars, 650 ± 30; reducing sugars, 9.2 ± 0.4; crude lipids, 19.3 ± 2.7; phenols, 15.3 ± 2.4; hemicellulose, 172 ± 15; cellulose, 393 ± 24; lignin, 257 ± 14.; ash, 82 ± 4; total nitrogen, 19.8 ± 1.1; total carbon, 415 ± 8; hydrogen, 59.4 ± 2.1; sulphur, 2.2 ± 0.1. The DLF contained the following parameters (g L^{-1}): total sugars, 0.72 ± 0.02; reducing sugars, 0.36 ± 0.01; protein, 0.82 ± 0.22; N-NH$_4$, 1.09 ± 0.01; total amino acids, 0.57 ± 0.024; phenols, 0.76 ± 0.03; potassium, 1.2 and assimilable phosphorus, 0.35. Low molecular weight commercial chitosan from Fluka BioChemika was purchased from Sigma-Aldrich-Fluka (Milan, Italy).

2.2. Medium Development from Digestate's Fractions

As a first approach to developing a culture medium from the DSF and DLF components, we conducted a series of experiments, designed to verify the possibility of using solid-liquid extraction alone or assisted by hydrolysis under mild alkaline or acid conditions. DLF was used as the liquid extractant of the DSF. The experiments were conducted by varying the solid-liquid ratios (1:10, 1:15 and 1:20, w/v) and temperatures (60, 75, 90 and 120 °C) at a fixed extraction time (60 min). In the case of acid- or alkali-assisted extractions, sulfuric acid or sodium hydroxide was added to the DLF extractant to yield a concentration equal to 6% (w/v), and incubations were performed at 120 °C. The outcome of the experiments was determined by the concentrations of total sugars, reducing sugars, pentoses, ammonium nitrogen, protein and free amino acids in the enriched liquid phase (ELP) arising from the sole solid-liquid extraction or acid- or alkali-assisted extraction. Our thorough data analysis included the volumetric concentration of the component x in the ELP, and the recovery yield percentage (RY, %) for each component x_i, calculated by Equation (1):

$$RY(\%) = \left[\frac{C_{x(ELP)} - C_{x(DLF)}}{C_{x(TE)}}\right] \times 100 \quad (1)$$

Where $C_{x(DLF)}$ is the initial concentration of component x in the digestate liquid extractant, $C_{x(ELP)}$ is concentration of component x in the ELP after extraction and $C_{x(TE)}$ is the theoretical concentration of component x in the ELP assuming quantitative extraction at that given solid-liquid ratio.

In the second phase, experiments based on the statistical approach of the response surface methodology were designed to derive an ELP from DSF. Due to the inefficacy of solid/liquid extraction and alkali-assisted extraction approaches, attention was focused on acid-assisted extraction. In particular, the experiment was a full factorial design that recruited two variables at three levels, namely H_2SO_4 concentration (2, 6 and 10%, w/v) and extraction time (30, 60 and 120 min). Consequently, the number of runs was 9, each replicated twice, plus three center points. The actual values of the variables were coded as dimensionless terms using Equation (2):

$$X_i = \frac{(A_i - A_o)}{\Delta A} \quad (2)$$

where X_i is the coded value and A_i the actual value of the variable, A_o the actual value of the same variable at the center point, ΔA the mid-range value of the variable. Data were subjected to the analysis of variance (ANOVA) and fitted according to a second order polynomial model shown by Equation (3):

$$Y = \beta_0 + \sum(\beta_i \cdot X_i) + \sum(\beta_{ii} \cdot X_i^2) + \sum(\beta_{ij} \cdot X_i \cdot X_j) \quad (3)$$

where Y is the predicted response variable, β_o is the intercept, β_i and β_{ii} linear and quadratic coefficients, respectively, β_{ij} is the interaction coefficient and X_i and X_j are the coded forms of the input variables. Statistical examination of results and generation of response surfaces were carried out by the statistical software package Modde 9.1 (Umetrics AB, Umeå, Sweden).

2.3. Microorganisms

Except for an environmental strain (DIBAF-E) isolated from an Arsenic-contaminated soil and identified as *Mucor moelleri* (Accession number KY678773), all the fungal strains used in the present study were obtained from the Culture Collection of the North Regional Research Laboratory (NRRL, Peoria, Illinois, USA). The strains used, listed in alphabetical order, were: *Absidia blakesleeana* NRRL 1304 and NRRL 2696; *Absidia coerulea* NRRL 1312, NRRL 1315 and NRRL A-9483; *Absidia corymbifera* NRRL 2798; *Absidia glauca* NRRL 1324, NRLL 2799 and NRRL 3010; *Absidia repens* NRRL 1337; *Aspergillus tubingensis* NRRL 4700; *Benjaminiella poitrasii* NRRL 2845; *Cunninghamella elegans* NRRL 1392; *Mucor rouxii* NRRL 1894; *Rhizopus oryzae* NRRL 1510 and NRRL 2625. Stock cultures were maintained on Potato Dextrose Agar (PDA) (Difco) at 4 °C and sub-cultured every month.

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2.4. Shaken Culture Experiments

Ten-day-old malt extract agar (Oxoid, Basingstoke, UK) cultures on Petri dishes were suspended in sterile deionized water (5.0 mL) and used as the inoculum for pre-cultures to obtain an initial spores' concentration of 1 × 10^6 mL^{-1}. Incubations were carried out at 28 °C for 72 h under orbital shaking at 160 rev min^{-1} in 250-mL Erlenmeyer flasks containing 50 mL of Malt Extract Broth (Difco, Detroit, USA).

2.4.1. Strains Screening for Biomass Producing Capability on ELP Medium

Pre-cultures (5.0 mL) of all the fungal strains were used to inoculate 250-mL Erlenmeyer flasks containing 50 mL of ELP medium which were incubated at 28 °C for 96 h on a rotary shaker (160 rev min^{-1}). Culture samples were withdrawn at the end of the incubation (96 h). Fungal biomass concentration was determined gravimetrically: culture samples underwent filtration through pre-weighed Whatman GF/C discs (diameter, 47 mm), the harvested biomasses were washed twice with distilled water, lyophilized and then weighed. All cultures were performed in duplicate.

2.4.2. Growth and Chitosan Production Kinetics on ELP Medium

Pre-cultures (10.0 mL) of only those fungal strains that had exhibited good growth capacity on the ELP medium were used to inoculate 500-mL Erlenmeyer flasks containing 100 mL of ELP medium, which were incubated at 28 °C for 96 h on a rotary shaker (160 rev min^{-1}). Sacrificial flasks were withdrawn every 32 h. Fungal biomass concentration was determined gravimetrically before chemical analyses and chitosan extraction. The filtrate was stored at -20 °C until use. All cultures were performed in triplicate.

2.5. Analytical Methods

2.5.1. ELP Medium Characterization

The enriched liquid phase (ELP) was characterized by determining several parameters. Total sugars were determined by the method of Dubois et al. [15], while reducing sugars by the dinitrosalicylic acid method [16]. Pentose sugars were determined by the *p*-bromoaniline-thiourea method [17]. Ammonium nitrogen, total amino acids, and soluble protein were determined by the methods of Anderson and Ingram [18], Bradford [19] and Sun et al. [20], respectively. Total phenols were determined by the procedure of Ainsworth and Gillespie [21].

2.5.2. Chitosan Extraction

To extract chitosan, a slight modification of the procedure described by Rane and Hoover [22] was used. Finely ground dry mycelial biomass (200-500 mg) was transferred to centrifuge tubes, added with $NaBH_4$ (5.0 mg) and NaOH 1.0 M (4.0 mL) and autoclaved (30 min at 121 °C). After cooling, the cell suspension was centrifuged (12000×g, 30 min) and the pellet washed sequentially with deionized water, absolute ethanol, and deionized water again. Each washing was followed by centrifugation under the same conditions and subsequent pellet recovery. After the last wash, the pellet was lyophilized, thus yielding the alkali-insoluble material (AIM) and finally, weighed. The AIM was extracted with 4.0% acetic acid solution in water at 95 °C for 4 h and then centrifuged at 12000×g for 15 min. The pellet thus obtained was extracted and centrifuged again as above. The two supernatants were pooled, and the solubilized chitosan precipitated by adding NaOH 2.0 M to reach pH 10.0. Chitosan was then recovered by centrifugation (12000×g, 15 min) and the pellet subsequently washed with absolute ethanol and then with deionized water. Each washing step was followed by centrifugation as above and subsequent pellet recovery. At the end of the washing procedure, the pellet was lyophilized, and the weight was determined.

2.5.3. Determination of Minimum Inhibitory Concentration of Fungal Chitosan

The Minimum Inhibitory Concentration (MIC) of fungal chitosan was determined using the microdilution method (CLSI 2012) in 96-well polystyrene microplates (Greiner Bio-One™ CELLSTAR).

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Chitosan obtained from the previous tests was dissolved in a 1% acetic acid aqueous solution to obtain a concentration of 1 mg mL^{-1}, adjusting the pH to about 5.5 with NaOH, and subsequently subjecting the solution to microfiltration (diameter, 0.45 µm). Low molecular weight chitosan (Fluka BioChemika, Milan, Italy) solution was also analyzed at the same concentration as the samples. The solutions were diluted in Mueller Hinton Broth (MHB, Oxoid) and 100 µL of each dilution was dispensed into each well. The inoculum was adjusted to obtain a density of 5×10^5 CFU mL^{-1} in each well. The plates were incubated at 28 °C for 24 h. The absorbance was measured at 595 nm using a microplate reader (DTX 880 Multimode detector, Beckman Coulter, Indiana, USA). The MIC was also determined visually by adding 10 µL of an aqueous solution of TTC (2,3,5-triphenyl tetrazolium chloride) (2 g L^{-1}) to each well and incubating for 30 minutes. The lowest concentration at which no bacterial growth is observed was taken as the MIC value. The test bacteria used are *Pseudomonas syringae* DSM 21482 (Gram-negative), *Escherichia coli* ATCC 9637 (Gram-negative) and *Bacillus subtilis* DSM 10 (Gram-positive).

2.5.4. Fourier Transform Infrared (FT-IR) Spectroscopy, Elemental Analysis and Thermogravimetric Analysis (TGA) of Chitosan Samples

FT-IR spectra of the chitosan powder were recorded in transmission mode, using a Jasco FT-IR 615 spectrometer (Tokyo, Japan). The samples were measured using a KBr-pellet method in the 400 – 4.000 cm^{-1} wavenumber range, with a resolution of 4 cm^{-1}. The spectra thus obtained were compared with commercial chitosan (Fluka). FT-IR was performed to evaluate the functional groups of samples and the degree of deacetylation (DD%). For this purpose, among different calculation options based on various absorbance ratios between different spectral bands, the A_{1320}/A_{1420} ratio was used which, in a study conducted by Brugnerotto et al. [23], showed to provide the smallest deviation between expected and estimated values. In particular, the first band (1320 cm^{-1}) is characteristic of an acetylated amine or an amide function while the second band (1420 cm^{-1}), relating to the C-H bending signal, is chosen as the reference band. DD% was calculated using the Equation (4):

$$DD(\%) = 100 - \left[\left(\frac{A_{1320}}{A_{1420}} - 0.3822\right) \cdot \frac{1}{0.031}\right] \tag{4}$$

The Vario MacroCube elemental analyzer for solids (Elementar GmbH, Langelselbold, Germany) enabled the determination of the percentage contents in carbon, hydrogen, nitrogen and sulfur in the purified chitosan samples, and the calibration of the measurements relied on sulfonamide, used as the standard. The degree of deacetylation (DD%), based on C/N ratios, was calculated according to the Equation (5) [24]:

$$DD(\%) = 100 \cdot \left[4 - \left(0.5831 \cdot \frac{C}{N}\right)\right] \tag{5}$$

Thermogravimetric analysis (TGA) of the samples was performed by using a Seiko Exstar 6300 system (TA Instruments, Tokyo, Japan). The sample powders (*ca.* 10 mg) were placed in an open platinum pan and heated from 30 to 500 °C at the rate of 10 °C min^{-1} under dynamic nitrogen atmosphere (250 mL min^{-1}). The mass loss (TG) and the derivative mass loss (DTG) curves were calculated.

2.5.5. Intrinsic Viscosity and Average Molecular Mass Determination

The viscosity of chitosan was measured using Lovis 2000 M/ME microviscometer (Anton Paar) with a glass capillary with a diameter of 1.59 mm, a steel ball with a diameter of 1.50 mm, at 25 °C and an inclination of 30°. Stock chitosan solutions (1.0 mg mL^{-1}) were prepared in 1.0% aqueous acetic acid solution and filtered through a 0.45 µm membrane. The intrinsic viscosity [η] of each fungal chitosan was calculated graphically by extrapolating to zero the curve of reduced viscosity versus polymer concentration. The molar mass was calculated using the Mark- Houwink-Sakurada equation:

$$[\eta] = K \cdot (M_v)^a \tag{6}$$

where $[\eta]$ is the intrinsic viscosity, *K* and *a* are constants describing the polymer-solvent combination at the given temperature and M_v the viscosimetric average molecular weight. The values of the former and the latter constant for chitosan in 1% acetic acid solution at 25 °C are 0.0474 mL g^{-1} and 0.723, respectively [25].

3. Results and Discussion

3.1. Medium Development from Digestate's Fractions

The objective of the first set of experiments was to evaluate the effectiveness of a simple solid-liquid extraction compared to an acid- or alkali-assisted extraction, under mild conditions, for obtaining a carbohydrate-enriched liquid fraction. Since DLF contains significant levels of potassium and phosphorus but low concentrations of organic components, we used it as the liquid extractant for the DSF. Table 1 summarizes the results comparing the concentrations and recovery yields of total and reducing sugars in the ELP. Aqueous extraction was ineffective for total sugars: even though higher temperatures increased recovery yields, the values remained below 5.5%, resulting in low TS concentrations in the ELP. The recovery yields of aqueous extractions towards reducing sugars also increased with temperature, leading to values that generally exceeded 100% presumably due to the contribution of reducing ends of extracted oligosaccharides. However, because DSF contains intrinsically low levels of reducing sugars, their concentrations in the ELP remained unsatisfactory. Even mild alkali extraction gave rise to limited recovery yields of TS and reducing sugars; in fact, although alkaline extraction at 120 °C for 60 min significantly increased TS recovery yield as compared to the best aqueous extraction condition (8.2 *vs.* 4.5%), the volumetric concentrations of total sugars and reducing sugars (6.0 ± 0.3 and 2.6 ± 0.1 g L^{-1}, respectively) were deemed inadequate to support substantial fungal growth. Conversely, acid-assisted extraction at 120 °C yielded a TS recovery of 36.1%, resulting in a volumetric concentration of 24.1 ± 1.3 g L^{-1}. As for reducing sugars, their recovery yield under the same conditions was around 24 times higher than theoretical value, presumably due to acid-catalyzed hydrolysis of structural polysaccharides.

Table 1. Effect of different treatments of digestate on volumetric concentrations (VC) and percent recovery yields (RY) of total sugars and reducing sugars.

Treatment [a]	T [b] (°C)	SLR [c]	Total Sugars		Reducing Sugars	
			VC (g L^{-1}) [d]	RY (%)	VC (g L^{-1}) [d]	RY (%)
Aqueous extraction	**60**	1:20	1.2±0.1	1.4±0.4	0.9 ± 0.1	115.2±10.9
Aqueous extraction	90	1:20	1.3±0.1	1.9±0.1	1.2± 0.1	192.0±16.5
Aqueous extraction	120	1:20	2.4±0.1	5.3±0.2	1.5 ± 0.1	236.2±19.7
Aqueous extraction	60	1:10	1.5±0.3	1.3±0.0	1.2 ± 0.1	95.3±4.9
Aqueous extraction	90	1:10	2.1±0.3	2.1±0.3	1.5 ± 0.1	127.2±5.4
Aqueous extraction	120	1:10	3.7±0.1	4.5±0.1	2.0 ± 0.1	179.7±5.5
Mild alkaline hydrolysis 6% NaOH (w/v)	90	1:10	4.4±0.2	5.6±0.2	1.4±0.0	108.7±1.1
Mild alkaline hydrolysis 6% NaOH (w/v)	120	1:10	6.0±0.3	8.2±0.4	2.6±0.1	242.0±12.5
Mild acid hydrolysis of 6% H_2SO_4 (w/v)	90	1:10	7.4±0.4	10.3±0.6	4.4±0.1	433.5±14.9
Mild acid hydrolysis of 6% H_2SO_4 (w/v)	120	1:10	24.1±1.3	36.1±2.1	22.5±1.4	2412.0±153

[a] Regardless of the treatment type, the digestate liquid fraction was the aqueous phase; [b] temperature conditions of the treatment; [c] solid-liquid ratio between the solid and the liquid fractions of the digestate; [d] Data are the mean ± standard deviation of three parallel experiments.

Based on these results, mild acid hydrolysis appeared to be the most effective approach in yielding a carbohydrate-enriched ELP. To optimize this pretreatment, a full factorial design was used to statistically evaluate the impact of acid concentration and incubation time (X_1 and X_2, respectively). Table 2 reporting the structure of the experimental design and the volumetric concentrations obtained for each individual combination of the quantitative variables under study, shows that the response ranged widely from 4.38 to 25.98 g L^{-1} depending on the selected factor combination.

Table 2. Full factorial design showing the actual values and, between square brackets, the coded values of the independent variables H_2SO_4 concentration (X_1) and incubation time (X_2) and observed and predicted values of the volumetric concentrations of total sugars.

Run Number	H_2SO_4 Concentration (X_1) (%, w/v)	Incubation Time (X_2) (min)	Concentrations of Total Sugars (g L^{-1})	
			Observed	Predicted
1	**2 [-1]**	30 [-1]	4.38	4.87
2	2 [-1]	30 [-1]	4.49	4.87
3	6 [0]	30 [-1]	16.80	15.89
4	6 [0]	30 [-1]	12.01	15.89
5	10 [1]	30 [-1]	19.30	16.87
6	10 [1]	30 [-1]	17.5	16.87
7	2 [-1]	60 [-0.333]	12.38	12.74
8	2 [-1]	60 [-0.333]	14.22	12.74
9	6 [0]	60 [-0.333]	25.36	23.76
10	6 [0]	60 [-0.333]	25.15	23.76
11	10 [1]	60 [-0.333]	24.40	24.74
12	10 [1]	60 [-0.333]	24.24	24.74
13	2 [-1]	120 [1]	10.89	11.48
14	2 [-1]	120 [1]	11.84	11.48
15	6 [0]	120 [1]	25.98	22.49
16	6 [0]	120 [1]	21.83	22.49
17	10 [1]	120 [1]	20.61	23.48
18	10 [1]	120 [1]	24.14	23.48
20	6 [0]	75 [0]	23.02	25.56
21	6 [0]	75 [0]	24.89	25.56
22	6 [0]	75 [0]	25.89	25.56

Data were fitted by a second order polynomial equation, leading to a high fraction of variation explained by the model (R^2_{adj} = 0.925). Since the $X_1 \bullet X_2$ interaction coefficient was not statistically significant ($P = 0.324$), data were refitted by excluding the insignificant term from the model (Table 3).

Table 3. Least squares estimate of coefficients of the variables H_2SO_4 concentration (X_1) and incubation time (X_2) in the original and refined model. Statistical parameters measuring fitting power (R^2), predictive power (Q^2) and probability of lack of fit (F_{ME}) are also shown.

Model’s Terms [a] and Parameters	Original Model	Refined Model
Constant (β_0)	25.6 ± 0.8 ***	25.6 ± 0.8 ***
First order coefficient of X_1 (β_1)	5.9 ± 0.6 ***	6.0 ± 0.5 ***
First order coefficient of X_2 (β_2)	3.3 ± 0.5 ***	3.3 ± 0.5 ***
Second order coefficient of X_1 ($\beta_1 \bullet \beta_1$)	-5.01 ± 0.9 ***	-5.01 ± 0.9 ***
Second order coefficient of X_2 ($\beta_2 \bullet \beta_2$)	6.38 ± 0.9 ***	6.38 ± 0.9 ***
Interaction coefficient X_1X_2 ($\beta_1 \bullet \beta_2$)	-0.63 ± 0.7 n.s.	n.i.

R^2	0.944	0.941
R^2_{adj}	0.925 (DF = 16)	0.926 (DF = 16)
Q^2	0.890	0.895
F_{ME}	1.63 (P = 0.230)	1.54 (P = 0.256)

[a] Estimated coefficient ± standard error and significance level: ***, $P < 0.0001$; n.s., not significant; n.i., not included; R^2, coefficient of determination; R^2_{adj}, coefficient of determination adjusted for degrees of freedom (DF); F_{ME}, ratio between mean squares of model error and replicate error.

As an additional check, the Fisher-Snedecor's model error values (F_{ME}), calculated by the ratio between mean squares of model error and replicate error, showed that the probability for lack of fit of the model was not statistically significant ($P = 0.256$). Figure 1, reporting the contour diagram for saccharification yield as a function of different combinations of the two factors, shows that the attainment of TS concentrations as high as 25 g L^{-1} by mild acid hydrolysis with 6% H_2SO_4 required an incubation time of 68 min. Above that combination of variable levels, the TS values were all positioned on the same isocurve, probably indicating that the maximum extraction condition had been reached and, thus these hydrolysis conditions were adopted to derive the ELP. In the ELP thus derived, most of the TS pool were pentoses and reducing sugars with respective volumetric concentrations amounting to 23.02 and 22.82 g L^{-1}. Under the same condition, the concentration of total phenols and N-NH_4^+ amounted to 0.72 g and 1.14 g L^{-1}.

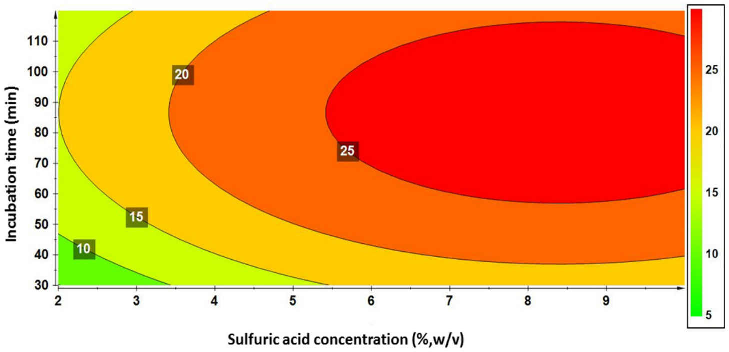

Figure 1. Contour plot of total sugars concentrations (g L^{-1}) in the enriched liquid phase as a function of incubation time and H_2SO_4 concentrations.

3.2. Screening of Strains for Biomass- and Chitosan-Producing Ability on ELP Medium

Table 4 shows biomass production, percentage of total sugars (TS) consumption and values of biomass yield referred to the substrate consumed ($Y_{X/S}$) in liquid cultures of several Zygomycota strains grown in the ELP derived from the mild acid hydrolysis of the digestate.

Table 4. Biomass production, percentage of total sugars (TS) consumed, and biomass yield referred to TS consumed ($Y_{X/S}$) obtained in 96-h-old cultures of Zygomycota strains grown on non-supplemented enriched liquid phase.

Fungal Strains	**Biomass (g L^{-1})**	**TS Consumption (%)**	**$Y_{X/S}$ (g g^{-1})**
Absidia blakesleeana NRRL 1304	0.55 ± 0.06 ^{E}	15.12 ± 0.22 ^{FG}	0.15 ± 0.01 ^{FG}
Absidia blakesleeana NRRL 2696	7.58 ± 0.08 ^{AB}	75.23 ± 2.12 ^{BC}	0.42 ± 0.02 ^{AB}
Absidia coerulea NRRL 1312	2.59 ± 0.06 ^{D}	29.42 ± 3.28 ^{E}	0.37 ± 0.05 ^{BC}
Absidia coerulea NRRL 1315	0.76 ± 0.04 ^{E}	14.23 ± 0.11 ^{FG}	0.22 ± 0.01 ^{EF}
Absidia coerulea NRRL A-9483	0.59 ± 0.02 ^{E}	10.11 ± 0.16 ^{G}	0.24 ± 0.01 ^{DE}
Absidia corymbifera NRRL 2798	0.61 ± 0.04 ^{E}	11.20 ± 2.23 ^{FG}	0.22 ± 0.06 ^{EF}
Absidia glauca NRRL 1324	0.74 ± 0.03 ^{E}	10.03 ± 1.12 ^{G}	0.31 ± 0.05 ^{CD}
Absidia glauca NRRL 2799	1.02 ± 0.08 ^{E}	92.45 ± 3.08 ^{A}	0.05 ± 0.01 ^{H}

Absidia glauca NRRL 3010	0.61 ± 0.04 ^{E}	20.22 ± 1.08 ^{F}	0.13 ± 0.02 ^{G}
Absidia repens NRRL 1337	0.61 ± 0.02 ^{E}	8.05 ± 0.09 ^{G}	0.31 ± 0.01 ^{CD}
Aspergillus tubingensis NRRL 4700	6.63 ± 0.15 ^{B}	91.23 ± 1.34 ^{A}	0.30 ± 0.01 ^{CD}
Benjaminiella poitrasii NRRL 2845	4.46 ± 0.13 ^{C}	72.47 ± 0.52 ^{C}	0.26 ± 0.01 ^{DE}
Cunninghamella elegans NRRL1392	0.70 ± 0.16 ^{E}	14.3 ± 1.10 ^{FG}	0.20 ± 0.04 ^{EFG}
Mucor moelleri As. Col E	4.29 ± 0.41 ^{C}	77.23 ± 2.67 ^{BC}	0.26 ± 0.03 ^{DE}
Mucor rouxii NRRL 1894	3.95 ± 0.16 ^{C}	81.34 ± 3.02 ^{B}	0.20 ± 0.02 ^{EFG}
Rhizopus oryzae NRRL 1510	8.12 ± 0.21 ^{A}	77.11 ± 2.01 ^{BC}	0.44 ± 0.02 ^{AB}
Rhizopus oryzae NRRL 2625	6.89 ± 0.67 ^{AB}	55.54 ± 2.12 ^{D}	0.52 ± 0.07 ^{A}

Same uppercase letters denote the absence of statistically significant differences among column means ($p < 0.05$).

With the notable exceptions of *A. glauca* NRRL 2799 and *A. blakesleeana* NRRL 2696 cultures, yielding TS consumptions of 92 and 75%, respectively, the strains belonging to the *Absidia* genus showed a limited ability to grow on the ELP medium, and this also concerned *C. elegans* NRRL 1392 cultures. *A. blakesleeana* NRRL 2696 and *Rhizopus oryzae* NRRL 1510 were the best biomass producers on the ELP medium (7.58 and 8.12 g L^{-1}, respectively). The only strain not belonging to the Mucorales order, namely *A. tubingensis* NRRL 4700, also showed a satisfactory biomass-producing capability on the ELP medium (6.63 g L^{-1}). Nine of the initial 17 strains under study were selected for the secondary screening phase since their cultures yielded biomass levels higher than 2.5 g L^{-1}, an amount considered compatible with accurate gravimetric quantification of the purified chitosan. The results are summarized in Table 5, showing the amounts of alkali-insoluble material (AIM), chitosan volumetric production, daily productivity (r_c), and chitosan yield referred to biomass dry weight ($Y_{C/X}$), obtained after 96 h of incubation.

Table 5. Values of alkali-insoluble material (AIM), chitosan yield ($Y_{C/X}$), volumetric production (CVP) and daily productivity (r_c) in fungal cultures grown on the enriched liquid phase for 96 h.

Fungal Strains	**AIM (mg g^{-1} Biomass)**	**Chitosan (% on AIM)**	**$Y_{C/X}$ (mg g^{-1} Biomass)**	**CVP (mg L^{-1})**	**r_c (mg L^{-1} d^{-1})**
A. blakesleeana NRRL 2696	245.9±12.2 ^{C}	21.0±1.0 ^{A}	59.0±3.0 ^{A}	443.5±17.7 ^{A}	110.9±4.4 ^{A}
A. coerulea NRRL 1312	610.0±21.0 ^{A}	7.0±0.3 ^{C}	44.0 ± 5.0 ^{AB}	49.1±4.9 ^{E}	12.3±1.2 ^{E}
A. tubingensis NRRL 4700	354.5±3.0 ^{B}	4 .2±0.3 ^{C}	13.0±1.1 ^{D}	86.0±6.9 ^{DE}	21.5±1.7 ^{DE}
B. poitrasii NRRL 2845	128.0±9.1 ^{E}	16.2±1.0 ^{AB}	20.1±2.0 ^{CD}	91.0±12.0 ^{DE}	22.8±3.0 ^{DE}
M. moelleri As. Col E	155.1±42.0 ^{DE}	20.4±.11 ^{A}	31.3±6.2 ^{BC}	134.3±14.4 ^{CD}	33.6±3.6 ^{CD}
M. rouxii NRRL 1894	205.0±19.1 ^{DE}	13.9±1.1 ^{B}	29.1±1.4 ^{BCD}	139.0±7.4 ^{CD}	34.7±1.1 ^{CD}
R. oryzae NRRL 2625	147.4±26.2 ^{DE}	18.1±3.4 ^{AB}	25.2±1.1 ^{CD}	176.2±22.3 ^{C}	44.1±5.6 ^{C}
R. oryzae NRRL 1510	219.0±6.9^{CD}	18.4±1.3 ^{AB}	40.0 ± 8.1 ^{AB}	324.1 ± 34.2 ^{B}	81.0±8.5 ^{B}

Data are the mean ± standard deviation of duplicate cultures. Multiple pairwise comparisons were performed via the post-hoc Tukey test. Same uppercase letters denote the absence of statistically significant differences among column means ($p < 0.05$).

The ELP medium was conducive to a tangible chitosan accumulation with $Y_{C/X}$ values ranging from 20 to 59 mg g^{-1} biomass for the Mucorales strains. In this respect, the performance of the tested species was higher than those grown on a non-optimized liquid medium made of 1% corn-steep liquor and 25% cassava peel juice, where $Y_{C/X}$ ranged from 5 and 15 mg g^{-1} of fungal biomass, depending on the species [26]. In the present study, the highest AIM values per gram of biomass, observed in *A. coerulea* NRRL 1312 and *A. tubingensis* NRRL 4700 (610 ± 21 and 355 ± 3 mg g^{-1}, respectively) cultures, were counterbalanced by the lowest percentage of chitosan on AIM (around 7 and 4%, respectively), leading to low chitosan volumetric productions (49 ± 5 and 86 ± 7 mg L^{-1}, respectively) and r_c values (12 ± 1 and 22 ± 2 mg L^{-1} d^{-1}, respectively).

The best $Y_{C/X}$ and r_c values (59 ± 3 mg g^{-1} and 111 mg L^{-1} d^{-1}, respectively) were observed in *A. blakesleeana* NRRL 2696 cultures attaining a chitosan volumetric production of 444 ± 18 mg L^{-1}. Among the other strains screened, *R. oryzae* NRRL 1510 cultures gave promising results with chitosan volumetric productions, r_c and $Y_{C/X}$ being 324± 34 mg L^{-1}, 81 mg L^{-1} d^{-1} and 40 mg g^{-1} biomass, respectively. Figure 2 displays the process kinetics of *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510 shaken cultures grown on the non-supplemented ELP medium.

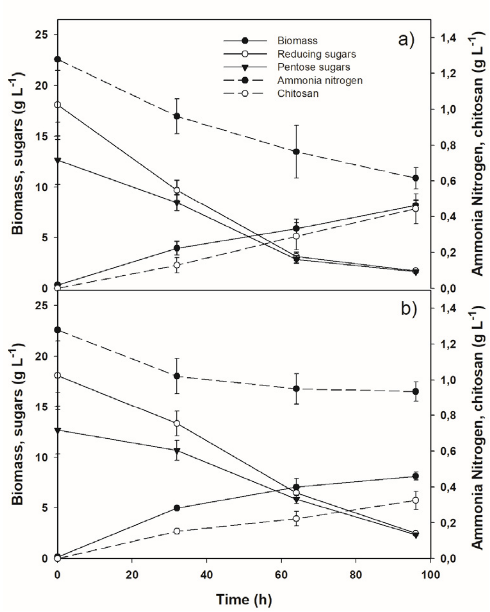

Figure 2. Time courses of biomass and chitosan productions and consumptions of reducing sugars, pentoses and ammonia nitrogen in shaken liquid cultures of the selected strains: (**a**) *A. blakesleeana* NRRL 2696 and (**b**) *R. oryzae* NRRL 1510.

The process endpoint was set at 96 h since preliminary experiments showed that, at that time, the cultures of both strains had reached the late exponential phase. Several studies pointed out that this phase is the optimal harvesting time since, during the subsequent stationary phase, chitosan molecules establish cross-links with other cell wall constituents, making extraction less effective [27-29]. Both fungal cultures removed reducing sugars and pentoses from the ELP medium to similar extents at the end of the fermentation. *A. blakesleeana* NRRL 2696 cultures consumed ammonia nitrogen at a faster rate and to a much greater extent than those of *R. oryzae* NRRL 1510. Although this disparity did not result in significant differences in biomass formation at the end of the process, it did impact chitosan production.

For comparison, Table 6 shows the volumetric biomass and chitosan production, and the average daily productivity (r_c) values obtained with the two strains *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510, along with values from other studies that investigated fungal chitosan production efficiency in shaken cultures on chemically defined liquid media and various wastewater and industrial by-products.

Table 6. Comparing figures of merit of the developed work with the reported ones for chitosan production in shaken cultures of the same or similar fungal species.

Strain	Growth Medium	CVP^a (mg L^{-1})	r_c^b (mg L^{-1} d^{-1})	$Y_{C/X}^c$ (mg g^{-1} Biomass)	Reference
A. blakesleeana NRRL 2696	ELP^d	444	111	59	This study
A. blakesleeana NRRL 1340	GPY^e (2.0-1.0-0.1 g L^{-1}) added with 5.0 g L^{-1} $(NH_4)_2SO_4$	282	141	170	[22]

A. butleri NCIM977	GPY^e (20.0-10.0-1.0 g L^{-1})	570	190	55	[31]
A. coerulea ATCC14076	GPY^e (10.0-5.0-10.0 g L^{-1})	1860	624	300	[32]
A. glauca	GPY^e (20.0-10.0-1.0 gL^{-1})	650	324	74	[7]
A. coerulea CTCC93105	Glucose-based medium (20 g L^{-1}) added with 5% soybean pomace	4110	684	267	[33]
R. oryzae NRRL 1510	ELP^d	324	81	40	This study
R. oryzae USBD 0602	GPY^e (20.0-10.0-1.0 g L^{-1}) added with $(NH_4)_2SO_4$	280	55	49	[28]
R. oryzae USBD 0263	GPY^e (20.0-10.0-1.0 g L^{-1}) added with $(NH_4)_2SO_4$	220	71.4	44	[28]
R. oryzae USBD 0602	GPY^e (5.0-1.0-0.1 g L^{-1})	56	19	49	[34]
R. oryzae MTCC262	DW^f added with gibberellic acid (0.1 mg L^{-1})	1130	377	136	[35]
R. oryzae PAS17	Molasse-based medium (7%) added with $MgSO_4$	1500	187	140	[36]
R. oryzae ME-F12	Corn straw acid hydrolysate	580	n. r.	112	[37]
R. oryzae MTCC262	DW^f added with $(NH_4)_2HPO_4$ (8 g L^{-1}) and yeast extract	620	n. r.	100	[38]
R. oryzae NRRL395	Ground corn grains-based liquid medium (10%)	406	135	n. r.	[40]
R. oryzae NRRL395	Ground rice-based liquid medium (10%)	700	233	n. r	[40]
R. oryzae SWERI	Sugar cane molasses	940	118	77	[41]
R. oryzae SWERI	Sugar beet molasses	713	89	65	[41]

[a] Chitosan volumetric production; [b] average daily productivity; [c] Chitosan yield to dry biomass weight; [d] Enriched liquid phase; [e] Glucose-peptone-yeast extract; [f] Deproteinized whey.

Noteworthy, *A. blakesleeana* NRRL 1340 performed well as a chitosan producer in the pioneering study of Rane & Hoover [22], and a subsequent study reported it as a species of great interest to produce chitin and chitosan [30]. Despite these findings, we only found one pertinent study regarding this species, mentioned with its synonym *Lichtheimia blakesleeana* [26], that uniquely reported the value of $Y_{C/X}$ (12.04 mg g^{-1} biomass) obtained in a medium made of a mixture of corn steep liquor (CSL) and papaya peel juice (PPJ). For this reason, Table 6 also includes data on chitosan production by other Absidia species, most of which obtained on glucose-peptone-yeast extract (GPY) media [7,22,31,32] except for the study of Jiang et al. [33] that used a glucose-based medium added with soybean pomace (GBMSP) to investigate the chitosan-producing ability of *A. coerulea* CTCC93105 cultures. Among these studies, *A. coerulea* cultures grown on GPY [32] and GBMSP [33] media stood out due to the high $Y_{c/x}$ and r_c values, which were about 6-fold higher than those obtained by *A. blakesleeana* NRLL 2696 cultures grown on the ELP medium.

For *R. oryzae*, in addition to studies conducted on synthetic media mostly consisting of GPY added or not with ammonium sulphate [28,34], which attained similar chitosan production results to those obtained with *R. oryzae* NRRL 1510 on ELP medium, considerable efforts have been devoted to the development of low-cost production media [35-41]. Among these studies, the best performance was attained by *R. oryzae* MTCC62 and PAS17 cultures grown on deproteinized whey added with gibberellic acid [35] and a molasse-based medium [36], respectively.

The most similar medium to the ELP one was that used by Tai et al. [37], which cultivated *R. oryzae* ME-F12 cultures on a corn straw hemicellulose hydrolysate (HH)-based medium, obtained by dilute acid hydrolysis, and supplemented with urea and $MgSO_4$ (2.0 and 0.5 g L^{-1}, respectively). When the HH was used in lieu of a glucose-based medium with the same supplements, biomass and chitosan productions increased by 10 and 82%, respectively [37]. This hydrolysate mostly contained xylose, which was confirmed to be a preferential sugar source over glucose in the production of biomass and chitosan by *R. oryzae* [39]. In this respect, although the ELP medium composition resembled that used by Tai et al. [37], the chitosan volumetric production and $Y_{C/X}$ obtained therein by *R. oryzae* NRRL 1510 were 1.4- and 1.8-fold lower than those of *R. oryzae* ME-F12, likely because the ELP medium was not supplemented with additional organic or inorganic nutrients. A further reason might be the undetectable presence of putative inhibitors (e.g., furfural and acetic and formic acids) in the ELP medium, the joint addition of which to a xylose-based medium, at the same concentrations found in HH, increased chitosan production by around 64% [37]. In this respect, the same authors postulated that such an increase might be a compensative fungal response to counteract the toxic effects of these compounds. Irrespective of these considerations, all these comparisons must be interpreted not only with respect to the differences in the growth medium but also with respect to the variability of the strains used, the cultivation conditions, and the chitosan extraction methods used.

3.3. Characterization of Fungal Chitosans

The infrared spectroscopic characterization of samples produced by *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510, from here onwards referred to as *Ab*-Cht and *Ro*-Cht, respectively, is reported in Figure 3. FT-IR spectra were used to identify and confirm the characteristic functional groups of the produced chitosan. Both samples exhibited similar trends in their FT-IR spectra, showing the same peaks, with slight differences in their intensities, with the typical peaks assigned to polysaccharide structures.

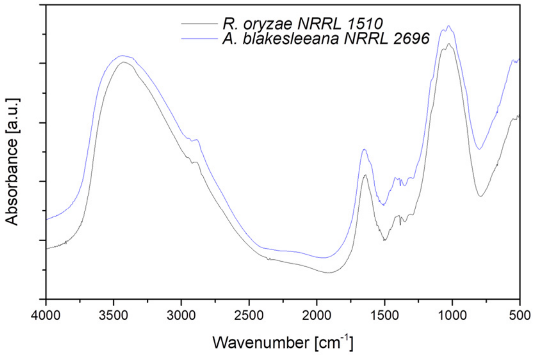

Figure 3. Absorbance FT-IR spectra of chitosan samples (*A. blakesleeana* NRRL 2696 in blue, *R. oryzae* NRRL 1510 in black) in the 4000-500 cm^{-1} range collected in transmission mode.

A broad absorption band in the range of 3000 – 3500 cm^{-1} was found in both samples, and it was attributed to O–H and N–H stretching, centered at 3440 cm^{-1}, while a small absorption band was registered at 2870 – 2880 cm^{-1}, due to C–H aliphatic stretching vibration. The distinctive absorption peaks of amide I, centered at 1650 cm^{-1} and due to axial C=O stretching, and amide II, centered at 1558 cm^{-1} due to N-H in plane deformation coupled with C-N stretching, were clearly visible in the fingerprint region. Furthermore, between 1250 cm^{-1} and 750 cm^{-1}, a broad band was present, extremely useful for evidencing chitosan presence even in trace amounts, due to the vibrations of glycosidic bonds, C–O and C–O–C stretching. FT-IR was also used to determine DD% value of the chitosan samples, and the chosen bands were at 1420 and 1320 cm^{-1}. The DD% values calculated with the Equation (4), proposed by Brugnerotto et al. [23], were 79.0 and 84.2% for *Ab*_Cht and *Ro*_Cht, respectively.

Table 7 shows the elemental analysis of *Ab*_Cht and *Ro*_Cht samples and the respective DD% values, calculated according to the Equation (5), proposed by Dos Santos et al. [24] and relying on the C/N ratios. The DD% value for the *Ab*_Cht was higher than that determined by FT-IR spectroscopy (86.5 *vs*. 79.0%), while the DD% value of *Ro*_Cht was very similar to that determined from FT-IR spectroscopy.

Table 7. Elemental analysis of the chitosan preparations derived from the late exponential phase of *A. blakesleeana* NRRL2696 and *R. oryzae* NRRL 1511 shaken cultures grown on non-supplemented enriched liquid phase derived from the digestate. Data are the means of duplicate cultures, and the relative standard deviations of data were less than 7%.

Samples	C (%)	H (%)	N (%)	S (%)	DD (%)
A blakesleeana NRRL 2696	32.91	6.61	6.12	1.25	86.45
R oryzae NRRL 1510	36.94	6.65	6.84	0.08	84.18
Low Molecular Weight chitosan (Fluka)	39.69	7.18	7.49	0.19	90.69

Regardless of the technique employed, these DD% values are well above the minimum threshold suggested by various authors for a clear separation between chitin and chitosan [4-6]. The DD% is an important parameter affecting the physicochemical properties of this polymer since a high degree of deacetylation enhances its polycationic nature and water-solubility, thus making it very suitable for both environmental and food applications as a coagulating or a clarifying agent [2-6].

The determination of the intrinsic viscosity through a rolling-ball viscometer and the subsequent application of the Mark-Houwink-Sakurada equation showed that the average molecular weights of *Ab*-Cht and *Ro*-Cht samples were 20 and 5.39 kDa, respectively (Table 8).

Table 8. Intrinsic viscosity values [η], R^2 values of the fitting lines and average molecular weights (M_v) of chitosans obtained from shaken cultures of *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510 shaken cultures grown on the ELP medium.

Chitosan Source	Regression Method	R^2	Intrinsic Viscosity (ml g^{-1})	M_v (Da)
A. blakesleeana NRRL 2696	Huggins	0.985	61.0 ± 2.3	19996
	Kraemer	0.952	61.1 ± 2.1	20030
R. oryzae NRRL 1510.	Huggins	0.998	23.6 ± 1.2	5385
	Kraemer	0.997	24.1 ± 1.8	5525

These findings indicate that the cultivation of both strains on the ELP medium yielded low molecular weight chitosans (LMWCh), a group of polymers which have attracted considerable attention due to their strong bactericidal activities [42], and potential in DNA delivery systems as a carrier [43]. Until now, the primary processes to obtain LMWCh include thermally assisted acid hydrolysis [44] or biochemical hydrolysis [42] of high molecular weight chitosans. Alternatively, Niederhofer and Muller [45] were able to obtain *A. coerulea* LMWCh with average MW of 45 kDa and polydispersity of 2.63 though a radical modification of the extraction procedure. Wang and collaborators [46], instead, used *Absidia coerulea* AF93105 solid-state cultures on potato waste as a direct production method leading to a chitosan with an average molecular weight of 6.4 kDa and a very low degree of polydispersity.

Thermogravimetric analysis (TGA) was employed to evaluate the thermal degradation profile and thermal stability of the fungal chitosans. Figures 4a,b illustrate the residual weights (TG) and derivative thermogravimetric (DTG) curves *vs.* temperature (30-500°C) for *Ab*-Cht and *Ro*-Cht samples, respectively, in an oxygen-free atmosphere. The TGA curves revealed that both chitosan samples underwent a two-stage thermal decomposition process within the 30-500°C range, consistent with previous findings [47].

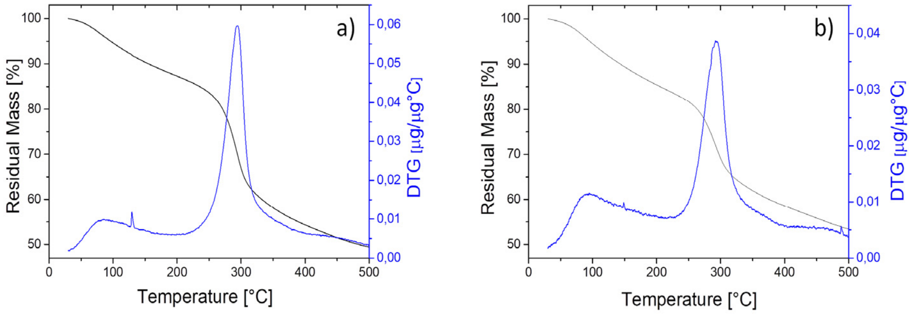

Figure 4. Residual mass (black line) and derivative thermal gravimetry (blue line) profiles under Nitrogen atmosphere, in the 30-500 °C temperature range, of two chitosan preparations extracted from: (**a**) *A. blakesleeana* NRRL 2696 and (**b**) *R. oryzae* NRRL 1510 cultures.

In the first degradation stage (30-200°C), *Ab*-Cht and *Ro*-Cht samples demonstrated weight losses of 11% and 13%, respectively. This initial weight reduction can be attributed to the evaporation of absorbed moisture, as chitosan's hydrophilic functional groups (-OH and -NH_2) readily form hydrogen bonds with water molecules [26]. The second, more substantial decomposition stage occurred between 200-400 °C, with weight losses of 34% for *Ab*-Cht and 37% for *Ro*-Cht. This stage corresponds to the complex thermal degradation of the chitosan polymer, including deacetylation, depolymerization, and dehydration of saccharide rings, followed by pyranose ring decomposition [48]. The onset of significant thermal degradation began at approximately 250 °C, with maximum decomposition rate (T_{max}) observed at 290 °C, as indicated by the DTG peak. While the thermal profile patterns of both fungal chitosans resembled those of commercial chitosan (data not shown), a slight shift toward lower temperatures was noted for the main thermal events, suggesting reduced thermal stability. After 350 °C, the decomposition rate notably decreased, likely due to crosslinking reactions or the formation of thermally stable carbonaceous residues [48]. The observed thermal behavior aligns with previous reports on chitosan thermal properties [47,49].

3.4. Antibacterial Activity of Fungal Chitosans

Chitosan has demonstrated antimicrobial activity against a broad range of target organisms, including bacteria, fungi, yeasts and algae [4]. Among its physico-chemical properties, the degree of deacetylation (DD%) and the molecular weight exert the most significant influence on chitosan's antimicrobial activity. The positively charged amino groups of chitosan can compete with bivalent ions, such as Ca^{2+} and Mg^{2+}, for binding to phosphate and carboxyl groups commonly found in lipopolysaccharide; this interaction destabilizes the Gram-negative cell wall [50]. However, other studies suggest that chitosan exhibits stronger antimicrobial activity on Gram-positive bacteria than Gram-negative ones [4,51]. In this case, the polycationic nature of chitosan could bind to anionic glycerophosphate units of lipoteichoic acids present in the Gram-positive wall, disrupting the structural stability of the cell wall [50]. Table 9 shows the minimum inhibitory concentration (MIC) values for one Gram-positive and two Gram-negative bacterial species tested with fungal-derived chitosans, compared to a commercial LMWCh. Among the tested bacteria, *Pseudomonas syringae* DSM 21482 was the most sensitive, with the lowest MIC values (160 mg L^{-1} for chitosan extracted from both *Ab*-Cht and *Ro*-Cht). The MIC values of the former and the latter fungal chitosan for *Escherichia coli* ATCC 9637, were 1.9- and 2.6-fold higher than those for *P. syringae* DSM 21482.

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Table 9. Minimum inhibitory concentrations (mg L^{-1}) of chitosan preparations from *A. blakesleeana NRRL 2696*, *R. oryzae NRRL 1510* and a commercial source. .

Chitosan Source	*Pseudomonas syringae* DSM 21482 (mg L^{-1})	*Escherichia coli* ATCC 9637 (mg L^{-1})	*Bacillus subtilis* DSM 10 (mg L^{-1})
A. blakesleeana NRRL 2696	160	300	360
R. oryzae NRRL 1510	160	420	480
Low Molecular Weight chitosan (Fluka)	120	180	220

The lowest antibacterial activity was detected against the Gram-positive strain *B. subtilis* DIBAF23-97, regardless of the chitosan source. Notably, the commercial chitosan preparation exhibited the highest antibacterial activity across all tested bacterial species (Table 9). The commercial chitosan had a molecular weight of 70 kDa, significantly higher than that of the two fungal-derived preparations, while its percentage of deacetylation (88%) was only slightly higher. Although studies suggest that antibacterial activity increases as MW decreases and is positively correlated with the DD% [52, 53], the reasons behind the superior antimicrobial activity of the commercial chitosan remain unclear. Nevertheless, it is worth noting that the MIC values obtained for the tested fungal-derived chitosans were much lower than those reported for other fungal-sourced chitosans, which ranged between 750 and 2000 mg L^{-1} against the same tested bacterial species [54, 55].

4. Conclusions

This study pioneers the innovative valorization of both liquid and solid fractions of digestate, demonstrating their potential as growth substrate for producing fungal chitosan. Through mild acid hydrolysis, optimized using the response surface methodology, a liquid production medium was developed that, even in its non-supplemented form, proved highly effective for fungal cultivation. Out of 17 fungal strains tested, nine exhibited robust growth on this medium, achieving satisfactory biomass yields. Notably, *Absidia blakesleeana* NRRL 2696 and *Rhizopus oryzae* NRRL 1510 emerged as standout performers in chitosan production, achieving CVP values of 444 mg/L and 324 mg/L, respectively. The extracted chitosans displayed desirable characteristics, including high degrees of deacetylation (79% for *A. blakesleeana* and 84.2% for *R. oryzae*) and remarkably low viscosimetric molecular weights (20 kDa and 5.4 kDa, respectively). Such low-molecular-weight chitosans (LMWCh) are exceptionally rare to obtain directly through fungal fermentation and are highly sought after due to their superior solubility, bioavailability, and enhanced biological activities compared to their high-molecular-weight counterparts. Furthermore, both chitosan preparations exhibited potent antibacterial properties against Gram-positive and Gram-negative bacteria, with minimum inhibitory concentrations (MICs) significantly lower than those reported for other fungal-derived chitosans. This underscores their potential as effective antimicrobial agents.

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Abbreviations

The following abbreviations are used in this manuscript:

*Ab*_Cht	*Absidia blakesleeana* chitosan
AIM	Alkali-insoluble material
CVP	Chitosan volumetric production
DD	Deacetylation degree
DLF	Digestate liquid fraction
DSF	Digestate solid fraction
DTG	Derivative thermogravimetry
ELP	Enriched liquid phase
FT-IR	Fourier transform infrared
GPY	Glucose-peptone-yeast extract
HH	Hemicellulose hydrolysate
LMWCh	Low molecular weight chitosan
MIC	Minimum inhibitory concentration
M_v	Viscosimetric average molecular weight
r_c	Average daily productivity
*Ro*_Cht	*Rhizopus oryzae* chitosan
RY	Recovery yield
TGA	Thermogravimetric analysis
TS	Total sugars
$Y_{c/x}$	Chitosan yield referred to biomass dry weight
$Y_{x/s}$	Biomass yield referred to the substrate consumed

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