*fermentation*

MDPI

*Article*

# 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]**

[1] Department for Innovation in Biological, Agro-Food and Forest Systems (DIBAF), University of Tuscia, Via S. Camillo De Lellis snc, 01100 Viterbo, Italy; crognale@unitus.it (S.C.); cristina.russo@hotmail.it (C.R.); a.cimini@unitus.it (A.C.); petrucci@unitus.it (M.P.)

[2] Department of Economics, Engineering, Society and Business Organization (DEIM), Largo dell'Università snc, University of Tuscia, 01100 Viterbo, Italy; ilaria.armentano@unitus.it

* Correspondence: dannib@unitus.it

**Abstract**

This study investigated the feasibility of using both the solid and the liquid fractions of waste from the anaerobic digestion process—the digestate—as a possible liquid growth medium for fungal production of chitosan. An enriched liquid phase (ELP), combining both fractions, and derived from mild acid hydrolysis treatment at 120 °C with 6% $H_2SO_4$ (w/v) for 70 min, 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

check for updates

Academic Editors: Ying Liu and Wen Huang

Received: 14 July 2025
Revised: 4 August 2025
Accepted: 11 August 2025
Published: 15 August 2025

**Citation:** Crognale, S.; Russo, C.; Carota, E.; Armentano, I.; Di Gregorio, F.; D'Annibale, A.; Cimini, A.; Petruccioli, M. Digestate-Based Liquid Growth Medium for Production of Microbial Chitosan. *Fermentation* **2025**, *11*, 469. https://doi.org/10.3390/fermentation11080469

**Copyright:** © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

## 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]. In contrast to chitosan derived

from crustaceans, fungal chitosans do not contain protein residues that can trigger allergic reactions in sensitive individuals [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 making 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 waste derived from anaerobic conversion processes—the digestate—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. The workflow of the study is summarized in Figure 1, detailing our experimental approach. To the best of our knowledge, this is the first study reporting the use of a digestate-based liquid medium for fungal chitosan production.

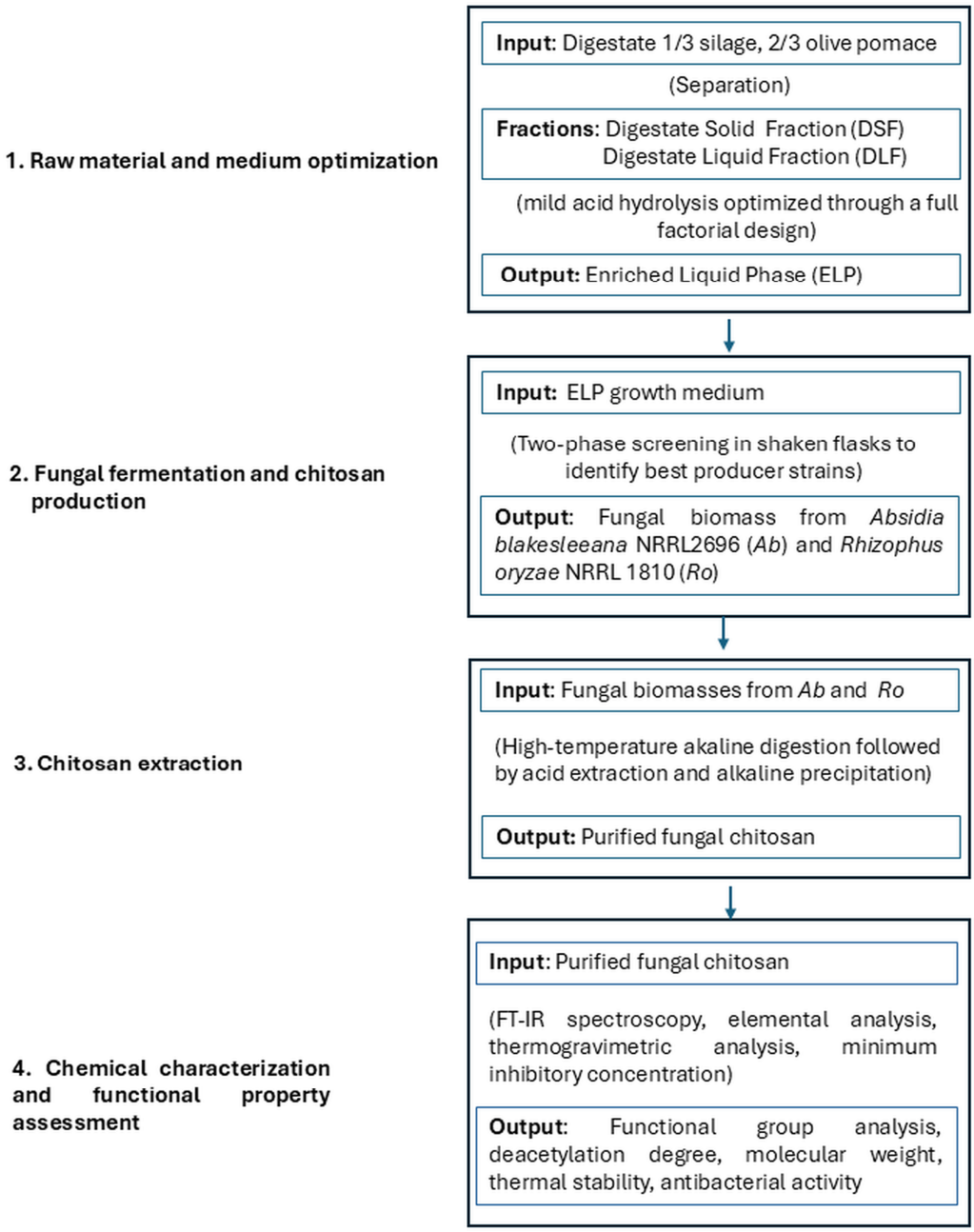


**Figure 1.** Overview of the experimental process for producing microbial chitosan from anaerobic digestate.

## 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, Milan, Italy) 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; sulfur, 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 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):

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

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, $\Delta A$ 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):

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

where $Y$ is the predicted response variable, $\beta_o$ is the intercept, $\beta_i$ and $\beta_{ii}$ linear and quadratic coefficients, respectively, $\beta_{ij}$ 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). Analysis of variance (ANOVA) allowed us to calculate the model's Fisher–Snedecor coefficient ($F_{MOD}$), verify the significance of each model term, and determine the model's lack of fit (LOF). The *p*-value associated with the ratio of the mean squares of the model error to the replicate error ($F_{ME}$) allowed us to determine whether the LOF was significant or not.

### *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, IL, 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, Detroit, MI, USA) at 4 °C and subcultured every month.

### *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 spore concentration of $1 \times 10^6$ 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], and 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 Jüttnerer [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. This procedure relies on high-temperature alkaline digestion to break down non-chitosan cell wall components (e.g., proteins, glucans and lipids) and solubilize them, leaving alkali-insoluble chitosan and other insoluble structural polysaccharides in the solid residue. The chitosan in the alkali-insoluble material (AIM) is extracted with a dilute aqueous acid solution since, due to its polycationic nature (amino groups), it is soluble under these conditions, while most other cell wall components are not. The chitosan in the separated acid-soluble fraction is then recovered through alkaline precipitation. Sodium borohydride ($NaBH_4$) was added to the biomass in the first extraction step, since it is a mild reducing agent used to prevent unwanted chemical modifications, specifically alkaline-induced deamination of chitosan. Finely ground dry mycelial biomass (200–500 mg) was transferred to centrifuge tubes, to which was added $NaBH_4$ (5.0 mg) and NaOH 1.0 M (4.0 mL), then autoclaved (30 min at 121 °C). After cooling, the cell suspension was centrifuged (12,000× *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 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 12,000× *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 (12,000× *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 [23]) in 96-well polystyrene microplates (Greiner Bio-One™ CELLSTAR, Cassina de Pecchi, Italy). 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 that of the samples. The solutions were diluted in Mueller Hinton Broth (MHB, Oxoid, Garbagnate Milanese, Italy) and 100 µL of each dilution was dispensed into each well. The inoculum was adjusted to obtain a density of $5 \times 10^5$ 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, IN, 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 min. TTC in its oxidized form is colorless; however, when introduced to a medium containing metabolically active microorganisms, it is reduced by cellular dehydrogenases to form intensely colored, insoluble formazan compounds (e.g., red formazan with TTC). The production of this colored compound is directly proportional to the amount of viable (metabolically active) bacteria. The lowest

concentration at which no bacterial growth is observed was taken as the MIC value. The test bacteria used were *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 400–4000 $cm^{-1}$ wavenumber range, with a resolution of 4 $cm^{-1}$. The spectra thus obtained were compared with those of commercial chitosan (Fluka, Buchs, Switzerland). 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. [24], was shown to provide the smallest deviation between expected and estimated values. In particular, the first band (1320 $cm^{-1}$) was characteristic of an acetylated amine or an amide function, while the second band (1420 $cm^{-1}$), relating to the C-H bending signal, was chosen as the reference band. DD% was calculated using Equation (4):

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

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 Equation (5) [25]:

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

Thermogravimetric analysis (TGA) of the samples was performed 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 Italia S.r.l., Rivoli, Italy) 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 $[\eta]$ 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:

$$[\eta] = K \cdot (M_v)^a \quad (6)$$

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 were 0.0474 mL $g^{-1}$ and 0.723, respectively [26].

## 3. Results and Discussion

### *3.1. Production of Growth Medium from Digestate 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 percentage recovery yield (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 $X_1 \cdot X_2$ 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 Terms [a] and Parameters | Original Model | Refined Model |
|---|---|---|
| Constant ($\beta_o$) | 25.6 ± 0.8 *** | 25.6 ± 0.8 *** |
| First order coefficient of $X_1$ ($\beta_1$) | 5.9 ± 0.6 *** | 6.0 ± 0.5 *** |
| First order coefficient of $X_2$ ($\beta_2$) | 3.3 ± 0.5 *** | 3.3 ± 0.5 *** |
| Second order coefficient of $X_1$ ($\beta_1 \cdot \beta_1$) | −5.0 ± 0.9 *** | −5.0 ± 0.9 *** |
| Second order coefficient of $X_2$ ($\beta_2 \cdot \beta_2$) | 6.4 ± 0.9 *** | 6.4 ± 0.9 *** |
| Interaction coefficient $X_{1\times2}$ ($\beta_1 \cdot \beta_2$) | −0.63 ± 0.7 n.s. | n.i. |
| $F_{MOD}$ | 50.6 ± 1.4 *** | 63.4 ± 1.9 *** |
| $R^2$ | 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); $Q^2$, predictive squared correlation coefficient; $F_{MOD}$, ratio between mean squares of the regression and the residuals; $F_{ME}$, 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 refined model was not statistically significant ($p$ = 0.256). The contour diagram for saccharification yield as a function of different combinations of the two factors (Figure 2) supported the absence of significant first-order interaction, as also determined primarily through statistical hypothesis testing based on ANOVA (analysis of variance) and regression analysis. The contour lines were mainly ellipsoidal and regularly spaced, showing parallelism and indicating additive effects of both variables. Moreover, there was no strong bending or twisting of contour lines, which is usually a sign of interaction between variables. Figure 2 also 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 these conditions efficiently broke down hemicellulose, revealed by the plateau in sugar yield at intermediate conditions, but left much cellulose intact due to its high crystallinity and resistance to acid attack under mild conditions. 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 $L^{-1}$.

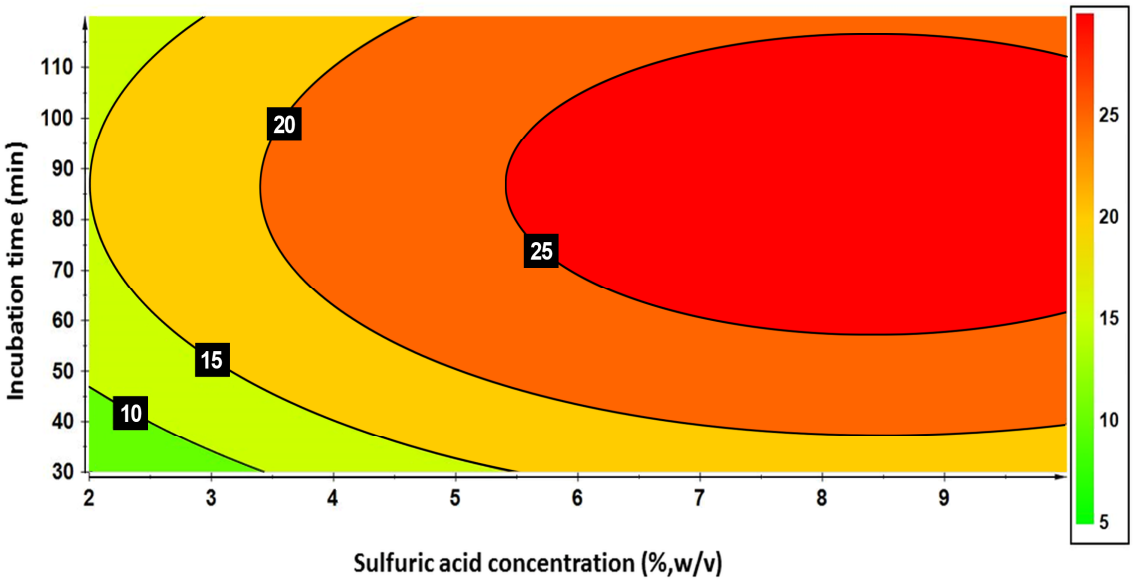


**Figure 2.** Contour plot of total sugars concentrations (g $L^{-1}$) in the enriched liquid phase as a function of incubation time and $H_2SO_4$ concentrations.

### *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.

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 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 ($p < 0.05$).

**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 ± 1.1 $^{A}$ | 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 supported significant 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 [27]. 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 3 displays the process kinetics of *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510 shaken cultures grown on the non-supplemented ELP medium.

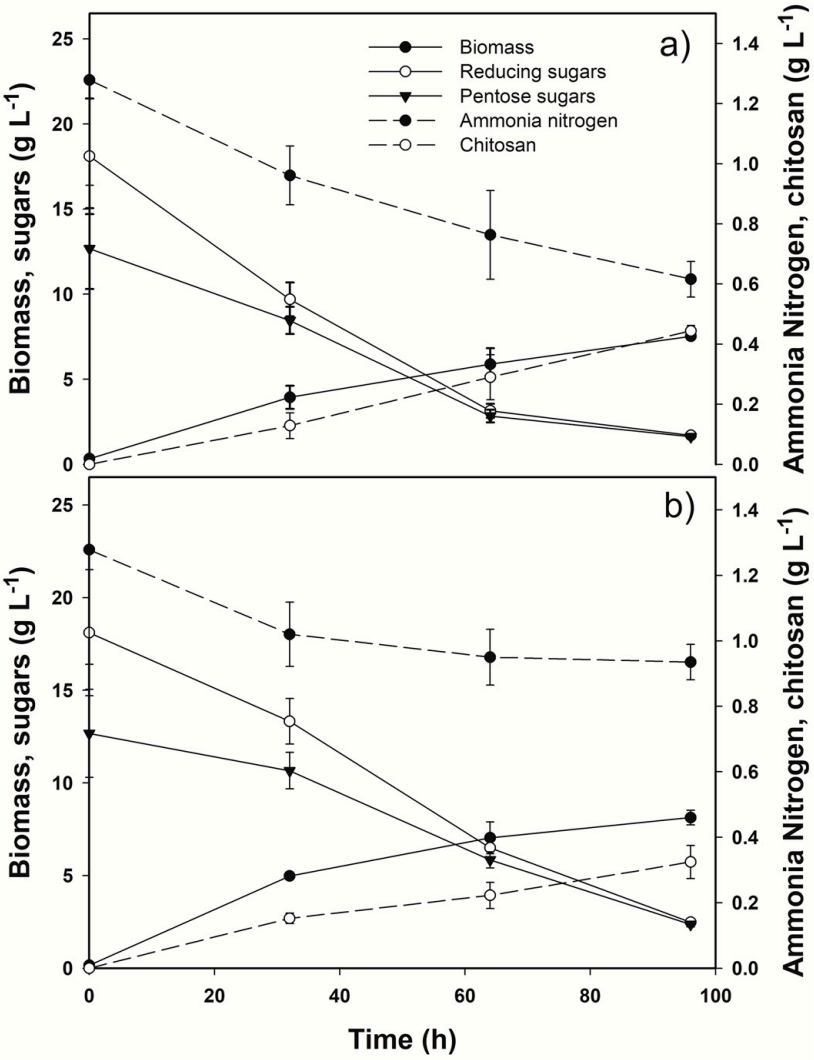


**Figure 3.** Time courses of biomass and chitosan productions and consumptions of reducing sugars, pentoses and ammonia nitrogen in shaken liquid cultures of the selected strains: (**a**) *A. blakesleeana* NRRL 2696 and (**b**) *R. oryzae* NRRL 1510.

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 [28–30]. 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.

Notably, *A. blakesleeana* NRRL 1340 performed well as a chitosan producer in the pioneering study of Rane and Hoover [22], and a subsequent study reported it as a species of great interest to produce chitin and chitosan [31]. Despite these findings, we only found one pertinent study regarding this species, mentioned with its synonym *Lichtheimia blakesleeana* [27], 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 were obtained on glucose-peptone-yeast extract (GPY) media [7,22,32,33], except for the study of Jiang et al. [34], which used a glucose-based medium with added soybean pomace (GBMSP) to investigate the chitosan-producing ability of *A. coerulea* CTCC93105 cultures. Among these studies, *A. coerulea* cultures grown on GPY [33] and GBMSP [34] 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 to which was added (or not) ammonium sulfate [29,35], 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 [36–42]. Among these studies, the best performance was attained by *R. oryzae* MTCC62 and PAS17 cultures grown on deproteinized whey with addition of gibberellic acid [36] and a molasses-based medium [37], respectively.

The most similar medium to the ELP one was that used by Tai et al. [38], which cultivated *R. oryzae* ME-F12 cultures on a corn straw hemicellulose hydrolysate (HH)-based medium, obtained by dilute acid hydrolysis, 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 [38]. 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* [40]. In this respect, although the ELP medium composition resembled that used by Tai et al. [38], 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.

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.

**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 $L^{-1}$) with added 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 | [32] |
| *A. coerulea* ATCC14076 | GPY [e] (10.0–5.0–10.0 g $L^{-1}$) | 1860 | 624 | 300 | [33] |
| *A. glauca* | GPY [e] (20.0–10.0–1.0 g $L^{-1}$) | 650 | 324 | 74 | [7] |
| *A. coerulea* CTCC93105 | Glucose-based medium (20 g $L^{-1}$) with 5% added soybean pomace | 4110 | 684 | 267 | [34] |
| *R. oryzae* NRRL 1510 | ELP [d] | 324 | 81 | 40 | This study |
| *R. oryzae* USBD 0602 | GPY [e] (20.0–10.0–1.0 g $L^{-1}$) with added $(NH_4)_2SO_4$ | 280 | 55 | 49 | [29] |
| *R. oryzae* USBD 0263 | GPY [e] (20.0–10.0–1.0 g $L^{-1}$) with added $(NH_4)_2SO_4$ | 220 | 71.4 | 44 | [29] |
| *R. oryzae* USBD 0602 | GPY [e] (5.0–1.0–0.1 g $L^{-1}$) | 56 | 19 | 49 | [35] |
| *R. oryzae* MTCC262 | DW [f] with added gibberellic acid (0.1 mg $L^{-1}$) | 1130 | 377 | 136 | [36] |
| *R. oryzae* PAS17 | Molasses-based medium (7%) with added $MgSO_4$ | 1500 | 187 | 140 | [37] |
| *R. oryzae* ME-F12 | Corn straw acid hydrolysate | 580 | n. r. | 112 | [38] |
| *R. oryzae* MTCC262 | DW [f] with added $(NH_4)_2HPO_4$ (8 g $L^{-1}$) and yeast extract | 620 | n. r. | 100 | [39] |
| *R. oryzae* NRRL395 | Ground corn grains-based liquid medium (10%) | 406 | 135 | n. r. | [41] |
| *R. oryzae* NRRL395 | Ground rice-based liquid medium (10%) | 700 | 233 | n. r | [41] |
| *R. oryzae* SWERI | Sugar cane molasses | 940 | 118 | 77 | [42] |
| *R. oryzae* SWERI | Sugar beet molasses | 713 | 89 | 65 | [42] |

[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.

### 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 4. 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.

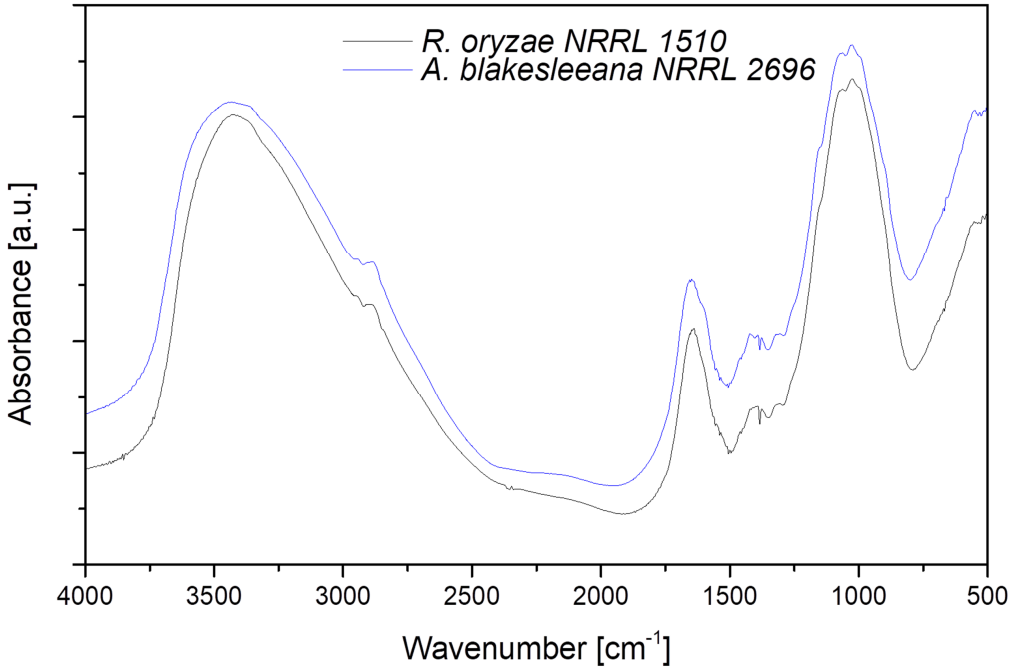


**Figure 4.** Absorbance FT-IR spectra of chitosan samples (*A. blakesleeana* NRRL 2696 in blue, *R. oryzae* NRRL 1510 in black) in the 4000–500 $cm^{-1}$ range collected in transmission mode.

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, which was 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 Equation (4), proposed by Brugnerotto et al. [24], 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. [25] 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.

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].

**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 |

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 [$\eta$], $R^2$ values of the fitting lines and average molecular weight ($M_v$) of chitosan 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 | 19,996 |
| | Kraemer | 0.952 | 61.1 ± 2.1 | 20,030 |
| *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 (LMWChs), a group of polymers which have attracted considerable attention due to their strong bactericidal activities [43], and potential in DNA delivery systems as a carrier [44]. Until now, the primary processes to obtain LMWCh have been thermally assisted acid hydrolysis [45] or biochemical hydrolysis [43] of high-molecular-weight chitosan. Alternatively, Niederhofer and Muller [46] were able to obtain *A. coerulea* LMWCh with average MW of 45 kDa and polydispersity of 2.63 through a radical modification of the extraction procedure. Wang and collaborators [47], 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 fungal chitosan. Figure 5a,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 [48].

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 [27]. The second, more substantial decomposition stage occurred at 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 [49]. 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 , 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 cross-linking reactions or the formation of thermally stable carbonaceous residues [49]. The observed thermal behavior aligns with previous reports on chitosan thermal properties [48,50].

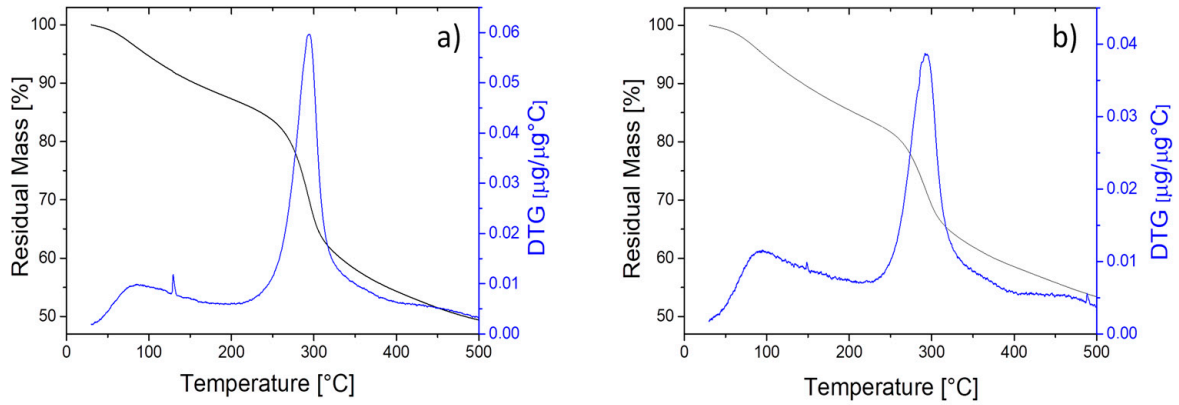


**Figure 5.** Residual mass (black line) and derivative thermal gravimetry (blue line) profiles under nitrogen atmosphere, in the 30–500 °C temperature range, of two chitosan preparations extracted from: (**a**) *A. blakesleeana* NRRL 2696 and (**b**) *R. oryzae* NRRL 1510 cultures.

### *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 $Mg^{2+}$, in binding to phosphate and carboxyl groups commonly found in lipopolysaccharide; this interaction destabilizes the Gram-negative cell wall [51]. However, other studies suggest that chitosan exhibits stronger antimicrobial activity on Gram-positive bacteria than Gram-negative ones [4,52]. In this case, the polycationic nature of chitosan binds to anionic glycerophosphate units of lipoteichoic acids present in the Gram-positive wall, disrupting the structural stability of the cell wall [51]. 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.

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 a general trend is reported where antibacterial activity increases with decreasing molecular weight and increasing DD% [53,54], the su-

perior antimicrobial performance of the commercial chitosan observed here suggests that additional factors influence activity. These include the charge density, which depends on the degree of protonation of amino groups under assay conditions and governs the strength of electrostatic interactions with the negatively charged bacterial surfaces [51]. Fungal chitosans can possess unique structural features and different acetylation patterns compared to commercial chitosans derived from crustaceans, potentially influencing their conformation and biological activities [55]. These combined factors likely contribute to the observed superior antibacterial activity of the commercial low-molecular-weight chitosan despite its higher molecular weight. Our MIC values for fungal chitosans (160–480 mg $L^{-1}$) were substantially lower than those reported for other fungal chitosans (750–2000 mg $L^{-1}$) against the same tested bacterial species [56,57], confirming the relatively strong performance of our samples within the fungal chitosan category. In summary, although MW and DD% are important determinants, the antibacterial effectiveness of chitosan is multifactorial, wherein charge density and acetylation patterns also play significant roles.

**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 |

## 4. Conclusions

This study pioneers the innovative valorization of both liquid and solid fractions of digestate, demonstrating their potential as a 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 (LMWChs) are obtained exceptionally rarely 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 [58]. 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.

**Author Contributions:** Conceptualization, S.C., M.P. and A.D.; methodology, S.C., A.D., A.C. and I.A.; validation, A.D., I.A. and S.C.; formal analysis, A.D.; writing—review and editing, A.D., S.C. and I.A.; investigation, E.C., C.R., F.D.G. and I.A.; visualization, I.A., C.R. and F.D.G.; supervision, M.P.; project administration, M.P.; funding acquisition, M.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was partially supported by the "Network 4 Energy Sustainable Transition—NEST" project (Ministero dell'Istruzione dell'Università e della Ricerca: MIUR project code PE000021, Concession Degree No. 1561 of 11 October 2022) in the framework of the Next Generation EU PNRR plan (CUP C93C22005230007).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The raw data supporting the conclusions of this article will be made available by the authors on request.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## 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 |

## References

1. Kim, S.K.; Rajapakse, N. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydr. Polym.* **2005**, *62*, 357–368. [CrossRef]
2. Elsoud, M.M.A.; El Kady, E.M. Current trends in fungal biosynthesis of chitin and chitosan. *Bull. Natl. Res. Cent.* **2019**, *43*, 43–59. [CrossRef]
3. Crognale, S.; Russo, C.; Petruccioli, M.; D'Annibale, A. Chitosan production by fungi: Current state of knowledge, future opportunities and constraints. *Fermentation* **2022**, *8*, 76. [CrossRef]
4. Dhillon, G.S.; Kaur, S.; Brar, S.K.; Verma, M. Green synthesis approach: Extraction of chitosan from fungus mycelia. *Crit. Rev. Biotechnol.* **2013**, *33*, 379–403. [CrossRef]
5. Chien, R.; Yen, M.; Mau, J. Antimicrobial and antitumor activities of chitosan from shiitake stipes, compared to commercial chitosan from crab shells. *Carbohydr. Polym.* **2016**, *138*, 259–264. [CrossRef]
6. Huq, T.; Khan, A.; Brown, D.; Dhayagude, N.; He, Z.; Ni, Y. Sources, production and commercial applications of fungal chitosan: A review. *J. Biores. Bioprod.* **2022**, *7*, 85–98. [CrossRef]
7. Hu, K.J.; Hu, J.L.; Ho, K.P.; Yeung, K.W. Fungal screening for chitosan producers and copper adsorption capabilities of fungal chitosan and chitosanaceous materials. *Carbohydr. Polym.* **2004**, *58*, 45–52. [CrossRef]
8. Streit, F.; Koch, F.; Laranjeira, M.; Ninow, J.L. Production of fungal chitosan in liquid cultivation using apple pomace as substrate. *Brazil. J. Microbiol.* **2009**, *40*, 20–25. [CrossRef] [PubMed]

9. Cardoso, A.; Lins, C.I.M.; Dos Santos, E.R.; Silva, M.C.F.; Campos-Takaki, G.M. Microbial enhance of chitosan production by *Rhizopus arrhizus* using agroindustrial substrates. *Molecules* **2012**, *17*, 4904–4914. [CrossRef] [PubMed]
10. Berger, L.R.; Stamford, T.C.; Stamford-Arnaud, T.M.; de Oliveira Franco, L.; do Nascimento, A.E.; Cavalcante, H.M.; Macedo, R.O.; de Campos-Takaki, G.M. Effect of corn steep liquor (CSL) and cassava wastewater (CW) on chitin and chitosan production by *Cunninghamella elegans* and their physicochemical characteristics and cytotoxicity. *Molecules* **2014**, *19*, 2771–2792. [CrossRef]
11. Namboodiri, M.M.T.; Pakshirajan, K. Sustainable and green approach of chitosan production from *Penicillium citrinum* biomass using industrial wastewater as a cheap substrate. *J. Environ. Manag.* **2019**, *240*, 431–440. [CrossRef]
12. Chozhavendhan, S.; Karthigadevi, G.; Bharathiraja, B.; Kumar, R.P.; Abo, L.D.; Prabhu, S.V.; Balachandar, R.; Jayakumar, M. Current and prognostic overview on the strategic exploitation of anaerobic digestion and digestate: A review. *Environ. Res.* **2023**, *216*, 114526. [CrossRef]
13. Fuchs, W.; Drosg, B. Assessment of the state of the art of technologies for the processing of digestate residue from anaerobic digesters. *Water Sci. Technol.* **2013**, *67*, 1984–1993. [CrossRef]
14. Carota, E.; Crognale, S.; Russo, C.; Petruccioli, M.; D'Annibale, A. Lignocellulolytic potential of the recently described species *Aspergillus olivimuriae* on different solid wastes. *Appl. Sci.* **2021**, *11*, 5349. [CrossRef]
15. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
16. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
17. Deschatelets, L.; Yu, E.K. A simple pentose assay for biomass conversion studies. *Appl. Microbiol. Biotechnol.* **1986**, *24*, 379–385. [CrossRef]
18. Jüttner, F. Interference with ammonium determination by the indophenol-type reaction of salicylate and dichloroisocyanurate. *Fresen. J. Anal. Chem.* **1999**, *363*, 128–129. [CrossRef]
19. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
20. Sun, S.W.; Lin, Y.C.; Weng, Y.M.; Chen, M.J. Efficiency improvements on ninhydrin method for amino acid quantification. *J. Food Comp. Anal.* **2006**, *19*, 112–117. [CrossRef]
21. Ainsworth, E.; Gillespie, K. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nat. Protoc.* **2007**, *2*, 875–877. [CrossRef]
22. Rane, K.D.; Hoover, D.G. Production of chitosan by fungi. *Food Biotechnol.* **1993**, *7*, 11–33. [CrossRef]
23. *Standard's CLSI Document M07-A9*; Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition. Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2012.
24. Brugnerotto, J.; Lizardi, J.; Goycoolea, F.M.; Argüelles-Monal, W.; Desbrieres, J.; Rinaudo, M. An infrared investigation in relation with chitin and chitosan characterization. *Polymer* **2001**, *42*, 3569–3580. [CrossRef]
25. Dos Santos, Z.M.; Caroni, A.L.P.F.; Pereira, M.R.; Da Silva, D.R.; Fonseca, J.L.C. Determination of deacetylation degree of chitosan: A comparison between conductometric titration and CHN elemental analysis. *Carbohydr. Res.* **2009**, *344*, 2591–2595. [CrossRef]
26. Amitaye, A.N.; Elemike, E.E.; Akpeji, H.B.; Amitaye, E.; Hossain, I.; Mbonu, J.I.; Aziza, A.E. Chitosan: A sustainable biobased material for diverse applications. *J. Environ. Chem. Eng.* **2024**, *12*, 113208. [CrossRef]
27. Berger, L.R.R.; Stamford, T.C.M.; de Oliveira, K.Á.R.; Pessoa, A.D.M.P.; de Lima, M.A.B.; Pintado, M.M.E.; Câmara, M.P.S.; de Oliveira Franco, L.; Magnani, M.; de Souza, E.L. Chitosan produced from Mucorales fungi using agroindustrial by-products and its efficacy to inhibit Colletotrichum species. *Int. J. Biol. Macromol.* **2018**, *108*, 635–641. [CrossRef] [PubMed]
28. Davis, L.L.; Bartnicki-Garcia, S. Chitosan synthesis by the tandem action of chitin synthetase and chitin deacetylase from *Mucor rouxii*. *Biochemistry* **1984**, *23*, 1065–1073. [CrossRef]
29. Tan, S.C.; Tan, T.K.; Wong, S.M.; Khor, E. The chitosan yield of zygomycetes at their optimum harvesting time. *Carbohydr. Polym.* **1996**, *30*, 239–242. [CrossRef]
30. El-Mekawy, A.; El-Baz, A.F.; Soliman, A.E.; Hudson, S. Statistical modeling and optimization of chitosan production from *Absidia coerulea* using response surface methodology. *Curr. Biotechnol.* **2013**, *2*, 125–133. [CrossRef]
31. Campos-Takaki, G.M.; Beakes, G.W.; Dietrich, S.M.C. Electron microscopic X-ray microprobe and cytochemical study of isolated cell walls of mucoralean fungi. *Trans. Brit. Mycol. Soc.* **1983**, *80*, 536–541. [CrossRef]
32. Vaingankar, P.N.; Juvekar, R.A. Fermentative production of mycelial chitosan from zygomycetes: Soil optimization and chemical-physical characterization. *Adv. Biosci. Biotechnol.* **2014**, *5*, 940. [CrossRef]
33. Muzzarelli, R.A.; Ilari, P.; Tarsi, R.; Dubini, B.; Xia, W. Chitosan from *Absidia coerulea*. *Carbohydr. Polym.* **1994**, *25*, 45–50. [CrossRef]
34. Jiang, L.; Pan, S.; Kim, J.M. Influence of nitrogen source on chitosan production by *Absidia coerulea* CTCC AF 93105. *Carbohydr. Polym.* **2011**, *86*, 359–361. [CrossRef]
35. Nadarajah, K.; Kader, J.; Mazmira, M.; Paul, D.C. Production of chitosan by fungi. *Pak. J. Biol. Sci.* **2001**, *4*, 263–265. [CrossRef]
36. Chatterjee, S.; Chatterjee, S.; Chatterjee, B.P.; Guha, A.K. Enhancement of growth and chitosan production by *Rhizopus oryzae* in whey medium by plant growth hormones. *Int. J. Biol. Macromol.* **2008**, *42*, 120–126. [CrossRef] [PubMed]

37. Tasar, O.C.; Erdal, S.; Taskin, M. Production of chitosan by psychrotolerant *Rhizopus oryzae* under non-sterile open fermentation conditions. *Int. J. Biol. Macromol.* **2016**, *89*, 428–433. [CrossRef]
38. Tai, C.; Li, S.; Xu, Q.; Ying, H.; Huang, H.; Ouyang, P. Production of chitosan from maize straw hemicellulose hydrolysate: Impact of degradation products on *Rhizopus oryzae* growth and chitosan fermentation. *Lett. Appl. Microbiol.* **2010**, *51*, 278–284. [CrossRef]
39. Chatterjee, S.; Guha, A.K.; Chatterjee, B.P. Evaluation of the quantity and quality of chitosan products from *Rhizopus oryzae* using processing waste from food, whey and molasses. *J. Environ. Manag.* **2019**, *251*, 109565. [CrossRef] [PubMed]
40. Yang, L.; Li, X.; Lai, C.; Fan, Y.; Ouyang, J.; Yong, Q. Fungal chitosan production using xylose rich of corn stover prehydrolysate by *Rhizopus oryzae*. *Biotechnol. Biotechnol. Equip.* **2017**, *31*, 1160–1166. [CrossRef]
41. Hang, Y.D. Chitosan production from *Rhizopus oryzae* mycelia. *Biotechnol. Lett.* **1990**, *12*, 911–912. [CrossRef]
42. Al-Sharnouby, S.; Abd-Elfatah, S.I. Fermentative production, characterization and antimicrobial activity of chitosan from some zygomycetes fungi. *Egypt. J. Chem.* **2022**, *65*, 1579–1589. [CrossRef]
43. Kittur, F.S.; Kumar, A.B.V.; Tharanathan, R.N. Low molecular weight chitosans preparation by depolymerization with Aspergillus niger pectinase, and characterization. *Carbohydr. Res.* **2003**, *338*, 1283–1290. [CrossRef]
44. Nimesh, S.; Thibault, M.M.; Lavertu, M.; Buschmann, M.D. Enhanced gene delivery mediated by low molecular weight chitosan/DNA complexes: Effect of pH and serum. *Mol. Biotechnol.* **2010**, *46*, 182–196. [CrossRef]
45. Gonçalves, C.; Ferreira, N.; Lourenço, L. Production of low molecular weight chitosan and chitooligosaccharides (COS): A review. *Polymers* **2021**, *13*, 2466. [CrossRef]
46. Niederhofer, A.; Müller, B.W. A method for direct preparation of chitosan with low molecular weight from fungi. *Eur. J. Pharm. Biopharm.* **2004**, *57*, 101–105. [CrossRef] [PubMed]
47. Wang, W.; Du, Y.; Qiu, Y.; Wang, X.; Hu, Y.; Yang, J.; Cai, J.; Kennedy, J.F. A new green technology for direct production of low molecular weight chitosan. *Carbohydr. Polym.* **2008**, *74*, 127–132. [CrossRef]
48. Abdou, E.S.; Nagy, K.S.A.; Elsabee, M.Z. Extraction and characterization of chitin and chitosan from local sources. *Bioresour. Technol.* **2008**, *99*, 1359–1367. [CrossRef] [PubMed]
49. Rao, V.; Johns, J. Thermal behavior of chitosan/natural rubber latex blends TG and DSC analysis. *J. Therm. Anal. Calorim.* **2008**, *92*, 801–806. [CrossRef]
50. Fai, A.E.C.; Stamford, T.C.; Stamford-Arnaud, T.M.; Santa-Cruz, P.D.A.; da Silva, M.C.F.; Campos-Takaki, G.M.; Stamford, T.L. Physico-chemical characteristics and functional properties of chitin and chitosan produced by *Mucor circinelloides* using yam bean as substrate. *Molecules* **2011**, *16*, 7143–7154. [CrossRef]
51. Kong, M.; Chen, X.G.; Xing, K.; Park, H.J. Antimicrobial properties of chitosan and mode of action: A state-of-the-art review. *Int. J. Food Microbiol.* **2010**, *144*, 51–63. [CrossRef]
52. Li, J.; Zhuang, S. Antibacterial activity of chitosan and its derivatives and their interaction mechanism with bacteria: Current state and perspectives. *Eur. Polym. J.* **2020**, *138*, 109984. [CrossRef]
53. Mellegård, H.; Strand, S.P.; Christensen, B.E.; Granum, P.E.; Hardy, S.P. Antibacterial activity of chemically defined chitosans: Influence of molecular weight, degree of acetylation and test organism. *Int. J. Food Microbiol.* **2011**, *148*, 48–54. [CrossRef] [PubMed]
54. Younes, I.; Sellimi, S.; Rinaudo, M.; Jellouli, K.; Nasri, M. Influence of acetylation degree and molecular weight of homogeneous chitosans on antibacterial and antifungal activities. *Int. J. Food Microbiol.* **2014**, *185*, 57–63. [CrossRef] [PubMed]
55. Sahariah, P.; Másson, M. Antimicrobial chitosan and chitosan derivatives: A review of the structure–activity relationship. *Biomacromolecules* **2017**, *18*, 3846–3868. [CrossRef]
56. Tajdini, F.; Amini, M.A.; Nafissi-Varcheh, N.; Faramarzi, M.A. Production, physiochemical and antimicrobial properties of fungal chitosan from *Rhizomucor miehei* and *Mucor racemosus*. *Int. J. Biol. Macromol.* **2010**, *47*, 180–183. [CrossRef]
57. Tayel, A.A.; Moussa, S.; Opwis, K.; Knittel, D.; Schollmeyer, E.; Nickisch-Hartfiel, A. Inhibition of microbial pathogens by fungal chitosan. *Int. J. Biol. Macromol.* **2010**, *47*, 10–14. [CrossRef]
58. Boamah, P.O.; Onumah, J.; Agolisi, M.H.; Idan, F. Application of low molecular weight chitosan in animal nutrition, husbandry, and health: A review. *Carbohydr. Polym. Technol. Appl.* **2023**, *6*, 100329. [CrossRef]


**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.