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XXXV CYCLE

**MICROBIAL VALORIZATION OF WASTE DIGESTATE:
PRODUCTION OF CHITOSAN AND
LIGNOCELLULOLYTIC ENZYMES**

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GENERAL ABSTRACT

This PhD thesis research project aims to perform the microbial upgrading of the digestate, a residue of a biogas plant derived from anaerobic digestion. In particular, this waste has been used as a fungal growth substrate for the production of chitosan and lignocellulolytic enzymes.

In CHAPTER 1, an overall introduction on the microbial valorization of wastes is presented, defining the concept of biorefinery and presenting the characteristics of the digestate and its valorization strategies.

In CHAPTER 2, the lignocellulolytic potential of the recently described species *Aspergillus olivimuriae* on different solid wastes is investigated. The profiles of the glycosyl hydrolases and lignin-modifying enzyme activities of *A. olivimuriae* were determined in solid-state fermentation, carried out on waste from different supply chains, including residual thistle biomass, spent coffee grounds, digestate solid fraction and barley straw. Among the tested substrates, residual thistle biomass was the most conducive to endo- β -1,4-glucanase and cellobiohydrolase productions while the digestate solid fraction was poorly conducive to enzymatic production and the activity levels observed are far from those reported for hyper-producing strains belonging to the same genus. Consequently, the obtained results are not of potential commercial relevance.

For this reason, the research activities have been focused on the use of the digestate as the basis of a liquid medium for selected fungal strains capable of accumulating high levels of chitosan, a polymer of relevant commercial interest.

Besides CHAPTER 3, where a current state of knowledge, future opportunities and constraints on fungal chitosan production has been described, in CHAPTER 4 experimental procedures have been reported.

For the production of chitosan, priority was given to strains of some fungal species belonging to the subdivision Zygomycota, the cell walls of which predominantly contain chitosan. As growth substrate, a liquid medium derived from the digestate was used; in particular, the liquid fraction of the digestate was used as an extractant of the solid fraction, to recover an enriched liquid phase to be used as a substrate; the extraction conditions were optimized in order to maximize the extraction of carbohydrates with a simultaneous low extraction of potential microbial inhibitors.

After an initial screening phase, aimed at testing the growth and chitosan production capacity of the selected strains, the production process was transferred to laboratory

bioreactor level with the most promising strains. The chitosan obtained through microbial bioconversions was analyzed for its physicochemical properties (degree of deacetylation, viscosity, molecular weight) and, in the light of an important application of this polymer, for its antimicrobial properties. Then, the production process was transfer from shaken flask to stirred tank reactor for the most promising strains.

This Ph.D. thesis work ended with a brief conclusion, where final considerations and future perspectives were given and outlined.

CHAPTER 1

Microbial Valorization of Wastes

1.1 Introduction

Millions of tons of waste are generated worldwide every year, making their management and treatment a challenging ordeal (Arneil et al., 2013). This growing burden of waste disposal coupled with natural resource scarcity has renewed interest in the remediation, valorization, and/or repurposing of waste (Lad et al., 2022). Traditional approaches such as composting, anaerobic digestion, use in fertilizers or animal feed, or incineration for energy production extract very little value out of these waste streams (Lad et al. 2022). For this reason, the focus has also been on developing several sustainable technologies that could maximize the value of waste by producing industrially-important chemicals, materials, and fuels that not only reduce our dependency on fossil-based fuels and products but also help in mitigating environmental pollution (Amulya et al., 2016). For example, agricultural waste streams rich in lignocellulosic biomass, such as corn stover, rice and wheat straw, and sugarcane bagasse have been explored for biofuels and specialty chemicals production (Singhvi & Gokhale, 2019), but most industrial and agricultural wastes have received little attention.

This global problem needs an imperative solution where circular bioeconomy can play the major role. The term circular bioeconomy, also known as biobased circular economy, is an integrated concept of circular economy and bioeconomy: it denotes the cascading use of biomass from biological resources into a systemic approach for economic development. A circular bioeconomy offers an efficient utilization of biomass which include wastes and side streams for the sustainable production of high value-added products (Leong et al., 2021).

Most industrial wastes are complex mixtures that are better treated using microbial fermentation as a valorization approach. More specifically, microorganisms are able to metabolize structurally distinct compounds under mild conditions and route them toward a single product, a feature incredibly difficult using purely chemical technologies. Microbial fermentation reroutes carbon toward high-value products, such as pharmaceuticals, surfactants, chemicals, and biodegradable polymers, while detoxifying waste, as depicted in Figure 1, and will therefore be a critical technology for waste valorization and achieving a circular economy (Lad et al., 2022).

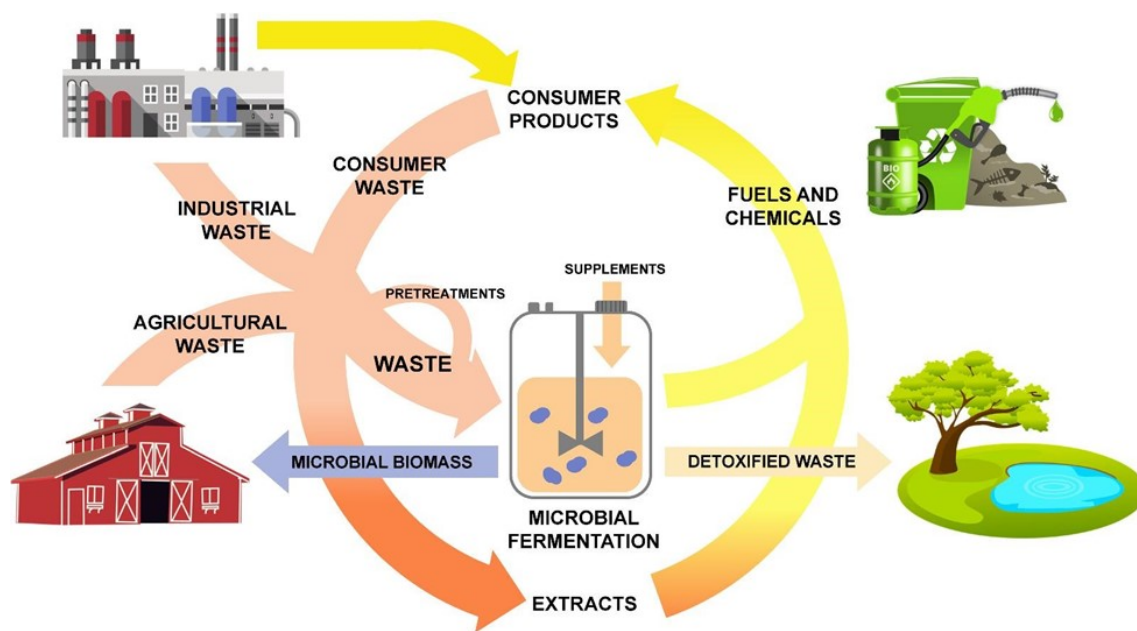


Figure 1 Graphical depiction illustrating the role of microbial fermentation in a circular economy. *Image from Lad et al., 2022.*

In addition to extracting value, microbial valorization also reduces the treatment costs traditionally associated with waste disposal. Recent advances in synthetic biology and metabolic engineering are expanding both the breadth of available waste streams and the uniqueness of downstream products (Lad et al., 2022).

1.2 Biorefinery

The biorefinery concept attempts to apply to biomass conversion the methods that have been applied to the refining of petroleum (Dermibas, 2009).

Biorefining is among one of the most primary facilitating strategies of the bio-based circular economy. It can be defined as the sustainable bioprocesses that efficiently utilize biomass resources for the production of various marketable products and metabolites (e.g., carbohydrates, proteins, lipids, bioactive compounds and biomaterials) (IEA Bioenergy, 2019).

Biomass is by definition the organic matter that derives from living sources and this raw material can be used to produce energy, thus representing an additional income for those who produce it. In fact, according to United Nations Environment Programme (UNEP, 2016), in 2011, 5 billion metric tons of agricultural biomass waste were produced

annually, representing about a quarter of the equivalent of the energy generated by fossil sources (Santos, 2016).

Waste biorefinery represents a better waste management approach (Dahiya et al., 2018; Mishra et al., 2019) and involves establishing a sustainable circular bioeconomy based on the philosophy of recycle, reuse and remanufacture (Carus et al., 2018; Ferreira et al., 2019).

Bioprocesses utilizing waste resources to produce biomaterials and biofuels can greatly elude fossil resources as the production feedstock, preventing the natural resources from complete depletion (Li et al., 2018). Moreover, they address energy and environmental security concerns and are eco-friendly and economically sound platforms as the production feedstock is sustainable and low in cost (Mohan et al., 2016).

Biorefineries are likely capable of producing fuels, chemicals or products, with processing methods that comprise biological, thermo-chemical or physical processes. In the last few years, millions of dollars have been invested in developing biorefineries worldwide. Companies like Alpena Biorefinery (USA), SECIL (Portugal), Steeper Energy (Denmark), Celtic Renewables Ltd (Scotland), UPM (Finland), Enerkem (Canada), Novamont (Italy) and many others are betting on these solutions. Pretty soon, biorefineries will be part of our daily lives (Santos, 2016).

1.3 Digestate

1.3.1 Digestate origin and properties

In the agricultural sector of many European countries, the production of biogas through anaerobic digestion is becoming a rapidly growing market (Monlau et al., 2015), and it is an increasingly applied biotechnology for the energy valorization of biomass and organic waste (Appels et al., 2011; Wang et al., 2013).

Anaerobic digestion is a simple and robust process that involves the degradation and stabilization of a wide range of waste substrates containing high concentrations of readily biodegradable organic matter in the form of carbohydrates, proteins and fats (i.e., industrial and municipal wastewaters and sludge, municipal solid wastes, manures, agricultural residues, energy crops). It converts them into biogas and digestate, the latter corresponding to the anaerobically non-degraded fraction, by a microbial consortium (hydrolytic-fermentative bacteria, fermentative bacteria, acetogenic bacteria and methanogenic archaea). (Appels et al, 2008; Ward et al, 2008; Monlau et al., 2015).

The degradation process occurs in digesters (Figure 2) designed to provide optimal growth conditions for microorganisms. Digesters are classified according to the feeding pattern, the number of stages, the temperature and the fluid-dynamic, which, in turn, depends on the feedstock concentration (Monlau et al., 2015).



Figure 2 Example of anaerobic digestion plant. Image from *Wikipedia*.

The digestate, the byproduct of the anaerobic digestion process, is a mixture of microbial biomass and undigested material. The composition and quality of this byproduct strongly depend on the raw materials used and the treatment conditions applied (Makàdi et al., 2012; Fuchs & Drosch, 2013). Generally, digestate is characterized by slightly-alkaline pH values caused by the degradation of volatile fatty acids, the production of ammonia during the process and the addition of strong bases or carbonates to control the pH and buffering capacity of the system (Ward et al., 2008; Tambone et al., 2010). Organic matter (OM), total organic carbon (TOC) and ash content are highly variable, and other macro-nutrients (i.e., P, K, S, etc.) and trace elements (i.e., Co, Fe, Se, Ni, etc.) can also be found in digestates (Albuquerque et al., 2012; Menardo et al., 2011; Seppälä et al., 2013).

Generally, digestate, produced in large quantities, is mechanically separated, at the farm scale, into liquid and solid fractions (Figure 3) that are stored separately for easy handling and transport (Lukehurst et al., 2010; Bauer et al. 2009). The liquid fraction contains a large part of nitrogen, potassium, phosphorous and minerals (Mehta &

Batstone, 2013). Conversely, the solid fraction contains significant amounts of residual fibres and phosphorous (Liedl et al., 2006).



Figure 3 On the left, solid fraction of digestate; on the right, liquid fraction of digestate.

During the last decades, the use of digestate as a fertilizer or soil amendment has generated great interest (Alburquerque et al., 2012; Tambone et al., 2010). However, improper land application practices, also requiring additional treatments, can lead to ammonia volatilization, pathogen contamination, over-fertilization, and nutrient runoff, sparking considerable debate about the environmental sustainability of this practice (Nkoa, 2014). Furthermore, the increasing number of biogas plants and their densification in some regions could lead to an oversupply of digestate on a local scale. The excess digestate should be transported to distant deficient areas (Rehl et al., 2011): considering transport and distribution costs, the value of the digestate can be close to zero and can even become an expense (Bridgewater & Quested, 2013).

To improve the economic and environmental sustainability of anaerobic digestion, alternative methods for digestate management are desirable (Sheets et al., 2015). Nowadays, there are emerging valorization routes for the utilization of digestate apart from the classical applications that involve: use as culturing media for algal growth, bioconversion into high-added value compounds and conversion into energy by biological and thermochemical processes (Monlau et al., 2015).

1.3.2 Digestate valorization strategies

Figure 4 summarizes the various value-added opportunities arising from the valorization of digestate nutrients. Replacing cost-intensive nutrients with digestate could

improve the bioprocesses' economic feasibility. This option fits in the advanced biorefinery model within the circular bioeconomy scheme by providing new and alternative markets for digestate while increasing the economic incentives for the anaerobic digestion (Kaur et al., 2020).

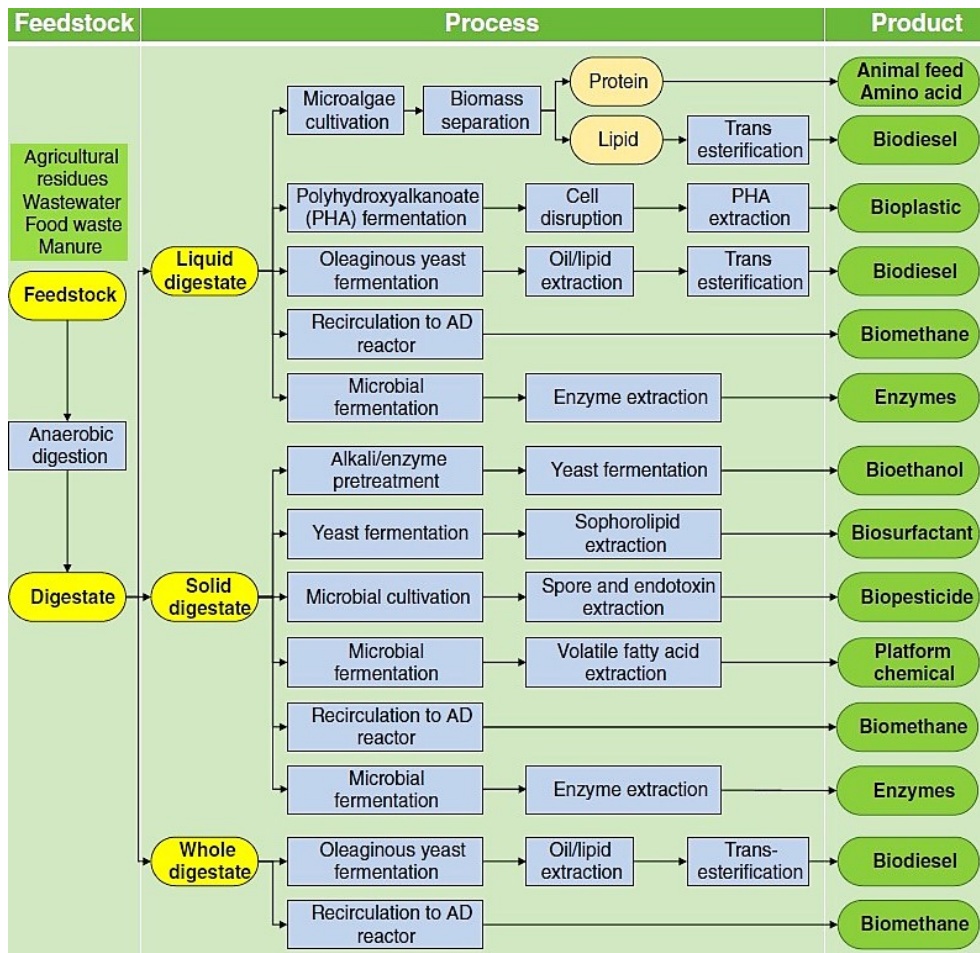


Figure 4 Valorization schemes for production of high-value bioproducts from digestate. *Image from Kaur et al., 2020.*

Digestate liquid fraction can provide water and nutrients in algae production. The combination of algal growth and anaerobic digestion, dating back to the 1950s, was first proposed by Golueke et al. (1959), who suggested that this process would allow the conversion of sunlight into chemical energy. The interest in microalgal cultivation has been driven by the need for alternative feedstocks to reduce the impact of first-generation biofuels on the food commodity market (Monlau et al., 2015). Available data suggest that digestate liquid fraction would be a suitable nutrient supply for algal culturing. However,

growth rates on this media are generally slower than those reached with synthetic culturing media (Fouilland et al., 2014; Sforza et al., 2012).

Bioethanol production through biological fermentation is another promising alternative route to valorizing digestates. In fact, due to its high content in cellulose fibres, solid digestate has recently attracted attention for bioethanol production. However, pre-treatments should be applied because fibres can present physicochemical barriers, such as lignin, that limit carbohydrate availability and degradation (MacLellan et al., 2013; Teater et al., 2011; Wang et al., 2014). In general rules, several studies have shown that digestate produced from an anaerobic digestion process presents several advantages for bioethanol production, as the digestate obtained is generally enriched in cellulose which is easily accessible (Teater et al., 2011; Yue et al., 2010; Yue et al., 2011). For example, Yue and collaborators (2011) observed that bioethanol productions in digestates from two different anaerobic digesters were similar to those in switchgrass bioethanol fermentation (Yue et al., 2010).

Enzymes are other important bioproducts derivable from digestate-based bioconversions. Some commercially-relevant enzymes produced *via* digestate bioconversion include manganese peroxidase and laccase (as ligninolytic enzymes), and β -glucosidase and endo- β -1,4-glucanase (as cellulases). These enzymes have a high market volume and their potential production using inexpensive and readily available feedstock is particularly attractive (Kaur et al., 2020; Santi et al., 2015; Fang et al., 2018).

Thermal conversion (i.e., combustion, hydrothermal carbonization process, pyrolysis) has also been investigated on agricultural anaerobic digestate to produce energy and improve the overall energy efficiency of the process (Hoffmann et al., 2013; Pedrazzi et al., 2015). The products obtained from these processes have several applications and include: hydrochar for energy purposes (Mumme et al., 2011; Funke et al., 2013), pyrochar as a soil amendment (Yao et al., 2011; Pituello et al., 2015), activated carbons for bio-adsorption of heavy metals, pesticides and other toxic organic molecules (Yuan et al., 2011; Wang et al., 2011).

In the future, digestate valorization should reach new horizons by considering energy production through novel biological and/or thermochemical pathways. Given current findings and state-of-the-art, digestate valorization and its integration in a sustainable biorefinery concept will become an important research field in the coming years due to the growing number of anaerobic digestion plants. (Monlau et al., 2015).

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CHAPTER 2

Lignocellulolytic Potential of the Recently Described Species *Aspergillus olivimuriae* on Different Solid Wastes

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2.1 Abstract

The genus *Aspergillus* encompasses several species with relevant lignocellulose-degrading capacity, and a novel species, denominated *A. olivimuriae*, was recently discovered after its isolation from table olive brine. The acquisition of insight into this species and the assessment of its potential relied on a bioinformatics approach, based on the CAZy database, associated with enzymatic activity profiles in solid-state cultures on four different types of waste, including residual thistle biomass (RTB), spent coffee grounds (SCG), digestate solid fraction (DSF) and barley straw (BS). The CAZy analysis of *A. olivimuriae* genome showed that the number of predicted genes for each family was close to that of other *Aspergillus* species, except for cellobiose dehydrogenase, acetyl xylan esterase and polygalacturonases. In *A. olivimuriae* solid-state cultures, hemicellulose degradation outperformed that of cellulose, and lignin removal did not occur, regardless of the growth substrate. This aligns with its CAZy content and the extent of hemicellulolytic, and ligninolytic activities detected in its solid-state cultures. RTB and barley straw were the substrates enabling the best glycosyl hydrolase production levels. The exception was SCG, the hemicellulose composition of which, mainly made of glucomannans and galactomannans, led to the highest β -mannanase and β -mannosidase production levels (3.72 ± 0.20 and 0.90 ± 0.04 IU g⁻¹ substrate, respectively).

2.2 Introduction

The characterization of new isolates and strains from public culture collections is essential for knowledge implementation and to provide the research and biotechnological industry with helpful information for conducting screenings to find microbial strains of

potential interest. There is a global abundance of lignocellulosic residues; for this reason, lignocellulolytic fungi are acquiring an ever-increasing role in biorefinery-based applications aiming to upgrade biomass through the production of value-added products (Troiano et al., 2020). Due to the structural heterogeneity and complexity that characterize plant biomass, fungi must produce a broad set of glycosyl hydrolase activities to break down plant polysaccharides into their monomeric constituents (Culleton et al., 2013; Ma et al., 2021). The Carbohydrate-Active EnZYme database (CAZy; <http://www.cazy.org>; accessed on 8 June 2021) has divided these enzymes into families based on the catalytic modules in their respective amino acid sequences. Several studies suggest that this database might be a robust tool, affording insights into the carbohydrate-degrading aptitude of a given fungal species and supporting the classification and functional prediction of novel genes (Culleton et al., 2013; Cantarel et al., 2009; Coutinho et al., 2009). The CAZy database also enables predicting Auxiliary Activity (AA) families involving lignin-modifying enzymes such as laccases (AA1) and ligninolytic peroxidases (lignin peroxidase, manganese peroxidases and versatile peroxidases) (AA2) (Levasseur et al., 2013).

Several members of the *Aspergillus* genus have a widely recognized capacity to degrade lignocellulose (Troiano et al., 2020; Culleton et al., 2013). In the last two decades, many studies have reported the presence of lignin-modifying enzymes in *Aspergillus* species (Kanayama et al., 2002; Fan et al., 2019).

In recent decades, the significant advances in molecular taxonomy have enabled the discovery of an ever-increasing number of species belonging to the *Aspergillus* genus (Jurjevic et al., 2019; Hubka et al., 2015), and extreme habitats have offered interesting isolation niches (Arzanlou et al., 2016; Martinelli et al., 2017). Recently, table olive brine, a liquid matrix commonly hosting lactic acid bacteria and yeast species capable of tolerating high salinity, offered the opportunity to isolate a new species, which, due to the isolation source, was denominated *A. olivimuriae* (Crognale et al., 2019). Although its whole genome has been sequenced, and the isolate is available from two public culture collections, NRRL 66783 and CCF 6208, this species' physiological information is still scant.

To fill this information gap, this study used a combination of bioinformatics and physiological approaches to predict and evaluate, at different levels, the lignocellulose-degrading potential of *A. olivimuriae*. Since most of the biochemical characterization of members of the CAZy families relies on synthetic (model) substrates (Coutinho et al.,

2009), using complex lignocellulosic matrices might be helpful to characterize the enzymatic potential of fungi, considering the large variability in the substrate specificity of polysaccharide-degrading enzymes.

Consequently, the profiles of the glycosyl hydrolases and lignin-modifying enzyme activities of *A. olivimuriae* were determined in solid-state fermentation, a culture technique simulating the conditions that saprophytic organisms encounter in nature. These fermentations relied on waste from different supply chains, including residual thistle biomass (RTB), spent coffee grounds (SCG), digestate solid fraction (DSF) of anaerobic bioconversions and barley straw (BS) as growth substrates. These matrices share the presence of a significant fraction of macromolecules of the plant cell wall (cellulose, hemicellulose and lignin). This characteristic makes these matrices ideal growth substrates for the expression of enzymes involved in the degradation of these polymers (Singhanian et al., 2009; El-Bakry et al., 2015). Determining fungal growth on the substrates under study and the chemical composition changes in the colonized growth substrates offered the necessary integration between genomic and enzymatic analyses.

2.3 Materials and Methods

2.3.1 Microorganism and Materials

The fungal strain used in the present study was *Aspergillus olivimuriae* 6C2 (NRRL 66783). The strain was maintained and routinely sub-cultured every month on Malt Extract Agar (MEA) slants. The waste residues used in this work, from four different supply chains, were: residual thistle biomass (RTB), spent coffee grounds (SCG), digestate solid fraction (DSF), and barley straw (BS). Expressly, SCG, kindly provided by Prof. Andrea Colantoni (University of Tuscia, Viterbo, Italy), came from the Gedap company (Viterbo, Italy), which deals with automatic coffee distribution. DSF derived from an anaerobic digestion plant, used for biogas production, located near Perugia. RTB, coming from the biorefinery of Porto Torres (Sassari, Italy), is represented by the calathide-deprived epiphytic biomass, from whose seeds the Novamont company extracts dicarboxylic acids used in the production of plasticizers. The barley straw was a kind gift from a farm located in the municipality of Gallese (Viterbo, Italy).

2.3.2. Annotation of Protein-Coding Genes for Holocellulose Degrading Enzymes

To ascertain the presence of putatively coding genes for carbohydrate-active enzymes (CAZymes) and ligninolytic ones, the genome of *A. olivimuriae* 6C2 (Accession number PRJNA498048) was annotated through the dbCAN2 meta-server (<http://bcb.unl.edu/dbCAN2/>; accessed on 8 June 2021) which integrates three tools for the annotation of all CAZymes of a genome: (i) HMMER search against the annotated CAZyme domain boundaries according to the dbCAN HMM (hidden Markov model) database (E-value $< 1e^{-15}$ and coverage > 0.35); (ii) DIAMOND search for blast hits in the CAZy pre-annotated CAZyme sequence database (E-value $< 1.0 \times 10^{-102}$); (iii) Hotpep search for short conserved motifs against the conserved CAZyme short peptide database (hits > 6 and the sum of conserved peptide frequencies > 2.6). The three outputs were combined, and the CAZymes found by only one tool were removed to improve the CAZome annotation accuracy (Zhang et al., 2018). The output generated by the dbCAN2 meta-server is shown in the Supplementary Materials titled “S1_dbCAN2_output_file_A_olivimuriae”. Moreover, to avoid misannotated genes, supplementary manual annotation was carried out, as described by Segato et al. (2014).

The Supplementary Materials titled “S2_Annotations” contains the details related to the manual annotation.

2.3.3. Inoculum Preparation

Six agar plugs (~ 7 mm Ø) from 7-d-old cultures on MEA were transferred into 500-mL Erlenmeyer flasks containing 100 mL of autoclave-sterilized (121 °C, 15 min) malt extract broth (Oxoid, Basingstoke, UK) and then incubated at 28 °C under orbital shaking (150 rpm) for 72 h. The mycelium was recovered by centrifugation at 4 °C (6000 $\times$ g, 15 min), and the pellet thus obtained suspended in physiological solution. The resulting suspension was then homogenized with an Ultra-Turrax T-18 (IKA Labortechnik, Staufen, Germany) at 50 Hz for 6 s.

2.3.4. Solid-State Cultures

Each substrate (20 g) was transferred into a 500-mL Erlenmeyer flask, and its moisture content adjusted to a mass fraction (w_m) of 0.70 using the Mandels and Sternberg’s medium (MSM), except for SCG, the moisture content of which was adjusted to a w_m equal to 0.55. The composition of MSM was (g L⁻¹): peptone, 1.0; (NH₄)₂SO₄, 1.4; KH₂PO₄, 2.0; urea, 0.3; CaCl₂, 0.3; MgSO₄·7H₂O, 0.3; FeSO₄·7H₂O, 0.005;

MnSO₄·H₂O, 0.002; ZnSO₄·7H₂O, 0.001, and COCl₂, 0.002. Moreover, an additional set of amended cultures was prepared by adding wheat bran (WB) to each substrate to yield a mass fraction (w_{WB}) equal to 0.20. Each non-amended and amended culture was inoculated with the homogenized inoculum (5 mL) to yield an inoculum density of 4.5×10^{-3} g mycelium g⁻¹ of solid substrate and incubated in the dark at 28 °C for 14 days under stationary conditions. For each amended and non-amended culture, a non-inoculated control was prepared and incubated as above. Each experiment was performed in duplicate.

2.3.5. Analytical Methods

Before chemical analyses, all the materials underwent grinding on a Cyclotec 1093 mill (Foss Analytics, Hilleroed, Denmark) endowed with a 1-mm sieve to make their granulometry uniform. The determination of the crude lipids was carried out gravimetrically after extracting the solid culture samples and the relative incubation controls (3.0 g) in the Büchi B-811 automated Soxhlet apparatus (Uster, Switzerland) with petroleum ether (boiling range 40-60 °C) for 6 h, as described by Cruz et al. (2012). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined on defatted samples in a Fibertec System M 1020 Tecator (Foss Analytics, Hilleroed, Denmark) using the method of Van Soest et al. (1991). Hemicellulose was calculated as NDF-ADF and cellulose as ADF-ADL (Brozzoli et al., 2010), while the ash content in ADL was subtracted from ADL to calculate Klason lignin. Total phenols were determined both on the liquid derived from the hydraulic pressing of solid-state cultures and the resulting residual solid. In the latter case, the material underwent extraction for 4 h at 50 °C with a water:methanol mixture, at a volume fraction of 0.50, using a solid:liquid ratio of 0.1 g mL⁻¹, before being analyzed with the procedure of Ainsworth and Gillespie (2007), using catechol as the standard. The determination of reducing sugars relied on a 3,5-dinitrosalicylic acid reagent (Miller, 1959). The elemental analyzer for solids Vario MacroCube of Elementar GmbH (Langenselbold, Germany) enabled the determination of the percentage contents in carbon, hydrogen, nitrogen and sulfur and the calibration of the measurements relied on sulfonamide, used as the standard. The ash content was determined after the ignition of samples in a muffle furnace at 550 °C for 8 h. The calculation of the organic matter losses (OML, %) relied on the variations in the ash content recorded in the solid phase cultures (x_{ssc}) compared to the

respective incubation controls (x_{ic}) according to equation suggested by Paredes et al. (2000):

$$\text{OML (\%)} = 100 - \left[\frac{x_{ic} \times (100 - x_{ssc})}{x_{ssc} \times (100 - x_{ic})} \times 100 \right]$$

The degree of colonization in solid-state cultures was inferred from both visual inspections and indirectly through a fungal-specific marker represented by D-glucosamine, a fungal wall polymers component. In particular, acid hydrolysis and deacetylation of the colonized material relied on the procedure described by Scotti et al. (2001), except for the extract's neutralization step, which used solid sodium carbonate. D-glucosamine was spectrophotometrically determined at 530 nm, as reported elsewhere (Sakurai et al., 1977). The same approach was applied to incubation controls to assess the possible presence of D-glucosamine in solid substrates. Soluble protein was determined in the crude extracts derived from the hydraulic pressing of both solid-state cultures and respective incubation controls by the dye-binding method, using bovine serum albumin as the standard (Bradford, 1976). Soluble protein contents in incubation controls were subtracted from those found in the respective solid-state cultures.

2.3.6. Recovery of the Enzymatic Extract from Solid-State Cultures

At the end of the incubation, 50 mL of sodium acetate buffer (0.01 mol L⁻¹) at pH 5.0 were added to each culture, and the system was incubated under orbital shaking (180 rpm) for 20 min on ice. Except for cultures on spent coffee grounds, in which centrifugation at 4 °C (8000×g, 20 min) followed the contact time, the other biomasses were subjected to hydraulic pressing to extract the extracellular enzymes. For this purpose, the material was loaded into an extraction cell (15 cm × 4.0 cm i.d.), and fluids were extracted at 0.68 MPa and 4 °C using a PS-10 press (Ravaglioli, Bologna, Italy). Following extraction, all the extracts underwent sequential filtration through Whatman paper n. 41 and centrifugation at 4 °C (8000×g, 20 min). The alcohol dehydrogenase activity was measured on the obtained extracts as a cytoplasmic marker to verify possible hyphal damage under the extraction conditions, as reported elsewhere (Giovannozzi-Sermanni et al., 1994).

2.3.7. Enzymatic Assays

Filter Paper Units (FPU) and endo- β -1,4-glucanase were determined by the method of Ghose (1987) using Whatman n. 1 paper and 10 g L⁻¹ carboxymethylcellulose (CMC) solution in sodium acetate buffer (0.05 mol L⁻¹) at pH 5.0, respectively. At the end of the incubation at 50 °C, 30 min and 60 min for the former and the latter substrate, Miller's method (1959) determined released the reducing sugars. Cellobiohydrolase, endo- β -1,4-xylanase and β -mannanase activities were determined by incubating appropriate volumes of extracts in 10 g L⁻¹ Avicel, 3.0 g L⁻¹, birchwood xylan and 5.0 g L⁻¹ carob galactomannan solutions at 50 °C for 30 min, in 0.05 mol L⁻¹ sodium acetate buffer pH 5.0, respectively, and determining the amounts of liberated reducing sugars (Wood & Bhat, 1988). β -glucosidase, β -xylosidase and β -mannosidase activities were determined by the method of Meijer et al. (2011), except for the volume of the reaction mixtures (1.0 mL in place of 0.1 mL) and incubation times, which varied in this study between 15 and 30 min. Polymethylgalacturonase (PMG) and polygalacturonase (PG) activities were determined as described by the method of Heerd et al. (2012). Pectinesterase activity was determined by the method of Vilariño et al. (1993). Laccase and Mn-peroxidase activities were determined using a modification of the method of del Pilar Castillo et al. (1997). The assay relies on the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic acid (DMAB) promoted by some phenol-oxidizing enzymes, leading to a purple chromophore which strongly absorbs in the red region. The reaction mixture used to determine laccase activity contained 7.0×10^{-5} mol L⁻¹ MBTH and 1.0×10^{-3} mol L⁻¹ DMAB in malonate buffer (0.05 mol L⁻¹) pH 5.0 and an adequate volume of extract. In the peroxidase assay, the same mixture contained hydrogen peroxide at a final concentration equal to 2.0×10^{-4} mol L⁻¹. The absorbance at 590 nm of the reaction mixture was measured immediately downstream of the addition of the enzyme and after predetermined periods of incubation at 50 °C by calculating the variation in optical density per minute (ODM), and data were converted using an extinction coefficient molar equal to 53,000 L mol⁻¹ cm⁻¹. The peroxidase activity was calculated by subtracting the ODM in the laccase mixture from that measured in the presence of H₂O₂. Regardless of the assay, incubation controls were run in parallel with heat-denatured extracts. Volumetric activities, originally expressed in International Units (IU), defined as the amount of enzyme releasing 1 μ mole of product per cm³ under the assay conditions, were converted into specific activities, referred to either the unit mass of the solid substrate or soluble protein.

2.3.8. Statistical Analysis

Data were analyzed by one-way ANOVA and post-hoc Tukey test at a significance level of 0.05, using the SigmaStat 3 software (Jandel Corp, San Rafael, CA, USA).

2.4 Results and Discussion

2.4.1 Chemical Characterization of the Residues

The first phase of the work focused on characterizing the chemical composition of the four residues and the wheat bran (WB) used in some solid-state cultures as the additive.

The choice of WB was made due to the inclusion, in this investigation, of spent coffee grounds (SCG), a matrix that, owing to its constituent particles' fine granulometry, has low porosity, making gas exchanges problematic. Due to its coarse texture, WB is well-suited to act as a bulking agent capable of increasing the material's overall porosity. Added to this is the fact that the bran has a relatively high nitrogen content (30.1 g kg^{-1}) (Table 1), being mainly composed of protein and amino acids (Baladrán-Quintana et al., 2015). Furthermore, WB contains microelements and cofactors which are essential for growth, such as B vitamins and folates (Bartłomiej et al., 2012). Consequently, this study set up and analyzed solid-state cultures on both non-amended and WB-amended residues to make the experimental design homogeneous.

Table 1 shows that the holocellulosic component, determined by the cellulose and hemicellulose contents' aggregation, constituted a significant fraction of the residues under study, since its mass fraction (w_{HC}) reached values equal to or well above 0.50 of the dry weight. In particular, the highest w_{HC} was found in barley straw, where the aggregate sum of the cellulose and hemicellulose concentrations gave a value equal to about 0.70, followed by RTB, in which the value of this parameter was slightly less than 0.60. Cellulose was the dominant polysaccharide in RTB, barley straw and DSF (405.9 ± 12.3 , 420.6 ± 2.2 and $395.4 \pm 24 \text{ g kg}^{-1}$, respectively). Conversely, SCG exhibited the lowest content of this parameter ($120.2 \pm 3.2 \text{ g kg}^{-1}$), and hemicelluloses were the most abundant polysaccharide fraction ($375 \pm 16.8 \text{ g kg}^{-1}$). The monomeric composition of the hemicelluloses in this matrix consists mainly of mannose, galactose and, to a lesser extent, arabinose residues forming galactomannans and arabinogalactans (Mussatto et al., 2011).

Table 1 Chemical composition of residual thistle biomass (RTB), spent coffee grounds (SCG), digestate solid fraction (DSF), barley straw and wheat bran (WB). The data, expressed as g kg⁻¹ of the dry weight, are the mean $\pm$ standard deviation of 3 determinations. Multiple pair-wise comparisons were made between the row means using the Tukey test; identical letters denote that row means were not significantly different ($p > 0.05$).

Parameter (g kg ⁻¹)	RTB	SCG	DSF	BS	WB †
Hemicellulose	189.2 $\pm$ 3.2 a	375.2 $\pm$ 16.8 c	172.2 $\pm$ 14.9 a	279.3 $\pm$ 3.0 b	187.6 $\pm$ 12.2 c
Cellulose	405.9 $\pm$ 12.1 c	120.2 $\pm$ 3.2 a	393.4 $\pm$ 24.4 c	420.6 $\pm$ 2.2 c	201.1 $\pm$ 24.3 b
Klason lignin	204.2 $\pm$ 4.6 c	201.7 $\pm$ 7.2 c	257.4 $\pm$ 14.2 d	165.2 $\pm$ 4.6 b	73.2 $\pm$ 3.9 a
Crude lipids	20.8 $\pm$ 1.4 a	139.8 $\pm$ 5.4 c	19.3 $\pm$ 2.7 a	16.2 $\pm$ 1.8 a	45.4 $\pm$ 4.3 b
Reducing sugars	3.6 $\pm$ 0.4 a	4.1 $\pm$ 0.2 a	9.2 $\pm$ 0.4 b	41.8 $\pm$ 2.7 c	49.3 $\pm$ 3.2 d
Phenols	17.2 $\pm$ 1.5 b	49.2 $\pm$ 2.4 d	15.3 $\pm$ 2.4 b	21.4 $\pm$ 1.6 c	9.2 $\pm$ 1.7 a
Ash	85.3 $\pm$ 7.0 d	20.4 $\pm$ 2.5 a	82.1 $\pm$ 3.6 d	70.1 $\pm$ 5.3 c	43.6 $\pm$ 2.1 b
Nitrogen	5.9 $\pm$ 0.3 a	22.8 $\pm$ 1.6 c	19.8 $\pm$ 1.1 b	5.4 $\pm$ 0.4 a	30.1 $\pm$ 1.0 d
Carbon	423.4 $\pm$ 8.0 ab	508.9 $\pm$ 11.1 d	415.3 $\pm$ 8.2 a	467.4 $\pm$ 5.4 c	441.2 $\pm$ 4.2 b
Hydrogen	62.1 $\pm$ 2.0 b	75.6 $\pm$ 1.8 c	59.4 $\pm$ 2.1 a	55.2 $\pm$ 4.1 a	69.3 $\pm$ 4.2 c
Sulphur	1.7 $\pm$ 0.0 b	1.0 $\pm$ 0.0 a	2.2 $\pm$ 0.1 b	0.1 $\pm$ 0.0 a	1.8 $\pm$ 0.0 b

† Starch, the content of which was 175.4 g kg⁻¹ in WB, was omitted in this table since this parameter was not detected in the other waste.

Moreover, xylose polymers are absent from this matrix, and this is a particular element that differentiates SCG from the remaining residues (Mussatto et al., 2011; Kwon et al., 2013). In addition to its relatively high hemicellulose content, three additional parameters significantly differentiated SCG from other residues in terms of composition. These were the lower ash content (20.4 ± 2.5 g kg⁻¹) and higher crude lipids (139.8 ± 5.4 g kg⁻¹) and total phenols (49.2 ± 2.4 g kg⁻¹) contents than the other three residues. Crude lipid contents in SCG are very similar to those reported in various studies conducted on this matrix (Cruz et al., 2012; Couto et al., 2009; Burniol-Figols et al., 2016). Concerning the phenolic content in SCG, some studies report concentrations ranging from (17 to 50) g kg⁻¹ (Cruz et al., 2012; Panusa et al., 2013), and the main components of this fraction fall within the family of chlorogenic acids within which the quantitatively most relevant compound is 5-O-caffeoylquinic acid (Burniol-Figols et al., 2016). Among the matrices investigated, the lowest phenolic content was in wheat bran (9.2 ± 1.7 g kg⁻¹), although it is considered a relevant source of hydroxy-substituted cinnamic acids (Stuper-Szablewska et al., 2019; Ferri et al., 2020). However, a significant fraction of these

aromatic acids is esterified to the side chains of arabinose residues in arabinoxylans (Bartłomiej et al., 2012) and, therefore, not present in its free form in WB.

Regarding other WB composition data, Table 1 shows that its holocellulosic fraction amounted, overall, to around 390 g kg^{-1} , in agreement with other works (Krawczyk et al., 2013; Merali et al., 2015). For the same matrix, however, the obtained Klason lignin content ($73.2 \pm 3.9 \text{ g kg}^{-1}$) was significantly lower than those reported by Merali et al. (2012) and Prinsen et al. (2014), and equal to (108 and 121) g kg^{-1} , respectively.

The Klason lignin contents in RTB and SCG were not significantly different from each other, providing values of about 200 g kg^{-1} while, in barley straw, this parameter amounted to $165.2 \pm 2.4 \text{ g kg}^{-1}$. This parameter's highest value, equal to 257 g kg^{-1} , was found in DSF, since stoned olive pomace represents a significant fraction of the feed biomass to the digester from which this matrix derives.

In SCG, the data relating to the total nitrogen content (22.8 g kg^{-1}) and the C/N ratio (22.3), determined by elemental analysis, are also in agreement with a survey conducted by Cruz et al. (2012) on a large variety of spent coffee grounds. From this perspective, the C/N ratios of spent coffee grounds and DSF did not significantly differ. This parameter's highest value, equal to about 86.6, was found in barley straw while, in RTB, it was equal to 71.8.

2.4.2. Fungal Colonization of Solid Substrates and Impact on Their Chemical Composition

One of the significant constraints inherent in solid-state bioconversions, compared to those in the liquid phase, is represented by the impossibility of directly determining the microbial biomass (Musaalbakri et al., 2018). Contrary to the submerged fermentation, in which the fungal biomass is easily separated from the culture medium and determined by gravimetry, this approach is not possible in solid-state conversions on natural substrates. Therefore, it is necessary to resort to indirect approaches based on either molecular or chemical markers (Musaalbakri et al., 2018). The most used chemical “markers” for fungi are ergosterol and D-glucosamine. In the first case, the solid material is subjected to saponification, followed by extraction with n-hexane and quantification of ergosterol by reverse-phase chromatography (Brozzoli et al., 2010). The second parameter's determination requires acid hydrolysis of the material, deacetylation, and quantification of the D-glucosamine, released by chromatography or spectrophotometry (Scotti et al.,

2001). However, D-glucosamine levels vary in fungal species in a range of from 67 to 125 mg g⁻¹ of biomass, depending on the culture's species and age (Singhania et al., 2009; Sharma et al., 1977). Although aware that cultivation conditions can also influence this parameter's levels, we adopted D-glucosamine as an indirect indicator of colonization levels, bearing in mind that the comparisons took place within the same fungal species and between cultures of the same age. We also considered the possible presence of D-glucosamine residues which are sometimes associated with glycoproteins in the lignocellulosic substrates (Singhania et al., 2009). Although this parameter's levels were significantly low in the incubation controls, varying in the range of from 0.03 to 0.18 mg g⁻¹ substrate (data not shown), these were subtracted from those determined in solid-state cultures.

Table 2 shows that, among the non-amended cultures, the lowest growth was observed on barley straw, while the colonization levels did not differ significantly from each other in the remaining substrates, ranging from 0.79 to 0.94 mg D-glucosamine g⁻¹ substrate. On the contrary, an element that emerged from the D-glucosamine data analysis was the stimulating effect of WB on fungal growth in the amended cultures compared to non-amended ones except those carried out on DSF. This stimulation effect was of variable magnitude depending on the substrate. In particular, in solid-state cultures on WB-amended barley straw, the colonization levels increased by a factor of more than four compared to non-amended counterparts (2.99 ± 0.5 vs. 0.70 ± 0.04 mg D-glucosamine g⁻¹), and visual inspections corroborated the observed data. A loose mycelium with a limited extension to the most peripheral layers of the substrate characterized non-amended cultures on barley straw. Conversely, in WB-amended cultures, the mycelium appeared to be more compact, and the colonization affected the entire substrate mass. In WB-amended cultures on RTB and SCG, the factors of increase in D-glucosamine levels compared to homologous non-amended cultures were equal to 2.2 and 2.7, respectively.

In *A. olivimuriae* solid-state cultures, colonization led, compared to incubation controls, to the slight acidification of some matrices, such as in RTB (6.13 ± 0.02 vs. 6.93 ± 0.03) and, to a greater extent, in SCG (4.02 ± 0.05 vs. 5.77 ± 0.04), and this effect was also observed in the respective WB-amended cultures. In those carried out on barley straw, the pH values did not differ significantly from those of the relative incubation control while, on the WB-amended ones, slight acidification took place (6.66 ± 0.02 vs. 6.88 ± 0.01). Although not experimentally demonstrated, the acidification observed in solid-state cultures was presumably due to the production of organic acids, a general

metabolic characteristic among the species belonging to the *Aspergillus* genus that can lead to the substrate pH being lowered by four units (Singhanian et al., 2009; Andersen et al., 2009; Mai et al., 2016).

Table 2 D-glucosamine and extracellular protein (EXP) contents, pH changes, organic matter losses (OML) and yield of fungal biomass per unit of organic matter consumed ($Y_{GLC/S}$) in 14-day-old *A. olivimuriae* solid-state cultures carried out on residual thistle biomass (RTB), spent coffee grounds (SCG), barley straw (BS) and solid fraction of digestate (DSF) in the presence and in the absence of wheat bran (WB). Data are mean $\pm$ standard deviation of 4 replicates. Multiple pair-wise comparisons were made with Tukey's test, and identical letters denote that column means were not statistically different ($p > 0.05$).

Solid-State Cultures on:	D-Glucosamine (mg g ⁻¹)	EXP (mg g ⁻¹)	Δ pH †	OML (%) ‡	$Y_{GLC/S}$ (mg g ⁻¹)
RTB	0.87 $\pm$ 0.05 ab	2.89 $\pm$ 0.24 b	- 0.80 (6.93 $\pm$ 0.03)	12.64 $\pm$ 0.40 c	6.88 $\pm$ 0.50 b
RTB + WB	1.89 $\pm$ 0.07 c	3.49 $\pm$ 0.11 c	- 0.56 (6.43 $\pm$ 0.04)	17.01 $\pm$ 0.60 e	11.11 $\pm$ 0.41 c
SCG	0.94 $\pm$ 0.06 b	2.00 $\pm$ 0.09 a	- 1.75 (5.77 $\pm$ 0.04)	10.77 $\pm$ 1.21 b	8.73 $\pm$ 0.46 b
SCG + WB	2.47 $\pm$ 0.08 d	1.89 $\pm$ 0.12 a	- 0.73 (5.34 $\pm$ 0.02)	14.70 $\pm$ 0.81 d	16.80 $\pm$ 0.54 d
BS	0.70 $\pm$ 0.06 a	5.46 $\pm$ 0.33 d	n. s. * (6.50 $\pm$ 0.04)	8.43 $\pm$ 0.34 a	8.30 $\pm$ 1.30 b
BS + WB	2.99 $\pm$ 0.21 e	5.04 $\pm$ 0.18 d	- 0.17 (6.83 $\pm$ 0.02)	12.24 $\pm$ 0.78 bc	24.44 $\pm$ 1.63 e
DSF	0.79 $\pm$ 0.04 ab	3.68 $\pm$ 0.07 c	+ 0.24 (6.60 $\pm$ 0.05)	17.73 $\pm$ 0.51 e	4.46 $\pm$ 0.49 a
DSF + WB	0.87 $\pm$ 0.03 ab	3.70 $\pm$ 0.38 c	+ 0.64 (6.92 $\pm$ 0.08)	17.03 $\pm$ 0.46 e	5.11 $\pm$ 0.7 ab

† The data in round brackets are the pH values found in the non-inoculated incubation controls; ‡ calculated from the ash content in solid-state cultures and related incubation controls using the equation of Paredes et al. (2002); * n. s., not significant (pH values of solid-state cultures did not significantly differ from the respective incubation controls).

The production of organic acids by these species seems to be a strategy implemented in nature to counteract the antagonistic effect exerted by bacteria (Poulsen et al., 2012). Conversely, on the DSF, the solid-state cultures' pH values were higher than those of the incubation controls, albeit with limited variations. The lack of acidification on this matrix may be due to the known buffering capacity of the digestate (Mejias et al., 2018).

Table 2 also shows the biotic losses of organic matter are attributable to mineralization and calculated based on the ash contents of the solid-phase cultures and the related incubation controls. The amendment's presence increased the extents of organic matter losses in thistle biomass, barley straw and SCG. Conversely, this parameter's values did not significantly differ in non-amended and amended cultures on DSF (17.73 $\pm$ 0.51 and 17.03 $\pm$ 0.46, respectively). The values of the biomass yields ($Y_{GLC/S}$), derived by relating D-glucosamine levels to the unit mass of organic matter

consumed, did not significantly differ in non-supplemented cultures, while the presence of the WB markedly increased this parameter in amended ones.

Table 3 shows the contents in cellulose, hemicellulose, lignin, reducing sugars, crude lipids and total phenols in the incubation controls and the respective cultures inoculated with *A. olivimuriae*. ANOVA was conducted on four homogeneous blocks represented by each substrate to verify the significance of the differences between each parameter's values in the cultures and the incubation controls in both the amended and non-amended cultures. Regarding the changes induced by the fungus on the cell wall polysaccharide contents, the percentage removal values of the hemicelluloses were significantly higher than those of cellulose, with the only exception being represented by cultures grown on WB-amended RTB; under these conditions, cellulose removal significantly increased compared to homologous non-amended cultures (23.5 vs. 12.7%). The percentage of hemicellulose removal varied widely, ranging from 12% in cultures grown on SCG to 59.7% in nonamended DSF ones. WB's general effect in amended cultures was to determine an increase in wall polysaccharides' removal levels compared to those observed in non-amended ones. RTB and DSF were exceptions to this rule, since non-amended cultures outperformed amended ones in hemicellulose removals (34 vs. 26.7% and 59.7 vs. 47.6%, respectively). The presence of WB generally improved the cellulose removal in the amended cultures, even though, in some cases, the extents of these increases were slight. In general, this study observed a higher susceptibility of hemicelluloses to degradation than cellulose, regardless of the substrate and presence or absence of WB. These results are in line with several studies which ascribe the higher recalcitrance of cellulose to biodegradation rather than hemicellulose, due to its partial crystallinity (Singhania et al., 2009; Hatakka et al., 2011).

The increase in reducing sugars observed on solid-state cultures on RTB and SCG, compared to the respective incubation controls, testifies to the partial degradation of the structural polysaccharides, although the concentrations of reducing sugars are the result of the balance between the hydrolysis of the polymers themselves and fungal uptake of the monosaccharides made available by the hydrolysis itself. In these cultures, comparing the yield in reducing sugars released by the hydrolysis of cellulose and hemicelluloses and those found in the extracts, it is evident that *A. olivimuriae* metabolized a high fraction of these (data not shown).

Table 3 shows that, with few exceptions, the Klason lignin contents in the cultures did not differ significantly from their respective incubation controls, apparently

suggesting that *A. olivimuriae* does not have ligninolytic aptitudes, as was widely thought for several years for most *Aspergillus* species (Cardoso et al., 1994). Some studies, conducted in solid-state fermentation and simultaneous saccharification and fermentation with other organisms, suggest that the preferential degradation of cellulose and hemicellulose inevitably leads to a shift in the relative percentage proportions of the wall macromolecules, thus leading to a lignin-enriched residue (Brozzoli et al., 2010; Santos et al., 2015). For this reason, some studies have proposed normalizing the lignin mass fractions to their respective ash contents (Brozzoli et al., 2010; Paredes et al., 2000). However, even after this normalization, no significant lignin removal was evident in *A. olivimuriae* solid-state cultures. In this respect, this species differs from other *Aspergilli*, such as *A. oryzae* (Fan et al., 2019) and *A. flavus* (Hasanin et al., 2019), which have shown ligninolytic potential.

Except for the non-amended cultures grown on DSF and barley straw, total phenol concentrations were significantly higher than those of the respective incubation controls. To explain these results, we could hypothesize the hydrolysis of glucosides by β -glucosidase, leading to the release of phenolic aglycones. Alternatively, we might invoke a partial lignin fragmentation, not translating into a substantial decrease in this parameter but leading to the liberation of phenolic hydroxyls or the release of hydroxycinnamic acids from WB in amended cultures due to the action of *A. olivimuriae* feruloyl esterase.

Crude lipids were minor components in RTB, BS, and DSF, reaching values in the incubation controls lower or slightly higher than 20 g kg^{-1} . The only exceptions were represented by the uncorrected and amended SCG incubation controls, where this parameter amounted to around (139 and 121) g kg^{-1} , undergoing a reduction of 24 and 37.3%, respectively, in solid-state cultures.

Table 3 Contents of cellulose, hemicellulose, Klason lignin, reducing sugars, total phenols and crude lipids in non-inoculated incubation controls (IC) and 14-day-old *A. olivimuriae* solid-state cultures (SSC) on residual thistle biomass (RTB), spent coffee grounds (SCG), barley straw (BS) and digestate solid fraction (DSF) in the absence and in the presence of wheat bran (WB). Data represent the mean $\pm$ standard deviation of 4 replicates. Multiple pairwise comparisons were carried out within each matrix using the Tukey test, and identical letters denote that column means within each block were not significantly different ($p > 0.05$).

Culture	Cellulose (g kg ⁻¹)	Hemicellulose (g kg ⁻¹)	Klason Lignin (g kg ⁻¹)	Reducing Sugars (g kg ⁻¹)	Crude Lipids (g kg ⁻¹)	Total Phenols (g kg ⁻¹)
RTB (IC)	405.6 $\pm$ 12.1 c	187.0 $\pm$ 3.3 c	201.1 $\pm$ 8.0 a	3.3 $\pm$ 0.6 a	20.9 $\pm$ 2.0 b	17.3 $\pm$ 2.3 a
RTB (SSC)	354.2 $\pm$ 4.0 b	121.9 $\pm$ 4.4 a	217.5 $\pm$ 2.2 a	17.7 $\pm$ 2.2 b	14.5 $\pm$ 1.3 a	38.3 $\pm$ 4.2 c
RTB + WB (IC)	365.6 $\pm$ 13.4 b	167.7 $\pm$ 6.2 b	181.2 $\pm$ 14.4 a	3.7 $\pm$ 0.5 a	26.5 $\pm$ 2.1 c	15.6 $\pm$ 3.4 a
RTB + WB (SSC)	279.6 $\pm$ 16.3 a	123.4 $\pm$ 12.1 a	204.8 $\pm$ 19.5 a	16.4 $\pm$ 0.6 b	15.3 $\pm$ 3.2 a	23.8 $\pm$ 2.8 b
SCG (IC)	119.2 $\pm$ 3.0 b	370.2 $\pm$ 16.3 c	199.4 $\pm$ 18.5 b	3.2 $\pm$ 0.0 a	139.0 $\pm$ 6.4 c	48.7 $\pm$ 3.2 b
SCG (SSC)	105.4 $\pm$ 4.4 a	326.3 $\pm$ 18.3 b	240.0 $\pm$ 21.3 c	7.6 $\pm$ 1.1 b	109.2 $\pm$ 4.1 b	56.0 $\pm$ 2.3 c
SCG + WB (IC)	136.2 $\pm$ 3.2 c	337.1 $\pm$ 8.6 bc	171.4 $\pm$ 5.2 a	3.1 $\pm$ 0.5 a	121.2 $\pm$ 8.2 b	40.8 $\pm$ 2.1 a
SCG + WB (SSC)	103.5 $\pm$ 3.1 a	255.4 $\pm$ 21.6 a	176.5 $\pm$ 5.2 ab	9.6 $\pm$ 0.4 c	75.8 $\pm$ 10.3 a	51.5 $\pm$ 2.6 bc
BS (IC)	420.1 $\pm$ 3.0 d	279.5 $\pm$ 11.2 c	165.0 $\pm$ 14.3 a	41.3 $\pm$ 2.8 a	15.6 $\pm$ 1.1 a	20.7 $\pm$ 2.1 ab
BS (SSC)	393.9 $\pm$ 3.2 c	237.0 $\pm$ 9.3 b	172.6 $\pm$ 8.2 a	51.3 $\pm$ 2.0 b	12.0 $\pm$ 1.9 a	27.1 $\pm$ 4.3 b
BS + WB (IC)	376 $\pm$ 8.3 b	262.7 $\pm$ 8.3 c	159.0 $\pm$ 14.8 a	43.4 $\pm$ 4.6 a	21.4 $\pm$ 2.4 b	18.4 $\pm$ 4.2 a
BS + WB (SSC)	339.7 $\pm$ 5.0 a	210.4 $\pm$ 7.6 a	170.6 $\pm$ 13.3 a	40.8 $\pm$ 5.4 a	20.3 $\pm$ 3.0 b	25.4 $\pm$ 3.0 b
DSF (IC)	393.2 $\pm$ 4.6 c	174.1 $\pm$ 12.3 c	257.2 $\pm$ 11.6 bc	10.9 $\pm$ 0.5 b	19.2 $\pm$ 3.1 b	15.6 $\pm$ 1.6 b
DSF (SSC)	363.0 $\pm$ 7.1 b	70.2 $\pm$ 9.1 a	272.0 $\pm$ 8.3 c	4.6 $\pm$ 0.3 a	21.4 $\pm$ 0.2 b	11.9 $\pm$ 2.0 a
DSF + WB (IC)	354.2 $\pm$ 4.1 b	177.3 $\pm$ 8.6 c	220.3 $\pm$ 4.8 a	17.4 $\pm$ 3.1 c	22.4 $\pm$ 3.1 b	14.3 $\pm$ 1.3 ab
DSF + WB (SSC)	321.3 $\pm$ 12.0 a	92.7 $\pm$ 8.3 b	236.1 $\pm$ 5.2 b	4.9 $\pm$ 0.1 a	4.4 $\pm$ 0.2 a	12.9 $\pm$ 1.1 a

2.4.3. Gene Prediction of Holocellulose Degrading Enzymes in *A. olivimuriae*

Genomes of *Aspergillus* species code for a high number of CAZymes involved in the degradation of plant polysaccharides, including cellulose, hemicellulose (xylan, xyloglucan, arabinoxylan and glucomannan) and pectin. Several glycosyl hydrolases (GH) families have been characterized and predicted in *Aspergilli*, along with carbohydrate esterases (CE) and polysaccharide lyases (PL) (Ma et al., 2021).

Different *Aspergillus* species (e.g., *A. niger*, *A. fumigatus*, *A. tamaraii*, *A. nidulans*) showed the occurrence of genes coding for lignin-modifying enzymes (Ma et al., 2021; Rytioja et al., 2014), classified in the family of auxiliary activity enzymes (AA). These include multi-copper oxidases, such as laccases, ferroxidases and laccase-like multi-copper (AA1), heme peroxidases, such as lignin peroxidases, manganese peroxidases and versatile peroxidases (AA2) and, finally, 1,4-benzoquinone reductases (AA6).

Recently, lytic polysaccharide monooxygenases (LPMO) have also received special attention due to their boosting effect on hydrolytic enzymes' activity, suggesting a fundamental role of redox reactions in lignocellulose conversion. In the CAZy database, LPMOs belong to six AA families: AA9, AA10, AA11, AA13, AA14 and AA15, among which, the most widely studied are AA9 and AA10 (Eijsink et al., 2019).

In *A. olivimuriae*, the genome annotation of CAZymes allowed identifying a total of 148 protein-coding genes involved in lignocellulose degradation, belonging to 42 families. Among them, 109 genes were assigned to GH, 6 to CE, 3 to PL and 30 to AA. CAZymes were further subdivided based on their substrate and the family they belong to, as suggested by Lombard et al. (2014), and compared to *Aspergillus* species already characterized for their CAZyme content (Table 4) (Ma et al., 2021).

Table 4 Comparison of predicted number of glycosyl hydrolases (GH), carbohydrate esterases (CE), polysaccharide lyases (PL) and of auxiliary activities (AA) found in the genome of *A. olivimuriae* (*Aol*) (this work), *A. fumigatus* (*Af*), *A. nidulans* (*And*), *A. niger* (*Ang*) and *A. oryzae* (*Aor*).

Substrate	Enzyme Activity	CAZyme Families	Number of Protein-Coding Predicted Genes				
			<i>Aol</i> †	<i>Af</i> ^a	<i>And</i> ^a	<i>Ang</i> ^a	<i>Aor</i> ^a
Cellulose	β-1,4-Endoglucanase	GH3, -5, -6, -7, -9, -12, -45	35 (22)	41	42	36	41
	Cellobiohydrolase	GH7	3 (3)	4	3	2	3
	β-1,4-Glucosidase	GH1, -3	17 (11)	23	23	22	26
	Cellobiose dehydrogenase	AA3, AA8	2 (2)	2	2	2	2
	Lytic polysaccharide monooxygenase	AA9	6 (6)	7	9	8	8
Xylan	β-1,4-Endoxylanase	GH10, -11, -30	6 (5)	8	5	6	8
	β-1,4-Xylosidase	GH3, -43	30 (15)	36	25	30	43
Galactomannan	β-1,4-Endomannanase	GH5	15 (5)	13	15	10	13
	β-1,4-Mannosidase	GH2	6 (2)	6	10	6	7
	β-1,4-Galactosidase	GH2, -35	9 (3)	11	14	11	14
	α-1,4-Galactosidase	GH27, -36	3 (2)	3	4	2	3
	α-Arabinofuranosidase	GH51, -54	3 (3)	3	3	5	4
Hemicellulose	β-1,4-endoglucanase	GH12, -74	3 (3)	6	3	4	4
	α-,β-Arabinofuranosidase	GH51, -54, -127	2 (3)	3	4	2	3
	α-Xylosidase	GH31	10 (1)	n.a.	10 ^b	7 ^b	10 ^b
	α-Fucosidase	GH29, -95	2 (0)	n.a.	3 ^b	3 ^b	3 ^b
	α-1,4-Galactosidase	GH27, -36	3 (2)	3	4	2	3
	β-1,4-Galactosidase	GH2, -35	9 (3)	11	14	11	14
	Arabinofuranohydrolase	GH62	2 (2)	2	2	1	2
	α-,β-Glucuronidase	GH67, -115, -154	4 (2)	2	2	1	5
	α-1,4-Galactosidase	GH27, -36	3 (2)	3	4	2	3
	β-1,4-Galactosidase	GH2, -35	9 (3)	11	14	11	14
Arabinoxylan	Acetyl xylan esterase	CE1, -5	3 (4)	10	7	8	10
	Feruloyl esterase	CE1	1 (1)	5	3	3	5

Substrate	Enzyme Activity	CAZyme Families	Number of Protein-Coding Predicted Genes				
			<i>Aol</i> †	<i>Af</i> ^a	<i>And</i> ^a	<i>Ang</i> ^a	<i>Aor</i> ^a
Pectin	Endo-polygalacturonases	GH28	3 (1)	14	9	22	21
	α -Rhamnosidase	GH78, -106	7 (0)	6	8	8	9
	α -Arabinofuranosidase, Arabinofuranohydrolase	GH51, -54, -62	4 (5)	5	5	6	6
	Endoarabinanase	GH43	14 (5)	18	15	11	20
	Exoarabinanase	GH93	3 (2)	3	2	1	3
	β -1,4-Endogalactanase	GH53	1 (1)	1	1	1	1
	Unsaturated glucuronyl hydrolase	GH88	1 (0)	n.a.	2 ^b	1 ^b	3 ^b
	Unsaturated rhamnogalacturonan hydrolase	GH105	2 (0)	n.a.	3 ^b	2 ^b	4 ^b
	β -1,4-Xylosidase	GH3, -43	30 (15)	36	35	30	43
	β -1,4-Galactosidase	GH2, -35	9 (3)	11	14	11	14
	Pectate lyase	PL1	2 (0)	6	8	7	12
	Rhamnogalacturonan lyase	PL4	1 (0)	3	4	2	4
	Pectin methyl esterase PME	CE8	1 (0)	6	3	3	5
	Rhamnogalacturonan acetyl esterase	CE12	2 (0)	3	2	2	4
Lignin	Feruloyl esterase	CE1	1 (3)	5	3	3	5
	Laccase/p-diphenol:oxygen oxidoreductase	AA1	3 (2)	1 ^c	2 ^b	2 ^c	4 ^b
	Lignin-modifying peroxidases	AA2	2 (0)	3 ^c	0 ^b	2 ^c	0 ^b
	1,4-benzoquinone reductase	AA6	1(1)	0 ^c	0 ^b	2 ^c	1 ^b

^a Data from [55], integrated with ^b Cazy database (<http://www.cazy.org/>; accessed on 8 June 2021); ^c data from [3]. † For *A. olivimuriae*, the number of genes that have been manually annotated is shown between round brackets; the supplementary material titled “Annotations” contains the details related to the manual annotation.

A significant number of enzymes related to cellulose, hemicellulose and pectin degradation was identified (Culleton et al., 2013; Ma et al., 2021). In particular, the number of predicted genes for each family was close to that found in other *Aspergillus* species, except for acetyl xylan esterase and endo-polygalacturonases, which are significantly lower in number in *A. olivimuriae* (Table 4). The most frequently found glycoside hydrolases were endo β -1,4-glucanase (GH3, GH5, GH6, GH7, GH9, GH12, GH45) followed by β -xylosidase (GH3, GH43).

The number of genes related to lignin-modifying enzymes in *A. olivimuriae* was low and CAZy analysis identified 2, 1 and 3 genes coding for laccase, 1,4-benzoquinone reductase and lignin-modifying peroxidases, which, however, is in line with other *Aspergillus* species (Ma et al., 2021).

The presence of putative CAZyme-encoding genes in *A. olivimuriae* genome provides insight into its lignocelluloses-degrading enzyme potential, but cannot be directly related to its real degradation ability. In fact, in several works, it has been observed that the number of genes related to the degradation of a given polysaccharide is not necessarily correlated to the extents of its degradation, since fungal species rely on different strategies. For instance, *Trichoderma reesei* relies on the high production levels of a limited set of glycosyl hydrolases, while the white-rot basidiomycete *Phanerochaete chrysosporium* has a large repertoire of these enzymes (Rytioja et al., 2014). For this reason, CAZy analysis is necessarily associated with functional approaches, such as enzymatic activity assays, and wet chemistry methods, to gain valuable insight into the actual behavior of the species of concern on specific lignocelluloses.

2.4.4. Solid-State Production of *A. olivimuriae* Extracellular Glycosyl Hydrolases and Lyases

As expected from the composition changes in the colonized materials and the CAZy prediction, we detected both glycosyl-hydrolase and lyase activities involved in the depolymerization of plant cell-wall polysaccharides in *A. olivimuriae* solid-state cultures. However, CAZyme production levels varied depending on the residue used and the presence or absence of the amendment.

Regarding cellulolytic enzymes, non-amended and amended cultures on RTB yielded the highest endo- β -1,4-glucanase activities (4.11 ± 0.81 and 4.40 ± 0.69 IU g⁻¹ substrate, respectively) (Figure 1A). The presence of WB significantly increased this activity compared to homologous non-amended ones, but only in cultures on barley straw

(4.40 ± 0.58 vs. 3.20 ± 0.59 IU g⁻¹ substrate, respectively). Amended and non-amended cultures on RTB exhibited the highest cellobiohydrolase activity, and the presence of WB resulted in a significant increase in this activity, but only for SCG cultures (2.51 ± 0.42 vs. 1.51 ± 0.28 IU g⁻¹ substrate) (Figure 1B). As was already observed for endo- β -1,4-glucanase and cellobiohydrolase, among non-amended cultures, those on RTB gave the highest β -glucosidase activity and, in this case, the presence of WB led to a doubling of production levels (2.68 ± 0.38 vs. 1.33 ± 0.15 IU g⁻¹ substrate) (Figure 1C). The total cellulolytic activity was also determined using Whatman filter paper n. 1, a substrate with the merit of the inclusion of amorphous and para-crystalline regions, whose use was proposed by Ghose (1987) and, later on, recommended by the IUPAC. For this reason, the activity of many commercial cellulolytic preparations is expressed in terms of Filter Paper Units (Eveleigh et al., 2009). Figure 1D shows that solid-state cultures on RTB and barley straw yielded the best activity values. The effect of WB was statistically significant in cultures carried out on RTB (2.27 ± 0.40 vs. 1.48 ± 0.34 FPU g⁻¹ substrate), barley straw (1.95 ± 0.26 vs. 2.98 ± 0.60 FPU g⁻¹ substrate) and in those on DSF (0.97 ± 0.12 vs. 0.47 ± 0.03 FPU g⁻¹ substrate) (Figure 1D). Notably, CMCase, FPU and β -glucosidase production levels by *Aspergillus coespitosus* NRRL 1929 and *A. tubingensis* NRRL 4700 solid-state cultures on RTB were very similar to those observed with the strain under study on the same substrate (Crognale et al., 2019). DSF was poorly conducive to cellulase production in line with Mejias' results (2018); in that study, in the solid-state cultures of *T. reseei* ATCC 26921, a well-known cellulase-producing strain, the enzymatic activity never exceeded 1.1 FPU g⁻¹ digestate.

However, *Aspergillus olivimuriae* 6C2 cellulase production levels do not compare with hyper-producing strains belonging to the same genus. To exemplify, *Aspergillus terreus* M11 solid-state cultures on different substrates, such as rape straw, wheat straw, wheat bran and corn stover, reached enzymatic CMCase and FPU activity levels ranging from 122 to 440 IU g⁻¹ and 12 to 198 IU g⁻¹, respectively (Gao et al., 2008). CMCase, β -glucosidase and FPU activities in *A. fumigatus* ABK9 grown on WB-rice straw mixed substrate led to 824.21 and 254.44 and 106.2 IU g⁻¹, respectively (Das et al., 2013).

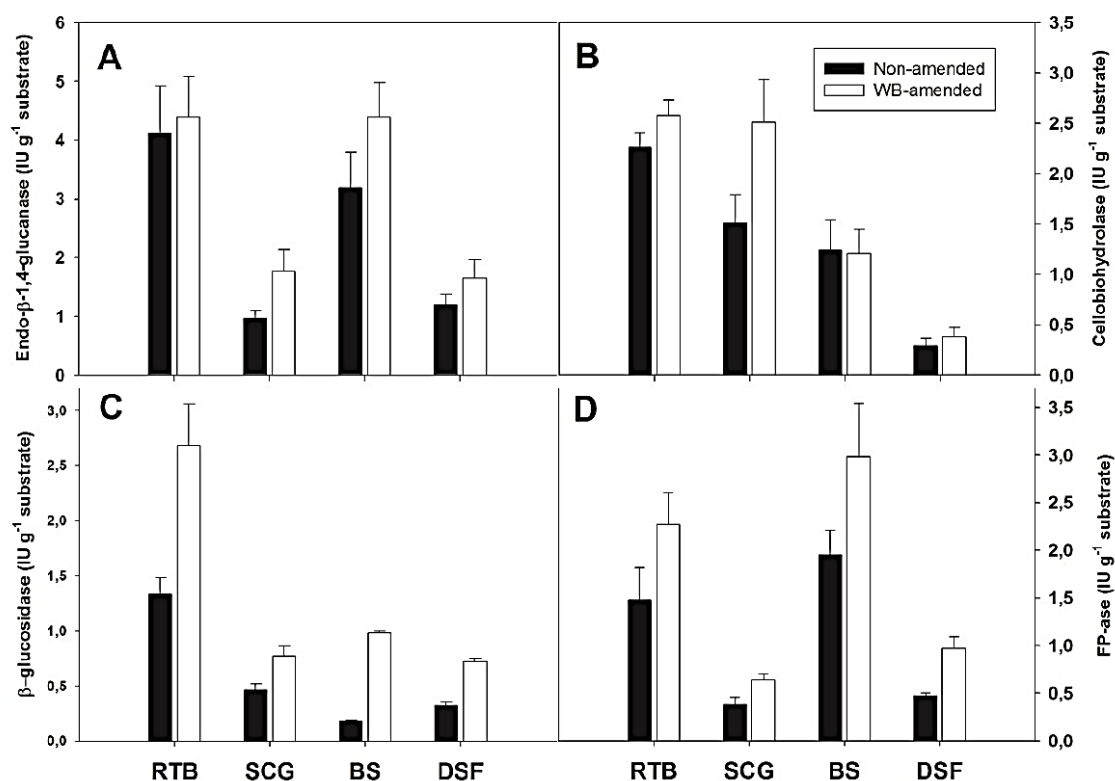


Figure 5 Specific activities, referred to unit mass of initial substrate, of endo-β-1,4-glucanase (A), cellobiohydrolase (B), β-glucosidase (C) and FP-ase (D) in 14-day-old solid state cultures conducted on residual thistle biomass (RTB), spent coffee grounds (SCG), barley straw (BS) and digestate solid fraction (DSF) in the presence and in the absence of wheat bran (WB) as the amendment. Data are the mean $\pm$ standard deviation of four replicates.

Concerning hemicellulases, this study determined the activities of enzymes involved in the hydrolysis of two ubiquitous hemicellulose components, namely xylan and galactomannan. In addition to the endo-β-1,4-xylanase and galactomannase activities, which hydrolyze the β (1→4) glycosidic bonds between adjacent residues within the chain and in a random manner, we determined the activities of two exo-enzymes acting on these two polymers, namely β-xylosidase and β-mannosidase. The former catalyzes the xylobiose hydrolysis and the detachment of xylooligosaccharides from the xylan's nonreducing end. The latter brings about the detachment of mannosyl residues from the mannans' non-reducing end.

Among the non-amended cultures, those on barley straw attained the endo-β-1,4-xylanase activity (7.56 ± 0.79 IU g⁻¹ substrate), but RTB cultures also led to significant activity levels (5.52 ± 1.17 IU g⁻¹ substrate) (Figure 2A). Although the presence of WB increased the activity levels of this enzyme in cultures on barley straw and RTB (8.95 and 6.65 IU g⁻¹ substrate, respectively), the only statistically significant increase occurred on

SCG (3.23 ± 0.76 vs. 1.04 ± 0.18 IU g⁻¹ substrate). It is likely that WB stimulated this enzyme's production, since this amendment brings xylose-based polymers to the growth substrate, whereas the hemicellulose fraction in SCG is devoid of these components (Mussato et al., 2011; Burniol-Figols et al., 2016). On the same growth substrate, the presence of WB also led to a 4.5-fold increase in β -xylosidase activity (Figure 2B). As evidence of the previous considerations about the hemicellulose composition in SCG, the highest β -mannanase activity was found precisely on this matrix (3.72 ± 0.2 IU g⁻¹ substrate) (Figure 2C), thus confirming the adequacy of coffee wastes to support the production of this enzyme by *Aspergillus* species (Favaro et al., 2020). The β -mannosidase activity was also the highest in cultures carried out on non-amended SCG (0.90 ± 0.04 IU g⁻¹ substrate) compared to other cultures (Figure 2D). Regardless of the growth substrate, the amendment's presence did not significantly affect the production of both β -mannanase and β -mannosidase (Figure 2C, D). As was already observed for cellulolytic enzymes, *A. olivimuriae* production levels of hemicellulases on the tested residues were significantly lower than those achieved by solid-state cultures of other *Aspergillus* species on different residues. For instance, de Oliveira Rodrigues et al. (2020) found that *A. niger* SCBM1 and *A. fumigatus* SCBM6, grown individually on a mixture of spent bagasse and WB, produced very high activity levels for both endo- β -1,4-xylanase (2271.7 and 1164.8 IU g⁻¹ substrate, respectively) and β -xylosidase (145 and 2.5 IU g⁻¹ substrate, respectively).

Although none of the used solid substrates is a pectin-rich material, the investigation was also extended to the determination of polymethylgalacturonase (E.C. 3.2.1.15), polygalacturonase (E.C. 3.2.1.67) and pectinesterase (E.C. 3.1.1.11) activities. Polymethylgalacturonase, ambiguously called pectinase, catalyzes the random hydrolysis of α (1 $\rightarrow$ 4) bonds between adjacent residues in pectate and other galacturonans and, depending on the microbial source, has different tolerances to the methyl esterification of galacturonic residues. On the contrary, polygalacturonase is specific to D-homogalacturonan oligomers and cannot degrade unsaturated or methyl esterified substrates. The role of pectinesterase is to facilitate the action of depolymerizing enzymes by hydrolyzing the carboxylic esters present in the D-galacturonic residues of the pectin, producing pectates and methanol.

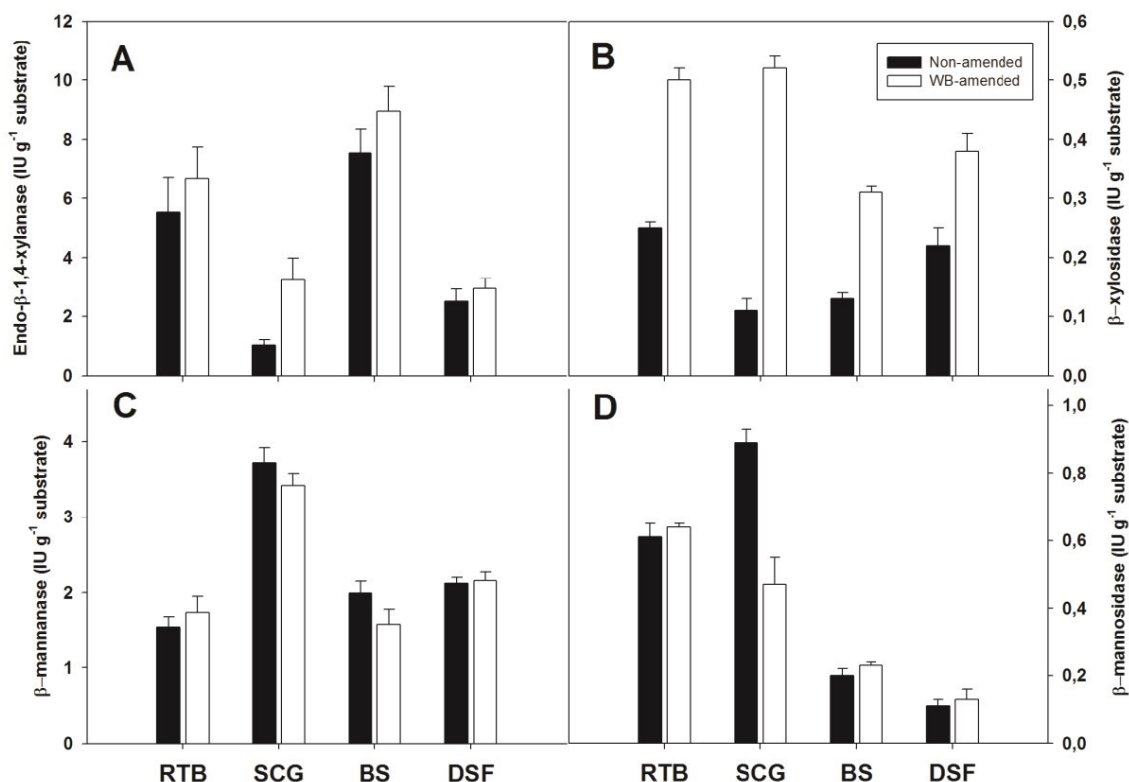


Figure 2 Specific activities, referred to unit mass of initial substrate, of endo-β-1,4-xylanase (A), β-xylosidase (B), β-mannanase (C) and β-mannosidase (D) in 14-day-old solid state cultures conducted on residual thistle biomass (RTB), spent coffee grounds (SCG), barley straw (BS) and digestate solid fraction (DSF) in the presence and in the absence of wheat bran (WB) as the amendment. Data are the mean $\pm$ standard deviation of four replicates.

In addition to these hydrolases' activities, the activities of pectin lyase (EC 4.2.2.10) and pectate lyase (E.C. 4.2.2.2) were also measured. These enzymes break the α (1 $\rightarrow$ 4) bond between D-galacturonic residues adjacent through a β -elimination mechanism that leads to the production at the non-reducing end of one of the two fragments of an unsaturated residue between C4 and C5 (4-deoxy- α -D-galact-4-en-uronosyl groups).

Table 5 shows that, among the non-amended substrates, the best polymethylgalacturonase, polygalacturonase and pectinesterase activities were found in cultures carried out on RTB (2.30 ± 0.18 , 1.27 ± 0.21 and 1.24 ± 0.20 IU g⁻¹ substrate, respectively). Significantly increased polymethylgalacturonase activities, due to the presence of WB compared to the non-amended counterpart, were found on RTB (2.99 ± 0.27 vs. 2.30 ± 0.18 IU g⁻¹ substrate) and barley straw (1.49 ± 0.10 vs. 0.99 ± 0.13 IU g⁻¹ substrate). In the case of polygalacturonase, the only cultures to benefit from the presence of WB were those carried out RTB (1.93 ± 0.09 vs. 1.27 ± 0.21 IU g⁻¹ substrate) and pectinesterase activities (2.16 ± 0.33 vs. 1.24 ± 0.20 IU g⁻¹ substrate). Although

detected in all the solid-state cultures, the lyase activities were significantly lower than those of hydrolases (Table 5).

Table 5 Specific activities, referred to unit mass of the solid substrate, of polymethylgalacturonase (PMG), pectin esterase (PE), polygalacturonase (PG), pectin lyase, and pectate lyase in *A. olivimuriae* solid-state cultures conducted for 14 days at 301.15 K on residual thistle biomass (RTB), spent coffee rounds (SCG), barley straw (BS) e digestate solid fraction (DSF) in the absence and presence of wheat bran (WB). Data are the means $\pm$ standard deviation of 4 replicates. Multiple pair-wise comparisons were made with Tukey's test, and identical letters denote that column means were not statistically different ($p > 0.05$).

Culture	PMG (IU g ⁻¹ substrate)	PE (IU g ⁻¹ substrate)	PG (IU g ⁻¹ substrate)	Pectin Lyase (IU g ⁻¹ substrate)	Pectate Lyase (IU g ⁻¹ substrate)
RTB	2.30 $\pm$ 0.18 c	1.24 $\pm$ 0.20 c	1.27 $\pm$ 0.21 c	0.33 $\pm$ 0.08 bc	0.11 $\pm$ 0.03 a
RTB + WB	2.99 $\pm$ 0.27 d	2.16 $\pm$ 0.33 d	1.93 $\pm$ 0.09 d	0.31 $\pm$ 0.01 b	0.08 $\pm$ 0.01 a
SCG	0.66 $\pm$ 0.05 a	0.39 $\pm$ 0.03 ab	0.54 $\pm$ 0.06 a	0.17 $\pm$ 0.05 a	0.12 $\pm$ 0.06 ab
SCG + WB	0.73 $\pm$ 0.08 a	0.53 $\pm$ 0.08 b	0.56 $\pm$ 0.11 a	0.15 $\pm$ 0.01 a	0.10 $\pm$ 0.02 a
BS	0.99 $\pm$ 0.13 a	0.10 $\pm$ 0.01 a	0.74 $\pm$ 0.11 ab	0.19 $\pm$ 0.04 a	0.20 $\pm$ 0.04 ab
BS + WB	1.49 $\pm$ 0.13 b	0.06 $\pm$ 0.02 a	0.91 $\pm$ 0.11 b	0.31 $\pm$ 0.02 b	0.25 $\pm$ 0.04 b
DSF	0.49 $\pm$ 0.12 a	0.14 $\pm$ 0.05 ab	0.52 $\pm$ 0.07 a	0.19 $\pm$ 0.04 a	0.22 $\pm$ 0.02 b
DSF + WB	0.71 $\pm$ 0.09 b	0.09 $\pm$ 0.02 a	0.73 $\pm$ 0.11 a	0.46 $\pm$ 0.05 c	0.26 $\pm$ 0.04 b

2.4.5. Solid-State Production of Lignin-Modifying Enzymes by *A. olivimuriae*

A further purpose of this work was to verify the presence of ligninolytic enzymes in *A. olivimuriae* 6C2 solid-state cultures and determine their activity. This strain, mistakenly identified in a previous study as *A. fumigatus* 6C2, based on ITS sequences and morphological characters, underwent a battery of agar plate tests that indicated the presence of lignin-modifying enzymes (formation of a brown halo in the Bavendamm's test with tannic acid, decolorization of Poly-R478, formation of ABTS oxidation halos using the underlay agar technique) (Crognale et al., 2012). In the same study, the strain produced detectable laccase and peroxidase activities in the same work, albeit at low levels. Following the correct identification of this species and sequencing of its genome, this study assessed the presence of putative genes coding for ligninolytic enzymes.

Therefore, it was reasonable to expect these enzymes' presence under a cultural condition, such as solid-state fermentation on lignocellulose-based substrates, which reproduces those encountered in natural habitats. As shown in Table 6, activity levels were very low and in line with those found in the previously mentioned work (Crognale et al., 2012). Among the non-amended cultures, the best laccase and peroxidase

production levels were found in RTB and SCG; in these growth substrates, the presence of WB led to significantly increased laccase and peroxidase activities. However, on the tested substrates, the production levels of these enzymes were markedly lower than those observed in solid-state cultures of other *Aspergillus* species, such as *A. fumigatus* SCBM6 and *A. niger* SCBM1 and ATCC 1004 conducted on different residues (de Oliveira Rodrigues et al., 2020; Dos Santos et al., 2016). For instance, solid-state cultures of *A. niger* ATCC 1004, grown on palm cactus husk, yielded laccase activity, which was around 173-fold higher than the best production achieved with *A. olivimuriae* (Dos Santos et al., 2016).

Table 6 Specific activities, referred to unit mass of the solid substrate and extracellular protein, of laccase and peroxidase in *A. olivimuriae* solid-state cultures conducted for 14 days at 301.15 K on residual thistle biomass (RTB), spent coffee rounds (SCG), barley straw (BS) e digestate solid fraction (DSF) in the absence and in the presence of wheat bran (WB). Data are the means $\pm$ standard deviation of 4 replicates. Multiple pair-wise comparisons were made with Tukey's test, and identical letters denote that column means were not statistically different ($p > 0.05$).

Culture	Laccase		Peroxidase	
	(IU g ⁻¹ substrate) ($\times 10^{-3}$)	(IU g ⁻¹ protein) ($\times 10^{-3}$)	(IU g ⁻¹ substrate) ($\times 10^{-3}$)	(IU g ⁻¹ protein) ($\times 10^{-3}$)
RTB	32.8 $\pm$ 0.4 c	11.3 $\pm$ 0.1	30.0 $\pm$ 1.6 b	10.4 $\pm$ 1.3
RTB + WB	49.2 $\pm$ 3.2 d	14.1 $\pm$ 2.5	47.4 $\pm$ 6.9 c	13.6 $\pm$ 1.0
SCG	32.9 $\pm$ 4.1 c	16.5 $\pm$ 0.4	23.6 $\pm$ 3.3 b	11.8 $\pm$ 0.6
SCG + WB	41.3 $\pm$ 3.0 d	21.9 $\pm$ 1.9	27.6 $\pm$ 3.8 b	14.6 $\pm$ 1.8
BS	20.0 $\pm$ 1.6 b	3.6 $\pm$ 0.1	13.8 $\pm$ 2.5 a	2.5 $\pm$ 0.8
BS + WB	18.6 $\pm$ 1.1 b	3.7 $\pm$ 0.2	15.6 $\pm$ 2.3 sb	3.1 $\pm$ 1.0
DSF	2.7 $\pm$ 0.6 a	0.7 $\pm$ 0.1	n. d. ‡	n. d. ‡
DSF + WB	3.6 $\pm$ 0.8 a	1.0 $\pm$ 0.2	n. d. ‡	n. d. ‡

‡ n.d., not detected.

2.5 Conclusions

The integrated use of different procedures made it possible to acquire information regarding the lignocellulolytic attitudes of *Aspergillus olivimuriae*, a recently described species. The choice of using production waste derived from four different supply chains increased the applicative value of this study. For new taxa, providing information of an applicative nature can be helpful to those who want to undertake screening procedures to evaluate changes to specific materials or productions. Among the tested substrates, RTB

was the most conducive to endo- β -1,4-glucanase and cellobiohydrolase productions, due to its high cellulose content, while β -mannanase production was highest in SCG, the hemicellulose of which are mainly composed of glucomannans and galactomannans. In general, the hemicellulolytic attitude of *A. olivimuriae* prevailed over the cellulolytic one, and this behavior was particularly evident in the DSF, where hemicellulose degradation was equal to 60%. Irrespective of the waste used, and as opposed to other *Aspergillus* species, the strain under study did not show ligninolytic potential.

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2.7 Supplementary Materials

The following Supplementary Materials are also available online at: <https://www.mdpi.com/article/10.3390/app11125349/s1>

Excel file, S1_dbCAN2_output_file_A_olivimuriae.xls

Gene ID	HMMER	Hotpep	DIAMOND	Signalp	#ofTools
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1001_5402_6739_+	GH47(47-425)	GH47	GH47	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1011_4756_6756_-	GH71(2-349)+CBM24(390-465)+CBM24(484-560)	GH71+CBM24	CBM24+GH71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1023_16219_17112_+	GT59(1-227)	GT59	GT59	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1026_11928_14108_+	GT2_Chitin_synth_1(301-463)	GT2	GT2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1055_3837_4760_-	GH30_3(64-300)	GH30	GH30_3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1064_3931_5268_+	GH13_31(35-411)	GH13	GH13_40	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1111_311_1582_+	AA11(20-229)	AA11	AA11	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1151_10769_12664_-	GH74(334-428)	CBM1	CBM1+GH74	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1181_4900_5727_+	GH28(17-261)	GH28	GH28	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1186_4876_5745_-	GT2_Chitin_synth_2(1-242)	GT2	GT2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1186_5649_8561_-	GT2_Chitin_synth_2(640-894)	GT2	GT2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1193_14333_14983_-	GH72(1-202)	GH72+CBM43	CBM43+GH72	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1199_16_3870_+	GT2_Chitin_synth_2(642-1149)	GT2	GT2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1201_12885_13991_-	GH130(58-366)	GH130	GH130	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1212_19915_21675_+	GH47(119-583)	GH47	GH47	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1214_5067_7394_+	GH95(3-732)	GH95+CBM13+CBM32+CBM51	GH1+GH95	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1214_7641_9113_+	GH1(11-447)	GH1	GH1+GH95	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1227_12411_14123_+	GH32(14-160)+CBM38(185-302)	GH32+CBM38	CBM38+GH32	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1291_959_2428_+	GH49(31-471)	GH49	GH49	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1293_3855_5651_-	GT20(216-505)	GT20	GT20	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1343_11114_13066_-	AA3_2(176-647)	AA3	AA3_2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1343_7566_8855_+	GH15(21-388)	GH15+CBM20	GH15	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1364_5554_6939_+	GH71(1-384)	GH71+CBM24	CBM24+GH71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1397_155_3274_+	GT1(239-565)	GT1	GT1	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1418_820_2595_-	GH78(66-565)	GH78+CBM67	GH78	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1419_6715_8001_-	CE2(201-422)	CE2+CBM1	CBM1+CE2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1469_7888_9054_+	GH72(2-169)	GH72	GH72	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1508_814_2364_-	GT69(144-390)	GT69	GT69	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1566_3188_4270_-	AA9(9-232)	AA9+CBM1	AA9	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1573_14468_15454_-	GH5_9(93-312)	GH5	GH5_9	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1648_971_2848_-	GH17(371-609)	GH17	GH17	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1901_1977_3917_-	GT3(51-643)	GT3	GT3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_2513_1_1504_+	CBM38(115-232)	GH32+CBM38	CBM38+GH32	N	3

Gene ID	HMMER	Hotpep	DIAMOND	Signalp	#ofTools
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_869_16111_17661_-	GH43_36(23-287)	GH43	GH43_36	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_869_18532_19518_+	GH62(28-298)	GH62+CBM1+CBM13	GH62	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_869_20078_20905_-	CE1(43-169)	CE1+CBM2	CE1	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_871_8422_9438_+	GH5_12(20-331)	GH5	GH5_12	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_874_21788_22990_-	AA1_3(11-154)	AA1	AA1_3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_899_2099_3238_-	AA3_2(3-368)	AA3	AA3_2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_899_697_1413_+	CE9(1-206)	CE9	CE9	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_906_1435_3315_-	GH146(48-563)	GH146+CBM13	GH146	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_912_12230_14179_-	GH18(14-350)	GH18+CBM18	CBM18+GH18	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_920_16940_19618_-	GH18(60-409)	GH18+CBM18+CBM2	CBM18+GH18	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_921_2177_3661_-	GH115(38-474)	GH115	GH115	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_926_1799_4888_-	GH78(434-986)	GH78	GH78	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_938_936_2579_-	GH43_14(30-314)	GH43	GH43_14	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_943_3792_4631_+	GH35(47-241)	GH35	GH35	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_969_4985_6325_+	GH43_29(22-301)+CBM6(322-445)	GH43+CBM2+CBM22+CBM42+CBM6	CBM6+GH43_29	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_989_6776_8560_+	GH49(26-592)	GH49	GH49	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_992_10493_11377_-	GH10(11-285)	GH10+CBM22	GH10	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_994_15338_17233_+	AA3_2(44-627)	AA3	AA3_2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_107_22927_24447_+	GT71(148-383)	GT71	GT71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_110_34939_36135_+	GT76(68-392)	GT76	GT76	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_112_24717_26618_+	GH37(15-604)	GH37	GH37	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_126_38700_39899_-	GH35(1-293)	GH35	GH35	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_139_4680_5570_+	AA3_2(1-289)	AA3	AA3_2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_139_8628_9722_+	GH105(41-361)	GH105	GH105	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_147_19340_20299_+	GH114(84-275)	GH114	GH114	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_149_18164_19069_+	GH18(11-189)	GH18+CBM1+CBM19	CBM1+GH18	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_155_4377_5339_+	GH62(22-290)	GH62+CBM1+CBM13	CBM1+GH62	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_158_26736_27839_-	AA9(8-219)	AA9+CBM14	AA9+CBM1	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_158_49393_50775_-	GH71(13-394)	GH71+CBM24	GH71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_173_1170_3641_-	GH55(2-267)+GH55(402-820)	GH55	GH55	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_174_83447_85131_-	GH7(44-478)+CBM1(528-556)	GH7+CBM1	CBM1+GH7	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_188_1332_2330_+	GT2_Chitin_synth_1(4-86)	GT2	GT2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_188_15469_16503_-	AA9(10-234)	AA9+CBM1	AA9+CBM1	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_188_44109_45098_+	GH93(57-315)	GH93	GH93	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_20_23582_25330_+	GT71(371-552)	GT71	GT71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_215_13947_15608_+	GH127(2-462)	GH127	GH127	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_222_41112_42425_-	GT2_Chitin_synth_2(1-239)	GT2	GT2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_222_42483_46055_-	GT2_Chitin_synth_2(923-1189)	GT2	GT2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_223_12293_13444_-	GT66(7-383)	GT66	GT66	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_226_22960_24294_+	GH2(12-438)	GH2	GH2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_22_35545_38415_+	GT24(653-901)	GT24	GT24	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_235_11952_13613_+	GH81(1-533)	GH81+CBM6	GH81	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_240_23895_29888_-	GH13_22(3-321)+GH13_22(1001-1465)	GT5	GH13_22+GT5	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_240_33067_34110_+	GH13_1(65-338)	CBM20	GH13_1	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_253_1767_3179_-	GH125(83-449)	GH125	GH125	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_264_14143_16860_-	GH2(89-691)	GH2	GH2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_26_25688_27163_-	GH2(11-434)	GH2	GH2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_278_41148_42548_-	GH2(18-463)	GH2	GH2	N	3

Gene ID	HMMER	Hotpep	DIAMOND	Signalp	#ofTools
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_283_24495_26009_-	GT20(177-481)	GT20	GT20	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_283_27592_28389_+	GT20(2-256)	GT20+CBM20	GT20	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_284_14171_15709_-	GT21(6-326)	GT21	GT21	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_28_3947_7765_+	GH32(5-155)+CBM38(185-312)+CBM38(356-470)+GH32(788-1113)	GH32+CBM38+CBM66	GH32	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_302_5690_11674_-			GH13_22+GT5	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_319_7745_8728_-	GH43_1(5-324)	GH43	GH43_1	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_327_34243_36993_-	CBM67(135-316)+GH78(338-868)	GH78+CBM67	GH78	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_333_2931_4133_+	GT4(195-365)	GT4	GT4	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_334_5607_10703_-	GT48(735-1514)	GT48	GT48	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_345_3486_5486_-	GH31(259-646)	GH31	GH31	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_351_737_2716_-	GT90(357-648)	GT90	GT90	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_358_58848_60863_-	AA3_2(36-653)	AA3	AA3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_361_27749_30277_-	GH67(23-693)	GH67	GH67	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_363_2236_3672_-	GT57(15-454)	GT57	GT57	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_379_2335_3243_-	GT15(84-290)	GT15	GT15	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_382_20771_21589_-	GH3(95-257)	GH3	GH3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_38_13740_18161_+	GH18(499-842)	GH18+CBM18+CBM5+CBM55	CBM18+CBM50+GH18	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_403_2538_4292_+	GH43_14(34-316)			N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_418_28520_29227_-	GH76(2-222)	GH76	GH76	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_421_3324_6386_-	GH2(45-903)	GH2+CBM32+CBM67	GH2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_423_7012_8490_-	GT71(130-361)	GT71	GT71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_431_5819_6511_+	GH92(2-218)	GH92	GH92	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_434_8390_10183_-	GH92(261-596)	GH92	GH92	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_437_1_1061_+	GT62(53-317)	GT62	GT62	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_45_13615_14256_-	GH43_26(10-195)	GH43	GH43_26	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_45_33492_35837_+	GH3(124-326)	GH3	GH3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_467_45897_47318_+	GT22(4-222)	GT22	GT22	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_469_327_1928_-	GT4(256-458)	GT4	GT4	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_473_35396_36703_+	GH76(1-373)	GH76	GH76	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_509_13966_15255_-	GT71(206-387)	GT71	GT71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_51_11639_13039_+	GH72(2-263)+CBM43(312-396)	GH72+CBM43	CBM43+GH72	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_51_36804_37895_+	GH31(2-293)	GH31	GH31	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_520_30109_30720_+	AA13(2-202)	AA13+CBM20	AA13	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_522_5360_7054_-	GH54(86-401)+CBM42(418-559)	GH54+CBM13+CBM42	CBM42+GH54	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_529_13116_14759_+	GH31(234-533)	GH31	GH31	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_530_713_2884_-	GH3(2-122)	GH3	GH3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_533_46247_47662_-	GH7(30-399)+CBM1(439-467)	GH7+AA9+CBM1	CBM1+GH7	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_546_12263_13999_-	GH13_40(31-391)	GH13	GH13_40	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_548_19485_21977_-	GT20(117-520)	GT20	GT20	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_557_8053_10431_+	GH3(2-201)	GH3+CBM1	GH3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_564_9737_10660_-	AA13(2-164)+CBM20(206-296)	AA13+CBM20	AA13+CBM20	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_567_254_1117_-	GH13_8(106-232)	GH13+CBM48	CBM48+GH13_8	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_567_3128_4045_-	GH28(66-303)	GH28	GH28	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_567_9646_10392_-	GH92(1-248)	GH92	GH92	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_571_28262_29707_+	GT71(117-342)	GT71	GT71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_575_18931_21408_-	GH38(219-477)	GH38	GH38	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_583_1169_3574_-	GH3(94-343)	GH3	GH3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_583_8310_9266_+	GT4(42-174)	GT4	GT4	N	3

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66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_588_25160_29479_-	GH13_25(973-1415)	GH133	GH13_25+GH133	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_599_15031_16869_+	AA1_2(44-382)	AA1	AA1_2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_611_3398_5212_-	GH29(22-394)	GH29+CBM13	GH29	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_612_23625_25613_-	GH3(9-210)	GH3	GH3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_613_19469_20992_-	AA3_2(1-499)	AA3	AA3_2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_632_10106_12670_+	GH47(217-854)	GH47	GH47	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_633_27109_28971_+	GH76(138-507)	GH0	GH0	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_63_49335_50084_+	GT1(16-191)	GT1	GT1	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_63_8472_9641_-	PL4_3(2-374)	PL4	PL4_3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_661_3844_5961_+	GH20(166-501)	GH20	GH20	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_665_4882_7410_+	AA8(8-727)	AA3+AA8+CBM1	AA3_1+AA8	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_670_17941_18930_-	GH18(107-271)	CBM18	CBM18+GH18	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_695_113_1567_+	AA3_2(120-480)	AA3	AA3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_702_5064_5852_+	CBM1	GH3(2-85)	GH3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_702_9462_11024_-	GH36(6-501)	GH36	GH36	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_704_32408_34108_+	AA3_2(139-562)	AA3	AA3_2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_725_20426_22201_-	GH3(2-140)	GH3	GH3	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_727_1334_2542_+	GH71(3-352)	GH71+CBM24	GH71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_735_54692_57160_-	GH92(309-816)	GH92	GH92	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_73_22668_24695_-	GH31(80-527)	GH31	GH31	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_743_6150_7412_+	GH55(5-416)	GH55	GH55	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_748_19467_20738_+	GH7(24-415)	GH7+CBM1	GH7	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_756_24609_27431_-	PL26(40-932)	PL26	PL26	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_756_2746_3876_+	CE15(19-345)	CE15+CBM1	CE15	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_766_8451_9122_-	GT15(7-213)	GT15	GT15	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_772_21887_23035_-	GT35(1-356)	GT35	GT35	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_774_6434_7801_-	GT71(190-372)	GT71	GT71	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_790_446_2170_-	GH43_11(42-332)	GH43	GH43_11	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_791_7021_7866_-	GT33(1-276)	GT33	GT33	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_803_9354_11156_+	GH20(175-544)	GH20	GH20	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_817_44056_44964_-	GH51(2-300)	GH51	GH51	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_817_8238_9422_+	CE8(54-357)	CE8	CE8	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_832_15813_17195_-	GT22(10-318)	GT22	GT22	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_843_10205_11530_+	GT50(149-364)	GT50	GT50	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_85_30903_33281_-	CBM67(29-204)+GH78(233-754)	GH78	GH78	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_85_5174_6316_-	GT32(113-193)	GT32	GT32	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_861_34277_35647_+	AA3_2(14-417)	AA3	AA3_2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_862_279_2534_-	GH65(398-671)	GH65+CBM32	GH65	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_866_26877_29003_-	CBM21(319-427)	CBM21	CBM21	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_89_21439_23016_+	AA3_2(9-510)	AA3	AA3_2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_89_40605_42179_-	GT2_Chitin_synth_1(139-302)+GT2_Chitin_synth_2(280-484)	GT2	GT2	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_8_18700_19776_+	GH31(1-357)	GH31	GH31	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_91_12382_13071_-	GT8(19-164)	GT8	GT8	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_91_16880_18709_-	GT90(327-592)	GT90	GT90	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_96_1569_4277_-	GT1(496-896)	GT1	GT1	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_9_74732_75640_-	GH47(1-282)	GH47	GH47	N	3
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1023_15003_15983_+	GT59(94-297)	GT59	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001](single)_contig_1032_7804_8391_+	GT34(24-169)	GT34	N	N	2

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66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1044_6931_7812_-	AA9(1-119)	AA9+CBM18	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1055_3232_3834_-	GH30_3(2-197)	GH30	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1110_13254_14057_+	GH128(50-256)	GH128	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1123_8693_10480_-	GT39(2-193)	N	GT39	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1145_6017_6364_-	GT15(5-115)	GT15	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1151_9443_9955_+	CE5(60-150)	CE5+CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1179_3362_4336_+	AA3_2(14-310)	AA3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1185_15871_16731_+	AA3_2(1-282)	AA3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1186_18803_19647_-	GH17(43-277)	N	GH17	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1227_5865_7463_-	GH32(1-306)	N	GH32	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1305_9302_9811_+	AA1_3(8-145)	AA1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1319_1108_1584_-	GT8(19-124)	GT8	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1371_3023_3406_-	CBM63(40-109)	CBM63	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1402_1_962_-	GH43_6(47-315)	N	GH43_6	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1432_27_686_-	GH12(87-218)	GH12	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1433_490_1176_-	GT34(9-188)	GT34	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1448_3962_4849_+	GT2_Glyco_tranf_2_3(38-278)	GT2	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1512_1_510_+	GH93(12-146)	GH93	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1516_3855_4436_+	GH5_4(9-188)	GH5	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1516_4494_5453_+	CBM46(136-222)	GH5+CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1625_2497_4599_+	GH47(8-204)	N	GH47	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1646_890_3481_+	GH3(24-249)	N	GH3	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1650_364_1092_+	GT62(69-239)	GT62	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1677_869_2155_+	GH28(49-410)	N	GH28	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1772_9497_10147_-	GH43_30(1-203)	N	GH43_30	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1779_3041_4012_-	GT90(59-289)	N	GT0	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1782_442_1515_+	GH3(86-311)	GH3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1801_4858_5844_+	GH5_5(1-147)	GH5+CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1811_673_1467_-	AA9(9-225)	AA9	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1878_4089_4888_+	AA11(18-211)	AA11	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2013_1_685_-	GH43_36(21-228)	GH43	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2149_687_1571_-	AA16(20-189)	AA16	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2169_873_1439_-	GH43_34(1-188)	GH43	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2242_2254_2919_+	GH76(16-188)	GH76	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_881_5899_6774_+	GH72(1-151)	GH72+CBM43	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_892_15821_17107_+	GT4(208-376)	N	GT4	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_960_5199_6074_-	GT109(40-279)	N	GT109	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_969_27971_28693_+	GH76(2-222)	GH76	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_969_3356_3889_-	GH27(1-144)	GH27	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1693_6883_8370_+	N	GH0	GH0	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1693_8637_9386_+	N	GH0	GH0	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1962_96_743_+	N	GH13+CBM48	CBM48+GH13_8	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1212_12547_13581_-	N	GH3	GH3	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1269_6042_7832_-	N	GH3+CBM6	GH3	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_943_4752_5684_+	N	GH35	GH35	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_920_23437_24329_+	N	GH55	CBM50+GH55	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1188_3902_5944_+	N	GH55	GH55	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1250_1806_3734_+	N	GT2	GT2	N	2

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66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1293_2726_4009_-	N	GT20	GT20	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1014_17088_18530_+	N	GT31	GT31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_983_527_2140_+	N	GT31	GT31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1101_11420_15775_-	N	GT41	GT41	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_106_12933_13664_-	GT62(30-199)	GT62	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_108_50651_51019_+	GH3(1-122)	GH3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_10_1042_1842_-	GH16_18(68-223)	N	GH16_18	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_139_3896_4600_+	AA3_2(2-216)	AA3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_13_18928_19335_-	AA6(1-120)	AA6	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_142_18029_18745_-	GH93(12-210)	GH93	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_144_28239_29078_-	AA11(20-227)	AA11	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_144_30454_31077_+	GH18(21-201)	GH18	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_147_14305_16497_+	CBM87(111-328)+CE18(329-680)	N	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_147_24512_25135_-	GH135(51-180)	GH135	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_149_8035_8634_+	GH128(1-196)	GH128	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_155_14804_15316_+	GH43_6(2-154)	GH43	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_155_30154_30405_-	PL1_7(1-68)	PL1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_174_80798_81547_-	GH5_5(1-147)+CBM1(217-245)	GH5+CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_176_47023_48099_-	GH55(2-357)	N	GH55	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_176_58552_59055_+	GH3(1-147)	GH3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_178_22373_23707_+	AA3_2(2-429)	N	AA3_2	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_180_24254_24985_-	GH5_7(62-230)	GH5+CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_182_23205_24119_+	AA3_2(6-264)	AA3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_182_24127_25041_+	AA3_2(1-287)	N	AA3_2	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_195_4524_5183_+	GH13_5(3-201)	GH13	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_201_7082_7963_+	GT69(59-195)	GT69	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_202_2932_3378_+	CE3(1-125)	CE3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_204_25694_26560_-	GH32(6-164)	GH32	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_208_24251_24817_-	GH92(1-167)	GH92	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_20_2388_3371_-	CE4(2-126)	CE4+CBM18	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_21_16441_17739_-	GT109(8-418)	N	GT109	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_231_36741_37616_+	GH31(1-172)	N	GH31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_238_5635_6120_+	GH154(21-161)	GH154	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_244_39599_40330_-	GH5_7(58-228)	GH5+CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_266_15895_16704_+	GH78(2-225)	N	GH78	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_282_2913_3260_+	GH11(1-113)	GH11+CBM1+CBM2+CBM36+CBM6+CBM60	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_309_1626_3863_-	GH31(254-732)	N	GH31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_309_26995_28596_+	GH79(39-383)	N	GH79	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_318_12174_13331_+	GH5_23(78-357)	N	GH5_23	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_329_3433_3957_-	GT39(1-161)	GT39	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_333_8321_8830_-	GH53(7-159)	GH53	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_334_18300_18941_+	GH72(1-205)	GH72+CBM43	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_334_829_1839_-	GH17(90-334)	GH17	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_337_39558_40358_+	GT1(5-203)	GT1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_33_54728_55123_+	PL3_2(31-116)	PL3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_340_46942_47526_+	GH18(14-187)	GH18	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_354_4468_5604_-	GH13_40(15-194)	N	GH13_40	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_357_29889_31637_-	CE3(3-210)	CE0	N	N	2

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66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_362_448_1455_-	GH5_27(5-224)	N	GH5_27	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_381_40719_41474_+	GH71(62-241)	GH71+CBM24	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_382_2082_2621_+	GH18(2-165)	GH18	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_388_10978_11766_+	GT1(6-214)	GT1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_400_2041_3711_+	GT90(292-554)	N	GT90	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_406_2726_3688_-	GH18(12-256)	N	GH18	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_414_20291_21427_+	GT22(32-249)	GT22	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_425_3060_3647_-	GH18(24-165)	GH18+CBM18	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_427_6328_6789_+	GH25(1-135)	GH25	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_434_7684_8292_-	GH92(1-175)	GH92	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_451_8024_8896_+	GH31(1-167)	N	GH31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_457_13999_15315_+	AA3_2(2-434)	N	AA3_2	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_464_17042_18328_+	GH106(11-421)	N	GH106	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_464_25637_26032_+	GH43_24(1-124)	CBM35	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_471_21507_21884_+	PL3_2(30-118)	PL3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_482_126900_127682_+	GH5_7(79-248)	GH5+CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_485_33286_34092_+	GH5_7(3-144)	GH5	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_491_103751_104446_+	GH15(1-183)	GH15+CBM20	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_491_104470_105117_+	CBM20(14-204)	GH15+CBM20	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_504_37731_38318_-	GH71(1-171)	GH71+CBM24	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_519_30696_31229_-	GH88(9-166)	N	GH88	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_519_67155_67622_-	GH43_13(2-134)	GH43	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_519_6966_7850_-	GH132(126-290)	GH132	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_524_23610_25529_+	GH16_1(87-320)	N	GH16_1	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_524_50939_51355_-	CE5(22-131)	CE5	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_537_7457_8419_+	AA2(53-283)	N	AA2	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_544_30374_30835_-	GH76(1-147)	GH76	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_562_5579_6892_+	GH43_14(9-202)	GH43	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_578_6794_7429_-	CE16(2-202)	CE0	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_615_16722_17135_-	AA11(1-124)	AA11	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_630_2367_3032_+	AA2(2-205)	N	AA2	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_636_15689_16450_-	CE12(27-220)	CE12	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_63_26141_26539_-	GH3(2-103)	GH3	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_649_37918_39237_-	GT2_Glyco_tranf_2_3(46-292)	N	GT2	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_661_26383_26961_+	PL1_7(39-131)	PL1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_661_6772_7458_+	CE16(2-191)	CE16	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_665_2227_2862_+	GH72(28-201)	GH72	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_665_2885_3652_+	GH72(3-130)	GH72	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_680_2651_2998_+	GH11(1-112)	GH11	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_702_4420_5067_+	GH3(77-184)	CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_729_3630_4235_-	GT58(3-165)	GT58	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_729_4253_4780_-	GT58(1-173)	GT58	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_741_1295_2428_+	GH5_23(79-359)	N	GH5_23	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_751_7373_8212_-	CE12(53-257)	CE12	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_774_14829_15089_+	GH11(1-84)	GH11+CBM36+CBM60	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_785_42157_42828_+	GH105(37-201)	GH105	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_79_1457_1948_+	GH16_19(62-146)	CBM18	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_811_7706_8392_+	GH18(15-188)	GH18	N	N	2

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66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_820_36035_36547_-	GH5_5(1-147)	GH5+CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_826_12990_13607_+	AA9(5-189)	AA9+CBM1	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_833_8886_9488_+	GT69(59-198)	GT69	N	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_91_32545_33693_+	GH5_49(2-249)	N	GH5_49	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_98_8574_10436_-	GT2_Chitin_synth_2(265-556)	N	GT2	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_9_75697_76419_-	GH47(7-222)	N	GH47	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_238_6613_7764_+	N	GH154	GH154	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_843_2214_3074_-	N	GH2	GH2	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_63_24800_26092_-	N	GH3+CBM1	GH3	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_8_17652_18773_+	N	GH31	GH31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_4_44530_47112_-	N	GH36	GH36	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_270_89795_90721_-	N	GH63	GH63	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_522_8006_10465_-	N	GH74	GH74	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_266_14553_15536_+	N	GH78	GH78	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_485_24855_25706_-	N	AA3+AA8+CBM1	AA3_1+AA8	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_283_23140_24498_-	N	GT20	GT20	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_156_30593_31939_-	N	GT31	GT31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_7_17586_19250_+	N	GT31	GT31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_172_40252_41610_-	N	GT31	GT31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_701_29638_31065_-	N	GT31	GT31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_248_15666_16889_+	N	GT31	GT31	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_360_33161_34573_-	N	GT8	GT8	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_108_52059_52871_+	N	CBM1	GH3	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_551_745_1806_-	N	CBM13	CBM1	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_272_6456_8384_-	N	CBM18	CBM18	N	2
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1028_20913_21401_-	GH16_18(2-149)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1035_7207_7740_+	CE3(16-123)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1055_867_1361_-	GH16_10(33-147)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1100_17590_18828_+	AA7(58-254)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1118_4604_5803_+	GH16_18(66-222)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1178_3111_3533_+	CE5(18-106)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1178_3588_3839_+	CE5(7-81)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1193_13604_14089_-	CBM43(7-92)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1194_3072_3581_-	GH13_40(23-169)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1200_8624_9643_-	GT8(73-299)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1215_20499_21524_-	CE4(125-228)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1250_1290_1715_+	GT2_Chitin_synth_1(38-123)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1261_4787_5848_+	GH43_6(96-341)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1278_2444_3172_-	AA7(40-209)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1281_1280_2332_-	AA7(45-230)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1364_7002_7748_+	CBM24(37-109)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1418_2640_3293_-	CBM67(19-154)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1499_11369_11603_-	GH12(1-76)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1504_4780_5406_+	GH18(2-195)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1512_836_1212_+	GH93(2-120)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1664_1020_2003_+	AA7(2-310)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1682_392_1093_-	CE8(50-210)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1819_620_1111_+	GH5_31(25-149)	N	N	N	1

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66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2036_1_1020_+	AA7(1-329)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2092_77_513_-	GH16_1(1-110)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2161_1_573_+	GT109(10-187)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2171_1_425_-	GH32(3-138)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2196_369_1436_+	AA7(19-226)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2600_112_554_+	GH5_31(1-128)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_869_3720_5486_+	AA4(116-316)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_896_18937_19278_-	CE4(10-100)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_948_6394_7131_+	GT109(19-236)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_953_5106_5435_-	GH16_1(1-87)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_953_5470_6030_-	GH16_1(32-173)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_960_6055_6618_-	GT109(21-186)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1151_8622_8894_+	N	CE0	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1035_6639_7184_+	N	CE3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1682_1_327_-	N	CE8	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_920_20819_21754_+	N	GH0+GH55	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1577_1259_1940_-	N	GH12	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1194_3550_4095_-	N	GH13	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1298_1658_1906_+	N	GH132	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1542_5031_5192_+	N	GH17	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1316_406_1169_+	N	GH17	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1605_4342_4695_+	N	GH18	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_888_16177_16707_-	N	GH2	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_939_3718_4167_-	N	GH2+CBM42	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1181_4443_4859_+	N	GH28	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_885_11577_11930_+	N	GH31	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1227_11952_12404_+	N	GH32	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1227_10684_11235_-	N	GH32	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_879_7341_7598_-	N	GH35	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2290_1_743_+	N	GH35	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1536_1842_2678_-	N	GH43	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1625_1331_1720_+	N	GH47	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1625_1778_2332_+	N	GH47	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1516_3578_3868_+	N	GH5+CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_881_5542_5874_+	N	GH72	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1151_13274_13402_-	N	GH74+CBM1+CBM2	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1578_4164_4358_-	N	GH75	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1534_510_998_-	N	GH76	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_969_28830_29312_+	N	GH76	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1418_4238_4555_-	N	GH79	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1418_3654_4121_-	N	GH79	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_899_3390_3866_-	N	AA3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1776_112_345_+	N	PL1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1145_5364_5804_-	N	GT15	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1147_1598_1936_-	N	GT32	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1801_902_1645_+	N	GT32	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1433_1208_1411_-	N	GT34	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1101_10780_11415_-	N	GT41	N	N	1

Gene ID	HMMER	Hotpep	DIAMOND	Signalp	#ofTools
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1650_1205_1561_+	N	GT62	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1319_1592_1873_-	N	GT8	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_869_14220_14366_-	N	CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1151_12753_13175_-	N	CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1239_426_2159_-	N	CBM57	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1779_5336_10159_-	N	CBM57	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1278_3709_6372_-	N	CBM57	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_883_9398_10900_-	N	N	GH55	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_881_11298_13508_+	N	N	GH72	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_895_10699_13485_+	N	N	GH6	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_895_25708_26547_-	N	N	GH152	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_908_4917_6536_+	N	N	GT8	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_879_4524_6347_-	N	N	GH35	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_921_593_1582_-	N	N	GH115	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_926_1_1217_+	N	N	PL7_4	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_920_22485_23477_+	N	N	CBM50+GH55	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_965_404_2050_+	N	N	GT31	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_993_1_4021_-	N	N	CBM48+GH13_8	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1015_5358_6698_-	N	N	GT39	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1026_14141_15070_+	N	N	GT2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_970_49499_50476_+	N	N	GT1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1038_10342_14604_+	N	N	CBM48+GH13_8	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1041_12028_13149_-	N	N	GH76	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1041_13684_14478_-	N	N	GH76	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1034_1256_3850_-	N	N	GH2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1156_3156_4418_+	N	N	GH2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1145_491_3046_-	N	N	CE1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1151_1_1885_+	N	N	GT31	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1217_468_4085_-	N	N	GT7	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1189_1199_2761_+	N	N	GT57	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1189_2800_3882_+	N	N	GT57	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1211_8645_10135_-	N	N	GH27	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1229_1588_4014_+	N	N	GH51	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1352_11104_12924_-	N	N	GT22	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1234_13048_15087_-	N	N	AA3_2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1333_617_2257_+	N	N	GH47	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1305_9808_10926_+	N	N	AA1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1300_1509_4412_+	N	N	GH30_7	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1566_419_1381_-	N	N	AA9	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1430_10490_12220_+	N	N	GH31	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1595_3308_7813_+	N	N	CBM48+GH13_8	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1567_2409_3266_-	N	N	GT22	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1911_1_1061_+	N	N	GH1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_2001_214_2628_-	N	N	CBM12	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1875_892_2793_+	N	N	GH31	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1915_8133_8909_+	N	N	GH76	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_1915_9371_10522_+	N	N	GH76	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_125_10304_11560_+	AA7(2-412)	N	N	N	1

Gene ID	HMMER	Hotpep	DIAMOND	Signalp	#ofTools
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_129_17933_19393_-	AA7(49-465)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_133_53308_53658_-	AA9(36-115)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_133_53567_53953_-	AA9(8-114)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_188_948_1328_+	GT2_Chitin_synth_1(18-104)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_226_27449_27703_-	GH3(1-82)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_227_403_855_+	GH76(1-145)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_240_35890_36585_+	GH13_5(21-216)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_260_12323_13495_+	AA7(2-205)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_281_27674_28399_+	GH47(1-238)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_283_37265_38158_-	GH5_23(5-268)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_300_92374_92673_+	GH114(2-97)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_303_9957_10415_+	GH16_2(1-145)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_334_19018_19659_+	CBM43(56-144)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_341_13913_14635_+	GH76(1-229)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_359_6238_7209_-	AA3_2(1-274)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_388_16329_16808_+	GT4(6-90)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_431_6536_7102_+	GH92(1-168)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_470_7940_8623_-	GH5_9(8-197)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_471_6944_7564_-	GH76(5-185)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_475_10133_11641_-	AA7(36-252)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_504_38377_39273_-	GH71(83-297)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_520_32714_32959_+	CE5(6-81)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_520_67496_68038_+	GT109(14-179)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_520_67924_68841_+	GT109(54-293)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_544_32607_33620_+	GH16_18(80-187)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_592_13185_13427_+	PL1_4(2-70)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_5_84910_85794_-	AA7(17-223)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_60_29461_29916_+	CE8(46-151)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_650_4301_4816_-	CE3(2-101)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_661_18662_19825_-	AA7(2-211)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_662_3645_4094_+	PL1_2(6-110)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_670_26584_27204_-	GH16_18(2-60)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_692_15196_15747_+	PL1_4(1-94)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_728_1098_1580_-	GT4(7-91)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_735_36073_36372_-	GH131(2-95)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_759_20717_22240_+	AA7(27-489)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_785_42940_43533_+	GH105(1-130)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_79_1924_2745_+	GH16_19(15-101)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_820_6471_7034_-	GH43_6(6-162)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_834_10193_10906_+	GT34(29-139)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_836_11714_13477_+	AA7(32-210)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_861_48892_49626_+	GT2_Glycos_transf_2(9-193)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_89_23633_24595_-	AA7(1-310)	N	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_645_17009_17701_-	N	CE0	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_471_25549_25773_+	N	CE0	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_357_31679_31975_-	N	CE0	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_83_52319_52765_-	N	CE1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_83_52795_52968_-	N	CE1	N	N	1

Gene ID	HMMER	Hotpep	DIAMOND	Signalp	#ofTools
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_661_6419_6634_+	N	CE16	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_544_34756_35052_-	N	CE4+CBM18	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_662_3198_3563_+	N	CE8+PL1+CBM6	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_464_15157_15618_+	N	GH106	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_282_2404_2832_+	N	GH11+CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_680_2317_2577_+	N	GH11	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_240_35633_35878_+	N	GH13	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_195_4122_4487_+	N	GH13	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_354_5788_6198_-	N	GH13	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_567_33_314_-	N	GH13+CBM48	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_491_103275_103568_+	N	GH15+CBM20	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_601_2110_2837_+	N	GH17	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_340_46285_46731_+	N	GH18	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_382_1497_1775_+	N	GH18	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_144_29904_30296_+	N	GH18	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_527_35554_35967_-	N	GH18+CBM18	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_278_1309_1605_-	N	GH18+CBM12+CBM5	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_73_15635_16294_-	N	GH18+CBM1+CBM19	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_463_25218_25445_+	N	GH18+CBM18	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_843_1576_2094_-	N	GH2	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_26_24670_25650_-	N	GH2	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_278_40681_41244_-	N	GH2	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_427_5996_6247_+	N	GH25	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_46_38209_38601_+	N	GH28	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_816_3497_3778_-	N	GH28	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_348_10188_10697_-	N	GH28	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_348_9910_10146_-	N	GH28	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_360_11210_11665_-	N	GH28	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_567_2271_3047_-	N	GH28	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_702_6461_6877_+	N	GH3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_231_34883_35551_+	N	GH31	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_451_6222_6848_+	N	GH31	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_28_3299_3898_+	N	GH32+CBM38	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_204_27016_27231_-	N	GH32	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_215_9022_9324_+	N	GH39	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_45_14281_14676_-	N	GH43	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_519_65872_66615_-	N	GH43	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_281_26748_27539_+	N	GH47	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_485_32881_33222_+	N	GH5+CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_485_32483_32884_+	N	GH5	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_817_45494_45727_-	N	GH51	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_400_16216_16512_+	N	GH51	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_333_9163_9498_-	N	GH53	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_176_48187_49044_-	N	GH55	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_656_2310_2903_-	N	GH55	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_743_5265_5693_+	N	GH55	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_520_39097_39384_+	N	GH6+CBM1+CBM2	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_817_55673_56008_+	N	GH6+CBM1+CBM2	N	N	1

Gene ID	HMMER	Hotpep	DIAMOND	Signalp	#ofTools
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_520_39628_39861_+	N	GH6+CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_270_90803_91123_-	N	GH63	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_520_80520_81485_-	N	GH65+CBM32	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_353_39442_39867_+	N	GH7+CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_519_31409_32452_-	N	GH88	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_208_26057_26719_-	N	GH92	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_567_9042_9497_-	N	GH92	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_240_14026_14355_+	N	AA1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_240_14369_14545_+	N	AA1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_471_15498_15881_+	N	AA3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_485_23271_23453_-	N	AA3+AA8	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_485_24165_24779_-	N	AA3+AA8+CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_128_15244_15531_+	N	AA3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_613_21135_21458_-	N	AA3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_463_70056_70664_-	N	AA3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_463_69814_69990_-	N	AA3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_463_70686_71156_-	N	AA3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_89_23018_23233_+	N	AA3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_457_15409_15819_+	N	AA3	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_592_12240_12602_+	N	PL1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_63_9931_10617_-	N	PL4	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_456_6304_6456_-	N	PL9	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_158_20615_21232_-	N	GT1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_388_10039_10674_+	N	GT1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_63_48647_48919_+	N	GT1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_358_28222_28632_-	N	GT1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_158_21804_22442_-	N	GT1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_379_1910_2374_-	N	GT15	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_17_4351_4755_+	N	GT2	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_283_28417_28845_+	N	GT20	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_421_18641_18988_-	N	GT20	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_525_19714_20250_+	N	GT20	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_421_17677_17964_-	N	GT20	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_625_23616_23966_-	N	GT22	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_414_21397_22224_+	N	GT22	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_360_43075_44148_-	N	GT31	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_791_8276_8533_-	N	GT33	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_583_7945_8205_+	N	GT4	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_363_3627_3818_-	N	GT57	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_396_2839_3222_-	N	GT57	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_656_11191_12651_-	N	GT61	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_832_2775_4268_+	N	GT61	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_106_12634_12936_-	N	GT62	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_223_13431_14000_-	N	GT66	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_833_9864_10280_+	N	GT69	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_201_7985_8416_+	N	GT69	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_833_9485_9769_+	N	GT69	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_774_14279_14773_+	N	CBM1	N	N	1

Gene ID	HMMER	Hotpep	DIAMOND	Signalp	#ofTools
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_482_127871_128194_+	N	CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_702_6069_6440_+	N	CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_244_40406_40615_-	N	CBM1	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_743_19266_20275_+	N	CBM14	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_278_650_1126_-	N	CBM18	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_670_17619_18074_-	N	CBM18	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_464_25148_25627_+	N	CBM35	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_464_26848_27045_+	N	CBM35	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_107_19657_20289_-	N	CBM48	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_107_20320_21111_-	N	CBM48	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_524_1650_3410_+	N	CBM57	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_223_21002_21655_+	N	CBM57	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_469_2684_7390_-	N	CBM57	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_226_22260_22910_+	N	CBM67	N	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_22_34305_35177_+	N	N	GT24	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_37_17017_19323_-	N	N	CBM50	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_31_59137_69669_+	N	N	GT2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_15_14246_15049_+	N	N	GT1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_15_15494_16441_+	N	N	GT1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_44_36432_38834_-	N	N	GH18	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_51_28966_34500_+	N	N	GH23	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_51_35723_36400_+	N	N	GH31	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_59_3720_4727_-	N	N	GH31	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_51_69061_70341_-	N	N	GH125	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_60_25863_27881_+	N	N	GH18	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_79_13365_15800_-	N	N	GH12	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_73_6655_8364_+	N	N	GT22	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_147_3391_11880_-	N	N	GT4	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_96_4093_5565_-	N	N	GT1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_108_51081_51986_+	N	N	GH3	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_157_678_2210_-	N	N	GH1+GH95	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_120_38238_39770_-	N	N	GH55	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_130_495_2000_+	N	N	GT4	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_149_1_1053_+	N	N	AA0	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_126_37074_38210_-	N	N	GH35	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_207_28599_30680_-	N	N	GH47	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_222_49920_51419_+	N	N	GT2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_230_18228_28670_+	N	N	GT2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_198_17669_20080_+	N	N	GH6	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_215_15837_16844_-	N	N	GH78	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_176_59119_60405_+	N	N	GH3	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_327_31563_32336_-	N	N	GH51	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_327_32554_33423_-	N	N	GH51	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_244_11135_14932_+	N	N	GH18	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_262_6150_7124_+	N	N	CE5	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_245_4023_5711_-	N	N	AA3_2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_226_24381_25481_+	N	N	GH2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_304_19999_21828_-	N	N	GH27	N	1

Gene ID	HMMER	Hotpep	DIAMOND	Signalp	#ofTools
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_270_82708_84669_+	N	N	GH28	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_329_1956_2531_-	N	N	GT39	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_329_2558_3364_-	N	N	GT39	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_270_92747_93766_-	N	N	GH63	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_349_23987_25471_+	N	N	GT0	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_349_26601_28910_+	N	N	GT0	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_382_9776_11818_+	N	N	GT1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_382_18855_20411_-	N	N	GH3	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_369_13573_15534_+	N	N	GT1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_402_26524_27558_+	N	N	GT8	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_320_6017_7072_+	N	N	AA1	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_340_10860_13544_-	N	N	GH18	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_487_13111_13938_+	N	N	GH27	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_425_1036_2808_-	N	N	CBM18+GH18	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_401_3686_5638_-	N	N	GH10	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_414_23556_24290_+	N	N	GT22	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_525_34313_36733_+	N	N	AA3_2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_464_16178_17014_+	N	N	GH106	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_545_3525_5594_+	N	N	GT13	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_532_5101_8895_-	N	N	CBM48+GH13_8	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_504_35877_37439_-	N	N	GH71	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_529_14982_15800_+	N	N	GH31	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_559_8337_9671_-	N	N	GH2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_737_19541_20830_-	N	N	GH71	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_584_1_2341_+	N	N	GT31	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_550_11350_15672_-	N	N	CBM48+GH13_8	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_712_24896_25990_+	N	N	GH78	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_600_4810_14652_-	N	N	GT2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_588_21859_22830_-	N	N	GH13_25+GH133	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_588_22871_24073_-	N	N	GH13_25+GH133	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_752_5393_6712_+	N	N	GH131	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_837_4322_5353_+	N	N	GT34	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_670_1_1353_-	N	N	CBM18+GH18	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_670_19709_20764_-	N	N	GH18	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_613_4771_7524_+	N	N	GT7	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_774_19311_20558_-	N	N	GH18	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_643_11480_13786_-	N	N	CBM50	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_780_1079_6928_+	N	N	GH47	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_769_24705_26570_+	N	N	GH18	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_708_16669_17703_+	N	N	GT34	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_843_613_1350_-	N	N	GH2	N	1
66783_trimmed_un-mapped_reads_[66783_S3_L001_R1_001]_ (single)_contig_788_34285_35610_-	N	N	GH18	N	1

Excel file, S2_Annotations.xls

CaZY families subdivided for catalytic function	Reference gene	Reference microorganism	Aligned sequence in <i>A. olivimuriae</i>			HOMOLOGY			
	NCBI Gene ID/GenBank		Accession number	Contig	Range nt	QC %	E value	Identity %	
Cellulohydrolases									
GH6	4354942	<i>Aspergillus terreus</i> NIH2624	RHLO01000817.1	817	55348 to 56766	96	0E+00	74	
GH6	4354942	<i>Aspergillus terreus</i> NIH2624	RHLO01000520.1	520	39069 to 40116	100	3E-133	70,32	
GH7	4318706	<i>Aspergillus terreus</i> NIH2624	RHLO01000353.1	353	39776 to 40628	100	0E+00	80,42	
GH7	4318706	<i>Aspergillus terreus</i> NIH2624	RHLO01000174.1	174	39174 to 39777	82	2E-105	71,49	
Endo-β-1,4-glucanases									
GH5	4321100	<i>Aspergillus terreus</i> NIH2624	RHLO01000174.1	174	80798 to 82252 & 83448 to 83620	100	0E+00	74,98	
GH5	4321100	<i>Aspergillus terreus</i> NIH2624	RHLO01001801.1	1801	4304 to 5316	70	1E-119	70,58	
GH7	4323506	<i>Aspergillus terreus</i> NIH2624	RHLO01000748.1	748	19491 to 20707	96	0E+00	79,15	
GH12	4354153	<i>Aspergillus terreus</i> NIH2624	RHLO01001577.1	1577	1028 to 1816	93	2E-88	69,54	
Endo-β-1,6-glucanase									
GH30*	3504964	<i>Aspergillus fumigatus</i> Af293	RHLO01001055.1	1055	3239 to 3851	95	1E-124	76,18	
Lytic polysaccharide monooxygenases									
AA9	4322940	<i>Aspergillus terreus</i> NIH2624	RHLO01000158.1	158	26748 to 27839	98	0E+00	77,75	
AA9	4321074	<i>Aspergillus terreus</i> NIH2624	RHLO01001566.1	1566	3651 to 4134	64	5E-70	72,37	
AA9	4321074	<i>Aspergillus terreus</i> NIH2624	RHLO01000826.1	826	12804 to 13558	100	9E-130	73,79	
AA9	4315933	<i>Aspergillus terreus</i> NIH2624	RHLO01001044.1	1044	7033 to 8139	88	2E-146	70,93	
AA9	4323343	<i>Aspergillus terreus</i> NIH2624	RHLO01001811.1	1811	684 to 1433	93	2E-164	77,09	
AA9	4319653	<i>Aspergillus terreus</i> NIH2624	RHLO01000133.1	133	53061 to 53953	85	3E-176	75,81	
Cellobiose dehydrogenase									
AA3_1, AA8	4319513	<i>Aspergillus terreus</i> NIH2624	RHLO01000485.1	485	23272 to 25955	96	0E+00	69,62	
AA3_1, AA8	4353174	<i>Aspergillus terreus</i> NIH2624	RHLO01000665.1	665	4882 to 5774	100	7E-158	74,45	
α-glucosidase									
GH31*	XM_001393610.1	<i>Aspergillus niger</i> GN-3	RHLO01000309.1	309	1663 to 3773	95	0	73,51	
β-glucosidase									
GH1	4355442	<i>Aspergillus terreus</i> NIH2624	RHLO01000369.1	369	26133 to 27853	99	0E+00	77,73	
GH3	4322590	<i>Aspergillus terreus</i> NIH2624	RHLO01000702.1	702	4501 to 6868	97	0E+00	73,11	
GH3	4318025	<i>Aspergillus terreus</i> NIH2624	RHLO01000108.1	108	51144 to 53141	95	0E+00	76,84	
GH3	4354713	<i>Aspergillus terreus</i> NIH2624	RHLO01000063.1	63	24804 to 27402	99	0E+00	70,07	
GH3	4353984	<i>Aspergillus terreus</i> NIH2624	RHLO01001212.1	1212	12549 to 15559	99	0E+00	73,34	
GH3	4319381	<i>Aspergillus terreus</i> NIH2624	RHLO01000557.1	557	7758 to 10410	61	0E+00	79,08	
GH3	4353962	<i>Aspergillus terreus</i> NIH2624	RHLO01001269.1	1269	6042 to 8697	100	0E+00	82,96	
GH3	4353962	<i>Aspergillus terreus</i> NIH2624	RHLO01000176.1	176	58377 to 60329	73	4E-58	63,71	
GH3	4318366	<i>Aspergillus terreus</i> NIH2624	RHLO01000725.1	725	19947 to 22464	93	0E+00	79,02	
GH3	4317192	<i>Aspergillus terreus</i> NIH2624	RHLO01000045.1	45	33651 to 35497	84	0E+00	71,38	
β-xylosidase									
GH3	4320717	<i>Aspergillus terreus</i> NIH2624	RHLO01000583.1	583	1230 to 3574	91,00	0E+00	73,29	
GH43	4354850	<i>Aspergillus terreus</i> NIH2624	RHLO01000319.1	319	1154 to 3520	55,00	0E+00	77,47	

CaZY families subdivided for catalytic function	Reference gene NCBI Gene ID/GenBank	Reference microorganism	Aligned sequence in <i>A. olivimuriae</i>			HOMOLOGY		
			Accession number	Contig	Range nt	QC %	E value	Identity %
Endo-1,4-β-xylanase								
GH10	4318079	<i>Aspergillus terreus</i> NIH2624	RHLO01000537.1	537	32 to 1441	82	0E+00	72,63
GH10	4318885	<i>Aspergillus terreus</i> NIH2624	RHLO01000992.1	992	10493 to 11524	94	0	74,98
GH11	4322746	<i>Aspergillus terreus</i> NIH2624	RHLO01000282.1	282	2506 to 3260	100	0	79,13
GH11	4321208	<i>Aspergillus terreus</i> NIH2624	RHLO01000774.1	774	14471 to 15073	97	8E-137	75,76
β-mannosidase								
GH2	4323103	<i>Aspergillus terreus</i> NIH2624	RHLO01000843.1	843	612 to 3371	78	0	77,34
GH2	4354149	<i>Aspergillus terreus</i> NIH2624	RHLO01000026.1	26	24761 to 27287	94	0	69,74
Mannan endo-1,4-b-mannosidase A								
GH5	4323372	<i>Aspergillus terreus</i> NIH2624	RHLO01000482.1	482	126922 to 128152	95	3E-157	70,41
GH5	4323372	<i>Aspergillus terreus</i> NIH2624	RHLO01000244.1	244	39094 to 40256	88	2E-121	68,47
GH5	4323372	<i>Aspergillus terreus</i> NIH2624	RHLO01000180.1	180	23800 to 24869	83	2E-65	66,05
α-L-arabinofuranosidase								
GH51	4317956	<i>Aspergillus terreus</i> NIH2624	RHLO01000400.1	400	14892 to 16928	85	0	69,84
GH51	4322980	<i>Aspergillus terreus</i> NIH2624	RHLO01001249.1	1249	57 to 1801	75	0	73,77
GH54	4322793	<i>Aspergillus terreus</i> NIH2624	RHLO01000522.1	522	5375 to 6809	94	0	77,56
GH62	4319554	<i>Aspergillus terreus</i> NIH2624	RHLO01000869.1	869	18612 to 19508	91	1E-174	75,14
GH62	4354699	<i>Aspergillus terreus</i> NIH2624	RHLO01000155.1	155	4098 to 5339	100	0	79,79
Endo-1,5-L-arabinase								
GH43	4317970	<i>Aspergillus terreus</i> NIH2624	RHLO01000155.1	155	14480 to 15583	97	0	73,43
GH43	4318802	<i>Aspergillus terreus</i> NIH2624	RHLO01000820.1	820	6118 to 7366	98	2E-178	70,94
GH43	4316117	<i>Aspergillus terreus</i> NIH2624	RHLO01001261.1	1261	4988 to 6236	87	1E-157	69,69
GH43	4315588	<i>Aspergillus terreus</i> NIH2624	RHLO01001402.1	1402	20 to 962	95	4E-174	74,74
GH93	4321284	<i>Aspergillus terreus</i> NIH2624	RHLO01001512.1	1512	5 to 457	76	3E-129	82,56
GH93	4355652	<i>Aspergillus terreus</i> NIH2624	RHLO01000142.1	142	18044 to 19167	96	0	88,49
Arabinogalactanase								
GH53*	AJ305303	<i>Aspergillus niger</i> CBS 120.49	RHLO01000333.1	333	8328 to 9049	79	8E-108	72,33
Xylan α-1,2-glucuronidase								
GH67	4321653	<i>Aspergillus terreus</i> NIH2624	RHLO01000361.1	361	27753 to 30223	97	0	74,85
GH115	4320357	<i>Aspergillus terreus</i> NIH2624	RHLO01000921.1	921	597 to 3646	74	0	75,31
Xyloglucan endo-β-1,4-glucanase								
GH12	4318627	<i>Aspergillus terreus</i> NIH2624	RHLO01001432.1	1432	34 to 853	96	1E-159	74,85
Xyloglucanase								
GH74	4319894	<i>Aspergillus terreus</i> NIH2624	RHLO01001151.1	1151	10801 to 13615	98	0	70,76
β-galactosidase								
GH35*	KC175324.1	<i>Aspergillus niger</i> ATCC 1015	RHLO01000879.1	879	4528 to 6558	91	0	68,47
Endo-polygalacturonase								
GH28*	U05015	<i>Aspergillus flavus</i> strain 70	RHLO01000046.1	46	38152 to 39080	57	1E-76	67,08
α-galactosidase								
GH27*	FJ843023	<i>Aspergillus fumigatus</i> IMI 385708	RHLO01000900.1	900	10134 to 11680	90	4E-82	65,29
GH36*	AJ251873	<i>Aspergillus niger</i> CBS120.49	RHLO01000702.1	702	9476 to 11651	90	0E+00	67,43
Acetyl xylan esterase								
CE1	4322353	<i>Aspergillus terreus</i> NIH2624	RHLO01000869.1	869	20082 to 20832	91	6E-158	76,43
CE1	4354490	<i>Aspergillus terreus</i> NIH2624	RHLO01000083.1	83	52321 to 53347	99	6E-172	73,31
Feruloyl esterase								
CE1	4322286	<i>Aspergillus terreus</i> NIH2624	RHLO01000061.1	61	31426 to 33051	64	0	76,54

CaZY families subdivided for catalytic function	Reference gene	Reference microorganism	Aligned sequence in <i>A. olivimuriae</i>			HOMOLOGY		
	NCBI Gene ID/GenBank		Accession number	Contig	Range nt	QC %	E value	Identity %
Acetyl xylan esterase								
CE5	4319895	<i>Aspergillus terreus</i> NIH2624	RHLO01001151.1	1151	9608 to 10574	93	1E-129	70,87
CE16	4353509	<i>Aspergillus terreus</i> NIH2624	RHLO01000661.1	661	3844 to 5964	91	0	74,11
Laccase								
AA1	3509957	<i>Aspergillus fumigatus</i> Af293	RHLO01001305.1	1305	10060 to 10903	75	2E-48	65,29
AA1	3509608	<i>Aspergillus fumigatus</i> Af293	RHLO01000874.1	874	21790 to 23280	74	3E-97	66,4
1,4-benzoquinone reductase								
AA6	5989775	<i>Aspergillus oryzae</i> RIB40	RHLO01000013.1	13	18813 to 19520	88	3E-149	77

Aspergillus terreus NIH2624 reference genes were derived from Segato et al. 2014. MMBR, 78(4), 588. For all genes analyzed, sequences were considered homologous when the E Value (Blastn) was equal or lower than 1.00E-45 and the query cover (QC) length higher than 55%. *Reference genes derived from MycoCLAP (<http://mycoclap.fungalgenomics.ca/mycoCLAP> - Database (2011) 2011:bar020). For GH9, GH29, GH45, GH88, GH95, GH105, GH106, GH154, PL1, PL4 and CE12 no reference genes were found in MycoCLAP. For AA6, the reference gene was derived from *Aspergillus oryzae* RIB40. For GH78 and CE8 no alignment was found with any of the *Aspergillus* reference genes reported in MycoCLAP.

CHAPTER 3

Chitosan Production by Fungi: Current State of Knowledge, Future Opportunities and Constraints

A slightly modified version of this chapter has been published as: Crognale S., Russo C., Petruccioli M., D'Annibale A. (2022). **Chitosan Production by Fungi: Current State of Knowledge, Future Opportunities and Constraints** *Fermentation*, 8, 76; doi: <https://doi.org/10.3390/fermentation8020076>

3.1 Abstract

Conventionally, the commercial supply of chitin and chitosan relies on shellfish wastes as the extraction sources. However, the fungal sources constitute a valuable option, especially for biomedical and pharmaceutical applications, due to the batch-to-batch unsteady properties of chitin and chitosan from conventional ones. Fungal production of these glycans is not affected by seasonality, enables accurate process control and, consequently, more uniform properties of the obtained product. Moreover, liquid and solid production media often are derived from wastes, thus enabling the application of circular economy criteria and improving the process economics. The present review deals with fungal chitosan production processes focusing on waste-oriented and integrated production processes. In doing so, contrary to other reviews that used a genus-specific approach for organizing the available information, the present one bases the discussion on the bioprocess typology. Finally, main process parameters affecting chitosan production and their interactions are critically discussed.

3.2 Introduction

Chitin, a structural glycan composed of randomly distributed N-acetyl-D-glucosamine (GlcNAc) residues (Figure 1a) (Islam et al., 2017), is the second most abundant biopolymer on earth (more than 100 billion tons) (Tharanathan et al., 2003). Chitosan, a linear heteroglycan mainly made of β -(1-4)-linked D-glucosamine (GlcN) units (Figure 1b), is often derived from chitin deacetylation, the extent of which, however, is never quantitative. Consequently, chitosan is a copolymer made of GlcNAc and GlcN residues, where the latter account for at least 60% of total residues (Islam et al., 2017;

Dhillon et al., 2013a; Akila et al., 2014). However, the degree of deacetylation (DD) of commercially available chitosans generally amounts to or exceeds 80% (Dhillon et al., 2013a).

Over a 2020-2027 period, the Global Industry Analysts Inc. (2021) estimated growth of the market of chitin and chitosan from 106.9×10^3 to around 282×10^3 tons, with a compounded annual growth rate equal to 14.8%; this growth estimate was attributed to the ever-increasing applications of these polymers in various end-use sectors.

Chitin occurs in nature in different crystalline forms denominated α -, β - and γ -chitin, exhibiting distinct physicochemical properties (Figure 1c). The differences among these polymorphs are due to the mode with which crystalline regions' chains are reciprocally arranged. In the α and β forms, all the chains are arranged in an antiparallel and parallel mode, respectively, while in the γ form there is an alternation of sets of two parallel strands with single anti-parallel ones (Jang et al., 2004). Among these allomorphs, α -chitin is the most widespread being found in arthropods and fungi, β -chitin generally occurs in cephalopods, while γ -chitin is rather rare.

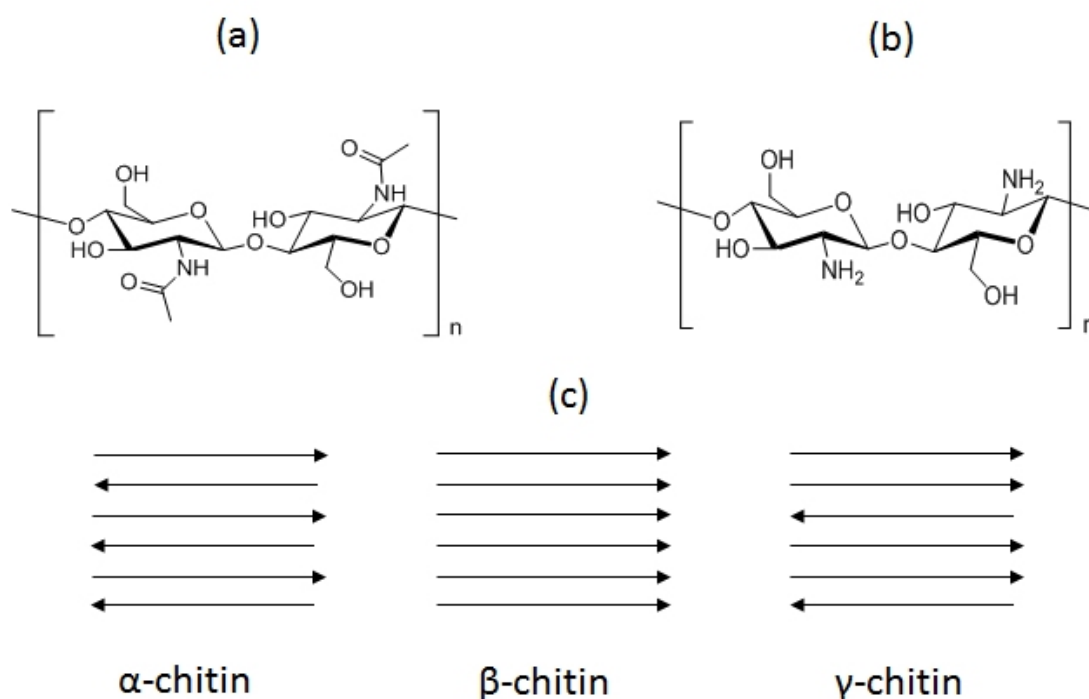


Figure 1 Basic structures of chitin (a) and chitosan (b) and chitin allomorphs (c). The tips of the arrows indicate the positions of the reducing ends of the chains.

At present, the industrial production of chitin and chitosan rely on crustacean wastes. Chitin contents in crab and shrimp processing wastes range from 13 to 15% and 14 to 27% dry weight, respectively (Kurita et al., 2006). The heterogeneous composition of crustacean shell wastes requires stepwise chemical methods to extract chitin and chitosan from these sources (Kumari et al., 2020).

The extraction scheme generally involves a demineralization step with strong acids, followed by alkaline deproteinization and, frequently, a decolorization step. The first step, generally relying on HCl solutions (concentration ranges 0.6-11.0 M), is generally conducted at room temperatures, while the second uses NaOH solutions (concentration ranges 0.12-5.0 M) and temperature up to 160 °C; the final step, frequently added to remove pigments, such as β -carotene and astaxanthin, generally relies on acetone as the extraction solvent (Younes & Rinaudo, 2015). The chitin thus obtained undergoes alkaline deacetylation under very harsh chemical conditions (NaOH solutions from 30 to 60%) under variable temperature and contact times to yield chitosan (Oyatogun et al., 2020). The variability of the raw materials and the harsh conditions that characterize some extraction steps can lead to unsteady physicochemical properties of chitosan from batch to batch (Younes & Rinaudo, 2015).

The occurrence of chitin and chitosan in fungi has opened the door to a promising alternative route for their productions (Chien et al., 2016). Since fungal chitin has a lower ash content than crustacean shell wastes, the demineralization step is not required during its processing (Teng et al., 2011; Elsoud & El Kady, 2019). Moreover, chitin and chitosan of fungal origin give a non-seasonal and reliable source of these polymers and consistent properties of the product. The extraction of a value-added product, such as chitosan, may afford a profitable solution to mushroom growers and biotechnological industries considering the vast quantities of fungal-based wastes accumulated and the ensuing expense in waste management.

3.3 Physicochemical and Functional Properties of Chitin and Chitosan

The chemical structures of chitin and chitosan resemble that of cellulose, a glycan composed of hundreds of D-glucose residues connected by β -(1-4) linkages (Islam et al., 2017). In chitin and chitosan, however, an acetamide or amino group replaces the hydroxyl group at the C-2 position of glucose residues. Thus, the nitrogen content of

chitin and chitosan ranges from 5 to 8%, and the presence of amino groups gives these glycans distinctive biological functions and susceptibility to chemical modification reactions (Chisty et al., 2020); chitosan, in particular, owing to the presence of free amino-groups is susceptible to N-acylation and Schiff's reactions paving the way to a variety of chemical modifications. Moreover, the joint presence of the amino and hydroxyl groups on each deacetylated unit renders chitosan more water-soluble and chemically reactive than chitin. Due to their pK_a values (≈ 6.3), the free amino groups in GlcN residues are protonated at slightly acid pHs, making chitosan the only naturally-occurring cationic glycan (El Knidri et al., 2020). A further consequence is the polymer's solubility in slightly acid aqueous solution as opposed to chitin (Akpan et al., 2020).

The majority of the biological properties of these glycans are due to their physicochemical features, such as solubility, deacetylation degree, molecular weight, and inherent moisture content (Aranaz et al., 2009). For instance, the inhibition of fungal and bacterial growth exerted by chitosan relies principally on the extent of positively charged groups and molecular mass. Two main mechanisms have been suggested to explain the antimicrobial activity of chitosan. The first outlines the importance of its molecular weight and postulates that the smaller chito-oligosaccharides can easily penetrate the cellular membrane, thus preventing cell growth via inhibition of DNA transcription (Zheng & Zhu, 2003; Klaykruayat et al., 2010). The second suggests that the positively charged groups of chitosan interact with anionic components of the microbial cell membrane resulting in cell death (Hosseinnejad & Jafari, 2016; Duan et al., 2019). Moreover, chitosan can operate as a chelator of essential elements (Goy et al., 2009). Chitosans with a degree of deacetylation (DD) larger than 97.5% have a higher positive charge density and an ensuing stronger antibacterial activity than those with moderate DD (83.7%), as shown by Kong et al. (2010). Some properties of chitin and chitosan, such as non-toxicity, biodegradability, biocompatibility and non-allergenicity, associated with bioactivity and good adsorption properties, render them appropriate alternative options to artificial polymers (Tan et al., 2009; Croisier & Jérôme, 2013). Another reason underlying their success is that they can be manufactured to yield several forms, including beads, flakes, membranes, gels and fibers (El Knidiri et al., 2020). As a consequence, they have been exploited as carriers for enzyme immobilization (D'Annibale et al., 1999; Amorim et al., 2003), coagulating agents in effluents treatment, as food preservatives (Tayel et al., 2014), hypocholesterolemic and wound healing agents, and as components of several drug delivery systems (Lang et al., 2020; Shafabakhsh et al., 2020).

3.4 General Aspects of Chitin and Chitosan Production from Fungal Sources

Since the beginning of this century, many countries have focused attention on using fungal sources for the commercial production of chitin and chitosan due to the remarkable disadvantages that burden the conventional process. Table 1 comparatively summarizes the advantages and disadvantages of chitin and chitosan production from fungal and crustacean sources (Teng et al., 2001; Elsoud & El Kady, 2019).

Among the advantages of the fungal approach, there is the possibility of obtaining chitosans with different properties by varying species and culture conditions (Jaworska & Konieczna, 2001; Joseph et al., 2021). For instance, the chitosan derived from shellfish wastes has a high molecular mass (around 1.5×10^6 Da), while the MW of chitosan from fungal sources widely ranges from 6.4×10^3 to 1.4×10^6 Da (Arcidiacono et al., 1992; Wang et al., 2008). High MW chitosans are sparingly soluble in neutral pH aqueous solutions and yield high viscous solutions that limit their exploitation in the food, health and agricultural sectors (Wibowo et al., 2007). Fungal-derived chitosan with medium-low MW can be used as hypocholesterolemic agents in healthcare products and as a thread or membrane in a variety of biomedical applications (Sumiyoshi & Kimura, 2006; Sathiyaseelan et al., 2017). Moreover, chitosan extraction from fungal sources is more environmentally benign than that from shellfish wastes since the latter source requires highly concentrated acid and alkaline solutions for demineralization and chemical deacetylation that have to be disposed of. Another advantage associated with fungal chitosan encompasses the absence of allergenic proteins, such as tropomyosin (Younes & Rinaudo, 2015). Chitin and chitosan extraction from fungi can lower disposal costs of fungal-based waste materials in association with the production of value-added products, which may offer a lucrative opportunity to the biotechnological industries (Wu et al., 2005; Safaei et al., 2016; Zininga et al., 2019).

Table 1 Benefits and drawbacks of fungal chitosan compared to those from conventional sources with reference to a series of evaluation criteria.

Evaluation criterion	Benefits	Drawbacks
Biomass supply	Not affected by seasonal and geographical factors. Possible biomass supply from pharmaceutical and biotechnological industries.	Lower biomass amounts than those made available from the shellfish industry
Extraction process	Process scheme simpler (no demineralization and decolorization steps) and lower amounts of chemicals employed as compared to those used for crustacean sources.	Less established as compared to that from shellfish waste.
Environmental impact and disposal costs of process wastes	More environmental-friendly and lower disposal costs of effluents as compared to the shell-fish waste process.	Potential risks of dispersal of pathogenic fungi when dealing with species not satisfying the generally regarded as safe requirements.
Inorganic and organic contaminants in the product	Absence of heavy metals and allergenic proteins as opposed to chitosan preparations from shellfish waste.	Some chitosan preparations might contain residual phosphates
Production costs	They can be modulated by the choice of low-cost substrates and low equipment-intensive fermentation techniques, such as solid-state fermentation.	Not yet competitive in terms of production costs compared to the conventional process
Physico-chemical properties of the products	Molecular weights and degree of deacetylation of fungal chitosans frequently lower and higher, respectively, than those from conventional sources with ensuing positive impacts on their antimicrobial and antioxidant activities.	The lower MW of fungal chitosans than those from conventional sources make them less suitable as anti-lipidemic and hypocholesterolemic agents.

3.4.1 Chitin and Chitosan Biosynthesis and Their Biological Functions in Fungi

Arthropods and fungi share a common biosynthetic pathway that uses glucose and its storage carbohydrates, such as trehalose and glycogen, as the starting materials. The pathway of chitin biosynthesis is organized into three groups of reactions, the first leading to the formation of GlcNAc, the second yielding its activated counterpart uridine 5'-diphospho-N-acetylglucosamine (UDP-GlcNAc) through a modification of the Leloir pathway, and the third resulting in polymer formation using UDP-GlcNAc as the GlcNAc donor to the growing chitin chain (Figure 2) (Merzendorfer, 2011).

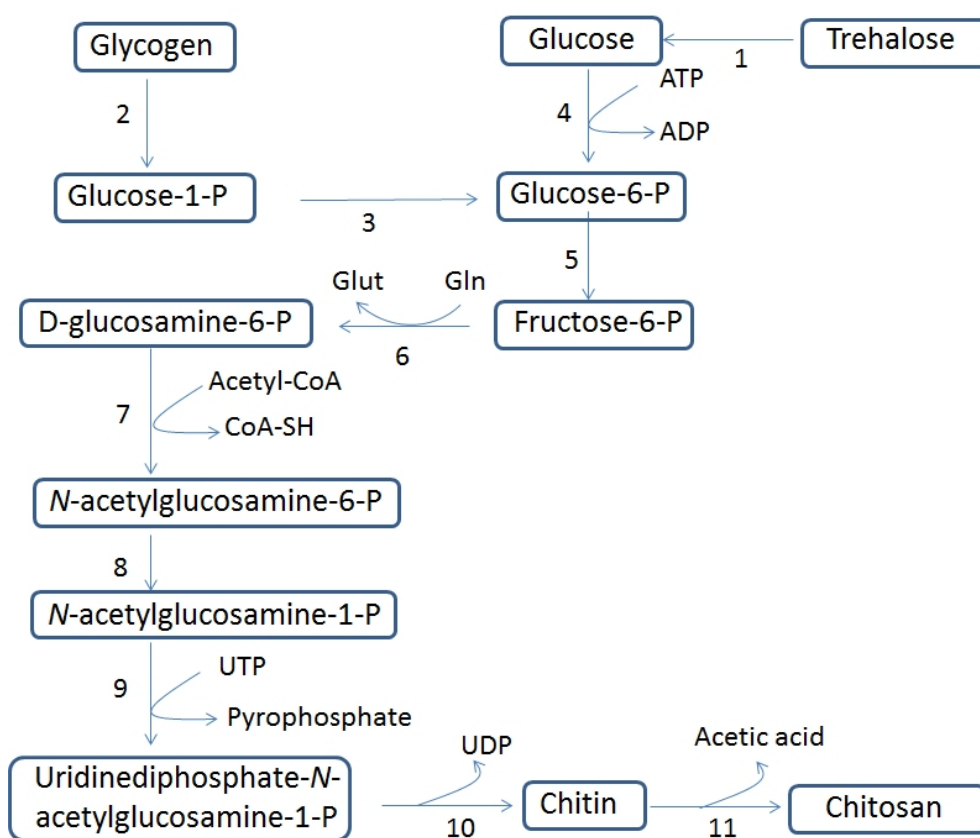


Figure 6 Biosynthetic pathway of chitin and chitosan. The following numbering has been assigned to the enzymes that catalyze each reaction: 1, trehalase; 2, glycogen phosphorylase; 3, phosphoglucomutase; 4, hexokinase; 5, glucose-6-phosphate isomerase; 6, glutamine-fructose-6-phosphate amidotransferase; 7, glucosamine-6-phosphate N-acetyltransferase; 8, N-acetylglucosamine-phosphate mutase; 9, UDP-N-acetylglucosamine pyrophosphorylase; 10, chitin synthase and 11, chitin deacetylase. The abbreviations used are as follows: Acetyl-CoA, acetyl coenzyme A; ADP, Adenosine diphosphate; CoASH, coenzyme A; Gln, L-glutamine; Glu, L-glutamate; UDP, uridine diphosphate; UTP, uridine triphosphate.

Free glucose or that derived from trehalase-catalyzed hydrolysis of trehalose is converted to glucose-6-phosphate (G-6-P) by hexokinase. If the starting material is glycogen, its depolymerization, catalyzed by glycogen phosphorylase, releases glucose-1-phosphate which is also converted to glucose-6-phosphate by phosphoglucomutase-catalyzed isomerization. Irrespective of its origin, G-6-P is then isomerized to fructose-6-phosphate (F-6-P) by phosphoisoisomerase. F-6-P is then converted to N-acetyl-D-glucosamine-6-phosphate (GlcNAc-6-P) through two consecutive transfer reactions of an amino and acetyl group where glutamine and acetyl CoA, respectively, act as the donors. Isomerization step of GlcNAc-6-P catalyzed by phospho-N-acetyl glucosamine mutase yields 1-phospho-N-acetyl-D-glucosamine (GlcNAc-1-P). The chitin precursor, UDP-GlcNAc is formed upon reaction of GlcNAc-1-P with uridine triphosphate (UTP) and serves as a N-acetylglucosamine (GlcNAc) donor for the sequential addition of GlcNAc units to the non-reducing terminus of the growing chain catalyzed by chitin synthase (Sitanggang et al., 2012). The linear chains spontaneously assemble to form microfibrils with varying diameters and lengths. In a further step, chitin deacetylase (CDA, E.C. 3.5.1.41) brings about the deacetylation of GlcNAc residues of chitin, thus leading to chitosan (Batista et al., 2018). The formation of GlcNAc and UDP-GlcNAc takes place in the cytosol, while chitin synthesis occurs in specialized domains of the cell membrane. Some enzymes involved in chitin synthesis, such as glutamine-fructose-6-phosphate amidotransferase (EC 2.6.1.16), UDP-N-acetylglucosamine pyrophosphorylase (EC 2.7.7.23), and chitin synthase (EC 2.4.1.16) are subjected to tight regulation and limit the rate of chitin production (Merzendorfer, 2011; Lenardon et al., 2010).

Table 2 summarizes the taxonomic classification of chitosan-producing species and range of chitosan contents (referred to dry biomass) and degree of deacetylation.

Table 2. Taxonomic classification of chitosan-producing species and range of chitosan contents referred to their biomass dry weight and degree of deacetylation (DD).

Genus and species	Class	Order	Family	Chitosan content (g kg ⁻¹)	DD (%)	References
<i>Absidia</i>						
<i>A. blakesleeana</i>	Zygomycetes	Mucorales	Cunninghamellaceae	10-170	85	Miyoshi et al., 1992; Rane & Hoover, 1993; Muzzarelli et al., 1994; Hu et al., 1999; Jaworska and Konieczna, 2001; Kim et al., 2001; Jiang et al., 2011; Vaingankar & Juvekar, 2014
<i>A. coerulea</i>				30-300	93-95	
<i>A. glauca</i>				52-59	75-80	
<i>A. orchidis</i>				18-69	68-85	
<i>Cunninghamella</i>						
<i>C. bertholletiae</i>	Zygomycetes	Mucorales	Cunninghamellaceae	55-128	87-90	Tan et al., 1996; Franco et al., 2004; Amorim et al., 2003; Berger et al, 2014a; Berger et al., 2014b; Amorim et al., 2006a; Stamford et al., 2007; de Oliveira et al., 2014; Vaingankar & Juvekar, 2014; Junior et al., 2016
<i>C. echinulata</i>				50-130	85	
<i>C. elegans</i>				35-78	72-90	
<i>C. ramose</i>				50-123	n.r.	
<i>Gongronella</i>	Zygomycetes	Mucorales	Cunninghamellaceae			Tan et al., 1996; New et al., 2002; Nwe & Stevens, 2002; Nwe & Stevens, 2004; Sharifia et al., 2008; Streit et al., 2009; Vendruscolo & Ninow, 2014
<i>G. butleri</i>				58-216	89-92	
<i>Benjaminiella</i>	Zygomycetes	Mucorales	Mucoraceae			Mane et al., 2017
<i>B. poitrasii</i>				51-78	94-95	
<i>Mucor</i>						
<i>M. racemosus</i>	Zygomycetes	Mucorales	Mucoraceae	12-117	70-84	Synowiecki & Al-Khateeb, 1997; Pochanavanich & Suntornsuk 2002; Amorim et al., 2003; Chatterjee et al., 2005; Wu et al., 2005; Chatterjee et al., 2009; Tajdini et al., 2010; Karimi & Zamani, 2013; Abasian et al., 2020; Zininga et al., 2019
<i>M. rouxii</i>				33-204	80-90	
<i>M. rouxianus</i>				181	80-90	
<i>M. indicus</i>				94-235	72-89	
<i>Rhizomucor</i>						
<i>R. pusillus</i>	Zygomycetes	Mucorales	Mucoraceae	80	n.r.	Zamani et al., 2007; Tajdini et al., 2010
<i>R. miehei</i>				14-137	81	
<i>Rhizopus</i>						
<i>R. oryzae</i>	Zygomycetes	Mucorales	Mucoraceae	44-138	85-89	Tan et al., 1996; Nadarajah et al., 2001; Pochanavanich & Suntornsuk 2002; Suntornsuk et al., 2002; Chatterjee et al, 2008; Chatterjee et al., 2019; Kleekayai & Suntornsuk, 2011; Berger et al., 2014b; Vaingankar & Juvekar, 2014; Cardoso et al., 2012; Tasar et al., 2016
<i>R. oligosporus</i>				32	n.r.	
<i>R. arrhiizus</i>				21-58	82	

Genus and species	Class	Order	Family	Chitosan content (g kg ⁻¹)	DD (%)	References
<i>Syncephalastrum</i> <i>S. racemosum</i>	Zygomycetes	Mucorales	Syncephalastraceae	74-152	72-77	Amorim et al., 2003; Amorim et al., 2006b
<i>Aspergillus</i> <i>A. niger</i> <i>A. terreus</i>	Eurotiomycetes	Eurotiales	Trichocomaceae	70-209 69-141	81-90 85-88	Pochanavanich & Suntornsuk 2002; Wu et al., 2005; Maghsoodi et al., 2009; Kumaresapillai et al., 2011; Tayel et al., 2014; Abdel-Gawad et al., 2017; Habibi et al., 2021
<i>Penicillium</i> <i>P. chrysogenum</i> <i>P. waksmanii</i>	Eurotiomycetes	Eurotiales	Trichocomaceae	29-57 297	84-86 65	Tianwei et al., 2002; Ebrahimzadeh et al., 2013; Aili et al., 2019; Namboodiri & Pakshirajan, 2019

In fungi, both vegetative and sporulating cells are capable of chitin synthesis and its secretion occurs in a polarized mode. As a matter of fact, chitin accumulation occurs at growth sites such as hyphal tips and cross-walls in filamentous fungi and emerging buds in yeasts. Chitin and chitosan accumulation mostly occurs in the cell wall's layers adjacent to the plasmalemma where these glycans play a fundamental role in maintaining the cell wall's shape and integrity; moreover, they provide protection against foreign materials (e.g., cell inhibitors) and environmental stressors to which fungi might be exposed (Adams, 2004; Banks et al., 2005; Baker et al., 2007; Goldman & Vicencio, 2012). Owing to its positive charge, chitosan is capable of retaining anionic storage materials, such as polyphosphates, which are highly abundant in the Zygomycetes' cell wall (Kamiri & Zamani, 2013). Chitosan also exerts a role in some pathogenic fungi such as *Colletotrichum graminicola* (El Gueddari et al., 2002), and *Magnaporthe oryzae* (Kuroki et al., 2017). During infection by these species, the chitin deacetylase-catalyzed conversion of chitin into chitosan seems to preserve the appressorium from the hydrolysis by plant chitinases (El Gueddari et al., 2002).

3.4.2 Fungal Producers of Chitin and Chitosan

In cell walls of fungal species belonging to the classes Deuteromycetes, Ascomycetes, and Basidiomycetes, chitin is regarded as the second most abundant component (Gow et al., 2017).

As opposed to chitin, chitosan is a less widespread cell wall constituent. However, Zygomycetes have significantly higher amounts of chitosan in their cell walls than other fungal classes. Within Zygomycetes, the most productive genera belong to the order of Mucorales and to two families, namely Cunninghamellaceae and Mucoraceae. The former family includes a variety of highly-producing genera, such as *Absidia* (Hu et al., 1999; Jaworska & Konieczna, 2001; Jiang et al., 2011), *Cunninghamella* (Franco et al., 2004; Berger et al, 2014a; Amorim et al., 2006a; de Oliveira et al., 2014) and *Gongronella* (New et al., 2002; Nwe & Stevens, 2002; Nwe & Stevens, 2004; Streit et al., 2009) while the latter includes the genera *Mucor* (Synowiecki & Al-Khateeb, 1997; Pochanavanich & Suntornsuk, 2002; Amorim et al., 2003; Chatterjee et al., 2005; Chatterjee et al., 2009; Tajdini et al., 2010; Karimi & Zamani, 2013; Zininga et al., 2019; Abasian et al., 2020), *Rhizomucor* (Zamani et al., 2007; Tajdini et al., 2010), and *Rhizopus* (Pochanavanich & Suntornsuk 2002; Suntornsuk et al., 2002; Chatterjee et al, 2008; Liao et al., 2008; Cardoso et al., 2012; Tasar et al., 2016).

Although chitosan production from fungal sources can be regarded as a greener alternative to shellfish-based process, it is not devoid of risks, depending on the selected fungal species. To exemplify, *Colletotrichum lindemuthianum* has been used as a valuable source of chitin deacetylase (Suresh et al., 2011) although this fungus is the causative agent of ‘anthracnose’, a plant disease which can affect a variety of crops grown in both temperate and tropical climates. Within the class of Zygomycetes, some species, belonging to *Absidia* and *Rhizopus* genera, can be pathogens either to animals or humans. Some species of *Absidia* are causative agents of mucormycosis in humans with low immune systems (Morales-Aguirre et al., 2004) and zygomycosis, causing spontaneous abortion in cows. Also, *Rhizopus oryzae* can act as an opportunistic human pathogen causing pulmonary mucormycosis (Gerlach et al., 2016). As a consequence, the handling of these potentially pathogenic strains requires the adoption of specific measures to prevent their accidental dispersal. The hope is that the research and development of the chitosan fungal production process can be oriented in the future only and exclusively on strains that meet the generally regarded as safe (GRAS) requirement.

3.4.3 Production Processes of Fungal Chitin and Chitosan

The chitin and chitosan contents are species-specific and are largely affected by the growth medium and fermentation system. Although a variety of chitosan production processes have been conducted in solid-state fermentation (SSF) (Table 3), the majority of studies have relied primarily on liquid submerged fermentation (LSF) (Tables 4 and 5).

The marked preponderance of studies conducted in liquid fermentation is likely due to several advantages of this technique compared to SSF. These advantages include facile control of process parameters, especially at the reactor level, higher amenability to scale transfer and straightforward biomass recovery from the growth medium (Elsoud and El Kady, 2019). In SSF, the fungal colonization occurs either on inert and homogeneous solid substrates or on natural ones moistened in such a way as to ensure the absence of free water (Kapilan, 2015). On the one hand, using homogenous solid substrates is advantageous as it allows better control of medium composition and improved oxygen and nutrients transfer; moreover, it facilitates the recovery of fungal biomass. However, no reports dealing with the chitin/chitosan production on inert substrates are available to the best of our knowledge. On the other hand, the natural use of solid substrates, due to their intrinsic heterogeneity, implies mass-transfer limitations, which do not enable

accurate process control and enhances the difficulty of recovering fungal biomass (Wang et al., 2008).

Contrary to other reviews that have used a genus-specific approach for organizing literature data (Dhillon et al., 2013a; Sebastian et al., 2020), a discussion based on the fermentation technique appeared to be more valuable and informative here (the present review). Considering the relative scarcity of studies on the solid-state production of chitosan and, in general, their poorly equipment-oriented character, only a single section of this review has been dedicated to this topic. Conversely, this review offers an articulated discussion of LSF production studies. These were divided based on the nature of the production medium into two sections, the first of which focused on studies conducted on synthetic media and the second on waste- or effluent-derived media. Finally, the present review devotes a section to process parameters that directly influence fungal chitosan production and properties. The reader is referred to the conclusions section for any comparative considerations between the production approaches in liquid and solid-state culture.

3.4.3.1 Solid-State Production of Fungal Chitosan

As mentioned in section 3.4.3, solid-state chitosan production studies have relied solely on naturally-occurring substrates. This approach has offered the opportunity to explore economically sustainable production solutions and, at the same time, to exploit processing residues that would otherwise have little economic value. A variety of plant residues have been used for this purpose, including wheat and rice straw (Crestini et al., 1996; Khalaf, 2004), soybean residues (Suntornsuk et al., 2002; Maghsoodi et al., 2008, Mondala et al., 2015), hardwood sawdust (Kannan et al., 2010), sweet potato pieces (New & Stevens, 2002a), potato chip processing waste (Kleekayai & Suntornsuk, 2011) and cottonseed hulls (Wang et al., 2008).

Table 3, summarizing SSF chitosan production studies, shows that this technique enabled the achievement of high product concentration, referred to dry weight of solid substrate, which widely ranged from 1.6 to 17 g kg⁻¹ depending on strain and substrate combinations.

The average volumetric productivities (r_p) were also interesting in some cases, such as in cultures of *A. niger* and *M. rouxii* grown on soy-derived solid matrices (59 and 119 mg kg⁻¹ h⁻¹, respectively) (Maghsoodi & Yaghmaei, 2010; Mondala et al., 2015); these

productivity values were attributable to a high product concentration rather than to a rapid growth since the product peak was obtained in 12-day-old cultures in both cases.

Different evaluation, on the other hand, can be done for potato- or sweet potato-derived substrates, which provided productivity values higher than those obtained on cereal residues owing to the comparatively lower time requirements to attain the product peak (Table 3). For example, the time required to achieve the product peak in *R. oryzae* TISTR3189 and *A. coerulea* CTCC AF 93105 cultures grown on solid potato-based matrices was 5 and 7 days (Kleekayai & Suntornsuk, 2011; Wang et al., 2008). Conversely, *L. edodes* SC-495, *R. oryzae* and *P. citrinum* ATCC 24095 cultures grown on wheat straw, corn straw and rice straw, respectively, reached the product peak 12 days after the inoculation (Crestini et al., 1996; Omogbai & Ikenebomeh, 2021; Khalaf, 2004).

Noteworthy, *Absidia coerulea* AF93105 solid-state cultures on potato waste provided a direct method of producing low molecular weight chitosan, which, due to its compatibility with agricultural and biomedical applications, is generally obtained by thermochemical or enzymatic depolymerization starting from high molecular weight chitosans (Wang et al., 2008); in particular, the polymer obtained with a yield of 6.1 g kg⁻¹ showed an average molecular weight of 6.4 kDa associated with a very low degree of polydispersity.

Twelve-day-old *R. oryzae* solid-state cultures on nutrient-supplemented rice straw yielded 5.63 g chitosan kg⁻¹ substrate; the chitosan thus obtained exerted a higher antibacterial activity towards a variety of pathogenic bacteria, such as *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella* sp. and *Escherichia coli* as compared to chitosan from crab shells (Khalaf, 2004).

However, all these studies were performed either on stationary flasks or using autoclavable plastic bags. Although a variety of solid-state reactors are available, unique exceptions are the studies of one research group (Nwe et al., 2002; Nwe & Stevens, 2002; Nwe & Stevens, 2004; Nwe et al., 2011) that used a perforated tray reactor to perform chitosan production (3.7-4.3 g kg⁻¹) with excellent DD (92-96%) from *Gongronella butleri* USDB0201 cultures grown on potato peel wastes. An additional exception is the study of Dhillon et al. (2013a) who used a rotary tumbling drum reactor to investigate the coproduction of citric acid and chitosan.

Table 3. Chitosan production yields (CPY), referred to unit mass of the solid substrate, average volumetric productivity (r_p), degree of deacetylation and viscosity of chitosan obtained in solid-state cultures of several fungal strains.

Fungal strain	Solid substrate	Cultivation mode	CPY (g kg ⁻¹)	r_p (mg kg ⁻¹ h ⁻¹)	DD (%)	Viscosity (cP)	References
<i>Absidia coerulea</i> CTCC AF 93105	Non-supplemented cotton seed hulls	Conical flask	1.62	9.64	85	n.r.	Wang et al., 2008
<i>Absidia coerulea</i> CTCC AF 93105	Potato pieces added with sucrose and urea	Conical flask	6.12	36.4	85	n.r.	Wang et al., 2008
<i>Aspergillus niger</i> n.s.	Rice straw	Plastic bag with sterile filters	5.26	24.4	84.2	59	Khalaf, 2004
<i>Aspergillus niger</i> TISTR3245	Mung bean residues	Conical flask	1.39	19.3	n.r.	n.r.	Suntornsuk et al., 2002
<i>Aspergillus niger</i> BBRC 20004	Soybean residues	Conical flask	17.03	59.1	n.r.	n.r.	Maghsoodi & Yaghmaei, 2010
<i>Gongronella butleri</i> USDB0201	Sweet potato pieces supplemented with urea	Tray reactor	3.7	22.0	92-96	n.r.	New & Stevens, 2002
<i>Gongronella butleri</i> USDB0201	Sweet potato pieces supplemented with urea	Tray reactor	4.31	25.7	n.r.	n.r.	New & Stevens, 2004
<i>Lentinus edodes</i> SC-495	Wheat straw	Plastic bag with sterile filters	6.18	21.5	87.5	n.r.	Crestini et al., 1996
<i>Mucor rouxii</i> ATCC 24905	Soybean meal	Autoclavable plastic bag with sterile filters	32.4	119.4	55-60	n.r.	Mondala et al., 2015
<i>Penicillium citrinum</i> n.s.	Rice straw	Plastic bag with sterile filters	5.12	17.8	78.5	4.6	Khalaf, 2004
<i>Penicillium expansum</i>	Corn straw	Conical flask	4.31	n.r.	80.2	4.8	Omogbai & Ikenebomeh, 2021
<i>Rhizopus oryzae</i> n.s.	Rice straw	Plastic bag with sterile filters	5.63	19.6	90.2	6.8	Khalaf, 2004
<i>Rhizopus oryzae</i> TISTR3189	Potato peel	Conical flask	6.6	55.0	87.5-90	3.1-6.1	Kleekayai & Suntornsuk, 2011
<i>Rhizopus oryzae</i> (local isolate)	Corn straw	Conical flask	8.57	29.8	91.5	7.2	Omogbai & Ikenebomeh, 2021
<i>Rhizopus oryzae</i> TISTR3189	Soybean residue	Conical flask	4.3	29.9	n.r.	n.r.	Suntornsuk et al., 2002

3.4.3.2 Fungal Chitosan Production in Liquid Submerged Bioprocesses

Several screening studies aimed at identifying valuable chitosan-producing strains relied on synthetic media, such as the yeast extract-malt extract medium (YM) (Miyoshi et al., 1992), potato dextrose broth (PDB) (Pochanavanich & Suntornsuk, 2002), yeast extract-peptone-dextrose (YPD) medium (Rane & Hoover, 1993; Amorim et al., 2003), glucose-peptone (Ebrahimzadeh et al., 2013) and glucose-peptone-yeast extract (GPY) (Nadarajah et al., 2001; Hu et al., 2004). Moreover, a variety of strain-oriented studies, summarized in Table 4, used these production media in original or slightly modified formulation. Particularly in the last decade, the high costs of these media have shifted the attention of researchers towards cheaper solutions. Moreover, the temporal distribution of studies conducted on synthetic media shows that a non-negligible part of them is far from recent. This review, however, considered it appropriate to reserve them a section. The reasons underlying this choice are many and include a high relevance from the production point of view, the provision of information relating to the choice of strains, physiology of production and reactor configuration.

3.4.3.2.1 Chitosan Production on Chemically Defined Liquid Media

Table 4 shows comparatively volumetric biomass and chitosan productions (X and CVP , respectively) and r_p values of chitosan in liquid cultures of several fungal strains grown on synthetic media either in the shaken flask or in the reactor.

By sorting the data shown in Table 4 based on the volumetric production, it is evident, with a few exceptions, that the best performing strains belong to the *Absidia* genus. This outcome does not change using the r_p of chitosan as the sorting criterion, and it is no coincidence that eight of the ten highest values are related to studies conducted in reactors. Furthermore, it is in agreement with the investigation of Shimahara et al. (1989) who had already concluded several years earlier at the end of a screening conducted on 125 strains of Zygomycetes, that those belonging to the genus *Absidia* were by far the most productive.

Table 4. Volumetric productions of biomass (X) and chitosan (CVP) and chitosan average volumetric productivity (r_p) in liquid cultures of several fungal strains grown on synthetic media either in shaken flask or in reactor.

Fungal strain	Culture condition and growth medium	X (g L ⁻¹)	CVP (g L ⁻¹)	r_p (mg L ⁻¹ h ⁻¹)	References
<i>Absidia butleri</i> NCIM977	Shaken cultures on GYT medium	6.78	0.57	7.9	Vaingankar & Juvekar, 2014
<i>Absidia coerulea</i> ATCC14076	Aerated shaken cultures on YM broth	6.2	1.86	26.0	Muzzarelli et al., 1994
<i>Absidia coerulea</i> ATCC14076	Batch cultures in 2.5-L STR at 250 rpm and 2 vvm with adaptive pH control at 4.5 on GY medium supplemented with (NH ₄) ₂ SO ₄	20	2.33	63.8	Kim et al., 2001
<i>Absidia coerulea</i> ATCC14076	Batch cultures in 20-L STR at 200 rpm and 1 vvm on PGY medium	13.9	0.55	11.5	ElMekawy et al., 2013
<i>Absidia coerulea</i> ATCC14076	Continuous cultures in 2.5-L STR with pH control at 4.5 on GY medium supplemented with (NH ₄) ₂ SO ₄ at a dilution rate of 0.05 h ⁻¹	7.0	1.04	50.0	Kim et al., 2001
<i>Absidia coerulea</i> ATCC14076	Continuous cultures in BioFlo C30 chemostat on GYP medium at a dilution rate of 0.025 h ⁻¹	2.3	1.37	41.0	Rane & Hoover, 1993
<i>Absidia coerulea</i> CTCC AF 93105	Shaken cultures on a glucose-based medium added with 0.5 g L ⁻¹ as (NH ₄) ₂ SO ₄	11.4	2.86	19.9	Jiang et al., 2011
<i>Absidia coerulea</i> CCRC 30897	Batch cultures in airlift with double net draft tube on GYP medium	30.8	3.16	65.8	Wu et al., 2001
<i>Absidia coerulea</i> CCRC 30897	Batch cultures in bubble column reactor on GYP medium	11.3	1.36	28.3	Wu et al., 2001
<i>Absidia glauca</i> (+)	Shaken cultures on GYP medium	8.8	0.65	13.5	Hu et al., 2004
<i>Absidia orchidis</i> NCAIM F 00642	Batch cultures in 5-L STR on GYP medium supplemented with ferrous ions	45.3	1.79	37.3	Jaworska & Konieczna, 2001
<i>Absidia orchidis</i> NCAIM F 00642	Batch cultures in 5-L STR on GYP medium supplemented with Mn ²⁺ ions	15.2	1.05	21.9	Jaworska & Konieczna, 2001
<i>Absidia repens</i> CBS 102-32	Batch cultures in 10-L STR at 350 rpm on a medium made of glucose, yeast extract and (NH ₄) ₂ SO ₄	12.9	2.8	58.3	Davoust & Persson, 1992
<i>Aspergillus niger</i> MTCC 872	Shaken cultures on a medium made of Potato Dextrose Broth (24 g L ⁻¹), glucose (80 g L ⁻¹), L-Asparagine (6 g L ⁻¹)	15.9	3.35	46.5	Kumaresapillai et al., 2011
<i>Aspergillus niger</i> BBRC 20004	Shaken cultures on Sabouro Dextrose Broth added with 2% glucose	5.17	0.84	17.5	Maghsoodi et al., 2009
<i>Aspergillus nidulans</i> NS	Shaken cultures on peptone-glucose-yeast extract (PGY) salt broth	5.15	0.20	4.19	Hu et al., 2004
<i>Benjaminiella poitrasii</i> CSIR isolate	Batch cultures in 2-L STR on medium containing (g L ⁻¹): yeast extract, 6.0; peptone, 10.0; soluble starch, 10.0	10.0	0.51	10.6	Mane et al., 2017
<i>Cunninghamella bertholletiae</i> IFM 46.114	Shaken cultures on yeast extract- peptone- dextrose medium	7.1	0.39	5.5	Amorim et al., 2006a
<i>Cunninghamella echinulata</i>	Shaken cultures on glucose-peptone- yeast extract medium added with (NH ₄) ₂ SO ₄	5.6	0.40	3.3	Tan et al., 1996

Fungal strain	Culture condition and growth medium	X (g L ⁻¹)	CVP (g L ⁻¹)	<i>r_P</i> (mg L ⁻¹ h ⁻¹)	References
<i>Cunninghamella elegans</i> IFM 46109	Shaken culture on a medium mainly made of glucose, asparagine and MgSO ₄ (60, 3.0 and 0.25 g L ⁻¹ , respectively)	11.0	0.86	8.9	Franco et al., 2004
<i>Cunninghamella elegans</i> UCP 542	Shaken cultures on Sabouraud-Sucrose medium	12.0	0.42	8.7	Stamford et al., 2007
<i>Gongronella butleri</i> USDB 0201	Shaken cultures on glucose-peptone- yeast extract medium added with (NH ₄) ₂ SO ₄	8.2	0.47	3.9	Tan et al., 1996
<i>Mucor racemosus</i> (soil isolate)	Shaken cultures on Sabouraud dextrose broth	3.8	0.45	2.6	Tajdini et al., 2010
<i>Mucor rouxii</i> ATCC24905	Shaken cultures on glucose-peptone- yeast extract medium	3.8	0.28	5.8	Synowiecki & Al-Khateeb, 1997
<i>Mucor rouxii</i> ATCC 24905	Shaken cultures on peptone-yeast extract-glucose (PYG) salt broth	5.6	0.21	4.4	Hu et al., 2004
<i>Mucor rouxii</i> DSM 1191	Batch cultures in 30-L STR on glucose-peptone- yeast extract medium	8.6	0.30	21.2	Gözke et al., 2011
<i>Rhizomucor miehei</i> ATCC 26282	Shaken cultures on Sabouraud dextrose broth	4.1	0.56	3.4	Tajdini et al., 2010
<i>Rhizopus oryzae</i> USDB 0602	Shaken cultures on glucose-peptone- yeast extract medium added with (NH ₄) ₂ SO ₄	5.7	0.28	2.3	Tan et al., 1996
<i>Syncephalastrum racemosum</i> UCP148	Shaken cultures on yeast extract- peptone- dextrose medium	8.0	1.26	26.1	Amorim et al., 2003

Wu et al. (2001) achieved the best r_P (65.8 mg L⁻¹ h⁻¹) ever reported with *A. coerulea* CCRC 30897 batch cultures grown in an airlift reactor modified with a double-net draft tube. This modified reactor enabled an excellent oxygen transfer to the liquid medium resulting in the achievement of a CVP value more than two times higher than that observed in a conventional bubble column reactor (3.16 vs 1.36 g L⁻¹). The same study also compared the performance of the modified airlift with that of a mechanically agitated reactor and found that CVP values were more than two-fold and 55% higher than those achieved in an STR with an impeller speed of 600 and 300 rpm, respectively.

Kim et al. (2001) also obtained very relevant results with another *A. coerulea* strain, viz. 14076, grown in a 2.5-L STR operated either in batch or continuous mode and using a glucose-YE medium supplemented with (NH₄)₂SO₄. In this study, the strategy of pH control at 4.5 led to higher maximal growth rate and smaller pellets than cultures where pH was left to fluctuate freely leading to 1.8- and 3.5-fold improvement in CVP and r_P , respectively. The same study claimed that when the STR was operated in continuous mode at a dilution rate of 0.05 h⁻¹, the r_P of the process was 52 mg L⁻¹ h⁻¹, a value higher than that obtained in a chemostat with the same strain by Rane and Hoover (1993). Noteworthy, batch cultures of the same strain grown in a 20-L STR in a similar medium

(ElMekawy et al., 2013) exhibited lower performance than those of Kim et al. (2001), presumably owing to either the larger process scale or omitted control of medium's pH.

Another species belonging to the genus *Absidia* which proved to be a good producer of chitosan was *A. repens* CBS 102.32; this strain, grown in a 10-L STR on a medium consisting of glucose and YE and added with $(\text{NH}_4)_2\text{SO}_4$, gave CVP and r_p values equal to 2.8 g L^{-1} and $58.3 \text{ mg L}^{-1} \text{ h}^{-1}$, respectively (Davoust & Persson, 1992). Although the CVP value was moderate (0.3 g L^{-1}), the study by Gözke et al. (2012), conducted in a 30-L STR reactor with *M. rouxii* DSM 1191 cultures on GYP medium, is noteworthy due to the scale of the reactor and the short process duration reaching maximum productivity after 14-16 h from the inoculation. The use of inorganic supplements, such as manganese and ferrous ions able to affect the chitin synthase and chitin deacetylase activities was tested by Jaworska and Konieczna (2001). This study conducted in a 4.5-L STR with *A. orchidis* NCAIM F 00642 grown on iron-supplemented yeast extract-peptone glycerol medium led to CVP and r_p values equal to 1.79 g L^{-1} and $58.3 \text{ mg L}^{-1} \text{ h}^{-1}$ which were several folds higher than those of non-supplemented cultures.

3.4.3.2.2 Chitosan Production on Waste- and Effluent-Based Liquid Media

As discussed in the previous section, the liquid media for fungal chitosan production often include organic components, such as YE, D-glucose, and peptone, which are costly growth substrates. For this reason, several studies investigated the exploitation of cheap carbon and nitrogen sources derived from wastes (Streit et al., 2009; Tasar et al., 2016; Namboodiri & Pakshirajan, 2019; Habibi et al., 2021) to mitigate the production costs and compete commercially with crustacean's shell-based processes (Batista et al. 2018).

With regard to their origin, these wastes derived from crop residues (Tai et al., 2010), corn wet-milling operations (de Souza et al., 2020) or were byproducts of the dairy industry (Chatterjee et al., 2008; Chatterjee et al., 2019), sugar manufacturing (Göksungur, 2004; Amorim et al., 2006a; Chatterjee et al., 2009; Tasar et al., 2016), fruit juice industry (Streit et al., 2009; Vendruscolo & Ninow, 2014) or distilleries (Yokoi et al., 1998; Ray & Ghangrekar, 2016). In several cases, the liquid medium was derived from a solid substrate either by acid hydrolysis, such as for corn straw (Tai et al., 2010) or *via* its aqueous extraction, such as in the case of apple pomace (Streit et al., 2009; Habibi et al., 2021) and date syrups (Tayel et al., 2014). Other studies, instead, relied on liquid byproducts, such as sugarcane or sugar beet molasses (Amorim et al., 2006a;

Amorim et al., 2006b; Tasar et al., 2016) and deproteinized whey (Chatterjee et al., 2008). Suitable liquid production media have been also derived from a variety of process effluents, such as cassava wastewater (Berger et al., 2014a), paper mill effluent (Namboodiri & Pakshirajan, 2019), thin stillage (Ray & Ghangrekar, 2016) and xylose-rich wastewater from a bioethanol plant (Zamani et al., 2007) (Table 5).

Table 5. Volumetric productions of biomass (X) and chitosan (CVP) and chitosan average volumetric productivity (r_p) in liquid cultures of several fungal strains grown on liquid byproducts or process effluents either in shaken flask or in reactor.

Fungal strain	Culture condition and growth medium	X (g L ⁻¹)	CVP (g L ⁻¹)	r_p (mg L ⁻¹ h ⁻¹)	References
<i>Absidia coerulea</i> CTCC AF 93105	Shaken cultures on a glucose-based medium (20 g L ⁻¹) added with 0.5 g L ⁻¹ nitrogen as soybean pomace	15.4	4.11	28.5	Jiang et al., 2011
<i>Aspergillus awamori</i> MTCC6995	Shaken cultures on thin stillage from rice-based distillery	5.2	0.39	4.0	Ray & Ghangrekar, 2016
<i>Aspergillus brasiliensis</i> ATCC16404	Shaken cultures on syrup from date waste	13.3	2.78	19.3	Tayel et al., 2014
<i>Aspergillus niger</i> NRRL567	Batch cultures in 7.5-L STR (impeller speed at 200 rpm; adaptive flow rate to ensure 20% dissolved oxygen saturation) on apple pomace sludge	12.6	0.64	4.9	Dhillon et al., 2013a
<i>Cunninghamella bertholletia</i> IFM 46.114	Shaken cultures on sugarcane juice (10.5 g L ⁻¹ sucrose) added with YE (3 g L ⁻¹)	4.2	0.53	11.1	Amorim et al., 2006a
<i>Cunninghamella elegans</i> UCP 0542	Shaken cultures on a medium made of cassava wastewater (CWW, 10%) and corn steep liquor (CSL, 4%)	5.7	0.33	4.6	Berger et al., 2014a
<i>Cunninghamella elegans</i> UCP 0542	Shaken cultures on a medium made of CWW (10%) and CSL (4%)	5.7	0.33	4.6	Sharifia et al., 2008
<i>Gongronella butleri</i> CCT4274	Shaken cultures on aqueous extract of apple pomace supplemented with NaNO ₃ (2.5 g L ⁻¹)	5.5	1.19	16.4	Streit et al., 2009
<i>Gongronella butleri</i> IFO8081	Shaken cultures on sweet potato Shochu distillery wastewater	6.2	0.73	6.1	Yokoi et al., 1998
<i>Gongronella butleri</i> CCT 4274	Batch cultures in 6.5-L airlift reactor with external loop circulation (aeration rate, 0.6 vvm) on apple pomace extract added with 5 g L ⁻¹ (NH ₄) ₂ SO ₄	6.7	0.93	62.2	Vendruscolo & Ninow, 2014
<i>Lichtheimia hyalospora</i> UCP1266	Shaken cultures on a medium made of CWW (4%) and CSL (6%)	11.9	0.75	6.3	de Souza et al., 2020
<i>Mucor rouxii</i> MTCC 386	Shaken cultures on molasses salt medium added with indole-3-acetic acid (1.0 mg L ⁻¹)	9.1	0.95	29.7	Chatterjee et al., 2009
<i>Mucor subtilissimus</i> UCP 1262	Shaken cultures on a medium made of CWW (4%) and CSL (6%)	4.8	0.16	1.3	de Souza et al., 2020

Fungal strain	Culture condition and growth medium	X (g L ⁻¹)	CVP (g L ⁻¹)	r _p (mg L ⁻¹ h ⁻¹)	References
<i>Penicillium citrinum</i> (local isolate)	Batch cultures in 3-L Stirred Tank Reactor (200 rpm and 2.0 vvm) on paper mill effluent added with 50 mg L ⁻¹ acetic acid	n.s.	0.14	2.9	Namboodiri & Pakshirajan, 2019
<i>Rhizopus arrhizus</i> UCP 0402	Shaken cultures on a medium made of CSL (4%) and honey (13%)	11.7	0.34	3.6	Cardoso et al., 2012
<i>Rhizopus oryzae</i> MTCC262	Shaken cultures on deproteinized whey added with gibberellic acid (0.1 mg L ⁻¹)	8.3	1.13	15.7	Chatterjee et al., 2008
<i>Rhizopus oryzae</i> 00.4367	Batch cultures in 7-L STR (340 rpm, 2.1 vvm) on untreated sugarbeet molasses (45.4 g L ⁻¹ total sugars)	10.7	1.06	14.7	Göksungur, 2004
<i>Rhizopus oryzae</i> AS 3.819	Batch cultures in 3-L Stirred Tank Reactor (200 rpm, 1 vvm) on corn stover hydrolysate supplemented with urea (4 g L ⁻¹)	11.0	0.99	13.8	Yang et al., 2017
<i>Rhizopus oryzae</i> PAS 17	Shaken cultures on medium made of molasses (7%, v/v) and supplemented with MgSO ₄	10.7	1.50	7.8	Tasar et al., 2016
<i>Rhizopus oryzae</i> ME-F12	Shaken cultures on corn straw hydrolysate	5.2	0.58	n.s.	Tai et al., 2010
<i>Rhizopus oryzae</i> MTCC262	Shaken culture on deproteinized whey supplemented with (NH ₄) ₂ HPO ₄ (8 g L ⁻¹) and YE (2 g L ⁻¹)	6.2	0.62	n.s.	Chatterjee et al., 2019
<i>Syncephalastrum racemosum</i> UCP148	Shaken cultures on sugarcane juice (10.5 g L ⁻¹ sucrose) added with YE (3 g L ⁻¹)	8.1	0.60	5.0	Amorim et al., 2006b
<i>Syncephalastrum racemosum</i> UCP148	Batch cultures in 5-L STR on sugarcane juice (10.5 g L ⁻¹ sucrose) added with YE (3 g L ⁻¹)	8.0	0.96	32.0	Amorim et al., 2006b

In several cases, the approach adopted involved only a partial replacement of the expensive organic components of the medium, intended to act as sources of carbon or nitrogen, with others derived from residues of agro-industrial origin. For example, Jiang et al. (2011) obtained excellent CVP (4.11 g L⁻¹) and r_p (28.54 mg L⁻¹ h⁻¹) values in *A. coerulea* CTCC AF 93105 cultures on a glucose-based medium by replacing commercial sources of inorganic nitrogen with soybean pomace.

S. racemosum UCP148 cultures grown in a 5-L STR containing sugarcane juice supplemented with 0.3% YE yielded CVP and r_p values as high as 0.93 g L⁻¹ and 32 mg L⁻¹ h⁻¹, respectively (Amorim et al., 2006b).

In other studies, instead, the approach was to integrally eliminate the expensive organic components of the medium with the liquid residue used as it was or, possibly, supplemented with inorganic nitrogen sources. For instance, *G. butleri* CCT 4274 cultures grown in a 6.5-L airlift reactor on an aqueous extract of apple pomace (AEAP) supplemented with an inexpensive source of nitrogen, such as ammonium sulfate (5 g L⁻¹) gave a CVP value of 0.93 g L⁻¹ but, above all, one of the highest r_p values of chitosan

ever reported for reactor cultures ($62 \text{ mg L}^{-1} \text{ h}^{-1}$) (Vendruscolo & Ninow, 2014). The use of the airlift enabling better gas exchanges associated with the use of a more readily available nitrogen source gave much better results than those reported by Streit et al. (2009) with the same strain grown on sodium nitrate-supplemented AEAP. Göksungur (2004) used untreated and non-supplemented sugar beet molasses as the liquid medium for chitosan production by *R. oryzae* 00.4367 in a 7-L STR; the use of a response surface methodology approach allowed the investigators to optimize statistically both agitation and aeration regimes and sugar concentration. In particular, under the optimization of these variables (impeller speed, 340 rpm; aeration rate, 2.1 vvm; sugar concentration, 45.4 g L^{-1}), the CVP and r_P amounted to 1.06 g L^{-1} and $14.5 \text{ mg L}^{-1} \text{ h}^{-1}$, respectively.

Whey, a by-product of the dairy industry, the world production of which amounts to 121 million tons (Petrucchioli et al., 2011), was also used as the basis for the development of a chitosan production medium. In particular, *R. oryzae* MTCC262 cultures, grown on deproteinized whey (DW) and added with 0.3% YE and 0.1 mg L^{-1} of gibberellic acid (GA3), gave CVP and r_P values equal to 1.13 g L^{-1} and $15.72 \text{ mg L}^{-1} \text{ h}^{-1}$ (Chatterjee et al., 2008). In a subsequent study, the same research group reported for the same strain on a DW-based medium, but differently formulated (Table 5), CVP and r_P values equal to 0.62 g L^{-1} and $8.6 \text{ mg L}^{-1} \text{ h}^{-1}$ (Chatterjee et al., 2019).

With regard to the use of process effluents for the development of chitosan production media, several studies focused the attention on that produced by the cassava (*Manihot esculenta*) processing industry, termed cassava wastewater (CWW). Recent estimates established that the processing of 1 ton of tubers generates around 60 m^3 of effluent (Achi et al., 2020). Several studies shared the combination of CWW with corn steep liquor, a byproduct of corn wet-milling, frequently used as a low-cost nitrogen source (Berger et al., 2014a; Sharifia et al., 2008; de Souza et al., 2020). Among these studies, best process performance was obtained with *Lichtheimia hyalospora* UCP1266 yielding CVP and r_P values amounting to 0.75 g L^{-1} and $4.56 \text{ mg L}^{-1} \text{ h}^{-1}$ (de Souza et al., 2020).

Grain-based distilleries generate thin stillage (TS) as the high-strength process effluent, the volume of which is ten-fold higher than that of the ethanol produced. Ray and Ghangrekar (2016) used *Aspergillus awamori* MTCC6995 to reduce the organic load of a rice-based TS and to extract chitosan from the residual fungal biomass; at its endpoint (96 h), the process yielded 60% reduction of the effluent's COD and CVP and r_P of chitosan amounted to 0.39 g L^{-1} and $4.02 \text{ mg L}^{-1} \text{ h}^{-1}$, respectively. Yokoi et al. (1998)

used sweet potato-shochu wastewater as the chitosan production medium for *G. butleri* IFO 8081, which attained a CVP of 0.73 g L⁻¹ after 120 h from the inoculation.

3.4.4 Relevant Factors in Chitin and Chitosan Production from Fungi

Production levels of these glycans can be achieved *via* an increase in the biomass yield or an increase of their contents in the cell wall (Jaworska & Konieczna, 2001), and several process parameters were found to be relevant to this goal. These parameters affect not only the production of these glycans but also their physicochemical properties (Jaworska & Konieczna, 2001; Chatterjee et al., 2005; Chatterjee et al., 2008).

3.4.4.1. Fungal morphology

Fungal morphology, especially during cultivation in reactors, frequently evolves in a way resulting in a decline in the growth rate (Veiter et al., 2018). The growth mode involving the formation of dispersed mycelium leads to a highly viscous medium with ensuing agitation and aeration problems (Quintanilla et al., 2015). Several strains, instead, have a marked propensity to form pellets and, in that instance, an increased pellet diameter might result in dropped growth owing to diminished mass transfer to the pellet's innermost part (Veiter et al., 2018). Several studies highlighted the importance of the pellet size, showing that diameters in the 4.0-5.0 mm were most conducive to the maximization of D-glucosamine yield in *R. oligosporus* (Sparringa & Owens, 1999) or chitosan production by *Lichtheimia hyalospora* UCP 1266 (de Souza et al., 2020). Similar results were obtained with *Absidia repens* CBS 102.32 (Davoust & Persson, 1992), grown at different stirring regimes (i.e., 350, 200, 200 for the early 24 h followed by 350 rpm) in a 10-L STR; growth at 350 rpm, leading to the formation of pellets with average diameter of 0.5 mm was the condition enabling best chitosan production (2.8 g L⁻¹) with a r_p of 58 mg L⁻¹ h⁻¹.

Macro-morphology can also largely affect chitosan production of several dimorphic species, such as *M. indicus* (Satari et al., 2016) and *M. subtilissimus* (de Souza et al., 2020). The growth mode in dimorphic fungi is affected by several factors, including initial spore concentration, sugar content in the growth medium, and oxygen availability; moreover, the shift to a yeast-like morphology can be promoted by the addition of compounds acting as cytochrome oxidase inhibitors. Noteworthy, de Souza et al. (2020) reported that the increase in concentrations of cassava wastewater, containing cyanides, promoted a yeast-like growth mode and negatively affected the chitosan production in *M.*

subtilissimus UCP 1262 cultures. The same study found that best chitosan production conditions were those leading to the mycelial form in agreement with other studies conducted with *M. indicus* CCUG 22424 (Satari et al., 2016a) and *M. rouxii* (Bartnicki-Garcia and Nickerson, 1962).

3.4.4.2. Harvesting time

Although chitin and chitosan productions are obviously growth-associated processes, their maximum yields do not necessarily occur at the biomass peak (Akila, 2014). With this regard, in fact, the amount of extractable chitosan was highest at the late exponential growth phase in *Asidia coerulea* (Jiang et al., 2011), *Rhizopus oryzae* 00.4367 (Göksungur, 2004), *R. oryzae* USDB 0602 (Tan et al., 1996) and *Cunninghamella bertholletiae* (Amorim et al., 2006a) to decline significantly thereafter. Tan et al. (1996) suggested that free chitosan molecules were largely abundant during the exponential phase, owing to active growth; during the stationary growth phase, instead, a higher proportion of chitosan bound to other cell's wall constituents, thus rendering extraction less effective. This effect was also evident in *R. oryzae* solid-state cultures on rice straw where the ratio between chitosan and alkali-insoluble material (AIM) dropped from 61%, in concomitance with biomass peak, to 42% when culture had entered the stationary phase (Khalaf, 2004). Albeit not yet proven *in vivo*, an alternative explanation might be a time-dependent increase in chitin crystallinity, due to the failure of the majority of CDAs to perform the *in vitro* deacetylation of crystalline chitin (Grifoll-Romero et al., 2018). Notable exception to this time-dependent trend in chitosan production was the study of Davoust and Persson (1992) who observed chitosan accumulation in the late stationary phase of *Absidia repens* cultures; they hypothesized that since the synthesis of chitin and chitosan occurs at the hyphal apex during the elongation process, the observed increase was due to continued apical growth at the expense of products derived from partial cell lysis.

3.4.4.3. Medium's pH

By using a modified GPY medium adjusted to several pH values (range: 3.0-6.5), Rane and Hoover (1993) found that the chitosan-producing ability and DD of chitosan were largely unaffected in *M. rouxii* DSM1191; the same study, however, found significant strain-dependent effect of the pH on the same descriptors in *A. coerulea* ATCC 14076 and *A. coerulea* NRRL 1315. Kim et al. (2001) observed that pH affected fungal

morphology in *A. coerulea* ATCC 14076 cultures and adaptive control at pH 4.5 enabled the formation of smaller pellets than those without pH control in batch cultures conducted in a STR. This pH control strategy enabled higher CVP and r_p as compared to cultures without pH control (2.3 vs. 1.3 g L⁻¹ and 63.9 vs. 36.1 mg L⁻¹ h⁻¹, respectively). Solid-state *G. butleri* cultures conducted on sweet potato pieces at various initial pH (i.e., 3.77, 4.92, 5.46 and 5.52) gave better chitosan yields at pH 5.46 and 5.52 and the chitosans obtained had higher average molecular weights than those at more acidic pHs (Nwe & Stevens, 2004).

3.4.4.4. Nitrogen source and concentration

Both the nitrogen source and its amounts are among the most relevant process parameters since chitosan is a nitrogen-containing glycan. In general, fungi are able to directly exploit ammonium ions, while other inorganic nitrogen sources have to be reduced to the redox level of ammonium (Jennings, 1989). Nwe and Stevens (2004) showed that urea was a valuable N source for chitosan production by *Gongronella butleri* solid-state cultures. By increasing the urea levels from 5 to 14 g per kg of solid substrate, in addition to obtaining a significant increase in the yield of chitosan (from 0.082 to 0.114 g g⁻¹ mycelium), there was a disproportionate increase in weight average molecular weight (M_w) as compared to number average molecular weight (M_n), thus increasing polydispersity (M_w/M_n) (Nwe & Stevens, 2004). A dose-dependent effect of the N source on molecular weight of *Mucor rouxii* chitosan was also observed by Arcidiacono and Kaplan (1992) by doubling either peptone or yeast extract concentration in an YPG medium. Several studies claimed that the impact of yeast extract, a costly organic nitrogen source derived from cells autolysis, was beneficial to chitosan production (Arcidiacono and Kaplan, 1992; Amorim et al., 2006a; Amorim et al., 2006b). To improve the economic feasibility of the chitosan production process, Abasian et al. (2020) replaced YE with an autolysis-derived *M. rouxii* extract in a glucose-based medium; this approach improved the yields of the AIM and led to increased GlcN and decreased GlcNAc concentrations in AIM in *M. rouxii* CCUG 22424 cultures.

3.4.4.5. Plant growth hormones

Some studies showed that addition of phytohormones to *R. oryzae* and *Mucor rouxii* liquid cultures grown on deproteinized whey (Chatterjee et al., 2008) and molasses-salt medium (Chatterjee et al., 2009), respectively, stimulated both fungal biomass and

chitosan productions although in a dose-dependent manner. Among the tested phytohormones (i.e., indole-3-acetic acid, IAA; indolebutyric acid, IBA; gibberellic acid, GA3 and kinetin, KIN), GA3 was the most effective. In fact, the addition of GA3 at 0.1 and 3.0 mg L⁻¹ concentration to *R. oryzae* (Chatterjee et al., 2008) and *M. rouxii* (Chatterjee et al., 2009) cultures resulted in a 50% and 69% increase in chitosan, respectively; however, in both studies, higher GA3 doses had an inhibitory effect on chitosan production. Chatterjee et al. (2008) also showed a 27% increase in the specific activity of chitin deacetylase, in GA3-added cultures as compared to control ones thus leading to increased chitosan/chitin ratio (0.72 vs. 0.49). Another impact of GA3 addition was an increase in MW of chitosan derived from hormone-added cultures as compared to control ones (Chatterjee et al., 2008; Chatterjee et al., 2009).

3.4.4.6. Organic stimulators

Another range of potential stimulators of chitosan biosynthesis emerged indirectly from a study reporting on the superiority of a corn straw hydrolysate for chitosan production by *R. oryzae* ME-F12 over glucose-, and xylose-based media (Tai et al., 2010). This acid hydrolysate, in addition to containing xylose, as the main component and other pentoses, also contained furfural, acetic acid and formic acid. The last three compounds added to a xylose medium exerted a dose-dependent stimulatory effect on both chitosan production and chitosan yield (Tai et al., 2010); the authors speculated that the enhanced chitosan synthesis was a compensatory response of the fungus to increase thickness and density of the cell wall to better cope with the presence of potentially-inhibitory compounds.

3.4.4.7. Inorganic supplements

Some studies have taken into consideration the effect deriving from the addition of some inorganic supplements, such as Mg²⁺, Co²⁺, Mn²⁺ and Fe²⁺ ions, capable of influencing the *in vitro* activity of chitin deacetylase and chitin synthetase (Gooday et al., 1997; Grifoll-Romero et al., 2018). For instance, the wide recognition of the stimulatory effect of Mg²⁺ ions on both CS and CDA have led to the inclusion of MgSO₄ in several chitosan-production media at concentration ranging from 0.5 to 5.0 g L⁻¹ (Rane & Hoover, 1993; Jaworska & Konieczna, 2001; Kim et al., 2001).

For some microelements such as cobalt, the effect was strongly concentration-dependent or even led to growth failure due to the intrinsic toxicity of this metal. In fact,

Rane and Hoover (1993) reported while the addition of 5 mg L⁻¹ to a modified GPY medium led to a 20% increase in chitosan production in *A. coerulea* ATCC 14076 cultures, a four-fold increase in its concentration led to inhibitory effects. In another study attempting to exploit the stimulatory effect of several ions, the addition of Co²⁺ (2.3 and 4.5 g L⁻¹) to a YPG medium led to a severe growth inhibition in *A. orchidis* NCAIM F 00642 cultures [31]. Conversely, the same study found that the addition of Mn²⁺ ions led to an improved CVP as compared to control cultures (1.03 vs 0.71 g L⁻¹), increased viscosimetric molecular weight (1156 vs 751 kDa) and decreased DD (69.3 vs 84.4%).

Phosphates are primary constituents in the cell walls of Zygomycetes and several studies indicate that their contents can vary from 8.3 to 23% (Bartnicki-Garcia & Nickerson, 1962; Jeihanipour et al., 2009). Phosphates interact with chitosan and other structural polysaccharides to yield complexes that are not broken down by conventional acid treatment. As a consequence, a significant amount of chitosan can remain associated with both acid- and alkali-insoluble materials during extraction. Several studies showed that low concentrations of phosphates in the growth medium led to increased GlcN yields (Mohammadi et al., 2013; Safaei et al., 2016). In Zygomycetes, the accumulation of anionic storage materials, such as polyphosphates, is thought to be performed by chitosan, due to its polycationic nature (Karimi & Zamani, 2013). Consequently, under P-limiting conditions, chitosan synthesis is probably boosted to enhance the uptake of the phosphates from the growth medium. Moreover, in media containing inorganic nitrogen sources, such as ammonium sulfate (AS), the limitation of inorganic phosphorus (Pi) stimulates the overproduction of chitin/chitosan in some Zygomycetes (Dzurendova et al., 2020). An acidic stress caused by the low concentrations of Pi in the growth medium was suggested to be the primary reason explaining this phenomenon in AS-Pi media at low Pi concentrations (Bignell, 2012). These findings are in agreement with a chitosan optimization study conducted with *Mucor indicus* CCUG22424 cultures, where a phosphate-free medium led to the highest chitosan production (Safaei et al., 2016).

3.5 Integrated Bioprocesses

In recent decades, significant advances have been made towards promoting a sustainable bio-based economy. A positive outcome of these endeavors primarily depends on the enactment of the concept of biorefinery, namely a facility able to integrate processes and equipment to yield a wide array of marketable products and energy (Demirbas, 2009). Pursuing the concept of co-production can increase the economic viability of the microbial processes currently in place (Dhillon et al., 2013a; Durendzova et al., 2020). Moreover, there is an undeniable urgency to promote integrative technology to exploit the vast amounts of mycelial waste from industrially-relevant processes. In this framework, the mycelial wastes from various bioprocesses can be exploited as feasible chitosan sources in compliance with the biorefinery concept. These fungal strains mostly belong to the *Aspergillus* and *Mucor* genera, the species of which are used in several relevant processes. Unfortunately, microbial productions are sometimes governed by very different factors and, therefore, the concept of co-production can be difficult to pursue. This may mean sacrificing one of the two co-products or, otherwise, using tools capable of safeguarding the production levels of both. Among these tools, we can mention the use of high-throughput culture techniques (Durendzova et al., 2020) and that of statistical optimization methods enabling the identification of variable combinations maximizing both coproducts such as in response surface methodology (Elsoud & El Kady, 2019; Zininga et al., 2019).

In a very recent study, the combination of high-throughput culture technique with Fast-Fourier transform infrared spectroscopy allowed a fast determination of the impact of two relevant variables (i.e., two nitrogen typologies and inorganic phosphate) on the coproduction of single cell oils, polyphosphates, and chitin/chitosan in 9 different Zygomycetes strains (Durendzova et al., 2020).

One relevant bioprocess is the annual production of citric acid by *Aspergillus niger* strains, estimated to be around 1.7 million tons, and generating 0.34 million tons of fungal waste (Dhillon et al., 2013a). The average chitin contents in *A. niger* strains are around 15-22% of the dry mycelium and the polymer can be easily extracted and converted into chitosan (Cai et al., 2006). Dhillon et al. (2013a) integrated citric acid (CA) production by *A. niger* NRRL567 cultures on apple processing wastes and subsequent extraction of chitosan from waste stream; under LSF conditions, 132-h-old cultures grown in a 7.5-L STR on apple pomace sludge, gave volumetric CA and chitosan productions of 18.4 and

0.64 g L⁻¹, respectively. Solid-state cultures on apple pomace performed well leading to CA and chitosan production of 182 and 64 g kg⁻¹ dry substrate, respectively, after 120 h from the inoculation (Dhillon et al., 2013b); the viscosity (1.02–1.18 mPa s⁻¹) and DD (78–86%) of chitosan preparations from SSF and LSF cultures resembled those of chitosan from crab shells. A previous study, conducted on the *A. niger* spent biomass from a production plant of citric acid, showed that the use of an enzymatic extraction method yielded a chitosan with higher MW and D-glucosamine content and a similar DD as compared to the conventional alkali-acid reflux method (Cai et al., 2006).

Liao et al. (2008) investigated the co-production of fumaric acid and chitin by *Rhizopus oryzae* ATCC 20344 using a N-nitrogen rich liquid fraction of dairy manure and a DM-derived hydrolysate from sequential alkaline peroxide and enzymatic treatment; under optimal conditions, volumetric fumaric acid and chitin productions were 31 and 2.4 g L⁻¹, respectively.

The lipid-accumulation ability of several members of the Mucorales order makes them valuable candidates in second-generation biodiesel production (Kumar et al., 2017). This feature combined with the greater proportion with which chitosan and chitin enter the constitution of the cell wall opens up the possibility of profitably using their residual biomass. Zininga et al. (2019) seized this opportunity by associating the production of biodiesel by *M. circinelloides* ZKT with the chitosan recovery from the spent biomass; the lipid and chitosan contents of the biomass grown on a glucose-YE-peptone medium were 21.4 and 11.2%, respectively.

Vinche et al. (2012) developed an anaerobic chitosan-ethanol coproduction process by *Rhizopus oryzae* cultures grown on wheat hydrolysate containing variable glucose amounts (15–190 g L⁻¹). The initial sugar concentration markedly affected chitosan and ethanol production. The yield of the latter ranged from 0.28 to 0.46 (g g⁻¹ sugar consumed), and the best value of this parameter was observed at 45 g L⁻¹ glucose. The AIM was a major fraction (17–25%) of the *R. oryzae* dry mycelium and GlcN and GlcNAc constituted 61–65% of this fraction. In particular, the GlcN content reached its maximum (0.46 g g⁻¹ AIM) at a glucose concentration of 45 g L⁻¹.

Penicillium chrysogenum is extensively exploited for the industrial production of antibiotics, thus generating large amounts of mycelial wastes mostly disposed of by landfilling or incineration. On average, the production of 1 ton penicillin generates 8–10 tons of waste biomass, the annual amount of which is estimated to amount to 1.2 million tons (Ghormade et al., 2017).

P. chrysogenum mycelial waste supplied by a pharmaceutical company (Tianqi et al., 2007) underwent an integrative extraction method aimed at recovering (1-3)- α -D-glucan, ergosterol and chitosan; since the presence of alkali was required for the chitin deacetylation and for saponification during ergosterol isolation, the extraction scheme integrated these two steps for simplification.

3.6 Conclusion

There are several companies on the market that commercialize chitosan-based products of fungal origin, such as Kitozyme (<http://www.kitozyme.com>), MycoDev Group Inc. (<https://mycodevgroup.com>) and Chibio (<https://www.chibiotech.com>). This suggests an autonomous production capacity or, alternatively, the exploitation of fungal biomass derived from mycobiotechnological processes. Although the scientific literature currently available witnesses several efforts towards process upscaling to a diverse range of LSF reactors, studies reporting process scales on the order of cubic meters are absent. The solid-state production of chitosan has been regarded as a promising route since it enables high product concentration and is less equipment oriented than LsF. However, the use of SSF is negatively affected by mass transfer limitation phenomena, heat dissipation problems above all, which become critical in large-scale processes.

Despite these limitations, there are several issues that witness in favor of fungal chitosan. The advantages of fungal approaches to chitosan production are well documented with relatively uniform physico-chemical properties of the product made possible by accurate bioprocess control. As discussed earlier, a variety of studies shows that MW, polydispersity and DD can be manipulated by deliberate variations in process conditions. Obtaining reproducible values of these parameters is fundamental to guaranteeing the acceptance of chitosan in critical sectors such as the medical and pharmaceutical sectors. The possibility of controlling MW and polydispersity of the polymer allows to satisfy specific application requirements. This target is made difficult with the conventional extraction process relying on shellfish wastes, due to the variability and non-uniformity of the raw material and the relative complexity of the extraction scheme. Switching from conventional to mushroom-based processes requires economic and environmental factors to be balanced and carefully evaluated. A likely scenario involves an increased environmental unacceptability of the conventional process

associated with raised pollution abatement costs. An ever-increasing adoption of integrated bioprocesses, that in addition to the primary product, yield chitosan as a co-product from waste mycelia, might boost and increase the diffusion of this technology.

3.7 References

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CHAPTER 4

Digestate-based Liquid Growth Medium for Production of Microbial Chitosan

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4.1 Abstract

This study evaluated the Enriched Liquid Phase (ELP), a liquid medium derived from the digestate waste, as a substrate for the cultivation of selected Zygomycetes fungi capable of accumulating high levels of chitosan, a polymer of relevant commercial interest. After an initial screening phase, aimed at testing the growth and chitosan production capacity of the selected strains, the production process was transferred to laboratory bioreactor level with the most promising strains. The chitosan obtained through microbial bioconversions was analyzed for its physicochemical properties, such as degree of deacetylation, viscosity and molecular weight, and, in view of an important application of this polymer, for its antimicrobial properties. Then, the production process was transferred from shaken flask to stirred tank reactor for the most promising strains.

4.2 Introduction

Chitin is one of the main constituents of crustaceans' shells and an abundant component of the exoskeletons of insects and mollusks. For several decades, the production of chitin and its deacetylated counterpart, chitosan, relies on the extraction of crab and shrimp and shell wastes, where chitin amounts range from 14 to 27% and 13 to 15%, respectively. Since protein, lipids, inorganic salts are also significant structural components of crustacean shell wastes, chitin and chitosan extraction from these sources require a series of sequential and harsh chemical stages (Kim & Rajapakse, 2005).

However, chitin and chitosan are not unique to the animal kingdom since they make up a significant fraction of the cell wall of different fungal classes (Elsoud & El Kady, 2019); this has opened the door to extensive research on these polymers' production through fungal bioprocesses. Several technical elements make the fungal route of

production an exciting alternative to the conventional extraction approaches from the shellfish residues mentioned above. First, during processing, the demineralization step is not needed since the crude chitin from fungal sources has significantly lower levels of ash than crustacean shell (Teng et al., 2001). In addition to offering a non-seasonal source of chitin and chitosan, fungal bioprocesses are susceptible to accurate control, thus potentially ensuring stable characteristics of the product obtained (Chien et al., 2016; Elsoud & El Kady, 2019). 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 (Chien et al., 2016).

Therefore, given the better quality of chitin and chitosan present in the fungal cell wall and the lower environmental impact of their extraction process from fungal sources compared to that from crustacean waste, recent research is now focused on mycobiotechnology, relying on fungal strains able to produce chitosan in their cell wall directly. The strains that meet this requirement belong to the Zygomycota division and have a high percentage of chitin and chitosan in their cell wall, and, for this reason, they are very promising sources for the production of chitosan (Hu, 2004; Chatterjee et al., 2005).

The economics of chitosan production using inexpensive raw materials can make industrial microbial processes competitive with chemical ones. For this reason, studies on the use of alternative low-cost carbon sources to make the fungal chitosan production process highly sustainable are of particular interest in this area. In particular, several studies claimed the attainment of promising chitosan levels in liquid cultures conducted on apple pomace (Streit et al., 2009), corn steep liquor (Cardoso et al., 2012), cassava wastewater (Berger et al., 2014), thin stillage (Ray & Ghangrekar, 2016), and paper-mill wastewater (Namboodiri & Pakshirajan, 2019).

The digestate, the waste derived from anaerobic conversion processes, might represent a potentially promising source of nutrients from which derive a fungal growth medium. The composition and quality of this by-product depend strongly on the raw materials used and the treatment conditions applied (Makádi et al., 2012; Fuchs & Drosch, 2013). The digestate derived from the anaerobic conversion of wastes arising from the agricultural or agro-industrial sector can represent an excellent source of carbohydrates due to the significant content of residual fibres.

The objective of the present study was to derive a digestate-based liquid growth medium and assess its adequacy in microbial chitosan production subsequently. To this

end, a digestate arising 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 optimized medium was then tested for its ability to support growth and chitosan production of 17 fungal strains belonging 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.

4.3 Materials and Methods

4.3.1 Materials

The digestate used in this study 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. The waste, at the end of the chain, was also subjected to a liquid / solid separation, from which a solid fraction, from here onwards referred to as digestate solid fraction (DSF) and a liquid one, referred to as digestate liquid fraction (DLF) were derived. Both fractions were subjected to a preliminary characterization aimed at determining 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 digestate fractions were performed as described elsewhere (Carota et al., 2021). The chemical composition of most relevant parameters in DSF, expressed in g kg⁻¹, were: total sugars, 653 ± 34; reducing sugars, 9.2 ± 0.4; crude lipids, 19.3 ± 2.7; phenols, 15.3 ± 2.4; hemicellulose, 172.2 ± 14.9; cellulose, 393.4 ± 24.4; lignin, 257.4 ± 14.2; ash, 82.1 ± 3.6; total nitrogen, 19.8 ± 1.1; total carbon, 415.3 ± 8.2; 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 ± 0.42; N-NH₄, 1.09 ± 0.01; total amino acids, 6.57 ± 0.24; phenols, 0.76 ± 0.03.

4.3.2 Medium Development from Digestate's Fractions

As a first approach to developing a culture medium from the DSF and DLF components, a set of experiments was set up to verify the possibility of using solid-liquid extraction alone or assisted by hydrolysis under 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 case of acid- or alkali-assisted extractions, sulfuric acid or sodium hydroxide were 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 relied on 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. In addition to the volumetric concentration of the component x in the ELP, the percent recovery yield (RY , %) for each component x_i was calculated by the following expression:

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

where $C_{x(DLF)}$ is the initial concentration of component x in the digestate liquid extractant, $C_{x(ELP)}$ is concentrations 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, a design of experiments, based on the statistical approach of the response surface methodology, was used to derive an ELP from DSF. Due to the inefficacy of solid/liquid extraction and alkali-assisted extraction approaches, the attention focused on acid-assisted extraction. In particular, the design of experiments was a full factorial design recruiting two variables both at three levels, namely H_2SO_4 concentration (2, 6 and 10%, w/v) and extraction time (30, 60 and 120 min). As a consequence, the number of runs was 9, each of which replicated twice, plus 3 center points. The actual values of the variables were coded as dimensionless terms using equation:

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

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:

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

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).

4.3.3 Microorganisms

Except for *Mucor moelleri* DIBAF-E (Accession number KY678773) which was an environmental isolate from an Arsenic-contaminated soil, 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, NRRL 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. The strains were maintained at 4°C and routinely sub-cultured every month on Malt Extract Agar (MEA, Oxoid) Petri dishes.

4.3.4 Culture experiments

4.3.4.1 Inoculum Preparation

Spores of ten-day-old MEA (Oxoid) cultures on Petri dishes were suspended in a aqueous solution of NaCl (0.85%) and Tween80 (0.05%) and used as the inoculum for pre-cultures to obtain an initial spores concentration of $1 \times 10^6 \text{ mL}^{-1}$. 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 autoclave-sterilized Malt Extract Broth (MEB, Difco). The mycelium was then homogenized with an Ultra-Turrax T-18 (IKA Labortechnik, Staufen, Germany) at 50 Hz for 5 seconds.

4.3.4.2 Screening for Biomass Producing Capability

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.

4.3.4.3 Growth and Chitosan Production Kinetics

Pre-cultures (10.0 mL) of only fungal strains with good growth capacity 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 24 h. Fungal biomass concentration was determined gravimetrically as above. Dried samples were weighed before chemical analyses and chitosan extraction. The filtrate was stored at -20 °C until use. All cultures were performed in duplicate.

4.3.4.4 Bench-top Stirred Tank Reactor Cultures

Bioreactor experiments were performed under batch conditions in 3-L jacketed bench-top stirred tank reactors (STR) (Applikon, Schiedam, NL) filled with 1.8 L of ELP medium and inoculated with 200 mL of pre-cultures of *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510. The reactors were equipped with a top stirrer bearing two six-blade Rushton-type turbines (diameter 4.5 cm, blade width 1.4 cm, blade length 1.4 cm) and three baffles (width 1.4 cm). Aeration was provided through a perforated pipe sparger located under the bottom turbine, while monitoring of the fermentation processes was ensured by the following probes installed on the top plate: dissolved oxygen and pH sensor (Applikon).

The fermentations conditions were as follows: aeration rate, 1.0 vvm; temperature, 28 °C; initial pH, 5.0; initial dissolved oxygen concentration, 100% of saturation. Silicon Antifoam 289 (1.0 mL L⁻¹) (Sigma Chemical Co.) was added before inoculation. The

fermentation parameters were monitored by an ADI 1030 (Applikon) adaptive/PID digital controller.

Three different impeller speeds (300, 500 and 700 rpm) were investigated, in order to evaluate the effect on the production of biomass and chitosan. Each condition was tested in duplicate and aliquots of each culture were sampled every 8 h for the analyses.

4.3.5 Determination of Yields and Rates

Biomass yield ($Y_{X/S}$), specific product yield ($Y_{P/X}$) and product yield referred to consumed sugars ($Y_{P/S}$) were calculated according the following equations:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad Y_{P/X} = \frac{\Delta P}{\Delta X} \quad Y_{P/S} = \frac{\Delta P}{\Delta S}$$

where ΔX is the amount of biomass, ΔP is the amount of chitosan produced and ΔS is the amount of substrate consumed.

The volumetric growth and production rates (r_X and r_P , respectively) were calculated by relating the amounts of biomass and chitosan, respectively, to the time set as the end of the fermentations (Δt), according to the following equations:

$$r_X = \frac{\Delta X}{\Delta t} \quad r_P = \frac{\Delta P}{\Delta t}$$

Similarly, substrate (r_S) and nitrogen (r_N) consumption rates were calculated by relating the amounts of reducing sugars and ammonium nitrogen consumed, respectively, to the time set as the end of the fermentations (Δt), according to the following equations:

$$r_S = \frac{\Delta S}{\Delta t} \quad r_N = \frac{\Delta N}{\Delta t}$$

4.3.6 Analytical Methods

4.3.6.1 ELP Medium Characterization

The enriched liquid phase (ELP) was characterized by determining key parameters for the subsequent phase of microbial bio-conversion. In particular, total sugars were determined by the method of DuBois (1956) while reducing sugars by the dinitrosalicylic acid method (Miller, 1959). Pentose sugars were determined by the *p*-bromoaniline-thiourea method (Deschatelets & Yu, 1986). Ammonium nitrogen was determined using the nitroprusside method described by Anderson and Ingram (1989) while total phenols were determined with the procedure of Singleton and colleagues (1971).

4.3.6.2 Chitosan Extraction

To extract chitosan, a slight modification of the procedure described by Rane and Hoover (1993) was used. In particular, finely ground dry mycelial biomass was transferred to centrifuge tubes, added with NaBH₄ (50 mg g⁻¹ biomass) and NaOH 1.0 M (40 mL g⁻¹ biomass) and autoclaved (30 min at 121 °C). After cooling, the cell suspension was centrifuged (12000×g, 15 min) and the pellet washed sequentially with deionized water, absolute ethanol, and deionized water again. Each washing step 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 subjected to extraction with 4.0% acetic acid solution in water (150 mL g⁻¹ AIM) 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, 4°C) and the pellet subsequently washed with absolute ethanol and then with deionized water. Each washing step was followed by centrifugation under the same conditions and subsequent pellet recovery. At the end of the washing procedure, the pellet was lyophilized and the weight was determined.

4.3.6.3 Chitosan Characterization

4.3.6.3.1 Determination of Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration (MIC) of chitosan obtained by the shaken flask cultures of fungal strains *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510 was determined using the microdilution method (CLSI 2012) in 96-well polystyrene microplates (Greiner Bio-One™ CELLSTAR™). Chitosan was dissolved in a 1.0% 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). Commercial chitosan (Fluka) 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* (Gram-positive).

4.3.6.3.2 Fourier Transform Infrared (FT-IR) Spectroscopy and Thermogravimetric Analysis (TGA)

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 500 – 4.000 cm⁻¹ wavenumber range, with a resolution of 4 cm⁻¹. The spectra thus obtained were compared with that of 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 A₁₃₂₀/A₁₄₂₀ ratio was used which, in a study conducted by Brugnerotto and colleagues (2001), showed to provide the smallest deviation between expected and estimated values. In particular, the first band is characteristic of an acetylated amine or an amide function while the second band,

relating to the C-H bending signal, is chosen as reference band. DD% was calculated using the equation:

$$DD(\%) = 100 - \left(\left(\frac{A_{1320}}{A_{1420}} - 0.3822 \right) \times \frac{1}{0.031} \right)$$

Thermogravimetric analysis (TGA) of the samples was performed by using a thermogravimetric analyzer (TA Instruments, Seiko Exstar 6300, Tokyo, Japan). The sample powders (ca. 10 mg) were placed in an open platinum pan and heated from 30 to 800 °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.

4.3.6.3.3 Elemental Analysis

The elemental analyzer for solids Vario MacroCube of Elementar GmbH (Germany) enabled the determination of the percentage contents in carbon, hydrogen, nitrogen and sulfur in chitosan samples. The calibration of the measurements relied on sulfonamide used as the standard.

The degree of deacetylation (DD) was calculated according to the following equation (Dos Santos et al., 2009):

$$DD(\%) = 100 \times \left(4 - 0.583093 \cdot \frac{C}{N} \right)$$

4.3.6.3.4 Determination of Viscosity and Average Molecular Weight

Dilute Solution Viscometry is a well-known analytical technique in polymer chemistry for characterizing polymers in solution.

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 mg mL⁻¹) were prepared in 1.0% acetic acid solution, stirred for 72 h and centrifuged (12000×g, 15 min, 4 °C) to remove insoluble materials. Then, the flowing time (*t*) of several dilute solutions of stock chitosan solution (*C*) and the solvent (*t*₀) were measured in triplicate for each sample.

Relative viscosity (*η_{rel}*), specific viscosity (*η_{sp}*), reduced viscosity (*η_{red}*) and

inherent viscosity (η_{inh}) were determined by the following equations:

$$\eta_{rel} = \frac{t}{t_0} \quad \eta_{sp} = \eta_{rel} - 1 \quad \eta_{red} = \frac{\eta_{sp}}{C} \quad \eta_{inh} = \frac{\ln(\eta_{rel})}{C}$$

The intrinsic viscosity $[\eta]$ was determined from the y-axis intercept of a plot of the known values of viscosity against concentration. Huggins, Kraemer and Martin equations were performed to determine the intrinsic viscosity $[\eta]$:

$$\text{Huggins} \rightarrow [\eta] = \lim_{c \rightarrow 0}(\eta_{rid})$$

$$\text{Kraemer} \rightarrow [\eta] = \lim_{c \rightarrow 0}(\eta_{inh})$$

$$\text{Martin} \rightarrow [\eta] = \lim_{c \rightarrow 0}(\ln(\eta_{rid}))$$

Using the value of the intrinsic viscosity $[\eta]$, the average molecular weights of chitosan (M_v) were determined through the Marck-Houwink equation, which provides the relationship between intrinsic viscosity and molecular weight (Kasaai et al., 2000):

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

where the constants K and a are literature values and describe the polymer-solvent combination at a given temperature. The value of K and a for chitosan in 1% acetic acid solution at 25 °C are 0.0474 mL g⁻¹ and 0.723, respectively (Purwanto et al., 2019).

4.3.7. Statistical Analysis

Data were analyzed by one-way ANOVA and post-hoc Tukey test at a significance level of 0.05, using the SigmaStat 3 software (Jandel Corp, San Rafael, CA, USA).

4.4 Results and Discussion

4.4.1. Medium Development from Digestate's Fractions

The objective of the first set of experiments was to assess the adequacy of a mere solid-liquid extraction approach or an acid- or alkali-assisted extraction to yield a carbohydrate-enriched liquid fraction. Given the good levels of potassium and phosphorus in the DLF associated with low concentrations of organic components, it seemed appropriate to use it as a liquid extractant of the DSF. Table 1 summarizes the results comparing the concentrations and recovery yields of total and reducing sugars in the ELP.

Table 2 Volumetric concentrations and percent recovery yields of total sugars and reducing sugars in the enriched liquid phase at different temperatures (T) and solid liquid ratios (SLR) obtained by aqueous extraction (AE) of the digestate solid fraction (DSF) with the digestate liquid fraction (DLF) or by mild alkali or acid hydrolysis. Data are the mean $\pm$ standard deviation of three parallel experiments.

Treatment	T (°C)	SLR	Total sugars		Reducing sugars	
			Volumetric concentration (g L ⁻¹)	Recovery yield (%)	Volumetric concentration (g L ⁻¹)	Recovery yield (%)
AE of DSF with DLF as the extractant	60	1:20	1.16 $\pm$ 0.12	1.38	0.89 $\pm$ 0.05	94.64
AE of DSF with DLF as the extractant	90	1:20	1.34 $\pm$ 0.08	1.94	1.24 $\pm$ 0.08	157.14
AE of DSF with DLF as the extractant	120	1:20	2.41 $\pm$ 0.22	5.29	1.45 $\pm$ 0.09	194.64
AE of DSF with DLF as the extractant	60	1:10	1.53 $\pm$ 0.30	1.25	1.23 $\pm$ 0.05	155.36
AE of DSF with DLF as the extractant	90	1:10	2.13 $\pm$ 0.30	2.18	1.53 $\pm$ 0.02	208.93
AE of DSF with DLF as the extractant	120	1:10	3.65 $\pm$ 0.10	4.54	2.01 $\pm$ 0.04	294.64
Mild alkaline hydrolysis of DSF with DLF added with 6% NaOH (w/v)	90	1:10	4.35 $\pm$ 0.20	5.62	1.37 $\pm$ 0.03	180.36
Mild alkaline hydrolysis of DSF with DLF added with 6% NaOH (w/v)	120	1:10	6.03 $\pm$ 0.30	8.22	2.59 $\pm$ 0.12	462.50
Mild acid hydrolysis of DSF with DLF added with 6% H ₂ SO ₄ (w/v)	90	1:10	7.40 $\pm$ 0.40	10.34	4.35 $\pm$ 0.13	712.50
Mild acid hydrolysis of DSF with DLF added with 6% H ₂ SO ₄ (w/v)	120	1:10	24.09 $\pm$ 1.23	36.19	22.51 $\pm$ 1.40	3955.36

The aqueous extraction was ineffective towards total sugars since although temperature enhanced the recovery yields, their values were invariably lower than 5% with ensuing low TS concentrations in the ELP. The recovery yields of aqueous extractions towards reducing sugars also increased with temperature, leading to values that exceeded 100% presumably due to the contribution of reducing ends of extracted oligosaccharides. However, due to their intrinsically low concentration in DSF, their concentrations in the ELP were unsatisfactory. Even mild alkali extraction gave rise to limited recovery yields of total sugars and reducing sugars; in fact, although alkaline extraction at 120 °C for 60 min significantly increased total sugars recovery yield as compared to the best aqueous extraction condition (8.03 vs. 4.54%), the volumetric concentrations of total sugars and reducing sugars (6.03 $\pm$ 0.3 and 2.95 $\pm$ 0.12 g L⁻¹,

respectively) were deemed inadequate to support substantial fungal growth. Conversely, acid-assisted extraction at 120 °C enabled a recovery yield of 36.2% of total sugars yielding a volumetric concentration of $24.09 \pm 0.49 \text{ g L}^{-1}$. As for reducing sugars, their recovery yield under the same conditions was 40-fold higher than the theoretical value presumably due to acid-catalyzed hydrolysis of structural polysaccharides.

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 4.4 to 25.9 g L^{-1} depending on the selected factor combination.

Data were fitted by a second order polynomial equation, leading to a high fraction of variation explained by the model ($R^2_{\text{adj}} = 0.925$). 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). 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, the large majority 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} .

Table 3 Full factorial design showing the actual values and, between square brackets, the coded values of the independent variables H₂SO₄ concentration (X₁) and incubation time (X₂) and observed and predicted values of the volumetric concentrations of total sugars.

Run number	H ₂ SO 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

Table 4 Least squares estimate of coefficients of the variables H₂SO₄ concentration (X₁) and incubation time (X₂) in the original and refined model. Statistical parameters measuring fitting power (R²), predictive power (Q²) and probability of lack of fit (F_{ME}) are also shown.

Model's terms ^a and parameters	Original model	Refined model
Constant (β ₀)	25.57 ± 0.78 ***	25.57 ± 0.78 ***
First order coefficient of X ₁ (β ₁)	5.93 ± 0.55 ***	5.99 ± 0.54 ***
First order coefficient of X ₂ (β ₂)	3.30 ± 0.54 ***	3.30 ± 0.54 ***
Second order coefficient of X ₁ (β ₁ •β ₁)	-5.01 ± 0.87 ***	-5.01 ± 0.87 ***
Second order coefficient of X ₂ (β ₂ •β ₂)	6.38 ± 0.94 ***	6.38 ± 0.94 ***
Interaction coefficient X ₁ X ₂ (β ₁ •β ₂)	-0.634 ± 0.66 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 (DF = 4; P = 0.230)	1.54 (DF = 5; P = 0.256)

^a Estimated coefficient ± standard error and significance level: ***, P < 0.0001; n.s., not significant; n.i., not included; R², coefficient of determination; R²_{adj}, coefficient of determination adjusted for degrees of freedom (DF); F_{ME}, ratio between mean squares of model error and replicate error.

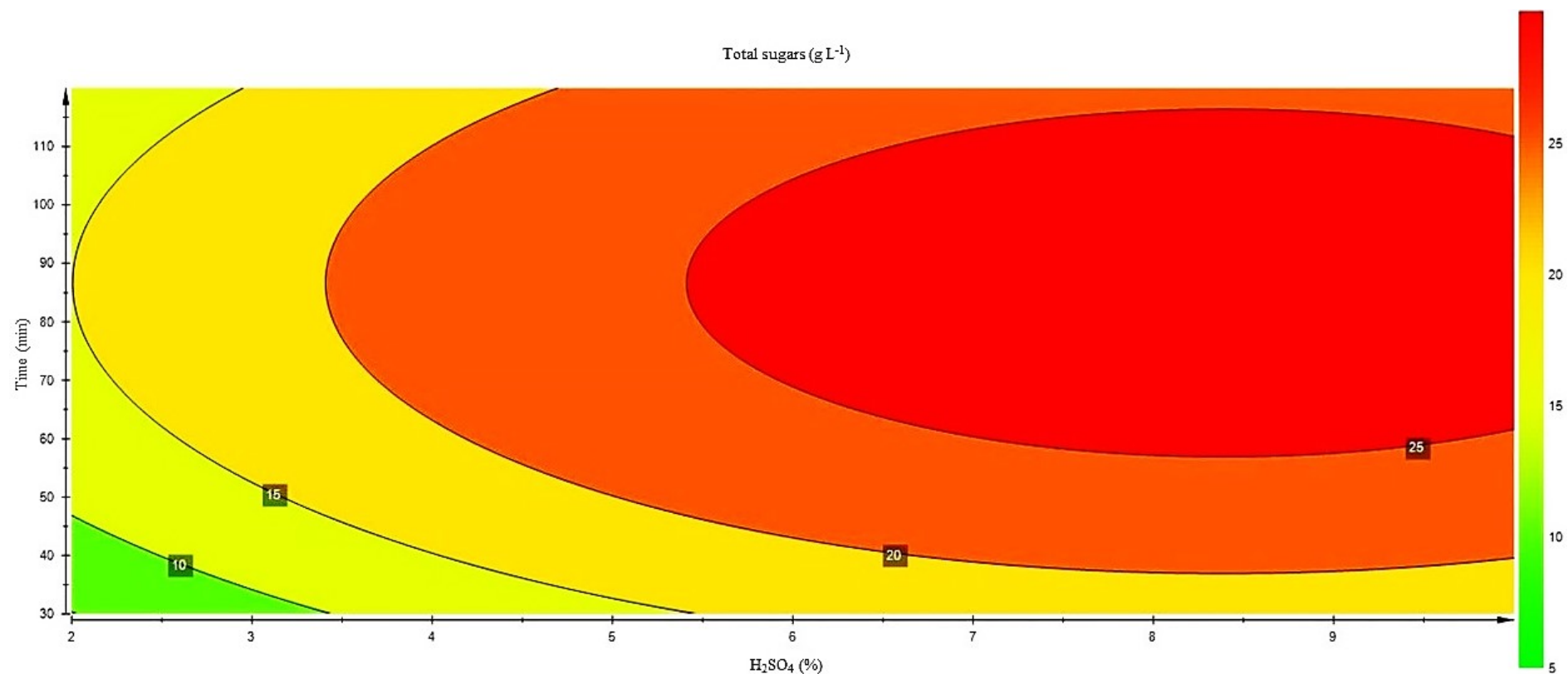


Figure 7 Contour plot showing the effect of the independent variables set in the factorial design on the extraction yield of total sugars.

4.4.2. Screening of Zygomycota Strains for Biomass and Chitosan-Producing Ability on ELP Medium in Shaken Flasks Experiments

Table 4 shows biomass production, percentage of TS consumption and biomass yield referred to the substrate consumed in liquid cultures of several Zygomycota strains grown in the enriched liquid phase derived from mild acid hydrolysis.

Table 5 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)
<i>Absidia blakesleeana</i> NRRL 1304	0.55 ± 0.06 ^E	15 ± 0 ^{FG}	0.16 ± 0.02 ^{FG}
<i>Absidia blakesleeana</i> NRRL 2696	7.58 ± 0.01 ^A	75 ± 0 ^{BC}	0.42 ± 0.01 ^B
<i>Absidia coerulea</i> NRRL 1312	2.59 ± 0.06 ^D	29 ± 3 ^E	0.37 ± 0.03 ^{BC}
<i>Absidia coerulea</i> NRRL 1315	0.76 ± 0.04 ^E	14 ± 0 ^{FG}	0.22 ± 0.01 ^{EF}
<i>Absidia coerulea</i> NRRL A-9483	0.59 ± 0.02 ^E	10 ± 0 ^G	0.24 ± 0.01 ^{DE}
<i>Absidia corymbifera</i> NRRL 2798	0.61 ± 0.04 ^E	11 ± 2 ^G	0.22 ± 0.02 ^{EF}
<i>Absidia glauca</i> NRRL 1324	0.74 ± 0.03 ^E	10 ± 1 ^G	0.31 ± 0.04 ^{CD}
<i>Absidia glauca</i> NRRL 2799	1.02 ± 0.01 ^E	92 ± 0 ^A	0.05 ± 0.01 ^H
<i>Absidia glauca</i> NRRL 3010	0.61 ± 0.02 ^E	20 ± 1 ^F	0.13 ± 0.01 ^G
<i>Absidia repens</i> NRRL 1337	0.61 ± 0.02 ^E	8 ± 0 ^G	0.31 ± 0.01 ^{CD}
<i>Aspergillus tubingensis</i> NRRL 4700	6.63 ± 0.15 ^B	91 ± 1 ^A	0.30 ± 0.01 ^{CD}
<i>Benjaminiella poitrasii</i> NRRL 2845	4.46 ± 0.13 ^C	72 ± 3 ^C	0.26 ± 0.00 ^{DE}
<i>Cunninghamella elegans</i> NRRL 1392	0.70 ± 0.16 ^E	14 ± 1 ^{FG}	0.20 ± 0.04 ^{EFG}
<i>Mucor moelleri</i> As. Col E	4.29 ± 0.41 ^C	77 ± 5 ^{BC}	0.26 ± 0.01 ^{DE}
<i>Mucor rouxii</i> NRRL 1894	3.95 ± 0.16 ^C	81 ± 0 ^B	0.20 ± 0.01 ^{EFG}
<i>Rhizopus oryzae</i> NRRL 1510	7.34 ± 0.01 ^{AB}	77 ± 2 ^{BC}	0.40 ± 0.01 ^B
<i>Rhizopus oryzae</i> NRRL 2625	6.89 ± 0.66 ^{AB}	55 ± 2 ^D	0.52 ± 0.03 ^A

Data are expressed as mean ± standard deviation. Multiple pairwise comparisons were performed via Tukey test. 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%, the strains belonging to the *Absidia* genus showed a limited ability to exploit the carbon source in ELP medium and this was also observed for *C. elegans* NRRL 1392 cultures. *A. blakesleeana* NRRL 2696

and *R. oryzae* NRRL 1510 were the best biomass producers on the ELP medium (7.58 and 7.34 g L⁻¹, respectively). In general, 9 out of the 17 strains under study yielded biomass levels higher than 2.5 g L⁻¹, namely an amount considered compatible with an accurate quantification of the extracted chitosan, being the latter a structural molecule of the fungal walls linked to growth.

Table 5 shows the results of chitosan production cultures, i.e., the amounts of the alkali-insoluble material (AIM), chitosan yield and its volumetric production and daily productivity (r_c).

Table 6 Values of AIM, chitosan and daily productivity of chitosan (r_c), obtained in 96-h-old cultures of Zygomycota strains with good growth capacity on non-supplemented enriched liquid phase.

Fungal strains	AIM (mg g ⁻¹ biomass)	Chitosan (% on AIM)	Chitosan yield (mg g ⁻¹ biomass)	Chitosan (mg L ⁻¹)	r_c (mg L ⁻¹ d ⁻¹)
<i>A. blakesleeana</i> NRRL 2696	246 ± 12 ^C	21 ± 1 ^A	52 ± 3 ^A	444 ± 18 ^A	111 ± 4 ^A
<i>A. coerulea</i> NRRL 1312	610 ± 21 ^A	7 ± 0 ^C	44 ± 5 ^{AB}	49 ± 5 ^E	12 ± 1 ^E
<i>A. tubingensis</i> NRRL 4700	341 ± 22 ^B	4 ± 0 ^C	13 ± 1 ^D	86 ± 7 ^{DE}	22 ± 3 ^{DE}
<i>B. poitrasii</i> NRRL 2845	128 ± 9 ^E	16 ± 1 ^{AB}	20 ± 2 ^{CD}	91 ± 12 ^{DE}	23 ± 3 ^{DE}
<i>M. moelleri</i> As. Col E	155 ± 42 ^{DE}	20 ± 1 ^A	31 ± 6 ^{BC}	134 ± 14 ^{CD}	33 ± 4 ^{CD}
<i>M. rouxii</i> NRRL 1894	205 ± 19 ^{DE}	14 ± 1 ^B	29 ± 1 ^{BCD}	139 ± 7 ^{CD}	35 ± 2 ^{CD}
<i>R. oryzae</i> NRRL 2625	147 ± 26 ^{DE}	18 ± 3 ^{AB}	25 ± 1 ^{CD}	176 ± 22 ^C	44 ± 5 ^C
<i>R. oryzae</i> NRRL 1510	220 ± 7 ^{CD}	17 ± 1 ^{AB}	43 ± 8 ^{AB}	285 ± 25 ^B	71 ± 6 ^B

Data are expressed as mean ± standard deviation. Multiple pairwise comparisons were performed via Tukey test. Equal letters next to the values indicate the lack of statistically significant differences within the same group of values.

The highest AIM per gram of biomass was observed in *A. coerulea* NRRL 1312 and *A. tubingensis* NRRL 4700 (610 ± 21 and 341 ± 22 mg g⁻¹, respectively), albeit with the lowest percentage of chitosan on AIM (7 ± 0 and 4 ± 0 %, respectively), volumetric production (49 ± 5 and 86 ± 7 mg L⁻¹, respectively) and productivity (12 ± 1 and 22 ± 3 mg L⁻¹ d⁻¹, respectively).

As for the strains belonging to Mucorales order (*Absidia*, *Benjaminiella*, *Rhizopus* and *Mucor*), the chitosan values ranged from a minimum of 13 to a maximum of 52 mg g⁻¹ of fungal biomass. In a paper for the production of fungal chitosan by Berger et al. (2014) performed with various species belonging to the Mucorales order, grown on a substrate deriving from a mixture of Corn Steep Liquor and papaya peels, after 96 hours

of fermentation, the chitosan values obtained ranged from 5 and 15 mg g⁻¹ of fungal biomass; this could demonstrate that the composition of the digestate-based medium used and the fungal strains selected in this study were more suitable for the production of chitosan.

The genus *Aspergillus*, in several bibliographical works, has produced high quantities of chitosan. Tayel et al. (2014) reported a chitosan yield of about 20 mg g⁻¹ of biomass with *A. brasiliensis* ATCC 16404 after 6 days in shaken flask at 28 °C, using a waste date syrup as substrate. Pochanavanich et al. (2002) observed a maximum chitosan production of 107 mg g⁻¹ of biomass with *A. niger* grown on PDB in shaken flask at 30 °C for 15 days. In the screening of this project, *A. tubingensis* NRRL 4700 had a maximum chitosan yield of about 13 mg g⁻¹ of biomass, placing itself at a lowest level respect to the two species mentioned.

The highest chitosan yield (52 ± 3 mg g⁻¹ biomass) and productivity (116 mg L⁻¹ d⁻¹) were observed in *A. blakesleeana* NRRL 2696 cultures, attaining a volumetric production of 444 ± 18 mg L⁻¹. This volumetric production was in line to that obtained with *Absidia butleri* NCIM977 (401 mg L⁻¹) (Vaingankar & Juvekar, 2014), but was evidently lower than those obtained with *Absidia coerulea* ATCC 14076 (1870 mg L⁻¹) (Muzzarelli et al., 1994) and *Absidia coerulea* CTCC AF 93105 (4110 mg L⁻¹) (Jiang et al., 2011).

The prevalence of pentose sugars in the medium, compared to synthetic ones, and the presence, albeit low, of phenols could be responsible for the lower chitosan volumetric production; in fact, it should be considered that, although Zygomycetes are able to consume pentose sugars, the consumption of hexoses is preferred and faster (Ferreira et al, 2013; Yang et al., 2017).

Among the other strains screened, *R. oryzae* NRRL 1510 cultures attained one of the highest chitosan volumetric productions (285 ± 25 mg L⁻¹), the percentage of which referred to the AIM fraction amounted to 17 ± 1 % and a chitosan yield of 43 ± 8 mg g⁻¹ biomass, a value which was lower than that obtained with *R. oryzae* ME-F12 (110 mg g⁻¹ biomass) grown on an acid hydrolysate of corn straw (Tai et al., 2010) and *R. oryzae* TISTR3189 grown on PDB (138 mg g⁻¹) (Pochanavanich et al. 2002). However, similar chitosan volumetric production was obtained with *Rhizopus oryzae* USDB 0602 grown on GYP added with (NH₄)₂SO₄ (280 mg L⁻¹) (Tan et al., 1996) and *R. oryzae* NCIM 1009 (274 mg L⁻¹) (Vaingankar & Juvekar, 2014).

The process kinetics during the 96 hours of shaken flasks fermentation was monitored for the two strains which have reached the highest volumetric productions, *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510 (Figure 2). If we compare them, *A. blakesleeana* NRRL 2696 is more performing than *R. oryzae* NRRL 1510 not only in terms of chitosan volumetric production and productivity (Table 5) but also for the ammonium nitrogen consumption rates (0.18 and 0.08 g L⁻¹ d⁻¹, respectively), despite almost the same biomass yields (0.50 and 0.52 g g⁻¹ of substrate, respectively) and reducing sugars consumption rates (4.1 and 3.9 g L⁻¹ d⁻¹, respectively).

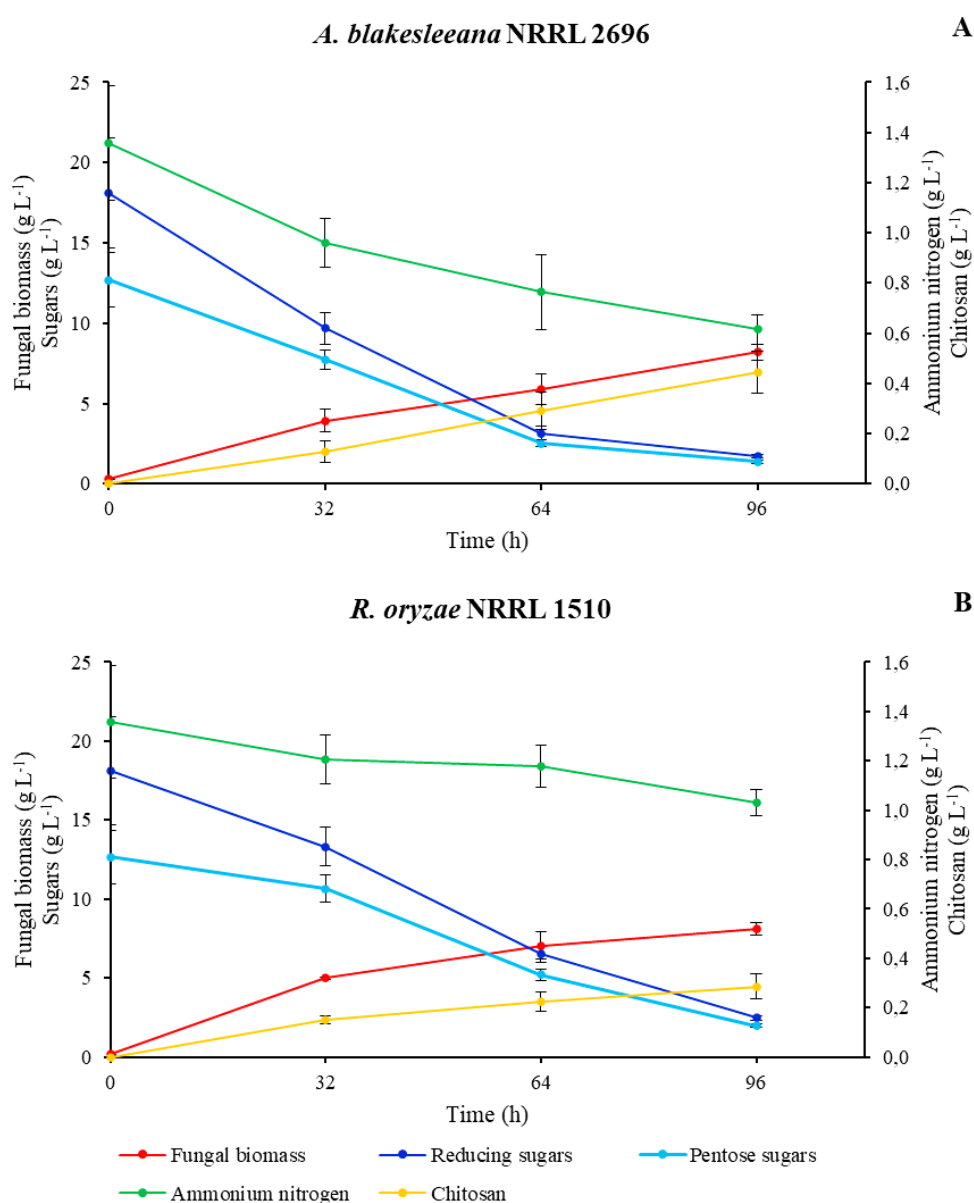


Figure 8 Time courses of biomass and chitosan productions, residual sugars and ammonium nitrogen concentrations in shaken flasks cultures of *A. blakesleeana* NRRL 2696 (Plot A) and *R. oryzae* NRRL 1510 (Plot B). Data are the means $\pm$ standard deviations of duplicate experiments.

4.4.3. Reactor experiments with *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510

To assess the feasibility of upscaling, the production processes with *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510 were performed in stirred tank reactors, and to evaluate the effect on biomass and chitosan productions, different impeller speeds were tested. Table 6 comparatively reports biomass and chitosan productions and related yields and rates parameters.

Regarding *R. oryzae* NRRL 1510, the stirring speed affected the biomass (Figure 3) and, consequently, the chitosan production. In fact, the impeller speed set at 700 rpm provided the best results both in terms of biomass and chitosan production (19.56 and 2.16 g L⁻¹, respectively).

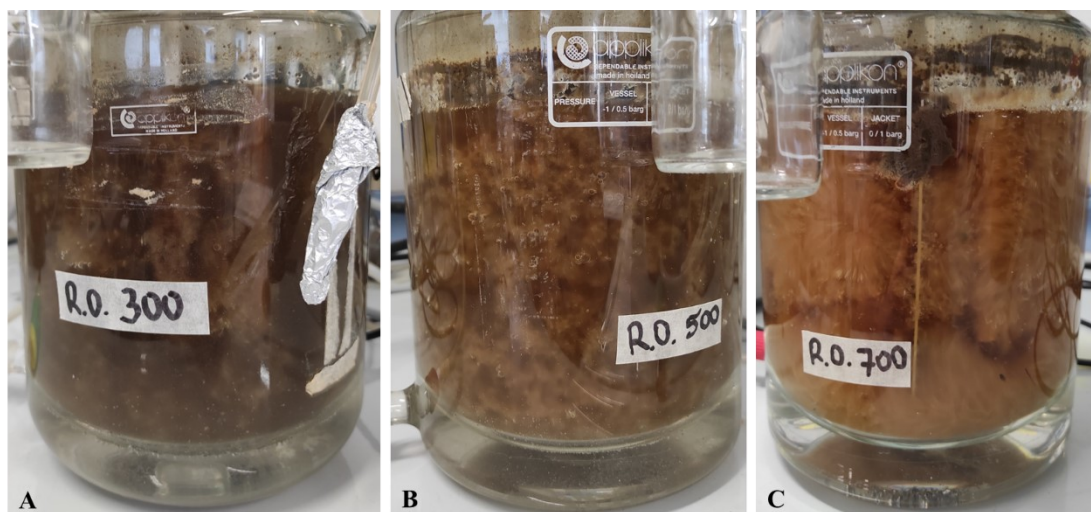


Figure 9 Cultures of *R. oryzae* NRRL 1510 in bioreactors at 300 rpm (A), 500 rpm (B) and 700 rpm (C).

At 300 and 500 rpm, a rapid limitation of dissolved oxygen was observed after 16 h of fermentation, which in the first case persisted until the end of fermentation (Figure 4, 1A and 1B) and was also associated with metabolically slowed strain and beginning of fouling on the internal components of the vessel. Conversely, switching to 700 rpm, the dissolved oxygen reached a minimum saturation of around 30% after 24 h and then raised up to 100% at the end of the fermentation, suggesting the achievement of the stationary phase (Figure 4, 1C). In all cases, the pH did not significantly change throughout the process, as well as the nitrogen consumption rates while reducing sugars consumption rates increased with the increase of the impeller speeds (Figure 4, 2A, 2B and 2C).

Table 6 Biomass (X) and chitosan (P) productions, yield parameters ($Y_{X/S}$, $Y_{P/X}$, $Y_{P/S}$), biomass and chitosan production rates (r_X and r_P , respectively), reducing sugars and ammonium nitrogen consumption rates (r_S and r_N , respectively). All values have been calculated at the time set as the end of the fermentations.

Fungal strains	Impeller speed (rpm)	X (g L ⁻¹)	P (g L ⁻¹)	Yields parameters			Rates parameters			
				$Y_{X/S}$ (g g ⁻¹)	$Y_{P/X}$ (g g ⁻¹)	$Y_{P/S}$ (g g ⁻¹)	r_X (g L ⁻¹ h ⁻¹)	r_P (g L ⁻¹ h ⁻¹)	r_S (g L ⁻¹ h ⁻¹)	r_N (g L ⁻¹ h ⁻¹)
<i>A. blakesleeana</i> NRRL 2696	300	9.46 ± 0.57 ^C	0.93 ± 0.05 ^C	0.83 ± 0.10 ^B	0.10 ± 0.00 ^C	0.08 ± 0.01 ^{BC}	0.13 ± 0.01 ^D	0.01 ± 0.00 ^F	0.16 ± 0.03 ^C	0.01 ± 0.00 ^A
	500	17.03 ± 0.85 ^{AB}	2.36 ± 0.14 ^A	1.46 ± 0.18 ^A	0.14 ± 0.00 ^A	0.20 ± 0.02 ^A	0.24 ± 0.02 ^{BC}	0.03 ± 0.00 ^B	0.16 ± 0.03 ^C	0.01 ± 0.00 ^A
	700	15.82 ± 0.78 ^B	1.73 ± 0.10 ^B	0.94 ± 0.08 ^B	0.11 ± 0.00 ^B	0.10 ± 0.01 ^B	0.22 ± 0.02 ^C	0.02 ± 0.00 ^C	0.23 ± 0.04 ^C	0.01 ± 0.00 ^A
<i>R. oryzae</i> NRRL 1510	300	9.83 ± 0.49 ^C	0.72 ± 0.04 ^C	0.73 ± 0.02 ^B	0.07 ± 0.00 ^D	0.05 ± 0.00 ^C	0.20 ± 0.01 ^C	0.01 ± 0.00 ^{DF}	0.28 ± 0.03 ^{BC}	0.01 ± 0.00 ^A
	500	14.48 ± 0.72 ^B	1.00 ± 0.06 ^C	0.80 ± 0.01 ^B	0.07 ± 0.00 ^D	0.06 ± 0.00 ^C	0.30 ± 0.02 ^B	0.02 ± 0.00 ^{CD}	0.38 ± 0.03 ^{AB}	0.01 ± 0.00 ^A
	700	19.56 ± 0.88 ^A	2.16 ± 0.13 ^A	0.96 ± 0.05 ^B	0.11 ± 0.00 ^B	0.11 ± 0.00 ^B	0.41 ± 0.03 ^A	0.04 ± 0.00 ^A	0.43 ± 0.05 ^A	0.02 ± 0.00 ^A

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

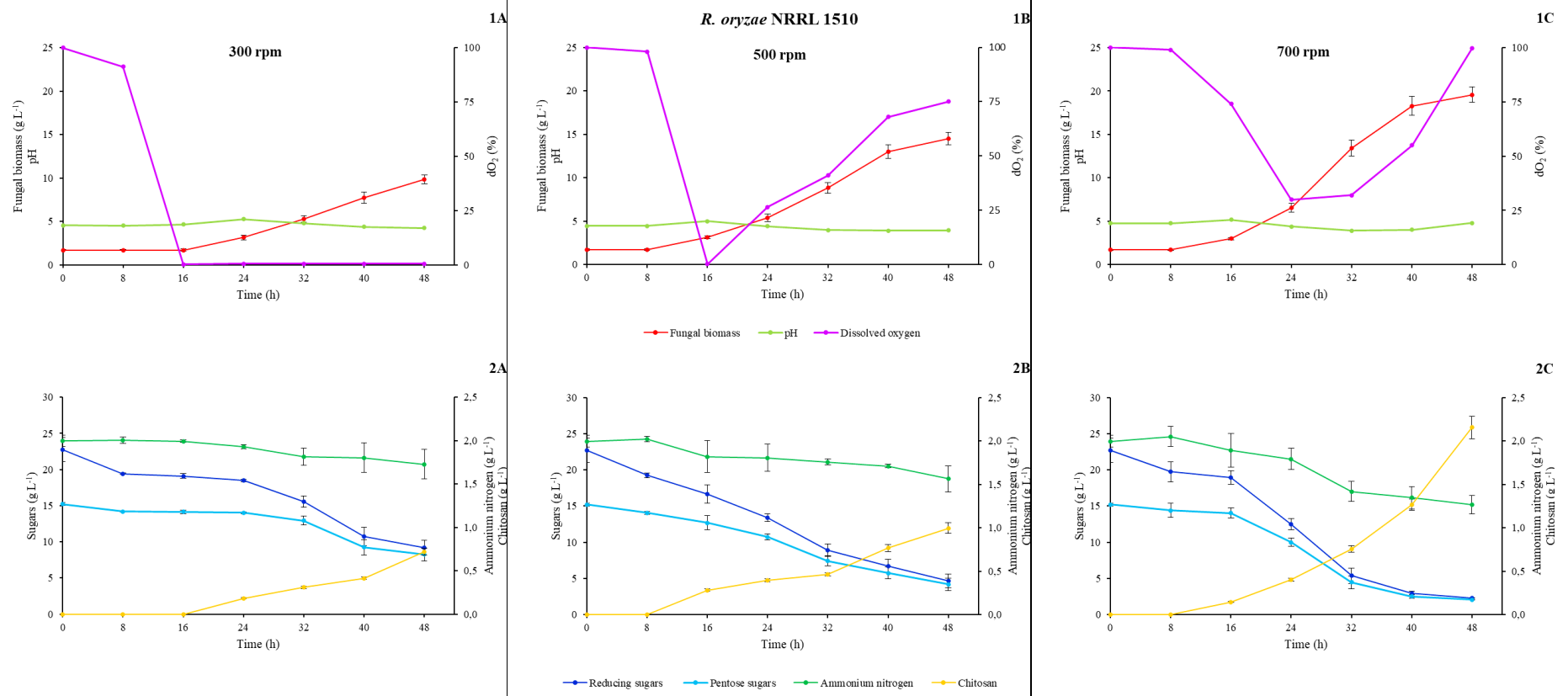


Figure 4 Time courses of biomass production, pH and dissolved oxygen (Plots 1) and chitosan production and residual sugar and nitrogen concentrations (Plots 2) in *R. oryzae* NRRL 1510 cultures grown in a 3-L stirred tank reactor at 300 rpm (Plots A), 500 rpm (Plots B) and 700 rpm (Plots C).

In a study of Göksungur (2004), aimed at optimizing the production of chitosan from beet molasses with *Rhizopus oryzae* 00.436, a maximum chitosan concentration of 1.1 g L^{-1} was obtained in 7-L stirred tank reactor with a medium containing an initial sugar concentration of about 45 g L^{-1} with an aeration rate and agitation speed of 2.1 vvm and 339 rpm, respectively, after 72 h of fermentation from a biomass concentration of 10.7 g L^{-1} . The biomass and chitosan production of this work are about two times lower than those obtained from *R. oryzae* NRRL 1510 with an aeration rate and agitation speed of 1 vvm and 700 rpm, respectively, after 48 h of fermentation with a medium containing an initial sugar concentration of about 22 g L^{-1} , confirming the suitability of the ELP as growth medium for this strain.

In another work (Yang et al., 2017), corn stover pre-hydrolysate was fermented by *R. oryzae* AS 3.819 for 72 h in a 3-L stirred tank reactor at 200 rpm and 1 vvm, obtaining biomass and chitosan productions of 10.96 g L^{-1} and 0.99 g L^{-1} , respectively. Also in this case, the results here obtained are higher and this comparison further confirms the suitability of ELP as medium for fungal chitosan production.

As regards the cultures of *A. blakesleeana* NRRL 2696, fouling problems immediately arose, irrespective of the agitation regimes. In fact, in the early phases the strain grew with a pellet morphology of uniform size (Figure 5A), but later the biomass developed in a form closely adherent to the internal components of the reactors (Figure 5B), making impossible to monitor biomass and chitosan production over time.

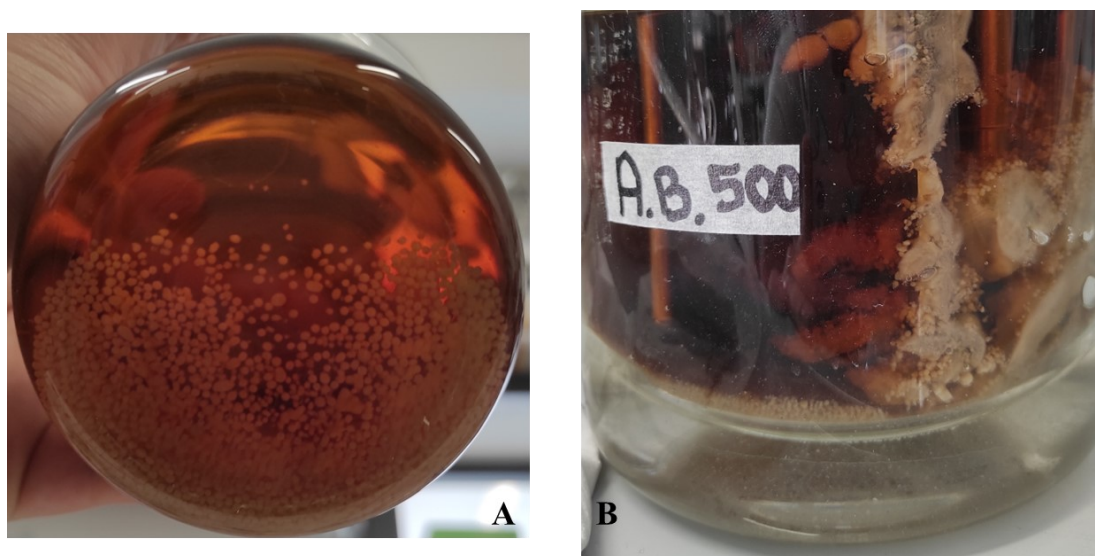


Figure 5 A: Pellet of *A. blakesleeana* NRRL 2696; B: Close-up of mycelial agglomeration occurred in bioreactor.

Pellet growth is very important in fungal fermentation because it eliminates the problems arising due to mycelial clogging of tubes and agglomeration around the impellers, baffles and walls of the reactor (Rane & Hoover, 1993). Therefore, modifications in bioreactor configurations are necessary.

For instance, a study conducted by Davoust & Hansson (1992) on various species of the genus *Absidia* showed how the use of an open paddle impeller, with a large diameter and a sufficiently high stirrer speed to avoid the formation of a viscous pulp, allowed most of the species to grow satisfactorily.

The issues of shear damage and mixing associated with stirred tank reactors have led to airlift fermenters being the choice of fungal fermentation involving fragile fungal mycelia (Namboodiri & Pakshirajan, 2020). For example, in a study of Wu and collaborators (2001), a modified airlift reactor, which had low shear stress and high oxygen transfer capability, provided a suitable environment for cultivation of *Absidia coerulea*, sensitive to shear stress, and the chitosan production was higher than those in stirred tank reactor.

Despite the growth-mode problems mentioned above and the incomplete consumption of substrates (Figure 6), the impeller speed set at 500 rpm provided the best results with 17.03 g L⁻¹ of biomass and 2.36 g L⁻¹ of chitosan production (Table 6). This condition is also associated to higher yield parameters compared to those obtained in the best condition with *R. oryzae* NRRL 1510. Even in this case, the pH did not significantly change throughout the process (Figure 6).

In a study of Kim et al. (2001), designed at optimizing culture conditions for the production of chitosan by *Absidia coerulea* ATCC 14076, a maximum chitosan concentration of 2.3 g L⁻¹ was obtained in 2.5-L stirred tank reactor with an aeration rate and agitation speed of 2 vvm and 250 rpm, respectively, after 72 h of fermentation with an optimized synthetic culture medium containing 20 g L⁻¹ of glucose. This result is comparable with the chitosan production achieved by *A. blakesleeana* NRRL 2696 at 500 rpm, despite the use of a low-cost growth medium, such as ELP.

In another work (Davoust & Persson, 1992), chitosan production reached 2.8 g L⁻¹ with *Absidia repens* CBS 102.32, grown on a defined medium consisting of glucose (20 g L⁻¹) and yeast extract (1 g L⁻¹), using a 10-L stirred tank reactor at 350 rpm and 1 vvm, which is quite similar to the result obtained here, proving once again the eligibility of ELP as medium for fungal chitosan production.

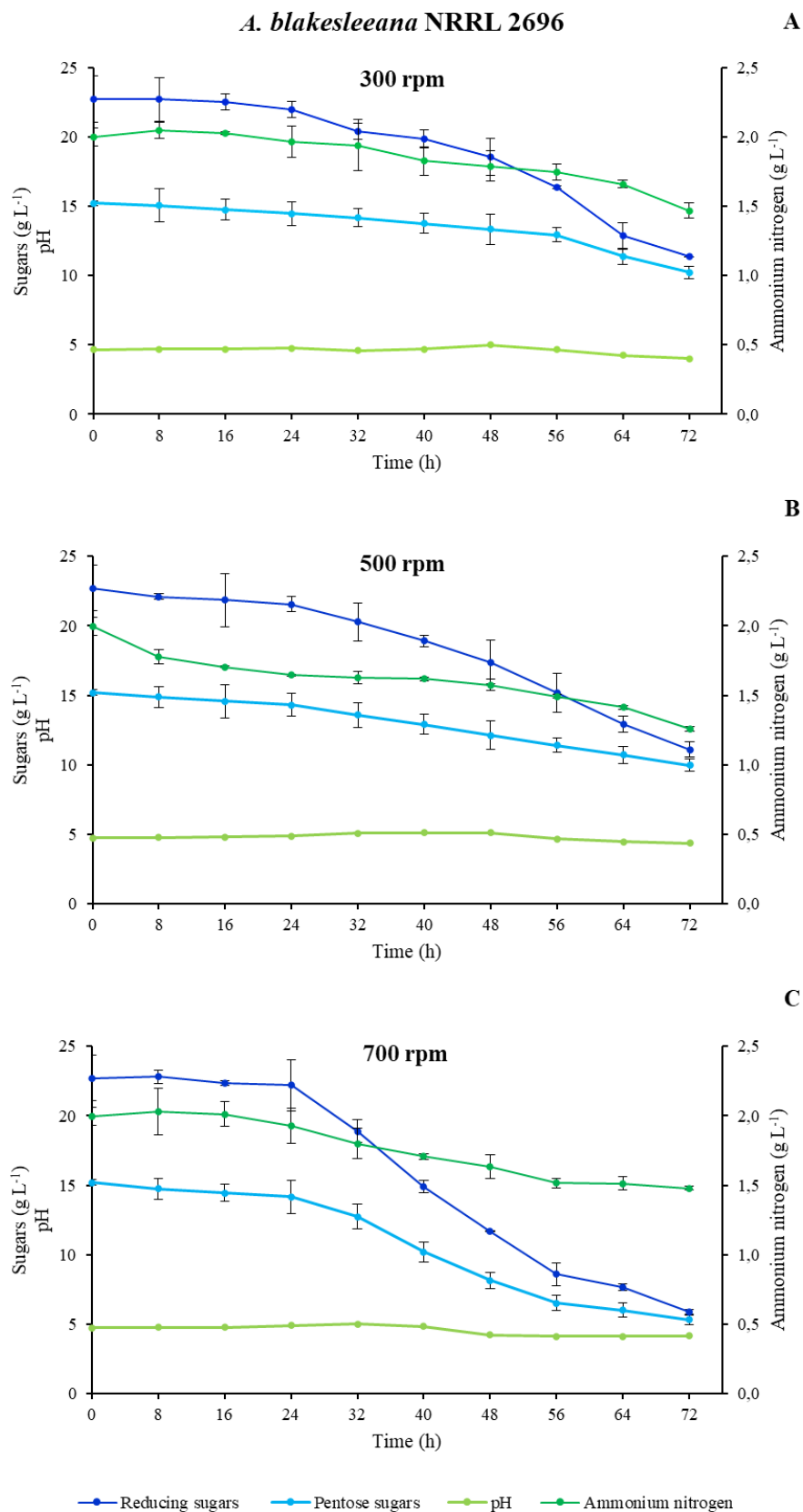


Figure 6 Time courses of pH and residual sugars and nitrogen concentrations in *A. blakesleeana* NRRL 2696 cultures grown in a 3-L stirred tank reactor at 300 rpm (Plot A), 500 rpm (Plot B) and 700 rpm (Plot C).

4.4.4 Antibacterial Activity of Fungal Chitosans

The antimicrobial activity of chitosan has been observed against a wide variety of microorganisms including fungi, algae and bacteria. However, the antimicrobial action is influenced by intrinsic factors, such as the type of chitosan, molecular weight, degree of polymerization and deacetylation, and by the type of microorganism (Sahariah & Masson, 2017; Matica et al., 2019).

Table 7 shows the MIC values in mg L⁻¹ of chitosan obtained from shaken flask fermentation of *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510. *Pseudomonas syringae* DSM 21482 turned out to be the most sensitive bacterial species, with the lowest MIC values. The tested chitosan preparations were more effective to inhibit the growth of Gram-negative bacteria than Gram-positive ones. Overall, data obtained showed that the antimicrobial activities of the fungal chitosans were less effective in comparison to the commercial chitosan, most likely due to the higher deacetylation degree (see next paragraphs) of the latter (Chang et al., 2019). However, these MIC values are well below those obtained with chitosans extracted from other Zygomycetes strains, the values of which ranged from 1000 to 2000 mg L⁻¹ against the same bacterial test (Tajidini et al., 2010). These differences may be attributed to various experimental methods, chitosan sources and characterization, degree of deacetylation and chitosan solvents (Hongpattarakere et al., 2008; Fujimoto et al., 2006).

Table 7 MIC values, expressed in mg L⁻¹, of chitosan obtained from shaken flask cultures of *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510, compared to commercial chitosan.

Fungal strains	<i>Pseudomonas syringae</i>	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>
	DSM 21482	ATCC 9637	
<i>A. blakesleeana</i> NRRL 2696	160	300	360
<i>R. oryzae</i> NRRL 1510	160	420	480
Commercial chitosan	120	180	220

4.4.5 FT-IR Characterization and TGA

The infrared spectroscopic characterization of samples produced by *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510 in shaken flask cultures is reported in Figure 7. FT-IR spectra was used to identify and confirm the characteristic functional groups of the produced chitosan. Both samples exhibited similar profiles in their FT-IR spectra, showing the same peaks areas with slight differences in their intensities, with the classic peaks assigned to polysaccharide structures. A broad absorption band in the range

of $3000 - 3500 \text{ cm}^{-1}$ is found in both samples, and it is attributed to O–H and N–H stretching, centered at 3440 cm^{-1} , C–H aliphatic stretching bands registered at $2870 - 2880 \text{ cm}^{-1}$, the absorption centered at 1650 cm^{-1} due to the axial C=O stretching of the acetamido groups (named amide I), the one at 1580 cm^{-1} attributable to the angular deformation of N–H bonds of the amino groups. Finally, finger print bands, extremely useful for evidencing chitosan presence even in low amounts, are due to skeletal signals (vibrations of glycosidic bonds, C–O and C–O–C stretching) absorbing between 1150 cm^{-1} and 900 cm^{-1} .

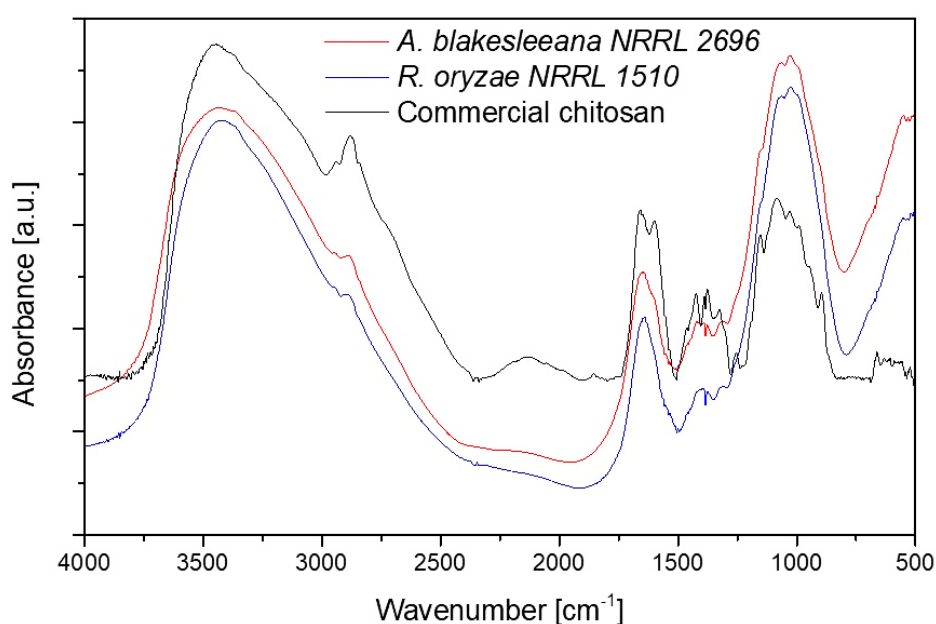


Figure 7 Absorbance FT-IR spectra of chitosan samples in the $4000 - 400 \text{ cm}^{-1}$ range collected in transmission mode.

The DD% values obtained with the equation previously reported were 80.3% and 79.8%, for chitosan from *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510, respectively. These values were well above the minimum threshold suggested by various authors for a clear separation between chitin and chitosan (Dhillon et al., 2013), but were found to be slightly lower than the reported values of DD of chitosan from other fungi (Arcidiacono & Kaplan, 1992; Crestini et al., 1996; Miyoshi et al., 1992).

The thermogravimetric analysis (TA) was used for measuring the thermal degradation profile and thermal stability of the samples. TG and DTG curves of chitosan

extracted from *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510 were compared with commercial chitosan and are shown in Figure 8.

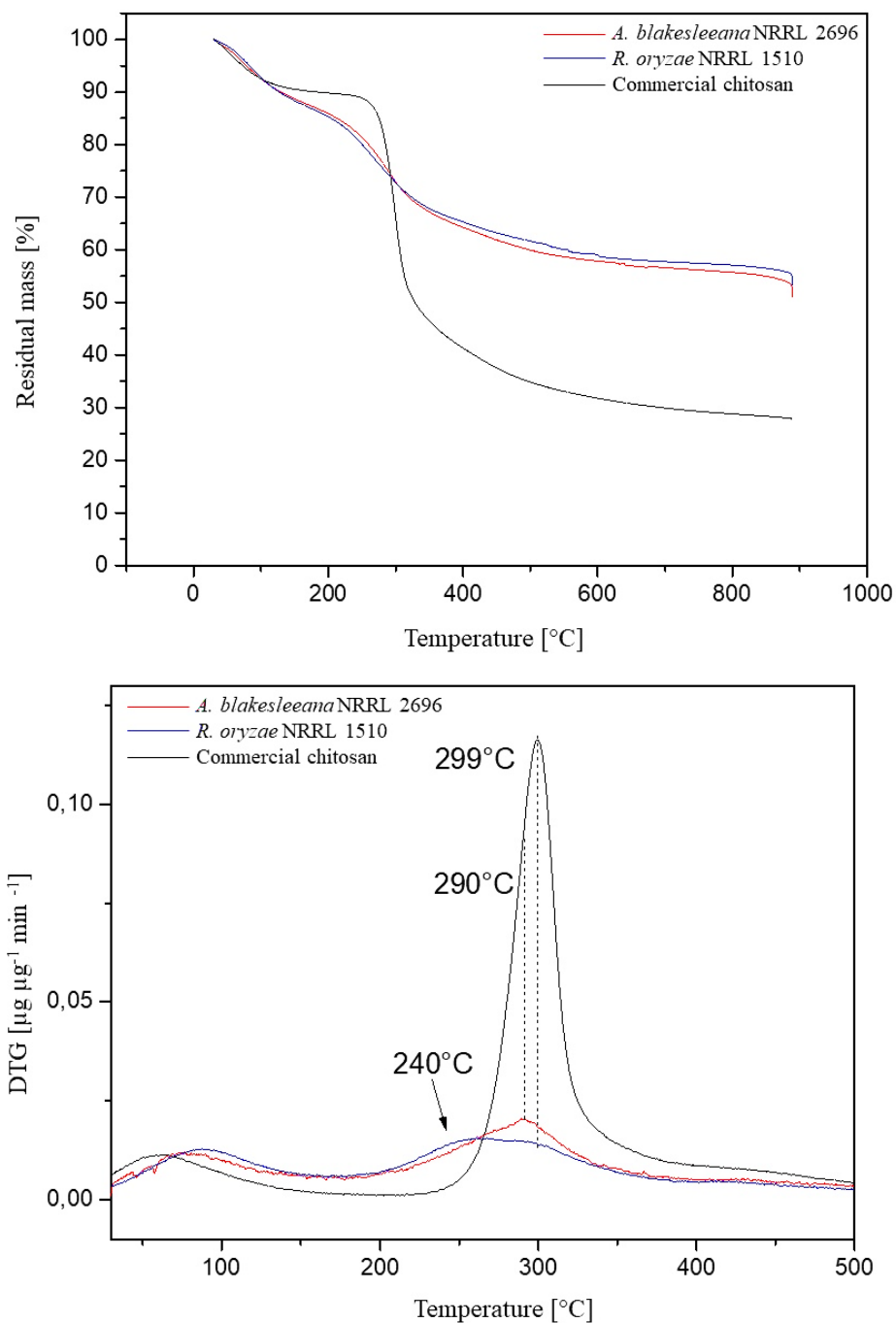


Figure 8 TG and DTG curves for thermal analysis of chitosan produced by *A. blakesleeana* NRRL 2696 (red line) and *R. oryzae* NRRL 1510 (blue line), compared to the commercial chitosan (black line).

It should be pointed out that the TA of polymeric materials provides information not only about their heat resistance and uses in various conditions but also about their chemical structure, phase transformations, polymorphic forms, relaxation processes,

composition and purity of compounds, constituent compatibility, degradation products, and kinetics. It is also a valuable tool for the quick identification of adsorbed volatile substances, e.g., moisture content (also the determination of crystallization water), residual solvents, unreacted monomers, and modifiers (Saikia et al., 2020; Pielichowski & Pielichowska, 2022).

Thermogravimetric measurements performed in nitrogen atmosphere indicates that the weight loss in chitosan samples took in two stages, in accordance with previous works (Sakurai, Maegawa & Takahashi, 2000; Abdou et al., 2008). The first one starts at 60 °C with a maximum at 80 °C and a second stage that starts at 200 °C and reaches a maximum at 300 °C. The first stage is assigned to the loss of water. Measurements reveal that the samples contain about 12% of adsorbed water, which is evaporated at relatively low temperature (below 130 °C). It means that this water is physically adsorbed and/or weakly hydrogen-bonded to chitosan molecules. The second thermal event corresponds to the decomposition of chitosan, vaporization and elimination of volatile products, also known as products of combustion (CO, CO₂, solvents, low-molecular-weight molecules, and functional groups) (Nieto et al., 1991).

The shape of the TG and DTG curves of samples is similar to commercial chitosan curves, but a slightly shift at lower temperature in the main determined thermal events is observed. The measured shift indicates a reduction in thermal stability. The main decomposition starts at 200 °C and proceeds at a maximum rate of 240 and 290 °C. This process can be connected with the simultaneous crosslinking of chitosan, as evidenced by a large residual mass not decomposed at 600 °C, higher than 55%. This can be explained by the higher thermal crosslinking degree, which the highest amount of carbonaceous residue (55.6%) indicates. It can be assumed that the carbon residue consists mainly of crosslinked and condensed aromatic structures resulting from formed covalent bonds between the macro-chains and the graphitization process, similarly as in commercial chitosan, as previously reported (Kaczmarek et al., 2010; Zawadzki et al., 2010).

4.4.6 Elemental Analysis of Chitosan

The composition of carbon, hydrogen, nitrogen, and sulfur elements of the samples of chitosan are provided in Table 8.

The results obtained in this study are in line with those obtained from other works. In fact, Tan and collaborators (2020) extracted chitosan from *Mucor circinelloides* ATCC 24905, after fermentation on various waste substrates, and reported carbon contents

ranging from 34.1 and 49.5 %, hydrogen amounts ranging from 6.6 and 7.0 % and nitrogen contents ranging from 5.0 and 8.5 %.

In another work (Muñoz et al., 2015), chitosan extracted from *Aspergillus niger*, showed average values of carbon 38.4 %, hydrogen 7.2 % and nitrogen 6.9 %.

The DD% values calculated in this way are a little bit higher than those obtained previously with FT-IR analysis.

Table 8 Elemental analysis of the samples of chitosan produced by *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510, in comparison with commercial chitosan, and DD values.

Samples	C (%)	H (%)	N (%)	S (%)	C/N	DD (%)
<i>A. blakesleeana</i> NRRL 2696	32.91	6.61	6.12	1.45	5.38	86
<i>R. oryzae</i> NRRL 1510	36.94	6.65	6.84	0.11	5.40	85
Commercial chitosan	39.69	7.18	7.49	0.19	5.30	91

4.4.7. Viscosity and Average Molecular Weight

The intrinsic viscosity values and average molecular weights of the chitosan obtained from STR fermentations of *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510 were reported in Table 9.

Table 9 Intrinsic viscosity values ($[\eta]$), average molecular weight (M_v) and R^2 of chitosan obtained from STR fermentations of *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510.

Impeller speed (rpm)	Regression method	<i>A. blakesleeana</i> NRRL 2696			<i>R. oryzae</i> NRRL 1510		
		$[\eta]$	M_v (Da)	R^2	$[\eta]$	M_v (Da)	R^2
300	Huggins	6.62	926	0.985	4.74	583	0.998
	Kraemer	6.65	932	0.985	4.74	584	0.998
	Martin	7.37	1074	0.991	4.82	598	0.997
500	Huggins	4.27	506	0.988	9.42	1510	0.990
	Kraemer	4.29	508	0.988	9.45	1517	0.989
	Martin	4.73	583	0.992	9.86	1609	0.987
700	Huggins	61.00	19996	0.985	23.63	5385	0.998
	Kraemer	61.08	20030	0.952	24.07	5525	0.997
	Martin	61.06	20022	0.986	26.81	6412	0.995

In present study, the molecular weights of chitosan were found to be between about 0.5 and 20×10^3 Da and were lower compared to commercial chitosan. Chitosan obtained from *R. oryzae* NRRL 1510 seemed affected by the impeller speed, increasing viscosity and molecular weight as the rpm increased.

Low molecular weight chitosan has better biodegradability, biocompatibility and bioactivity as well as lower toxicity compared to high molecular weight chitosan (Suryani et al., 2022). In addition, due to its higher solubility and lower viscosity, low molecular weight chitosan allows for a wider pharmaceutical application, such as drug delivery systems (Soares et al., 2016), hypocholesterolemic agents (Sumiyoshi et al., 2006) and antitumoral agents (Maeda & Kimura, 2004). Lastly, as compared to high molecular weight chitosan, low molecular weight chitosan exhibits better antimicrobial activity (No et al., 2007; Park et al., 2008).

4.5 Conclusion

Objective of this study was to investigate the possibility of chitosan production by *Zygomycetes* fungi grown on a digestate-base medium (i.e., ELP).

On the one hand, the screening performed with a variety of strains belonging to well-known chitosan-accumulating species confirmed that ELP medium was a potential substrate for the cultivation of *Zygomycetes* fungi for chitosan production purposes. Among the 17 fungal strains tested, 9 were able to grow on this substrate, reaching high biomass levels. Two of them, *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510, were outstanding for the production of chitosan, reaching volumetric productions of 444 and 285 mg L⁻¹, respectively, in shaken flask fermentations. Chitosans extracted showed deacetylation degrees of 80.3 % and 79.8%, respectively, and a good antibacterial activity against three bacterial strains.

On the other hand, despite good production levels achieved, the transfer of the chitosan production process of these two strains grown on ELP from shaken flask to STR needs to be improved and further work is required for the optimization of this process, especially for *A. blakesleeana* NRRL 2696. In this respect, the determination of the volumetric mass transfer coefficient (k_{La}), a parameter that indicates the rate at which O₂ can transfer between the gas phase and the liquid phase, could be very helpful in the scale-up and optimization of the bioprocesses.

Finally, low molecular weight chitosan obtained from these strains allows for a wider pharmaceutical application compared to high molecular weight chitosan.

4.6 References

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FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

Currently, the number of anaerobic digesters is increasing worldwide and, consequently, a huge amount of digestate is being produced. Hence, the necessity to determine innovative alternative solutions to integrate digestate in a sustainable biorefinery scheme, that can be defined as “a facility that converts waste materials into fuels, energy, chemicals and materials”, should become a major research issue in the future (Monlau et al., 2015; Amulya et al., 2016).

The replacement of cost-intensive pure nutrients by digestate could improve the economic feasibility of bioprocesses. This fits in the advanced biorefinery model within the circular bioeconomy scheme by providing new and alternative markets for digestate. The studies on digestate-based bioconversions to produce high-value products have been largely performed at laboratory scale. However, there have been few reports on pilot scale investigations (Kaur et al., 2020).

Moreover, incentives for digestate use, quality regulation, and social awareness are essential to promote digestate product commercialization as part of the organic waste circular economy model. Therefore, future studies about circular business models and standardized international regulations for digestate products are needed (Lamolinara et al., 2022).

Purpose of this PhD project was the microbial valorization of the digestate, using it as a fungal growth substrate for the production of chitosan and lignocellulolytic enzymes.

Wastes derived from four different supply chains, including residual thistle biomass, spent coffee grounds, digestate solid fraction and barley straw, have been used to acquire information regarding the lignocellulolytic attitudes of *Aspergillus olivimuriae*, a recently described species discovered after its isolation from table olive brine. In *A. olivimuriae* solid-state cultures, hemicellulose degradation outperformed that of cellulose, and this behavior was particularly evident in the digestate solid fraction, where hemicellulose degradation was equal to 60%; instead, lignin removal did not occur, regardless of the growth substrate. Therefore, irrespective of the waste used, and as opposed to other *Aspergillus* species, the strain under study did not show ligninolytic potential.

For this reason, the research activities moved to an alternative use of the digestate, as a growth substrate for the production of fungal chitosan.

Chitosan sourced from crustacean waste is not of a consistent quality owing to inherent variabilities in the raw materials used. Currently, several companies are commercializing chitosan-based products extracted from fungi, such as Kitozyme (<http://www.kitozyme.com>), MycoDev Group Inc. (<https://mycodevgroup.com>) and Chibio (<https://www.chibiotech.com>). This implies that these companies have an autonomous production capacity or, more likely, that they exploit fungal biomass arising from mycobiotechnological processes. Although the scientific literature currently available witnesses several efforts toward process upscaling to a diverse range of liquid submerged fermentation reactors, studies reporting process scales on the order of cubic meters are absent.

Being a versatile compound with applications in various fields, the demand for chitosan is destined to increase in the near future. Hence paths for alternative sources of chitosan, such as fungal culture, will have to be utilized to meet the demand. To this effect, the understandings provided by laboratory-scale studies need to be translated into the commercial production of chitosan. Moreover, to be economically feasible the culture medium used will have to be as low-cost. The use of wastes as cultures media can keep the cost of production low as well as prevent environmental pollution caused by the introduction of these organic residues into the environment. This necessitates further investigation to identify the suitable media for the production, besides ideal fungal strains and fermentation parameters to obtain maximum chitosan yield (Sebastian et al., 2020).

This PhD study evaluated a liquid medium derived from both solid and liquid fractions of the digestate, called Enriched Liquid Phase, as substrate for the cultivation of selected Zygomycetes fungi capable of accumulating high levels of chitosan in their cell walls. This culture media was found to be suitable for growth of 9 out of 17 selected strains, yielding biomass levels higher than 2.5 g L^{-1} .

Two of them, *A. blakesleeana* NRRL 2696 and *R. oryzae* NRRL 1510, were outstanding for the production of chitosan, reaching volumetric productions of 444 and 285 mg L^{-1} , respectively, in shaken flask fermentations. Chitosans extracted showed deacetylation degrees of 80.3 % and 79.8%, respectively, and a good antibacterial activity against three bacterial strains. The transfer of the chitosan production process of these two strains grown on ELP from shaken flask to STR needs to be improved and further work is required for the optimization of this process, especially for *A. blakesleeana* NRRL 2696. However, low molecular weight chitosan obtained from these strains allows for a wider pharmaceutical application compared to high molecular weight chitosan.

The results obtained in this work could therefore be the basis for stimulating the development of further studies that evaluate the scalability and competitiveness of the process compared to those based on the chemical extraction of chitosan from crustacean sources.

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Cristina

LIST OF PAPERS

Scientific publications produced during the PhD course:

- Carota E., Crognale S., Russo C., Petruccioli M., D'Annibale A. (2021). **Lignocellulolytic Potential of the Recently Described Species *Aspergillus olivimuriae* on Different Solid Wastes.** *Applied Science* 11(12): 5349; doi: <https://doi.org/10.3390/app11125349>
- Crognale S., Russo C., Petruccioli M., D'Annibale A. (2022). **Chitosan Production by Fungi: Current State of Knowledge, Future Opportunities and Constraints.** *Fermentation*, 8, 76; doi: <https://doi.org/10.3390/fermentation8020076>
- Russo C., Crognale S., Petruccioli M., D'Annibale A. (2023) **Digestate-based Liquid Growth Medium for Production of Microbial Chitosan.** Draft in preparation for submission.
- Crognale S., Russo C., Lelli D., Liberati D., De Angelis P. (2023) **From lab-to field: steps for in situ bioaugmentation approach in Phyto-bio requalification project of urban hydrocarbon polluted area.** Draft in preparation for submission.

Poster Communications:

- Crognale S., Russo C., Liberati D., De Angelis P. **Integrated biological approaches for the remediation and the requalification of a hydrocarbon polluted urban area.** SETAC Europe 30th Annual Meeting, Dublin, Ireland 3-7 May 2020.
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