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Chitosan and chitin production and extraction in isolates of *Cunninghamella* sp.

Aloisio Freitas Chagas Junior[1*], Lillian França Borges Chagas[1], Gessiel Newton Scheidt[2], Vanessa Mara Chapla[3], Brigitte Sthepani Orozco Colonia[4], Manuella Costa Souza[2] and Albert Lennon Lima Martins[5]

[1]Programa de Pós-graduação em Produção Vegetal, Curso de Agronomia, Universidade Federal do Tocantins, Chácara 69-72, Rua Badejos, Lote 7, s/n, 77410-530, Gurupi, Tocantins, Brazil. [2]Curso de Engenharia de Bioprocesso e Biotecnologia, Universidade Federal do Tocantins, Gurupi, Tocantins, Brazil. [3]Curso de Enenharia Química, Universidade Federal do Tocantins, Gurupi, Tocantins, Brazil. [4]Departamento de Engenharia de Bioprocesso e Biotecnologia da Universidade Federal do Paraná, Curitiba, Paraná, Brazil. [5]Empresa de Pesquisa Agropecuária de Minas Gerais, Prudente de Morais, Minas Gerais, Brazil. *Author for correspondence. E-mail: chagasjraf@uft.edu.br

ABSTRACT. The use of fungi to obtain chitin and chitosan has advantages such as handling, extraction, and production control, thus allowing to generate high quality chitosan. This study aimed to isolate and identify strains of *Cunninghamella* spp., and assess the production of biomass, chitin, and chitosan. We determined the macroscopic and microscopic phenotypic aspects of the superiors, mycelial growth rate index, consumption of glucose and nitrogen, as well as the pH variation, and the production of biomass, chitin, and chitosan. After assessing the mycelial growth speed index, considered fast, we found that the UFT-Ce08 and UFT-Ce09 isolates showed a higher growth speed. Therefore, we assessed the sporulation of the isolates, and all of them reached the concentration of 10^6 spores mL^{-1} in the period of 96 hours. The YPD was considered ideal for biomass production because it promotes an efficient consumption and synthesis of organic compounds by microorganisms. The assessment of the biomass production and the chitin and chitosan yield of nine isolates and the reference strain showed that the UFT-Ce08 isolate had the highest amount of biomass, the UFT-Ce11 isolate had the highest chitin yield, and despite having the second smallest biomass, the UFT-Ce09 isolate had the highest chitosan yield. Seven isolates showed higher chitosan yield than the reference strain. Therefore, chitosan production from this fungus can be further optimized to improve yield on a large-scale production. Chitosan production from this fungus can be further optimized to improve yield in large-scale production.

Keywords: fungal biomass; microbial growth; biopolymer production.

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Introduction

Chitin has a copolymer derived from its partial deacetylation, chitosan, which contains predominantly D-glucosamine units, thus being a soluble derivative in acidic solutions (Fráguas et al., 2015).

The interest in chitosan polymers has increased considerably in the last decades, having gained applications in the most diverse areas such as agriculture, food and pharmaceutical industries, development of cosmetics and biomaterials, water treatment, among others (Azevedo, Chaves, Bezerra, Lia Fook, & Costa, 2007).

Commercial chitosans are obtained from traditional sources for several purposes, basically crabs exoskeletons; however, the extraction process of these polymers uses the thermo-chemical deacetylation, which has economic and environmental disadvantages (Franco et al., 2004).

Soils with a high-water content cause an increase in the fungal population (Souto, Souto, Miranda, Santos, & Alves, 2008). Thus, areas of soil cultivation, in general, are constantly irrigated, being characterized as ideal habitat for these microorganisms to grow. These fungi are commonly found in this type of soil and belong the Zygomycetes class, especially the species belonging to the genus *Cunninghamella*, widely studied, and used in the biotechnological area, mainly due to its greater amounts of chitin and chitosan in their cell walls (Fai, Stamford, & Stamford, 2008; Campos-Takaki & Dietrich, 2009).

Cunninghamella spp. are filamentous fungi found in the soil and plant material. It is considered an important genus for medical mycology and biotechnological processes. The genus contains 14 species, among which *C. bertholletiae*, *C. elegans* and *C. echinulata* are the most common (Asha & Vidyavathi, 2009).

Besides the cell wall of fungi, chitin is present in the exoskeleton of insects and crustaceans, being the second polysaccharide most found in nature after cellulose. Its chemical structure is formed by a long linear

chain of successive units, joined by β (1-4) glycosidic bonds of amino monosaccharides called N-acetylglucosamine (Vázquez et al., 2013).

The use of fungi to obtain chitosan has advantages such as ease of handling, extraction, and production control, allowing to generate high quality chitosan, with lower molecular weight. and consequently, greater solubility (Niederhofer & Müller, 2004). Thus, this study aimed to isolate *Cunninghamella* sp. fungi in soils from cultivated areas and assess the production of biomass, chitin, and chitosan.

Material and methods

Collection area

The experiments were conducted at the Microbiology Laboratory of the *Universidade Federal do Tocantins*, Campus of Gurupi, Tocantins Brazil. For soil collection, cultivated areas were selected in the municipality of Gurupi, Tocantins, Brazil (latitude 11º43'45" S and longitude 49º04'07" W) at an altitude of 287m, and in the municipality of Formoso do Araguaia, Tocantins, Brazil (latitude: 11º47'48" S and longitude: 49º31'44" W) at an altitude of 240 m. Soil samples were collected in the municipality of Gurupi, near the *Universidade Federal do Tocantins*, from constantly irrigated experimental areas of corn, soybean, and cassava. In the municipality of Formoso do Araguaia, samples were collected in the experimental areas of rice cultivation. A total of 50 soil samples were collected with three repetitions each, with sub-samples at three points apart, respecting a radius of 1 m, taken from 0 to 15 cm deep, eliminating the burlap.

Isolation

The soil spreading method was applied for fungus isolation (Santos, Ferraz, & Muchovej, 1991), consisting of spreading 2 g of soil on the center of a Petri dish containing PDA culture medium plus antibiotic (250 g potato, 20 g dextrose, 20 g agar, 250 mg ampicillin per liter of water), and incubation at 28ºC in a BOD (Biochemical Oxygen Demand) incubator. After incubation, the dishes were checked daily, and upon detecting the presence of hyphae or mycelium characteristic of the genus *Cunninghamella,* the subculture was transferred to a new dish containing PDA culture medium plus antibiotics until the pure culture was obtained. The pure cultures were stored in a Petri dish under refrigeration (4ºC) for later macroscopic and microscopic identification and preserved in microtubes to form a culture bank.

To compare the macroscopic and microscopic phenotypic aspects of the isolates, a reference strain of *Cunninghamella elegans* CBMAIL 0843 was acquired from the Brazilian Collection of Environmental and Industrial Microorganisms of the *Universidade Estadual de Campinas* (UNICAMP), Brazil. The strain received in active repique was then transferred and maintained in Petri dishes in PDA medium, under a 96-hour inverted incubation at 28ºC.

The identification at genus level was performed in pure cultures of the fungi grown in Petri dishes containing PDA medium, maintained in BOD at 28ºC. The morphological characterization was conducted by observing the macroscopic characteristics of the colonies, such as coloration (back and front), aspect/texture, and growth topography. Each isolate was macroscopically compared with the reference strain to verify similarities between them.

For microscopic identification, slides were prepared for each pure culture grown in Petri dishes containing PDA medium by placing a drop of distilled water on a microscope slide containing a portion of the fresh material to be examined. The preparation was covered with a coverslip to be examined under a common optical microscope with 40x objective. Microstructures such as reproductive structures and spores were observed and compared with the reference strain.

Mycelial growth

To assess the mycelial growth speed, mycelium fragments from active monospore cultures of the isolated fungi and the reference strain were transferred to the center of Petri dishes containing PDA medium and conditioned in BOD at 28ºC for a 12 hours photoperiod until the entire dish diameter (95 mm) was completely covered. The diameter of the radial growth was measured with a digital pachymeter every 24 hours after incubation, based on the back of the dish.

The calculation of the Mycelial Growth Speed Index (MGSI) was based on growth measures in millimeters until the last assessment day by measuring the diameter of the colonies in three perpendicular axes. The

calculation followed the formula described by Pires et al. (2017): IVCM = {Σ (D-Da)} / N, where IVCM is the Mycelial Growth Speed Index (MSGI), D is the current average diameter of the colony, Da is the average diameter of the colony of the previous day, and N is the number of days after inoculation. Finally, the growth rate was classified as fast (<7 days), intermediate (8 to 14 days), or slow (> 15 days).

Metabolic profile and sporulation

To determine the metabolic profile and sporulation, 20 isolates and the reference strain were used. For sample maintenance and sporulation assessment, PDA medium was used with 250 mg of ampicillin per liter of water, poured on a Petri dish. As for batch culture, YPD medium was used with the following composition: 2 g yeast extract, 10 g peptone, and 20 g dextrose, diluted in distilled water (1 L), and pH corrected to 4.5. Cultivation in liquid medium started from a monospore culture of each fungus sample, standardizing the inoculum by counting spores in a Neubauer chamber at a final concentration of 10^6 spores mL^{-1}.

Standardized inoculums were added in 250 mL Erlenmeyer flasks, containing 50 mL of YPD medium. The incubation was then carried out at room temperature for 120 hours under constant orbital shaking at 100 rpm. Every 24 hours, aliquots of the metabolic medium were removed to monitor the consumption of glucose and nitrogen, as well as the pH variation. Each fungal sample studied received five study times.

For sporulation assessment, three plates of each fungus sample were removed from the incubation for spore counting procedure every 24 hours, generating five readings: 24, 48, 72, 96, and 120 hours. The number of spores was determined by adding 5 mL of distilled water in the cultured Petri dish and removing the spores using a round brush with soft bristles number 12. The suspension obtained in the dish was filtered on cotton gauze in a cloth type fabric in the format of compress with two folds and sterile. For the counting of spores, we used the Neubauer chamber with 10 µL of the filtered suspension of spores with the help of a single-channel micropipette of variable volume and three readings per dish in an optical microscope.

The spores present in the central quadrant of the Neubauer chamber were considered based on the small size of the spore and the need to establish a concentration of 10^6 spores mL^{-1} for subsequent cultivation in liquid medium. Thus, the five quadrants were counted, four lateral and one central, located in the central quadrant of the Neubauer chamber with a 40x objective. The concentration of the spores was determined by multiplying the total number of spores in the five quadrants by the formula below (Bastidas, 2008): Spores concentration = (total of spores in the 5 quadrants/number of quadrants) x 4x 10^6.

Glucose and nitrogen consumption

The colorimetric enzymatic method (Labtest®) was applied to establish glucose consumption. Aliquots were taken from the culture media and readings were performed in a digital spectrophotometer at 405 nm, following the recommendations established by the Labtest Manual for Glucose Liquiform (Ref. 133) (Labtest Diagnóstica, 2011).

Determination of nitrogen consumption was based on the total protein through the colorimetric enzymatic method. Aliquots were taken from the culture media and absorbance readings were performed in a digital spectrophotometer at 545 nm, according to the recommendations of the Labtest Manual for Total Proteins (Ref. 99) (Labtest Diagnóstica, 2009).

pH was determined using a digital pH meter immersed in the culture medium.

Chitin and chitosan production

Extraction samples from nine strains of *Cunninghamella* spp. and *Cunninghamella elegans* CBMAI 0843 were taken to produce biomass and chitin and chitosan using YPD medium at pH 4.5.

The liquid medium culture started from monosporic culture of each fungus sample with inoculum standardization by counting spores in a Neubauer chamber at the final concentration of 10^6 spores mL^{-1}. For cultivation, 250 mL Erlenmeyer flasks containing 50 mL of the YPD medium were used. Subsequently, incubation was carried out at room temperature for 120 hours under constant orbital shaking at 100 rpm, on an orbital shaking table.

Every 24 hours, Erlenmeyer stirring containing the mycelial biomass of each fungal sample was removed and the biomass was filtered under vacuum on a 0.45 µm qualitative filter paper, discarding the liquid medium. A total of five collections were prepared for each fungus sample, with one collection every 24 hours until the final collection in 120 hours. For all fungus samples, the experiment was carried out in three replications.

We subjected the collected and filtered biomass to an oven drying process at 50ºC, followed by grounding the dry mycelial mass to powder and weighed on an analytical balance to monitor growth.

Chitin and chitosan were extracted from the dried, dehydrated, and powdered mycelial mass according to the methodology described by Synowiecki and Al-Khateeb (1997). The authors recommend the use of 3 to 10 g of mycelial mass per extraction step. The general process involved deproteinization with 2% w v^{-1} sodium hydroxide solution (30:1 v m^{-1}, 90ºC, 2 hours); separation of the alkali-insoluble fraction (FIA) by centrifugation (4000 rpm, 15 min.); extraction of chitosan from the FIA with acetic acid 10% v v^{-1} (40:1 v m^{-1}, 60ºC, 6 hours); separation of chitin by centrifugation (4000 rpm, 15 min.); precipitation of chitosan from the extract, at pH 9.0, adjusted with a 4 M NaOH solution; washing chitin and chitosan with water, ethanol, and acetone and drying in air at 20°C.

Statistical analysis

The data were submitted to analysis of variance, and the means were compared through the Scott-Knott test at 5% significance on the statistical program Assistant 7.6 (Silva, 1998).

Results and discussion

Twenty pure monosporic cultures characteristic of the genus out of the 50 samples collected from cultivated soil were isolated in *Cunninghamella*. By the end of 96 hours of growth, a macroscopic observation for all isolates and for the reference strain revealed colonies with cottonseed mycelium, delicate white intertwined hyphae, easy to rupture, common features to all species of the genus *Cunninghamella* (Figure 1) (Campbell, Johnson, & Warnock, 2013). With a greater maturation degree of the culture, a gray to brown coloration started to appear, also peculiar to the genus, as highlighted by Severo, Guazzelli, and Severo (2010).

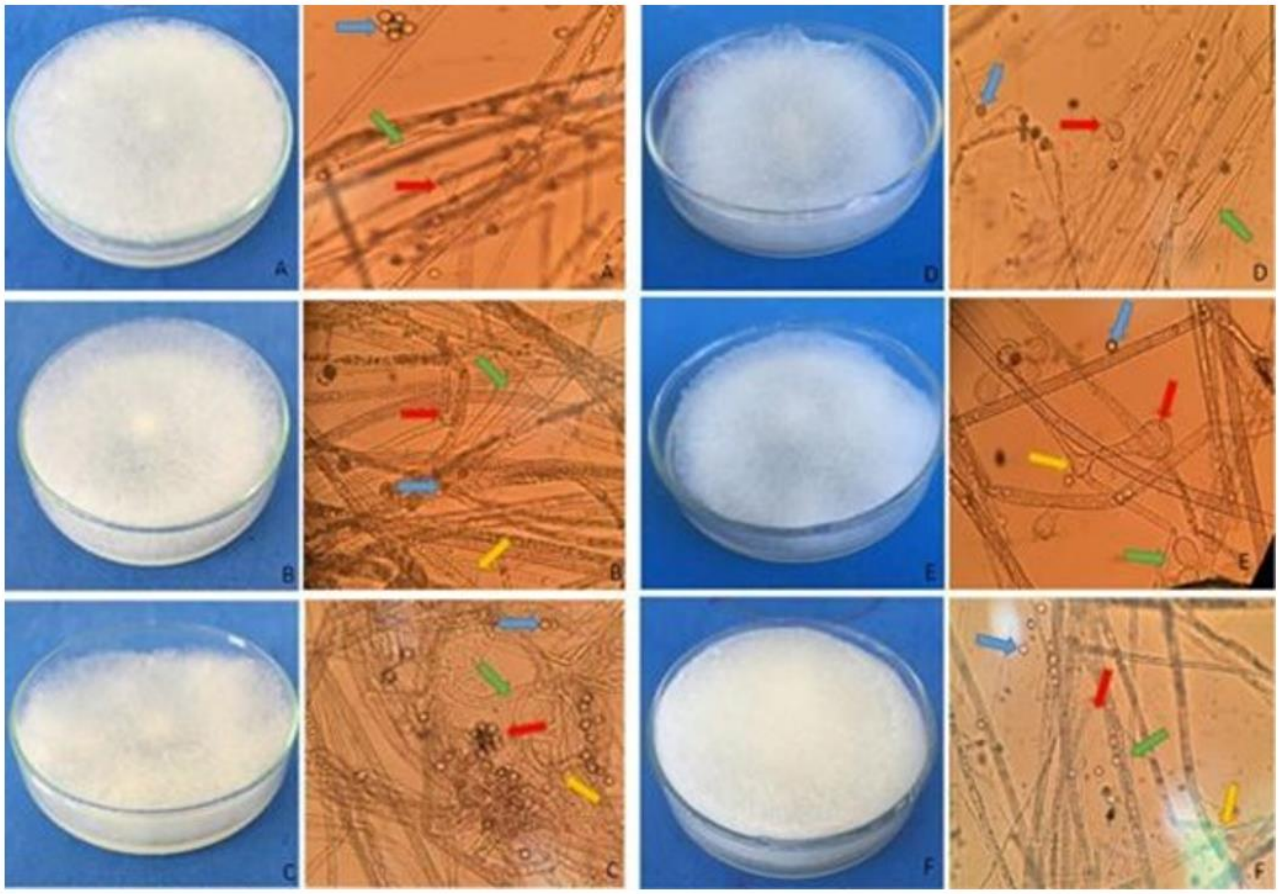

Figure 1. Macroscopic and microscopic aspects of the reference strain CBMAI 0843 (A), and the isolates UFT-Ce01 (B), UFT-Ce02 (C), UFT-Ce03 (D), UFT-Ce04 (E), UFT-Ce05 (F). Microscopy shows branched hyphae (yellow arrow), hyaline coenocytic hyphae (green arrow), sporangiophores ending in a globular vesicle with or without adherent spores (red arrow), hyaline spores (blue arrow).

As for the back side of the plate and the growth topography, all samples and the reference strain showed a characteristic beige color and homogeneous centrifugal circular growth. The microscopic identification was based on optical microscopy visualization of the microstructures of the vegetative mycelium and of the reproductive mycelium, such as hyphae, sporangia, sporangiophores, and sporangiospores.

The micromorphology demonstrated erect, simple, or branched sporangiophores, and the end of each branch showed globular vesicles (sporangiophores) in which the sporangiospores are inserted. The mycelium was not septate, most of the time, thus characterizing youth culture. The spores were hyaline, single-celled, globose, with equinulated membrane and smooth or spiky walls (Figure 1), as highlighted by Pitt and Hocking (2009).

Alexopoulos, Mims, and Blackwell (1996) report that the *Cunninghamella* species are very sensitive to small variations in temperature, humidity and culture medium, and the same isolate grown in different environments may present different phenotypic aspects. However, this study found macroscopic and microscopic characteristics that resemble the twenty isolates to the reference strain and to the literature.

Regarding the mycelial growth profile, all the isolates assessed covered the dish diameter in the period of 120 hours, and in the 96 hours period the reference strain and the UFT Ce07 and UFT Ce19 isolates did not fill the entire diameter of the Petri dish. This rapid growth within 96 hours period classifies the strains with rapid growth speed (<7 days).

The colony diameter from 24 to 72 hours was considered when calculating the MGSI since some isolates had already filled the diameter of the Petri dish during the period. We found that the isolates with the highest growth speed were UFT-Ce09 (25.77 mm day^{-1}), UFT-Ce08 (25.70 mm day^{-1}), UFT-Ce10 (24.27 mm day^{-1}), followed by UFT-Ce13 (23.90 mm day^{-1}), according to Table 1.

Table 1. Distribution of average mycelial growth rate of the *Cunninghamella* spp. isolates and the reference strain *C. elegans* CBMAI 0843 over 96 hours in Petri dishes with PDA medium.

Strains	Colony diameter (mm)[1]				MGSI[2]
	24 hours	48 hours	72 hours	96 hours	(mm day^{-1})
CBMAI 0843	21.7 c	48.7 c	68.7 b	93.5 a	15.67
UFT Ce01	27.8 b	55.8 c	90.5 a	95.0 a	20.90
UFT Ce02	32.7 b	72.5 a	95.0 a	95.0 a	20.77
UFT Ce03	40.7 a	82.5 a	95.0 a	95.0 a	18.10
UFT Ce04	30.8 b	79.2 a	94.5 a	95.0 a	21.23
UFT Ce05	29.9 b	60.8 b	86.3 a	95.0 a	18.80
UFT Ce06	28.3 b	68.5 b	95.0 a	95.0 a	22.23
UFT Ce07	24.2 c	44.5 c	69.2 b	93.5 a	15.00
UFT Ce08	17.9 c	59. 4 b	95.0 a	95.0 a	25.70
UFT Ce09	17.7 c	54.5 c	95.0 a	95.0 a	25.77
UFT Ce10	22.2 c	65.7 b	95.0 a	95.0 a	24.27
UFT Ce11	13.7 c	46.0 c	77.5 b	95.0 a	21.27
UFT Ce12	24.2 c	64.0 b	90.2 a	95.0 a	22.00
UFT Ce13	23.3 c	64.5 b	95.0 a	95.0 a	23.90
UFT Ce14	29.3 b	61.0 b	90.0 a	95.0 a	20.23
UFT Ce15	29.1 b	61.8 b	83,0 a	95.0 a	17.97
UFT Ce16	25.3 c	54.3 c	85.2 a	95.0 a	19.97
UFT Ce17	25.1 c	46.0 c	75.5 b	95.0 a	16.80
UFT Ce18	23.6 c	60.5 b	90.0 a	95.0 a	22.13
UFT Ce19	23.7 c	45.8 c	70.5 b	92.7 a	15.60
UFT Ce20	38.1 a	59.7 b	95.0 a	95.0 a	18.97
CV (%)[3]	21.6	10.7	5.6	0.94	-

[1]Averages followed by the same lower-case letter in the columns, do not differ by the Scott-Knott test at 5% significance. [2]MGSI (Mycelial Growth Speed Index), [3]CV = Coefficient of variation.

The reference strain had the third lowest MGSI (15.67 mm day^{-1}), ahead of only the isolates UFT-Ce07 (15.0 mm day^{-1}) and UFT-Ce19 (15.60 mm day^{-1}), thus indicating that out of the 20 isolates, 18 showed grown better than the reference strain. In the 24-hour period, the UFT-Ce03 and UFT-Ce20 isolates statistically differed the most showing the highest growth. As for the 48 hours period, the isolates with the most statistically significant growth were UFT-Ce02, UFT-Ce03, and UFT-Ce04. Within 72 hours, some strains had already filled the dish diameter (95 mm), thus generating a more uniform growth profile.

In the case of isolates of the same *Cunninghamella* genus and the same growth conditions, there were significant differences in MGSI between the assessed isolates. Souza, Souza and Mendes-Costa (2007) assessed the MGSI of seven isolates of the fungus *Colletotrichum lindemuthianum* and found that only two

isolates varied significantly in the speed of mycelial growth. Other studies used the MGSI to assess the growth of microorganisms at different temperatures and culture media (Maia, Armesto, Zancan, Maia, & Abreu, 2011; Ferreira, Nascimento, Neves, Gomes, & Nascimento, 2012).

As for the number of spores, the UFT-Ce09 and UFT-Ce14 isolates had the highest number of spores in the 96 hours period, with 9.19 x 10^6 spores mL^{-1} and 7.23 x 10^6 spores mL^{-1}, respectively, statistically differing from the other isolates assessed. In the 72 hours period, the UFT-Ce07 isolate showed greater sporulation, with 7.05 x 10^6 spores mL^{-1} (Table 2). Most isolates of *Cunninghamella* spp. had a behavior similar to the reference strain *C. elegans* (CBMAI 0843) regarding the period of greatest sporulation and number of spores.

Table 2. Average spore count of *Cunninghamella* spp. and the reference strain *Cunninghamella elegans* CBMAI 0843 grown in PDA medium at 28ºC for a period of 120 hours.[1]

Strains	Number of Spores mL^{-1}				120 hours
	24 hours	48 hours	72 hours	96 hours	
CBMAI 0843	0.18x10⁶ d	0.25x10⁶ d	0.54x10⁶ b	3.44x10⁶ c	1.19x10⁶ c
UFT Ce01	0.55x10⁶ c	0.80x10⁶ d	0.93x10⁶ b	1.95x10⁶ c	2.00x10⁶ b
UFT Ce02	0,43x10⁶ c	0.53x10⁶ d	1.08x10⁶ b	2,30x10⁶ c	1.40x10⁶ c
UFTCe03	0.00x10⁶ d	0.30x10⁶ d	1.55x10⁶ b	2.23x10⁶ c	1.30x10⁶ c
UFT Ce04	0.15x10⁶ d	0.65x10⁶ d	0.93x10⁶ b	1.25x10⁶ c	1.05x10⁶ c
UFT Ce05	0.04x10⁶ d	0.30x10⁶ d	1.03x10⁶ b	3.70x10⁶ c	1.90x10⁶ b
UFT Ce06	0.05x10⁶ d	0.60x10⁶ d	1.51x10⁶ b	3.66x10⁶ c	2.70x10⁶ b
UFT Ce07	0.40x10⁶ c	3.40x10⁶ b	7.05x10⁶ to	3.10x10⁶ c	2.55x10⁶ b
UFT Ce08	0.15x10⁶ d	0.33x10⁶ d	0.59x10⁶ b	5.03x10⁶ b	4.70x10⁶ a
UFT Ce09	0.29x10⁶ d	2.06x10⁶ c	2.29x10⁶ b	9.19x10⁶ a	4.94x10⁶ a
UFT Ce10	0.03x10⁶ d	2.15x10⁶ c	2.13x10⁶ b	2.65x10⁶ c	2.10x10⁶ b
UFT Ce11	0.88x10⁶ b	1.60x10⁶ d	1.68x10⁶ b	3.30x10⁶ c	1.33x10⁶ c
UFT Ce12	0.03x10⁶ d	0.55x10⁶d	2.00x10⁶ b	1.95x10⁶ c	1.95x10⁶ b
UFT Ce13	0.13x10⁶ d	0.55x10⁶d	2.53x10⁶ b	2.30x10⁶ c	1.53x10⁶ c
UFT Ce14	4.10x10⁶ a	4.83x10⁶ a	6.75x10⁶ a	7.23x10⁶ a	1.75x10⁶ b
UFT Ce15	0.14x10⁶ d	0.41x10⁶ d	1.13x10⁶ b	4.65x10⁶ b	1.93x10⁶ b
UFT Ce16	0.03x10⁶ d	0.13x10⁶ d	0.65x10⁶ b	1.20x10⁶ c	0.85x10⁶ c
UFT Ce17	0.08x10⁶ d	0.45x10⁶ d	0.63x10⁶ b	2.53x10⁶ c	1.50x10⁶ c
UFT Ce18	0.05x10⁶ d	0.10x10⁶ d	0.63x10⁶ b	1.50x10⁶ c	1.00x10⁶c
UFT Ce19	0.08x10⁶ d	0.71x10⁶ d	1.50x10⁶ b	5.54x10⁶ b	2.83x10⁶ b
UFT Ce20	0.13x10⁶ d	0.23x10⁶ d	0.50x10⁶ b	3.88x10⁶ c	0.48x10⁶ c
CV (%)[2]	38.76	40.17	50.10	37.16	26.34

[1]Averages followed by the same lower-case letter in the columns, do not differ by the Scott-Knott test at 5 % of significance. [2]CV = Coefficient of variation.

Even with the great biotechnological applicability of *Cunninghamella* spp., no studies were found demonstrating the sporulation profile of the fungus. Regarding cultivation conditions, Silva and Teixeira (2012) obtained good spore production by phytopathogens using the PDA culture medium, highlighting that PDA is one of the most nutritionally rich culture media with the highest amount of complex carbohydrates.

Luminosity favors the formation of spores and most fungi spores when exposed to continuous light, but some require a period in the dark followed by a period in the light (Pierobom, Santos, Rey, & Rosseto, 2005). The strains used in this study were sporulated in BOD over a 12 hours photoperiod.

According to Penariol, Monteiro and Pitelli (2008), the supplementation of the culture medium with peptone and yeast extract stimulates the growth and sporulation of the fungus. Thus, all the strains assessed reached a concentration of 10^6 spores mL^{-1} in the period of 96h, allowing the inoculum to transfer to the liquid medium YPD during the period in order to assess the metabolic profile of the strains.

Regarding glucose consumption from the YPD liquid medium, the results were homogeneous among the twenty isolates of *Cunninghamella* spp. and the reference strain (CBMAI 0843). The assessed isolates consumed approximately 15 g L^{-1} of glucose in the liquid medium in 24 hours. In the 96 hours period, differences in glucose consumption were more significant, in which the isolates UFT-Ce04, UFT-Ce05, UFT-Ce06, UFT-Ce08, UFT-Ce09 and UFT-Ce14 consumed a greater amount of glucose than the other fungi assessed, with emphasis to the UFT-Ce09 strain, which used all the remaining glucose in this period, whereas the strains UFT-Ce01, UFT-Ce03, UFT-Ce10 and UFT-Ce12 consumed smaller amounts of glucose (Table 3).

In the final assessment, 120 hours, in addition to the UFT-Ce04, UFT-Ce05, UFT-Ce06, UFT-Ce08, UFT-Ce09 and UFT-Ce14 isolates, the reference strain (CBMAI 0843) and the UFT-Ce02, UFT-Ce11 and UFT-Ce20 isolates showed lower residual glucose amounts in the culture medium (Table 3). From 20 g L^{-1} of glucose added in liquid

YPD medium, an average of 0.21 g L^{-1} remained, compared to the isolates that did not consume all glucose in the end of the fermentation process, thus suggesting no excess carbon source in the culture medium for fungi growth.

Table 3. Average glucose concentration in YPD broth culture of *Cunninghamella* spp. and the reference strain *C. elegans* (CBMAI 0843) within 120 hours.[1]

Strains	Concentration (g L^{-1})				120 hours
	24 hours	48 hours	72 hours	96 hours	
CBMAI 0843	5.87 a	4.57 a	3.00 a	0.66 e	0.01 e
UFT Ce01	5.67 a	4.07 a	3.65 a	2.35 b	0.32 d
UFT Ce02	5.49 b	4.75 a	2.84 a	1.52 c	0.15 e
UFT Ce03	5.42 b	4.38 a	3.34 a	2.28 b	0.25 d
UFT Ce04	5.40 b	4.69 a	1.65 b	0.19 f	0.08 e
UFT Ce05	5.64 a	4.82 a	2.33 b	0.33 f	0.07 e
UFT Ce06	5.87 a	4.00 a	2.23 b	0.30 f	0.04 e
UFT Ce07	5.77 a	4.63 a	2.73 a	1.28 d	0.24 d
UFT Ce08	5.77 a	3.94 a	2.87 a	0.01 f	0.00 e
UFT Ce09	5.63 a	4.75 a	3.99 a	0.00 f	0.00 e
UFT Ce10	5.77 a	4.50 a	3.29 a	2.08 b	0.27 d
UFT Ce11	5.77 a	4.26 a	2.13 b	0.45 e	0.04 e
UFT Ce12	5.67 a	4.25 a	3.84 a	2.75 a	0.79 a
UFT Ce13	5.77 a	4.32 a	2.53 b	1.32 d	0.30 d
UFT Ce14	5.58 a	4.38 a	1.70 b	0.12 f	0.03 e
UFT Ce15	5.67 a	3.88 a	2.30 b	1.20 d	0.28 d
UFT Ce16	5.68 a	4.69 a	3.21 a	1.07 d	0.42 c
UFT Ce17	5.87 a	4.19 a	1.89 b	1.21 d	0.64 b
UFT Ce18	5.30 b	4.82 a	2.94 a	1.77 c	0.27 d
UFT Ce19	5.49 b	4.07 a	1.70 b	1.16 d	0.22 d
UFT Ce20	5.35 b	4.13 a	1.66 b	0.71 e	0.06 e
CV (%)[2]	5.63	7.81	15.75	14.51	23.60

[1]Averages followed by the same lower-case letter in the columns, do not differ by the Scott-Knott test at 5% significance. [2]CV = Coefficient of variation.

Stamford, Stamford, Stamford, Barros Neto and Campos-Takaki (2007) obtained 1.27 g L^{-1} of residual glucose for *C. elegans* (UCP 542) cultivar with 10^8 spores mL^{-1} in a culture medium based on Jacatupé (*Pachyrhizus erosus* L. Urban) containing 11.14 g L^{-1} of glucose and 40.9 g L^{-1} of starch, for 96 hours. In 96 hours, this study obtained an average of 1.08 g L^{-1} of glucose remaining at lower concentrations of carbohydrate and inoculum in the culture medium.

In a study with *C. elegans* (URM 46109) and *Mucor racemosus* (IFM 40781) grown in YPD medium, Amorim, Souza, Fukushima, and Campos-Takaki (2001) observed a lower use of glucose by the strain of *C. elegans* in relation to *M. racemosus*; however, such factor did not interfere with the growth of the fungus biomass. The authors also report that *C. elegans* shows diauxic growth, that is, in a mixture of two substrates there is an initial lag phase, followed by a more easily assimilated substrate consumption, then a second lag phase of adaptation to the second substrate accompanied by its consumption, thus suggesting that the glucose source initially induces the synthesis of enzymes necessary for its use, repressing the synthesis of enzymes necessary for yeast extract use. This effect ceases when all sources of glucose are metabolized. Finally, Penariol et al. (2008) claim that using a culture medium supplemented with peptone (enzymatic hydrolyzate of proteins) provides greater biomass production, preservation of cell viability, and more efficient consumption of the carbon source.

Regarding the nitrogen concentration curve in the medium, as well as glucose, the highest consumption also occurred in the first 24 hours; however, this period was followed by an increase in the nitrogen concentration. In the period of 48 to 96 hours, the concentration of most strains decreased once again, later increasing after 96 hours of cultivation (Table 4). The higher nitrogen concentration after 96 hours was not observed in the culture media of UFT-Ce04, UFT-Ce07, UFT-Ce13, UFT-Ce16, and UFT-Ce17 isolates, showing a continuous decrease in nitrogen concentration after 48 hours (Table 4).

Nitrogen consumption was similar for most of the strains assessed and the reference strain (CBMAI 0843). Initially, the higher nitrogen concentration can be explained by the production of enzymes during growth and then by the production of nitrogenous compounds originated from the secondary metabolism of the microorganism, as reported by Amorim et al. (2001) and Stamford et al. (2007).

Stamford et al. (2007) found a residual concentration of 0.18 g L^{-1} of nitrogen in the culture medium based on Jacatupé containing 8.72 g L^{-1} of total proteins in 96 hours, whereas this study obtained an average of 3.0 g L^{-1}

of residual nitrogen in the same period, using 10 g L^{-1} of peptone. However, Franco et al. (2004) used strains of *C. elegans* (IFM 46109) for 10^7 spores mL^{-1} grown in synthetic medium for Mucolars containing 3 g L^{-1} of asparagine and found a continuous decrease in protein concentration in the culture medium throughout the assessment period, 96 hours.

Table 4. Average nitrogen concentration in cultivation in YPD broth from isolates of *Cunninghamella* spp. and the reference strain *C. elegans* CBMAI 0843 within 120 hours.[1]

Strains	Concentration (g L^{-1})				120 hours
	24 hours	48 hours	72 hours	96 hours	
CBMAI 0843	2.44 a	4.80 a	3.05 b	3.11 a	3.37 a
UFT Ce01	0.91 b	4.53 a	3.38 b	2.65 a	3.48 a
UFT Ce02	0.54 b	4.89 a	2.47 b	2.41 a	3.58 a
UFT Ce03	1.54 b	5.07 a	3.38 b	2,65 a	3.58 a
UFT Ce04	1.09 b	5.88 a	4.53 a	3.74 a	3.06 a
UFT Ce05	2.81 a	5.80 a	5.27 a	4.83 a	5.58 a
UFT Ce06	2.63 a	4.80 a	3.54 b	2.26 a	3.48 a
UFT Ce07	1.54 b	4.25 a	3.54 b	3.35 a	2.22 a
UFT Ce08	2.26 a	4.80 a	3.29 b	3.11 a	3.27 a
UFT Ce09	2.08 a	3.98 a	2.72 b	0.62 a	2.22 a
UFT Ce10	2.35 a	4.98 a	4,61 a	3.12 a	3.37 a
UFT Ce11	1.18 b	5.34 a	3.63 b	2.26 a	3.90 a
UFT Ce12	2.99 a	4.25 a	3.38 b	3.27 a	3.90 a
UFT Ce13	1.09 b	5.07 a	4.45 a	4.05 a	3.69 a
UFT Ce14	1.81 b	5.07 a	5.19 a	4.13 a	4.21 a
UFT Ce15	1.72 b	4.89 a	3.87 b	2.34 a	3.16 a
UFT Ce16	1.54 b	4.98 a	3.71 b	3.19 a	2.74 a
UFT Ce17	1.45 b	5.43 a	4.20 a	3.50 a	2.85 a
UFT Ce18	1.45 b	4.62 a	3.96 b	3.27 a	3.48 a
UFT Ce19	3.08 a	5.16 a	3.38 b	2.88 a	3.37 a
UFT Ce20	1.90 b	5.61 a	4.53 a	2.96 a	5.27 a
CV (%)[2]	19.84	11.43	13.23	16.46	17.06

[1]Averages followed by the same lower-case letter in the columns, do not differ by the Scott-Knott test at 5% significance. [2]CV = Coefficient of variation.

Penariol et al. (2008) reported that peptones contain a mixture of carbon and nitrogen sources that can be easily used by fungi, and the yeast extract is used as nitrogen source for fungal growth for containing amino acids, peptides, vitamins, and water-soluble carbohydrates.

The pH of the cultivation medium, initially at 4.5, showed a decrease in 24 hours in all isolates (Table 5). Such a decrease is characterized as the exponential phase of fungal growth due to the high metabolic exchange that causes cell ions to release and pyruvic acid to form from the glucose present in the culture medium. Then, the 'lag' phase begins with approximately constant pH values. Finally, the pH tends to neutralize, reaching its highest values, possibly due to the decrease in metabolic activities in the end of the cultivation time (Stamford et al., 2007).

After the exponential phase, the pH values oscillated between 4 and 6 (Table 5). Amorim et al. (2001) obtained pH values oscillating between 3 and 4, while Franco, Stamford, Stamford and Campos-Takaki (2005) found a pH varying between 4 and 5, and Stamford et al. (2007) reached a variation between 5 and 7. Amorim et al. (2001) and Franco et al. (2004) also reported constant pH values during the lag phase and a drop in the pH during the exponential phase.

For biomass production and extraction of chitin and chitosan, among the twenty isolates of *Cunninghamella* spp., the nine that showed the greatest similarities in the metabolic profile in relation to the reference strain were selected, namely: UFT-Ce02, UFT-Ce04, UFT-Ce05, UFT-Ce06, UFT-Ce08, UFT-Ce09, UFT-Ce11, UFT- Ce14, and UFT-Ce20. The mycelial masses collected during the 120 hours of cultivation were used to establish the growth profile from the dry biomass (Figure 2).

We found that for most of the assessed isolates, the period of greatest growth occurred within 48 hours of cultivation, and in this period, the UFT-Ce08 isolate had the highest amount of biomass, 20.17 g L^{-1}, followed by UFT-Ce20 isolate, with 12.64 gL^{-1}, the reference strain CBMAI 0843, with 10.78 g L^{-1}, UFT-Ce11, with 10.72 g L^{-1}, UFT-Ce02, with 9.42 g. L^{-1}, UFT-Ce09, with 9.34 g L^{-1}, and the UFT-Ce05 isolate, with lower biomass, 9.15 g L^{-1} (Table 6). The UFT-Ce04 and UFT-Ce14 isolates showed greater growth in the period of 72 hours, with 13.26 g L^{-1} and 10.50 g L^{-1}, respectively. The UFT-Ce06 isolate, in contrast, had greater growth in 96 hours, with 11.17 g L^{-1} of dry biomass (Table 6).

Table 5. Average pH variation in YPD broth cultivation of *Cunninghamella* spp. isolates and the reference strain *C. elegans* CBMAI 0843 for 120 hours. [1]

Strains	Ph				120 hours
	24 hours	48 hours	72 hours	96 hours	
CBMAI 0843	4.01 a	4.72 a	4.18 d	5.01 b	5.12 a
UFT Ce01	3.90 b	4.83 a	4.70 c	5.51 a	5.48 a
UFT Ce02	3.93 b	4.94 a	4.89 b	5.67 a	5.78 a
UFT Ce03	4.01 a	4.97 a	4.90 b	5.97 a	5.73 a
UFT Ce04	4.00 a	4.86 a	4.33 d	4.64 b	5.46 a
UFT Ce05	3.98 b	4.75 a	4.15 d	5.38 a	5.42 a
UFT Ce06	4.08 a	4.90 a	4.73 c	5.88 a	5.92 a
UFT Ce07	3.84 c	4.78 a	4.34 d	4.84 b	5.13 a
UFT Ce08	3.92 b	4.93 a	4.59 c	5.56 a	5.40 a
UFT Ce09	3.94 b	4.68 a	4.40 d	5.12 b	6.03 a
UFT Ce10	3.93 b	4.76 a	4.40 d	5.59 a	5.84 a
UFT Ce11	3.73 d	4.84 a	4.60 c	5.59 a	5.43 a
UFT Ce12	3.76 d	4.90 a	4.39 d	5.24 b	5.14 a
UFT Ce13	3.92 b	4.89 a	5.11 a	5.71 a	5.59 a
UFT Ce14	4.13 a	5.06 a	4.37 d	4.81 b	5.10 a
UFT Ce15	3.77 d	4.86 a	4.64 c	5.29 b	5.54 a
UFT Ce16	3.69 d	4.88 a	4.69 c	5.20 b	5.67 a
UFT Ce17	3.94 b	4.80 a	4.40 d	5.22 b	5.50 a
UFT Ce18	3.96 b	5.14 a	4.71 c	5.83 a	5.56 a
UFT Ce19	3.86 c	4.82 a	4.83 b	5.48 a	5.60 a
UFT Ce20	3.88 b	5.00 a	4.52 d	5.07 b	5.17 a
CV (%)[2]	1.35	2.30	2.35	4.79	9.45

[1]Means followed by the same lower-case letter in the columns do not differ by the Scott-Knott test at 5% significance. 2 CV = Coefficient of variation.

Figure 2. Aspect of the mycelial mass of *Cunninghamella* sp. in liquid medium forming multiple spheres (A) and aspect of crushed dry biomass (B).

Table 6. Average dry biomass of *Cunninghamella* spp. isolates and the reference strain *C. elegans* CBMAI 0843 every 24 hours of cultivation in YPD medium. [1]

Strains	Dry Biomass (g L^{-1})				120 hours
	24 hours	48 hours	72 hours	96 hours	
CBMAI 0843	7.29 a	10.78 c	9.80 a	9.07 c	7.62 c
UFT Ce02	8.52 a	9.42 d	9.35 a	8.09 c	4.69 d
UFT Ce04	5.07 a	12.20 b	13.26 a	11.02 b	10.87 a
UFT Ce05	8.39 a	9.15 d	8.69 a	8.07 c	5.68 d
UFT Ce06	7.52 a	9.36 d	10.81 a	11.17 b	8.41 b
UFT Ce08	8.52 a	20.17 a	12.24 a	13.15 a	8.99 b
UFT Ce09	8.95 a	9.34 d	8.50 a	6.98 c	7.07 c
UFT Ce11	9.73 a	10.72 c	8.40 a	9.31 c	4.88 d
UFT Ce14	7.13 a	10.36 c	10.50 a	8.03 c	7.61 c
UFT Ce20	8.33 a	12.64 b	11.83 a	10.30 b	8.65 b
CV (%)[2]	8.47	4.83	11.06	6.17	6.13

[1]Medias followed by the same lower-case letter in the columns, do not differ by the Scott-Knott test at 5% significance. [2]CV = Coefficient of variation.

The fungal cell wall comprises about 20-30% of the dry weight of the cell, its chemical composition, structure, and size vary considerably depending on environmental conditions and/or laboratory cultivation, a formation that is coordinated with the cell cycle (Klis, Boorsma, & Groot, 2006).

Stamford et al. (2007) also observed exponential growth in 48 hours and a decline in growth after this period, obtaining 20.4 g L^{-1} of biomass when cultivating *C. elegans* (UCP 542) in Jacatupé broth, a result close that is to the value found herein.

Franco et al. (2004) reported an exponential growth throughout the experimental period when culturing *C. elegans* in synthetic medium for mucorales, obtaining 11.6 g L^{-1} and 122.9 mg L^{-1} of dry biomass, respectively. Amorim et al. (2006) cultivated *Cunninghamella bertholletiae* (IFM 46.114) in YPD medium and found continuous mycelial growth in 120 hours; however, the dry biomass was only 9 g L^{-1}, a lower biomass amount than the one observed in another study by Amorim et al. (2001), with *C. elegans* (URM 46109) cultivation in YPD medium, reaching a higher value of dry biomass, 25 g L^{-1}.

Andrade, Barros Neto, Souza, and Campos-Takaki (2000) and Franco et al. (2005) also reported lower biomass values of *C. elegans* (IFM 46109) in a medium enriched with glucose, asparagine, and thiamine, corresponding to 11 g L^{-1} and 11.6 g L^{-1}, respectively. Santos et al. (2013) also obtained lower biomass values, 16.95 g L^{-1}, compared to this study, for *C. elegans* (UCP / WFCC 0542) culture in a medium composed of millhocin.

As for the extraction and yield of chitin and chitosan, the UFT-Ce11 isolate presented the highest chitin amount, 30.33%, and the second-best yield of chitosan, 5.91%. The UFT-Ce09 isolate showed the highest chitosan amount, 7.43%, followed by the UFT-Ce20 isolate, with 5.55%, UFT-Ce08, with 4.66%, and UFT-Ce14, with 4.26%. The chitin yields ranged from 15.64 to 30.33% and the chitosan yields ranged between 0.94 and 7.43% (Table 7).

Table 7. Average yield of chitin and chitosan by isolated strains of *Cunninghamella* spp. fungus and the reference strain *C. elegans* CBMAI 0843 grown on YPD medium.

Strains	Chitin Yield (%)	Chitosan Yield (%)
CBMAI 0843	24.29	1.89
UFT Ce02	21.42	2.69
UFT Ce04	18.54	1.66
UFT Ce05	22.90	2.43
UFT Ce06	15.64	0.94
UFT Ce08	30.28	4.66
UFT Ce09	16.02	7.43
UFT Ce11	30.33	5.91
UFT Ce14	16.69	4.26
UFT Ce20	22.38	5.55

The UFT-Ce06 isolate had the lowest yield of chitin and chitosan, 15.64% and 0.94%, respectively. The CBMAI 0843 reference strain had the third best chitin yield, 24.29%, but the third worst chitosan yield, 1.89%, that is, 7 strains isolated in this study showed higher chitosan yield than the reference strain. The UFT-Ce09 isolate showed higher chitosan yield, but less mycelial growth. Fukuda et al. (2009) described that during a fermentative process, the amounts of mycelial mass and polysaccharides are not necessarily proportional, being dependent on the different factors used in the culture.

Amorim et al. (2001) also found not proportional the biomass to chitosan ratio for *C. elegans*, since even reaching high biomass values, 25 g L^{-1}, the chitosan extraction yield was only 2.0%. Franco et al. (2004) found a chitin yield of 23.8% and chitosan of 7.8%. Franco et al. (2005) obtained 28 and 7.8% of chitin and chitosan, respectively. Stamford et al. (2007) reported high chitin yield, 40.09%, however, the chitosan yield remained within the average, 5.89%. Santos et al. (2013) reported an above-average yield with 2.14 g L^{-1} of chitosan, approximately 13.8%.

Amorim et al. (2006) obtained a low chitosan yield, 1.70% when culturing *Cunninghamella bertholletiae* in YPD medium. They reported that the maximum chitosan production occurred in the first three days of growth, declining after this period. Amorim et al. (2001) and Stamford et al. (2007) also reported greater chitosan production in the first days of mycelial growth. They mention that higher chitosan yields at early stages suggest that chitin deacetylase, an enzyme that converts chitin to chitosan, prevailed at these stages; in addition, the less crystalline nature of chitin at early stages makes it more susceptible to this enzyme.

Finally, the results presented by the respective study suggest that *Cunninghamella* sp. is a promising alternative source for chitin and chitosan extraction, stimulating the biotechnology industry based on advanced fermentation methods. Optimizing the production of chitin and chitosan in different media is an alternative to implement the production by these isolates, since they showed good yields in commercial YPD medium.

Conclusion

The isolated strains showed good mycelial growth, with emphasis to the isolate UFT-Ce08, which presented the highest value of dry biomass.

The yields of chitin and chitosan showed values close to those found in the literature, with the highest amount of chitin and chitosan for the isolates UFT-Ce11 and UFT-Ce09, respectively.

Therefore, chitosan production from this fungus should be further optimized to improve yield for a large-scale production.

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