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Green synthesis of chitosan by *Cunninghamella elegans* UCP 1306 using sustainable substrates mediated morphological changes

Síntese verde de quitosana por *Cunninghamella elegans* UCP 1306 usando substratos sustentáveis mediados por mudanças morfológicas

Síntesis verde de quitosano por *Cunninghamella elegans* UCP 1306 utilizando sustratos sostenibles mediados por cambios morfológicos

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Abstract
Currently, the commercial production of chitosan takes place through the thermochemical deacetylation of crustacean shells, which process requires the use of chemical substances, such as strong alkaline solutions, which are important sources of environment pollution. We present a method for the production of chitosan by *Cunninghamella elegans* UCP 1306 using a green metabolic conversion of agro-industrial residues, based on a 2^2 Factorial Design and describe the main morphological changes observed in the specie. The highest yield of biomass (6.375 g/L) and chitosan (101.7 mg/g) were observed in the assay 2 (Corn steep liquor -CSL 4%, Cassava wastewater-CWW 4%), where the specie presented loose and branched hyphae with the presence of septations, pellets of 0.4 to 0.5 mm and clumps of up to 0.1 mm. Statistical analysis showed that higher concentration of CSL contributed significantly to the growth of the species. The bands determined in Fourier transform infrared spectroscopy confirmed the degree of deacetylation of 84.61% of the chitosan obtained. This research showed that the use of CSL and CWW in association was very promising, being able to be used as sustainable sources of nitrogen and carbon in the production of fungal chitosan with a high degree of deacetylation and can be applied to the industrial process.
Keywords: Agroindustrial waste; Biopolymers; Fungal Chitosan; *Cunninghamella elegans*.

Resumo
Atualmente, a produção comercial de quitosana se dá pelo processo de desacetilação termoquímica a partir de carapaças de crustáceos, cujo processo requer o uso de substâncias químicas, como soluções alcalinas fortes, que constituem importantes fontes de poluição para o meio ambiente. O presente estudo apresenta um método para a produção de quitosana pela espécie *Cunninghamella elegans* (UCP 1306) usando uma conversão metabólica verde com resíduos agroindustrais, baseado em um Planejamento Fatorial 2^2 e descrevemos as principais alterações morfológicas observadas na espécie. O maior rendimento de biomassa (6,375 g / L) e quitosana (101,7 mg/g) foi observado no ensaio 2 (licor de maceração de milho-CSL 4% e água residual de mandioca- CWW 4%), onde a espécie apresentou predominância de hifas frouxas e ramificadas com presença de septações, pellets de 0,4 a 0,5 mm e clumps de até 0,1 mm. A análise estatística mostrou que a maior concentração de CSL contribuiu significativamente para o crescimento da espécie. As bandas determinadas na espectroscopia no infravermelho com transformada de Fourier confirmaram o grau de desacetilação de 84,61% da quitosana obtida. Esta pesquisa mostrou que a associação de CSL e CWW foi bastante promissora, podendo ser utilizadas como fontes sustentáveis de carbono e nitrogênio na produção de quitosana fúngica com alto grau de desacetilação e pode ser aplicada ao processo industrial.
Palavras-chave: Resíduos agroindustriais; Biopolímeros; Quitosana fúngica; *Cunninghamella elegans*.

Resumen

Actualmente, la producción comercial de quitosano se realiza a través del proceso de desacetilación termoquímica de caparazones de crustáceos, cuyo proceso requiere el uso de sustancias químicas, como soluciones alcalinas fuertes, las cuales son fuentes importantes de contaminación para el medio ambiente. El presente estudio presenta un método para la producción de quitosano por la especie *Cunninghamella elegans* (UCP 1306) utilizando una conversión metabólica verde con residuos agroindustriales, basado en un Diseño Factorial 2^2 y se describen los principales cambios morfológicos observados en la especie. El mayor rendimiento de biomasa (6,375 g/L) y quitosano (101,7 mg/g) se observó en el ensayo 2 (licor de maceración de maíz- CSL 4% y agua residual de yuca- CWW 4%), donde las especies presentaron predominio de hojas sueltas y ramificadas. hifas con presencia de tabicaciones, gránulos de 0,4 a 0,5 mm y macizos de hasta 0,1 mm. El análisis estadístico mostró que la concentración más alta de CSL contribuyó significativamente al crecimiento de la especie. Las bandas determinadas por espectroscopia infrarroja transformada de Fourier confirmaron el grado de desacetilación del 84,61% del quitosano obtenida. Esta investigación mostró que la asociación de CSL y CWW fue muy prometedora, pudiéndose utilizar como fuentes sostenibles de carbono y nitrógeno en la producción de quitosano fúngico con un alto grado de desacetilación y puede aplicarse al proceso industrial.

Palabras clave: Residuos agroindustriales; Biopolímeros; Quitosano fúngico; *Cunninghamella elegans*.

1. Introduction

Fungi biology has shown important advances in recent decades, allowing the extraction of molecules of biotechnological interest, such as chitosan, one of the most versatile and abundant biopolymers in nature (Souza et al., 2011). In addition to its applications in the medical and pharmaceutical fields, chitosan has been used in the environmental area and agricultural sectors, having as its main use the bioremediation of water and soil contaminated by heavy metals (Abdel-Gawad et al., 2017; Berger et al., 2020). In nature, chitosan is found in the formation of the exoskeleton of insects and crustaceans as well as in the cell wall of fungi belonging to the order Mucorales (Ghormade et al., 2017).

Currently chitosan is produced from the shell of crabs and shrimp discarded by the fishing industry (Hamed et al., 2016). Despite being economical and relatively sustainable, the production of chitosan from crustacean shell residues is not ecologically correct, since it produces large amounts of environmentally harmful effluents (Berger et al., 2018; Ghormade et al., 2017). About 6.3 kg of HCl and 1.8 kg of NaOH are needed to produce one kilogram of 70% deacetylated chitosan, in addition to nitrogen and large volumes of water for processing and cooling (Sebastian et al., 2020).

The production of chitosan from fungal sources has offered numerous advantages, as in addition to providing greater flexibility for the production of chitosan with consistent quality, it also requires less toxic ingredients, also allowing the use of different fungal strains and changes in culture conditions (Berger et al., 2020). However, the cost of producing fungal chitosan has been one of the main obstacles to be overcome, with the raw materials used in preparing the environment the main determining factor (Abdel-Gawad et al., 2017; Sebastian et al., 2020). Thus, it is imperative that the medium used is economical and its composition simple in order to improve viability, as well as to avoid problems during the further processing of fungal biomass to obtain chitosan (Batista et al., 2020).

In this sense, the present study proposes the use of a low cost and ecologically correct medium, using cassava wastewater and corn steep liquor as possible sources of carbon and nitrogen in the production of chitosan by *Cunninghamella elegans* UCP 1306, as well as its effect on the morphology of the species.

2. Methodology

2.1 Microorganism

In this study, the specie *Cunninghamella elegans* UCP 1306 from the Banco de Culturas UCP (Catholic University of Pernambuco), registered in the World Federation for Culture Collection -WFCC, was used, being kept in BDA (Potato dextrose agar) at 5 ºC until use.

2.2 Agro-industrial waste

The cassava wastewater (CWW), carbon source, came from the cassava press for the manufacture of flour from the indigenous village Pankará, in Carnaubeira da Penha, Pernambuco. The corn steep liquor (CSL), residue from the corn processing industry, kindly provided by Igredion Industries Ltd, municipality of Cabo de Santo Agostinho - Pernambuco, used as a source of nitrogen.

2.3 Cultivation conditions and biomass production

The pre-inoculum was prepared from *C. elegans* (UCP 1306) grown in BDA medium for 5 days. The spores were collected using swabs and transferred to sterile distilled water and then the number of spores was determined in a Neubauer chamber. 10 mL aliquots of spore suspension containing 10^7 spores / mL were transferred to 250 mL Erlenmeyer flasks containing 100 mL of the production media used, according to factorial planning 2^2. After inoculation, the flasks were kept under 150 orbital shaking rpm for a period of 96h. The mycelium was washed twice by filtration in distilled water on nylon membrane screen printing (120 F), and then lyophilized. Subsequently, the biomass was kept in a vacuum desiccator until constant weight (Berger et al. 2014). The biomass obtained was submitted to chitosan extraction. The pH of the collected samples was determined by potentiometry from the cell-free medium.

2.4 Morphological analysis

Biomass grown under different cultivation conditions were observed with the naked eye in petri dishes with the aid of a magnifying glass for macroscopic analysis, taking into account the appearance of the mycelium, its shape and size (in cases of formation of pellets or clumps). For the observation of microscopic morphological changes, slides were made from cultures grown under the conditions described above, which were stained with Amann blue (lactophenol) for direct observation under a light microscope, as to the hyphae branching pattern, its shape and the presence of hyphae structures. stress or resistance.

2.5 Chitosan extraction

The biomasses obtained from the factorial design 2^2 were weighed and subjected to the chitin and chitosan extraction process proposed by Hu et al. (1999). The steps of this process were the deproteinization of the lyophilized biomass with 1M NaOH in the proportion of 1:40 (w / v), followed by autoclaving (121 ºC, 15 minutes), centrifugation (4000 rpm, 15 minutes). The supernatant was discarded and the precipitate subjected to acid hydrolysis using 2% acetic acid (v / v), autoclaving (100 ºC, 10 minutes) and centrifugation (4000 rpm, 15 minutes). The precipitate corresponds to chitin, which was washed with cold distilled water until the pH was close to neutrality, and with the supernatant, the pH was corrected to 10-12, with the precipitation of the chitosan. Then, successive washings were carried out with cold distilled water until its pH was close to neutrality and, finally, it was dried at room temperature.

2.6 Infrared spectroscopy and degree of acetylation

The obtained chitosan was analyzed using an FT-IR infrared spectrometer, with Fourier transform (FT-IR), recorded in a Bruker IFS 66 device, using KBr tablets, the wave numbers being expressed in cm-1 in the region of 4000 at 400 cm -1. The degree of acetylation of the microbial chitosan was determined by infrared spectroscopy according to Baxter et al. (1992), using the A1655 / A3450 absorbance ratio. Two milligrams of the chitin and chitosan sample by fungi, were be dried during 60 °C under reduced pressure, and completely mixed with 100mg of KBr, to produce 0.5mm thick discs. The discs were be dried for 24 hours at 110 °C under reduced pressure. Infrared spectrometer was recorded with a Bruker 66 spectrometer, using a 100 mg KBr disk, for reference. The intensity of the maximum absorption bands was determined by the reference method.

2.7 Factorial planning

A complete factorial planning 2^2 was designed to assess the main effects and interactions of the independent variables (CSL and CWW), on the yield of biomass and chitosan, such as variable responses. Each independent variable was investigated at three levels, minimum (-1), central (0) and maximum (+1), according to Table 1. A total of 8 experimental assays were performed and the data obtained were analyzed using the Statistica® software, version 10.0 (StatSoft Inc., USA), testing the significance of the results ($p < 0.05$).

Table 1. Variables and levels evaluated in the full factorial design 2^2 to produce biomass, chitin, and chitosan by *Cunninghamella elegans* UCP 1306.

Variables	Levels		
	-1	0	+1
Corn Steep Liquor CSL (%, v/v)	0	2	4
Cassava waste water CWW (%, v/v)	4	5	6

Source: Authors (2021).

3. Results and Discussion

3.1 Effect of substrates on the production of biomass and chitosan by *Cunninghamella elegans UCP 1306*

Table 2 presents a comparison of the results obtained for the production of biomass and chitosan, obtained in each test of the 2^2 factorial design. A higher biomass yield (6.375 g/L) was achieved in the medium composed of CSL 4% and CWW 4%, in which *C. elegans* (UCP 13060) presented a predominance of loose and branched hyphae with the presence of septa and formed larger pellets than those observed in the other assays, with a diameter of 0.4 to 0.5 mm and clumps up to 0.1 mm.

Table 2. Effect of substrates on the biomass and chitosan production by *C. elegans* UCP 1306.

Assay	CSL (%)	CWW (%)	pH	Biomass (g/L)	Chitosan (mg/g)
1	0	4	7.0	0.671	73.02
2	4	4	7.5	**6.375**	**101.7**
3	0	6	7.5	0.954	75.47
4	4	6	8.0	5.774	80.36
5	2	5	8.5	3.611	86.67
6	2	5	8.6	4.023	65.37
7	2	5	8.6	3.473	82.64
8	2	5	8.6	2.959	80.09

[CSL: +1=4; 0=2 and -1=0] [CWW: +1=6; 0=5 and -1=4]. Source: Authors (2021).

A comparison of the results of this study was performed with the best results of other studies (Table 3) to biomass and chitosan produce by species of *C. elegans*. The data presented assume that the culture medium and the cultivation conditions influence the content of chitosan produced by fungi.

Table 3. Comparative analysis of obtaining biomass and chitosan produced by *C. elegans* UCP 1306 cultivated in different media with the results described in the literature in other species of *C. elegans*.

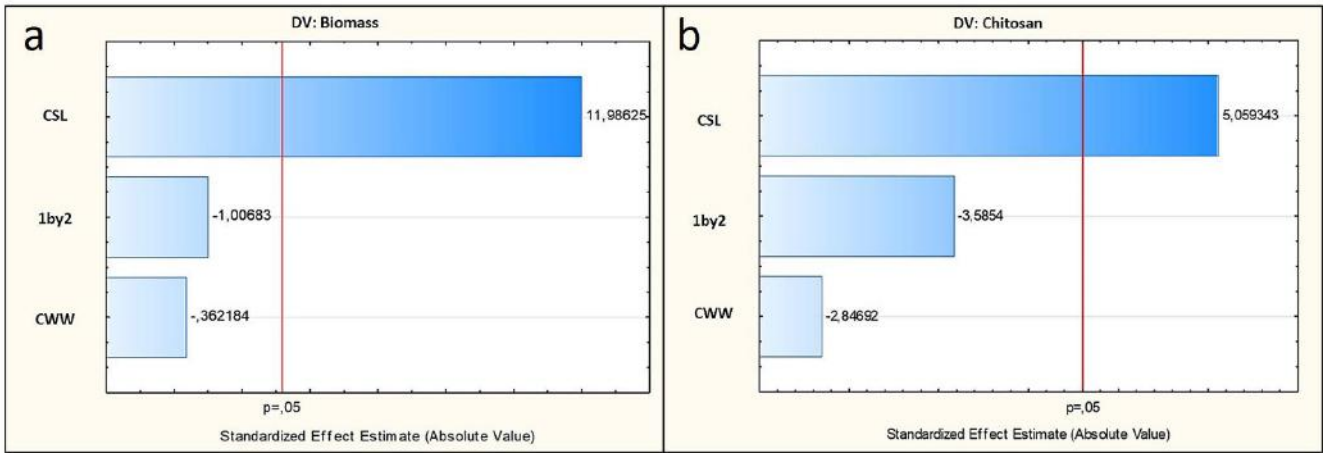
Fungi Strain	Medium Composition	Cultural Conditions	Biomass (g/L)	Chitosan (mg/g)	References
C. elegans (UCP 1306)	4% CSL, 4% CWW	SmF, 25 ºC, 150 rpm, 96 h	6.375	101.7	Present study
C. elegans (UCP 542)	Yam bean	SmF, 28 ºC, 150 rpm, 96 h	24.30	66.00	Stamford et al., 2007
C. elegans (UCP 0542)	10% CSL, 4% CWW	SmF, 28 ºC, 150 rpm, 72 h	5.67	57.82	Berger et al., 2014a
C. elegans (UCP 0542)	5% CSL and 2,5% molasses	SmF, 28 ºC, 150 rpm, 96 h	13.58	33.13	Berger et al., 2014b
C. elegans (SIS 41)	9,43% CSL, 42,5% papaya peel	SmF, 28 ºC, 150 rpm, 96 h	-	37.25	Berger et al., 2018
C. elegans (URM 46109)	Meio YPD	SmF, 28 ºC, 100 rpm, 96 h	25.00	20.50	Amorim et al., 2001

- Data not shown; SmF- submerged fermentation. Source: Authors (2021).

The analysis of variance showed a high coefficient of determination (R2 = 0.92136) with an adjustment of 0.91611, showing that the mathematical model described is in accordance with the experimental data. Figure 1 shows the influence and interaction (1 × 2) of the independent variables, corn steep liquor - CSL (1) and residual cassava - CWW (2) on the biomass production by *C. elegans* (UCP 1306) within the planning factorial, statistically significant of $p < 0.05$. ANOVA also indicated that the CSL was statistically significant on the biomass yield ($p = 0.00124$), for the experimental data obtained, when analyzed at the 95% confidence level. This was also confirmed by the Pareto diagram, where the point at which estimated effects showed statistical significance ($p = 0.05$), indicated by the dashed red vertical line. In the Pareto graph, it is possible to observe that the positive influence of the increase in the concentration of CSL (1) on the growth of the microorganism, as well as the interaction of the independent variables (1 × 2) which showed that the highest concentration of CSL (1) and the lowest concentration of CWW (2) showed an antagonistic interaction, with an important influence on the production of biomass by the microorganism.

As well as the statistical analysis of biomass production, the analysis of variance (ANOVA) for chitosan production demonstrated the high determination coefficient (R2 = 0.95806), demonstrating that the mathematical model used explains the obtained chitosan values. The model fit was 0.91611, showing that the mathematical model described is in agreement with the experimental data. The Pareto diagram (Figure 1) also indicated that only the variable CSL (1) was statistically significant on chitosan yield, when analyzed at the 95% confidence level. The Pareto diagram also shows that higher levels of the CSL variable (1) have a positive influence on chitosan production. However, CWW at higher levels negatively influences the production of the polysaccharide.

Figure 1. Pareto diagram obtained from the complete factorial design 2^2 to determine the influence of the independent variables: CSL (1) and CWW (2) on the biomass yield and chitosan, as dependent variables.

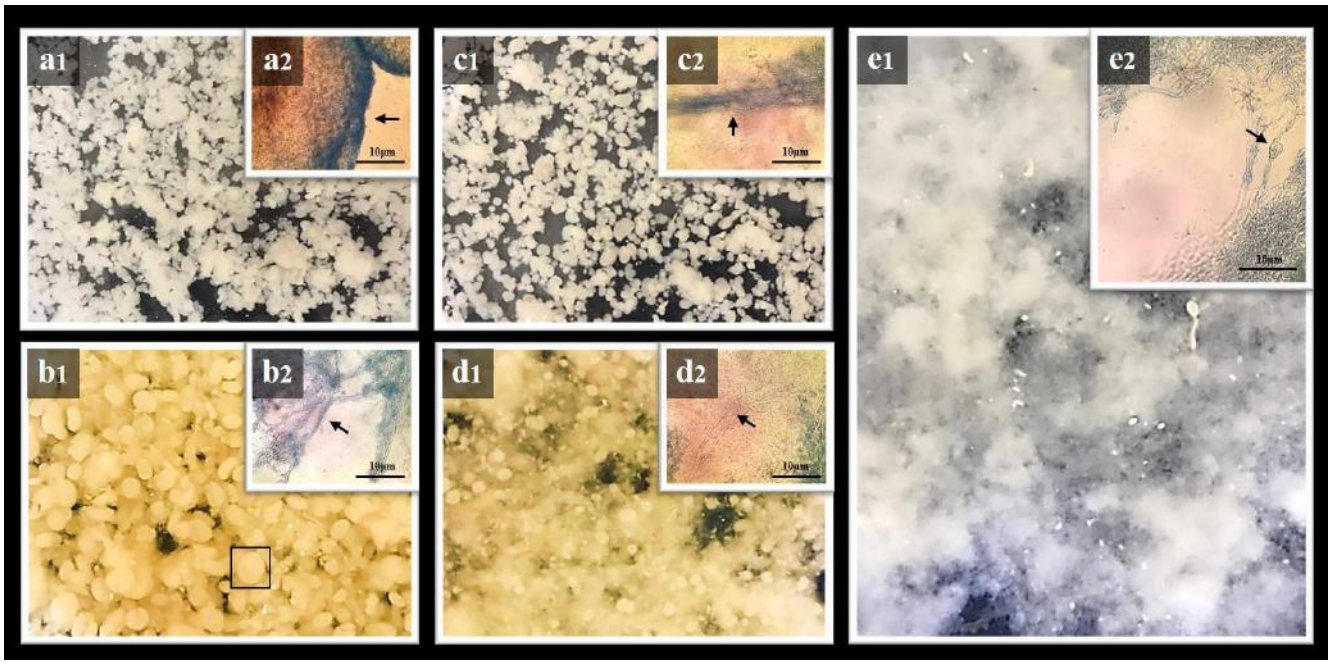


Source: Authors (2021).

3.2 Effect of substrate concentrations on fungal morphology

The effect of CSL and CWW concentrations used in a 2^2 factorial design on the morphological characteristics of the species was evaluated. Macroscopic images showed cell differentiation in *C. elegans* (UCP 1306), which exhibited three morphologies, as shown in Figure 2: dispersed mycelium, clumps and pellets with different sizes and shapes (Table 4), which differed according to the concentrations of agro-industrial residues used.

Figure 2. Morphological aspects of *Cunninghamella elegans* UCP 1306 observed in: assay 1 - (a1) macroscopic view, (a2) microscopic view; assay 2- (b1) macroscopic view, (b2) microscopic view; assay 3- (c1) macroscopic view, (c2) microscopic view; assay 4 - (d1) macroscopic view, (d2) microscopic view; central point- (e1) macroscopic view, (e2) microscopic view. Amann blue (lactophenol), 40X.



Source: Authors (2021).

Table 4. Relationship of fungal morphology in obtaining biomass and chitosan.

Assay	Macroscopic changes	Size of pellets Mean (SD)	Microscopic changes	Biomass (g/L)	Chitosan (mg/g)
1	Hollow smooth pellets	0.24 (0.03)	Cell lysis; compact hyphae without cell differentiation	0.671	73.02
2	Loose pellets	0.45 (0.03)	Branched loose hyphae, with presence of septations	**6.375**	**101.7**
3	Hollow smooth pellets	0.24 (0.03)	Compact hyphae with loss of individuality	0.954	75.47
4	Dispersed mycelium with granular growth	0.16 (0.02)	Broad hyphae with terminal chlamydospores	5.774	80.36
Central Point	Dispersed mycelium with granular growth	-	Loose hyphae, loosely compacted; presence of chlamydospores	3.611	86.67

SD- standard deviation. Source: Authors (2021).

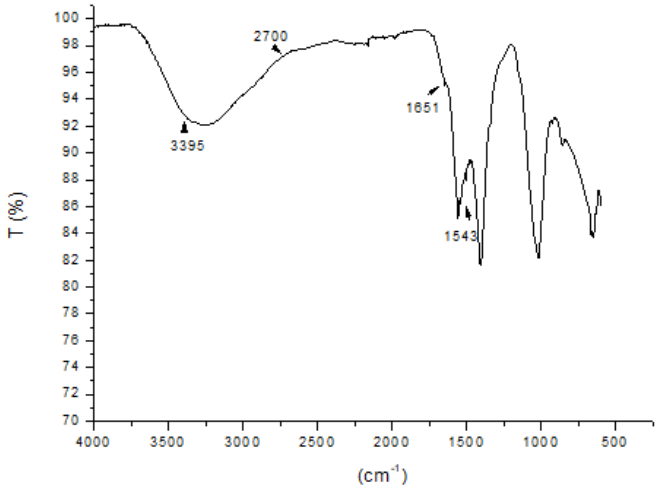
Hollow smooth pellets and clumps with diameters ranging from 0.1 mm to 0.2 mm were predominant in assays whose media had only CWW in their composition (Fig. 2 a1 and Fig. 2 c1). Assay 2 (Fig. 2 b1) presented loose pellets ranging from 0.4 to 0.5 mm and clumps of up to 0.1 mm. In assays 4 (Fig. 2 d1) and central point (Fig. 2 e1), the species presented dispersed mycelium with granular growth and, specifically in assay 4 (assay with the highest concentrations of the two residues used) clumps up to 0.2 mm were also observed.

Microscopically, important differences were observed in relation to the composition of the proposed culture medium. In assays that had only CWW in their composition, hyphae were extremely compacted and without cell differentiation (Fig. 2 a2 and Fig. 2 c2). Loose and branched hyphae with the presence of septations were observed in assay 2 (Fig. 2 b2), while in assay 4 (Fig. 2 d2) and also in the central point (Fig. 2 e2), broad hyphae with the presence of chlamydospores were observed.

3.3 Infrared spectroscopy (degree of deacetylation - DD%)

The properties of chitosan depend mainly on its apparent molar mass and the degree of deacetylation. The degree of deacetylation is a parameter that expresses the elimination of acetyl groups from N-acetylglucosamine units, generating D-glucosamine units in the polymer chain and has an important influence on the physical, chemical and biological properties of the chitosan obtained (Campana-Filho et al., 2007). In this study, chitosan obtained from the fungal biomass of *C. elegans* (UCP 1306) was characterized by FTIR spectroscopy and presented a degree of deacetylation of 84.61%, which is considered an excellent result (Figure 3). The bands amide I and chitosan amine extracted from *C. elegans* (UCP 1306) were found at 1651-1543 cm $^{-1}$. The values found are in agreement with the literature (Berger et al., 2014; Amorim et al., 2001; Amorim et al., 2003). The intensity of the bands at 1543 cm -1 (*C. elegans* UCP 1306) suggests a stable deacetylation in chitosan. When chitin is deacetylated, the amide I band (C = O-NHR) decreases while amide II growth occurs, which indicates the prevalence of NH groups. According to Zvezdova (2010), when the range of 1500-1700 cm^{-1} is emphasized, suggesting an intensification of deacetylation.

Figure 3. Infrared absorption spectrum of chitosan produced by *C. elegans* UCP 1306.



Source: Authors (2021).

Current advances in fermentation technology provide an alternative means of ecologically producing chitosan and its derived products. To ensure the standardization of the process, all parameters must be analyzed, including the possible morphological changes presented by the species during the fermentation process (Akila 2014; Tan 1996).

In submerged fermentation, filamentous fungi can grow in dispersed form or as spherical pellets, which consist of aggregated hyphal structures where pellet morphology, process control and productivity are intrinsically interlinked (Veiter et al., 2018). The mycelial pellet has characteristics of high biological activity, in addition to the ability to secrete metabolites and adsorb a large amount of pollutants, it has a fast sedimentation speed and easy solid-liquid separation, and can be an attractive alternative in industrial fermentation processes (Liao et al., 2007).

In this study, the composition of the medium seems to have been one of the factors that most influenced the morphological changes observed in *C. elegans* (UCP 1306) which, in turn, showed specific nutritional requirements to increase the response of a particular variable, as shown in Table II, the highest biomass and chitosan production being obtained in the presence of the highest levels of carbon and nitrogen sources (assay 2). In this same cultivation condition, the species formed pellets larger than those observed in the other assays, suggesting that the size of the pellets positively influences the yield of biomass and chitosan (Table 4). Therefore, it is possible to see that a culture medium containing CSL in higher concentration and CWW and lower concentration, can favor the growth of *C. elegans* (UCP 1306), showing that this is an important nutritional source for the growth of the fungus, since that CSL is a residue rich in carbohydrates and amino acids (Berger et al., 2020). Berger et al. (2014) found similar results, showing that higher concentrations of CSL have a positive influence on biomass production. Probably, the higher nitrogen and carbon contents of CSL compared to CWW favored this result (De Souza et al., 2020).

In assays where the concentration of CWW was higher than that of CSL (assay 4 and central point), it was possible to observe morphological changes such as granular growth, presence of chlamydospores and formation of clumps, which results in difficulty in oxygen transfer and consequent autolysis in the center of the hyphae clusters in these assays. Such morphological changes are expected in media containing higher concentrations of CWW, since it has substances such as acriflavine, cycloheximide and cyanide in its composition, which induce important morphological changes due to the inhibition of the synthesis or action of cytochrome oxidase (De Souza et al., 2020). Also in the assays containing only CWW (assays 1 and 3), the species seems to have suffered greater wear, with loss of cell individuality/differentiation, possibly caused by the low offer of carbon and nitrogen in the composition of these media.

4. Conclusion

In the present study, the species *C. elegans* (UCP 1306) showed great potential in converting agro-industrial residues into biomass and producing chitosan. The use of CSL and CWW residues in association, was very promising, and can reduce the cost of obtaining an effective production of chitosan polymer from Mucoralean species. The higher yields of chitosan in fermentation were a direct result of the higher production of cellular biomass. The diameter ranges of large pellets were related to a higher yield of chitosan by the fungal species. The investigation with *C. elegans* (UCP 1306) showed a sustainable and high quality alternative in the production of chitosan with a good degree of deacetylation. More studies should be carried out in order to investigate the potential application of this production route in industrial processes.

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