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SUSTAINABLE CHITOSAN PRODUCTION BY MUCORALEAN FUNGI USING WASTE POST-FRYING OILS AND CORN STEEP LIQUOR AS SUBSTRATES

Hugo Marques Galindo*[1], Adriana Ferreira de Souza[2], Erik Jonne Vieira de Melo[1], Sarah Signe do Nascimento[1], Neiva Tinti de Oliveira[3], Leonor Alves de Oliveira da Silva[4], Luciana de Oliveira Franco[5] and Norma B. Gusmão[6]

[1]Pos graduate Program of Fungus Biology, Federal University of Pernambuco (UFPE), Recife, PE, Brazil; [2]Postgraduate Program in Biotechnology, Federal Rural University of Pernambuco (UFRPE), Recife, PE, Brazil; [3]Micology Department, Federal University of Pernambuco (UFPE), Recife, PE, Brazil; [4]Department of Cellular and Molecular Biology, Federal University of Paraíba (UFPB), João Pessoa, PB, Brazil; [5]Department of Biology, Federal Rural University of Pernambuco (UFRPE), Recife, PE, Brazil; [6]Antibiotic Department, Federal University of Pernambuco (UFPE), Recife, PE, Brazil

ARTICLE INFO	ABSTRACT
	This study aimed to evaluate three industrial wastes as alternative sources of carbon and nitrogen in the production of chitosan by *Cunninghamella bertholletiae* URM 7658, *Rhizopus arrhizus* URM 7651 and *Mucor subtilissimus* URM 7659. A 2^3 full-factorial design (FFD) was used to determine the effect of wastes (oily emulsion from collectors wash -OECW-, mixture of waste post-frying oils -MWPO- and corn steep liquor -CSL-) in biomass and chitosan production by the Mucoralean fungi. The obtained biomasses were lyophilized and submitted to a chitosan extraction process using alkali-acid treatment. The polymers were characterized for degree of deacetylation using Fourier transform infrared spectroscopy (FT-IR), crystallinity index by X-ray diffraction and viscosity. The results showed that both *C. bertholletiae* URM 7658 and *M. subtilissimus*URM 7659 obtained the highest yields of biomass (25.95 and 8.74 g/L, respectively) and chitosan (69.05 and 41.90 mg/g, respectively) in condition 7 of FFD (1% OECW, 5% MWPO and 5% CSL). However, for *R. arrhizus* URM 7651, the highest biomass production (12.28 g/L) was found in assay 8 (5% OECW, 5% MWPO and 5% CSL) and chitosan (103.54 mg/g) in assay 5 of FFD (1% OECW, 1% MWPO and 5% CSL). The chitosans obtained presented 75-87% deacetylation degree.

INTRODUCTION

Among the biopolymers with higher biotechnological potential, chitosan stands out, being considered a biodegradable, biocompatible and low toxicity polysaccharide produced by natural sources (Dias *et al*., 2013; Souza *et al*., 2015; Gharieb *et al*., 2015;Tyliszczak*et al*., 2020). Chemically, chitosan is in the form of a copolymer formed by 2-amino-2-deoxy-D-glycopyranose (GlcN) and 2-acetamido-2-deoxy-D-glycopyranose (GlcNAc) structural units joined by glycosidic bonds of type β (1 → 4) (Balan; Verestiuc, 2014; Islam *et al*. 2017; Ghormade *et al*., 2017; Zininga *et al*., 2019). Chitosan is produced by partial deacetylation of chitin by physicochemical or natural processes. The deacetylated units in chitosan are present in greater proportion or the distribution

in the polymer chain is such that guarantees dissolution in aqueous acid solutions (Maity; Ray, 2014; Naghdi; Zamani; Karimi, 2014; Lizardi-Mendoza *et al*., 2016; Moreno-Vasquez *et al*., 2017). Thus, chitosan may present with different degrees of deacetylation, depending on the amount of acetyl groups that have been removed from the chitin polymer (Cardoso *et al*., 2012; Latha, Suresh, 2013; Berger *et al*., 2014a). Chitosan has unique properties and can be used in a wide variety of biotechnological applications, such as the removal and recovery of different residues in aquatic environments, biotransformation of pesticides, the adsorption of dyes, amino acids and proteins, such as bleaching juices and production of biodegradable films for packaging and cosmetics manufacturing. Moreover, it presents other biological properties as antimicrobial and healing, antitumor

and antioxidant (Barikani *et al*., 2014; Aljawish *et al*., 2015; Moreno-Vasquez *et al*., 2017; Islam *et al*. 2017; Bui; Park; Lee, 2017). Fungi belonging to the Mucorales order (subphylum Mucoromycotina) are considered viable sources of chitosan production and can present up to 50% of the polymer in the cell wall structure (Van Leeuwen, 2016). Mucorales specimens naturally produce chitosan during cell wall formation through the enzymatic action of chitin deacetylase, which catalyzes chitin deacetylation to form chitosan (Aljawish *et al*., 2015; Batista *et al*., 2018; Berger *et al*., 2018). The use of taxa of this order as a biotechnological tool to obtain chitosan is an interesting approach since the production occurs under controlled conditions, does not require high temperatures, is not affected by seasonal factors, has easy handling and extraction, as well as the uniformity of production and production. Also, high purity and deacetylation, resulting in a simpler and more advantageous process when compared to chitosan extraction from shellfish crustaceans (Barikani *et al*., 2014; Berger *et al*., 2014b; Nidheesh, Kumar, Suresh, 2015; Ghormade *et al*., 2017; Zininga *et al*., 2019). One of the main problems in the production of bioproducts in biotechnological processes is the high production costs. Many wastes generated in urban and agroindustrial activities have proven to be an alternative and economically viable nutritional source in fermentation processes to obtain high added value inputs (Silva *et al*., 2013; Martins *et al*., 2014; Berger *et al*., 2018).

The choice of the most profitable carbon source for the growth of microorganisms is important to make the production process viable. In this context, industrial wastes have been widely used as substrates because they are generally inexpensive, abundant, and rich in organic matter (Berger *et al*., 2014a; Mondala *et al*., 2015; Ravindran *et al*., 2018). Thus, fermentative processes to obtain fungal chitosan enable the reuse of agro-industrial wastes as a carbon and nitrogen source in microbial cultivation in substitution of the considerable cost synthetic culture media, representing great biotechnological potential, considering the significant chitosan content in its cell walls (Cardoso *et al*., 2012, Singh, Saini, 2014; Berger *et al*., 2014b; Berger *et al*., 2018). In this context, this study aimed to evaluate the effect of industrial wastes as substrates on chitosan production by three Mucoralean fungi.

MATERIALS AND METHODS

Materials

All reagents used were of analytical grade. Acetic acid and sodium hydroxide were obtained from Vetec (São Paulo, Brazil). The oily emulsion from collector's wash -OECW-, as well as the mixture of waste post-frying oils -MWPO- were kindly provided by ASA, Indústria e Comércio, located in Afogados, Recife-PE. Corn steep liquor (CSL), a by-product obtained from corn processing, was provided by the Brazilian Corn Products company, located in Cabo de Santo Agostinho-PE.

Micro-organisms and maintenance of cultures: Three fungi of the Mucorales order (*Cunninghamella bertholletiae* URM 7658, *Rhizopus arrhizus* URM 7651 and *Mucor subtilissimus* URM 7659), isolated from Caatinga biome soil (PE, Brazil) were used in this study. These strains were obtained from the Fungal Culture Collection Micoteca URM (Department of

Mycology, Federal University of Pernambuco-UFPE, Recife, Brazil). Fungal strains were kept on Potato Dextrose Agar (PDA, for 1 L water: 4 g potato starch, 20 g dextrose and 15 g agar) at 5°C and transferred to a new medium every four months to maintain viability.

Elemental analysis of industrial wastes: Elemental analysis of the industrial wastes was performed on an elemental analyzer (model EA 1110 from Carlo Erba Instruments) to verify the percentage of C, H, N and S.

Culture conditions and production of biomass: Fungi were cultured in Petri dishes containing PDA medium at 28 °C for 7 days until sporulation. Petri dishes containing PDA were inoculated with 1 mL of spore suspension (10^7 spores/mL) of *C. bertholletiae* URM 7658, *R. arrhizus* URM 7651 and *M. subtilissimus* URM 7659 and incubated in an oven for 18 h at 28°C. Then, 10 discs (1 cm in diameter) with young mycelium from each isolate tested were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of non-sterile medium containing the industrial wastes at concentrations pre-established by the 2^3 full-factorial design (FFD), initial pH 4,5. The flasks were incubated at 28°C in static condition for 96 h. After the fermentation, the biomasses were collected by vacuum filtration, washed with distilled water and then lyophilized.

Full-factorial design: A 2^3 FFD factorial design was carried out to analyze the effects of OECW, MWPO and CSL and the interactions between them on yield of biomass (g/L) and chitosan (mg/g) as response variables. Each independent variable was investigated at three levels, minimum (-1), zero (0) and maximum (+1), according to Table 1. Twelve experimental tests were performed, with four replicates in the central point, and the data obtained were analyzed by Statistica® software, version 8.0 (StatSoft Inc., USA), testing the significance of the results ($p \leq 0.05$).

Extration of Chitosan: Chitosan was extracted from biomasses lyophilized of *C. bertholletiae* URM 7658, *R. arrhizus* URM 7651 and *M. subtilissimus* URM 7659, as described by Hu *et al*. (2004). Briefly, biomasses were deproteinized with the addition of sodium hydroxide solution 1 M (1:30 w/v, 121°C, 15 min). The insoluble alkali fraction was separated by centrifugation (4000 *g*, 20°C, 10 min) and the precipitate was subjected to acid hydrolysis using 2% acetic acid (1:30 w/v, 100°C, 15 min), followed by centrifugation at 4000 *g*, 20 °C, 15 min. The precipitated chitin was considered an acid insoluble material, and the supernatant was separated and alkalized to pH 9, kept overnight at 5 °C and centrifuged (4000 *g*, 20°C, 10 min) for chitosan precipitation. The chitosan was washed in distilled water to pH 7 and lyophilized.

Chitosan Characterization

Infrared vibrational spectroscopy: To determine the structural characteristics of chitosan, the technique of infrared vibrational spectroscopy was performed according to the methodology of Santos *et al*. (2003). The infrared spectrum was obtained by means of a Bruker Model IF66 Fourier transform spectrophotometer in the region comprising the wavelengths from 4000 cm^{-1} to 400 cm^{-1}. Samples of two milligrams of chitosan, overnight dried at 60°C under reduced pressure, were mixed with 100 mg of potassium bromide

(KBr) to produce 0.5 mm thick discs. The discs were dried for 24 h at 110°C under reduced pressure. Disks with only KBR were used as reference. The maximum intensity of the absorption bands was measured by the baseline.
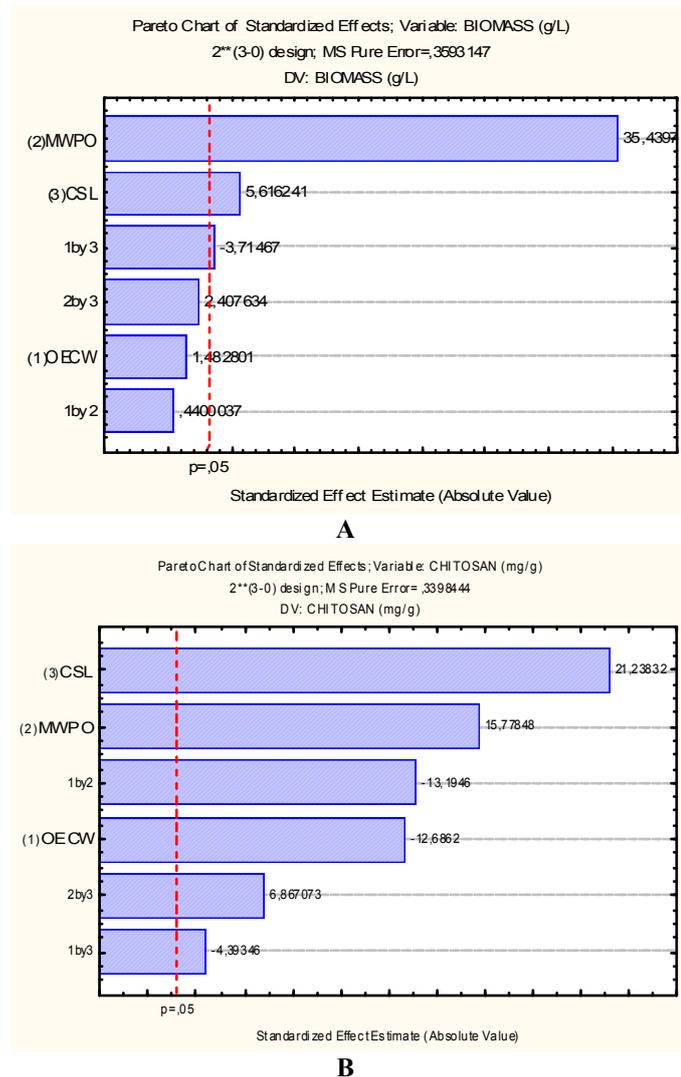

A

B

Fig. 1 Pareto Charts of standardized effects of (1) OECW, (2) MWPO, (3) CSL and the interactions between them on yield of biomass (A) and chitosan (B) by C. *bertholletiae*URM 7658. The point at which the effect estimates were statistically significant (p = 0.05) is indicated by dashed line

Degree of deacetylation (DD%): Degree of deacetylation (DD%) for chitosan was determined using infrared spectroscopy, according to Baxter *et al*. (1992), using the absorbance ratio A1655 / A3450 and calculated according to equation (1):

DD (%) = 100 - [(A1655 / A3450) × 115]   ……(1)

X-ray Diffraction: The crystallinity of the chitosan samples was observed by X-ray diffraction technique, according to the parameters: Cu-Kα radiation being λ= 1.5418A° in a scanning range between 3° and 80° with rate of 0.02° min^{-1}. The crystallinity index (CI) was determined using the following equation (2):

Crystallinity index (%) = 100{[I(θc) − I(θa)] / I(θc)}   (2)

Where I (θc) is the relative intensity of the lens (2θ = 20°) and I (θa) corresponds to amorphous regions (2θ = 12°) for chitosan.

Determination of viscosity: Viscosity was determined in an automatic viscometer (Brookfield - Middleboro, MA, USA; TC 500. Chitosan solution was prepared in 1% acetic acid at a concentration of 1% chitosan. Values were reported in centipoise units (cP).

RESULTS AND DISCUSSION

Elemental Analysis of Industrial Wastes: Table 2 shows the elemental composition (carbon, nitrogen, hydrogen, and sulfur concentrations) of industrial wastes used for formulation of production media. According to the percentages shown, the three residues contributed as carbon sources, however, CSL was the main source of nitrogen.

INFLUENCE OF INDUSTRIAL WASTES ON BIOMASS AND CHITOSAN PRODUCTION BY

Cunninghamella bertholletiae URM 7658, *Rhizopus arrhizus* URM 7651AND *MucorsubtilissimusURM 7659*

Table 3 shows the results obtained in the conditions established by the 2^3 FFD for biomass and chitosan production by the fungal isolates tested. The results showed that *C. bertholletiae* URM 7658 produced higher biomass (25.95 g/L) and chitosan (69.05 mg/g) in assay 7. Higher MWPO concentration (5%) in conditions 3, 4, 7 and 8 as well as higher CSL concentration (5%) in assays 7 and 8, provided high biomass yields. In contrast, higher chitosan yields were obtained in conditions 6, 7 and 8 with 5% CSL. Combinations of MWPO (5%) and CSL (5%) allowed to obtain the highest biomass and chitosan yields, as evidenced in tests 7 and 8. On the other hand, lower concentrations of OECW (1%) were favorable for higher yields of the response variables. Berger *et al*. (2014a) also reported the positive influence of CSL as a nutritional source for growth of *C. elegans* UCP/WFCC 0542. They obtained higher biomass production (9.93 g/L) at the maximum CSL concentration (8%), and higher chitosan production (57.82 mg/g) at the minimum CSL concentration (4%). CSL is an agro-industrial residue rich in carbohydrates, amino acids and vitamins that favors the growth of filamentous fungi and yeasts, suggesting its use as a carbon and nitrogen source in the composition of alternative culture media (Cardoso *et al*., 2012; Sobrinho *et al*., 2013; Berger *et al*., 2014a; Souza *et al*., 2016a). The Pareto diagrams presented in Figure 1 confirm the effect and interactions between the independent variables on *C. bertholletiae*URM 7658 biomass and chitosan production. MWPO (2) and CSL (3) showed a positive and statistically significant effect on biomass production, evidencing that high concentrations favored higher production. The interaction between them (2by3) was positive for biomass production, but there was no statistical significance (Fig. 1A). Chitosan production was positively influenced by the increase in CSL (3) and MWPO (2) concentrations, as well as by the positive and significant interaction between them (2by3) (Fig. 1B). However, OECW (1) and their interactions with MWPO (1by2) and CSL (1by3) had a negative effect on chitosan production, showing that the increase in concentrations was unfavorable to production.
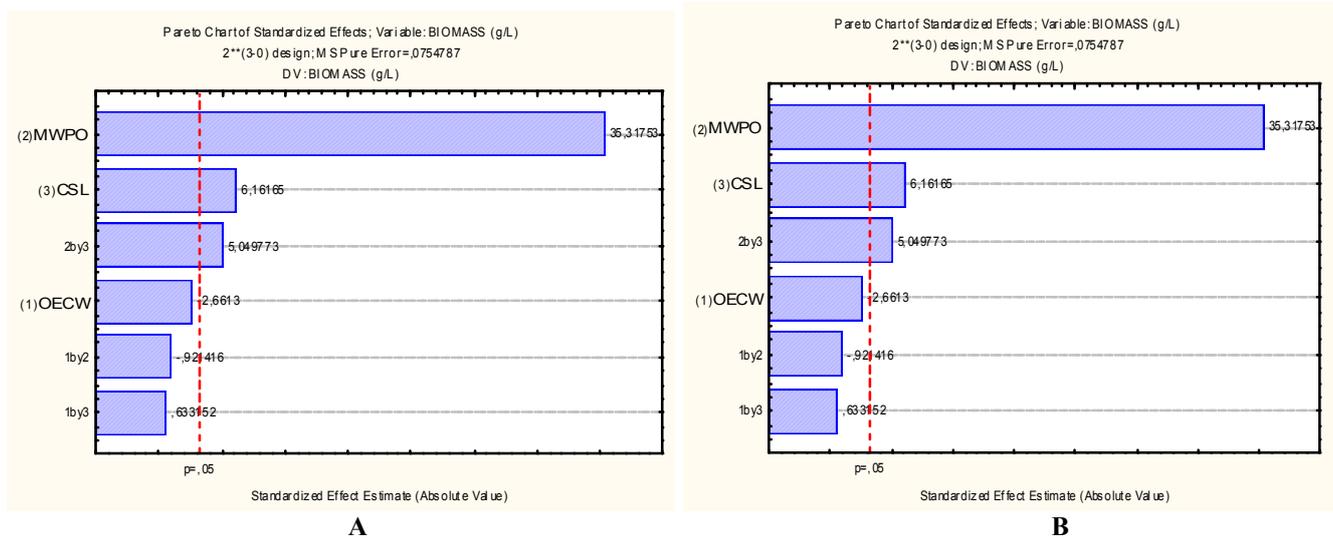
Figure 2. Pareto Charts of standardized effects of (1) OECW, (2) MWPO, (3) CSL and the interactions between them on yield of biomass (A) and chitosan (B) by *R. arrhizus*URM 7651. The point at which the effect estimates were statistically significant (p = 0.05) is indicated by dashed line
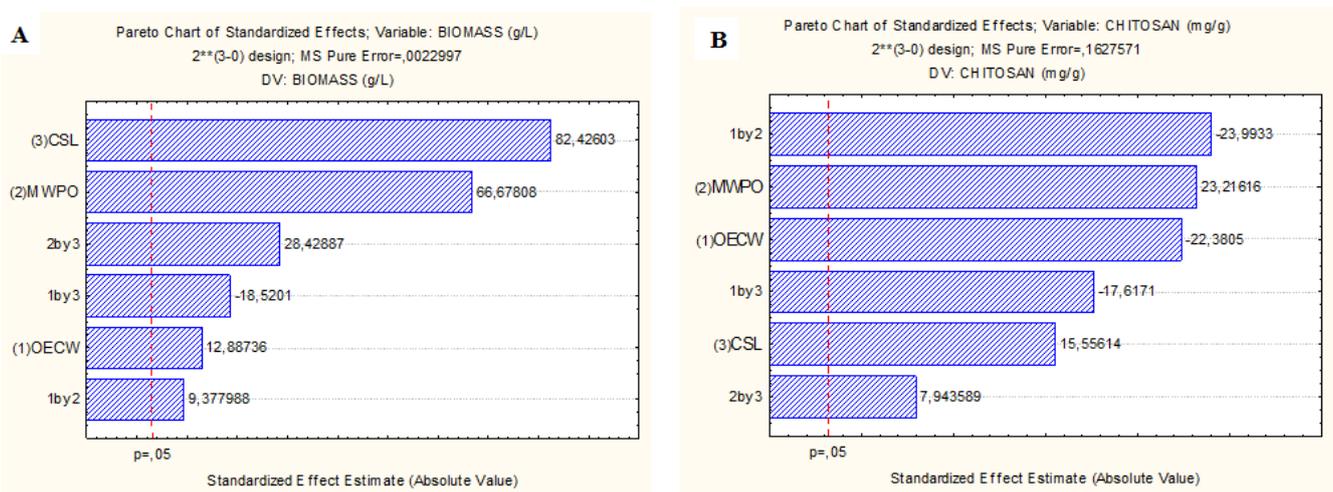

Figure 3. Pareto Charts of standardized effects of (1) OECW, (2) MWPO, (3) CSL and the interactions between them on yield of biomass (A) and chitosan (B) by *Mucor subtilissimus* URM 7659. The point at which the effect estimates were statistically significant (p = 0.05) is indicated by dashed line

Table 1. Variables and levels used in the 2^3 full-factorial design

Variables	Levels		
	-1	0	+1
OECW (%, v/v)	1	3	5
MWPO (%, v/v)	1	3	5
CSL (%, v/v)	1	3	5

Table 2. Elemental composition of industrial wastes used for formulation of media for chitosan production by Mucoralean fungi

Substrate	Carbon	Nitrogen	Hydrogen	Sulfur
OECW	44.87	0.19	8.03	0.71
MWPO	75.96	0.29	11.05	0.93
CSL	34.86	7.06	6.59	1.18

In the case of *R. arrhizus* URM 7651, the results shown in Table 3 showed the highest biomass production (12.28 g/L) in assay 8. Higher MWPO concentration (5%) was favorable to higher biomass yields, as evidenced in conditions 3, 4, 7 and 8. The combination of higher concentrations of MWPO and CSL (5%) obtained the highest biomass yields in assays 7 and 8. For chitosan the highest yield (103.54 mg/g) was obtained in the assay 5, where only CSL was at maximum concentration (5%).

MWPO and CSL combined at 5% concentrations allowed high chitosan yields, as evidenced in conditions 7 and 8. Cardoso *et al.* (2012) obtained higher chitosan production (29.30 mg/g) with lower biomass production (11.71 g/L) in an assay containing 6% of CSL and 13.24% of honey, when cultivated *R. arrhizus* UCP/WFCC 0402 in submerged fermentation. In this study we obtained superior value of chitosan production using similar concentration of CSL (5%) under static condition. According to the Pareto diagrams presented in

Figure 2, biomass production was positively influenced by MWPO (2) and CSL (3), as well as by the interaction between them (2by3), being statistically significant (Fig. 2A). As for chitosan production, MWPO (2) and CSL (3) had a positive and significant effect (Fig. 2B). The interaction between them (2by3) was significant but negative, not favoring production. OECW (1) had negative and significant effect for biomass and chitosan production. Interactions between OECW (1) with the other variables (1by2, 1by3) also had negative and significant effect only for chitosan. Thus, the production of response variables was increased when the concentration of MWPO (2) and CSL (3) were at the highest levels (5%). For *M. subtilissimus* URM 7659, the results in Table 3 show that the highest biomass production (8.74 g/L) and chitosan (41.90 mg/g) was obtained in assay 7. Biomass production was high in conditions 4, 7, 8 and in the central points, where MWPO was in maximum concentration (5%).

concentrations of MWPO and CSL (5%) promoted increase in production. Tajdini *et al.* (2010), when cultivating *Mucor racemosus* in Sabouraud medium by submerged fermentation, obtained maximum chitosan yield of 11.72 mg/g, much lower result of chitosan production by *M. subtilissimus* URM 7659 in assay 7 in this study. Therefore, the adaptation of the studied isolate to the alternative medium for growth and chitosan production was evident. Other studies show that Mucoralean fungi are efficient in chitosan production, and their production is dependent on factors such as culture medium composition, fermentation type, fungal strains, and growing conditions (Berger *et al.*, 2014b; Ghormade, Pathan and Deshpande, 2017). According to Pareto diagrams presented in Figure 3, the increase in concentration of all independent variables (1-3) provided higher biomass production (Figure 3A).

Table 3. Full-factorial design applied to biomass and chitosan production by *Cunninghamella bertholletiae* URM 7658, *Rhizopusarrhizus* URM 7651 and *Mucor. subtilissimus* URM 7659

Assays	Substrates			*C. bertholletiae* URM 7658		*R. arrhizus* URM 7651		*M. subtilissimus* URM 7659	
	OECW (%)	MWPO (%)	CSL (%)	Biomass (g/L)	Chitosan (mg/g)	Biomass (g/L)	Chitosan (mg/g)	Biomass (g/L)	Chitosan (mg/g)
1	1	1	1	7.16	46.57	3.94	32.51	3.374	18.94
2	5	1	1	7	44.86	4.28	39.01	3.934	20.64
3	1	5	1	18.80	51.92	10.79	85.96	4.166	26.33
4	5	5	1	23.01	46.82	9.18	64.50	5.736	21.98
5	1	1	5	7.92	50.53	4.83	103.54	5.646	22.34
6	5	1	5	8.96	52.66	3.82	69.94	5.324	21.58
7	1	5	5	25.95	69.05	12.05	89.56	8.74	41.90
8	5	5	5	23.37	53.71	12.28	79.45	8.68	19.84
9-12*	3	3	3	16.01±0.8	51.79±0.6	8.84±0.5	62.81±0.6	7.39±0.8	23.93±1.0

* Four replicates at center point.

Table 4. Chitosan production by *C. bertholletiae* URM 7658, *R. arrhizus* URM 7651 and *M. subtilissimus* URM 7659 in industrial wastes compared with other studies with Mucoralean fungi using alternative substrates

Microorganisms	Substrates	Fermentation conditions	Chitosan (mg/g)	Degree of deacetylation (%)	References
Cunninghamellabertholletiae	1% OECW, 5% MWPO and 5% CSL	Static fermentation, 28°C, 96 h	69.05	87.00	Present study
Rhizopus arrhizus	1% OECW, 1% MWPO and 5% CSL	Static fermentation, 28°C, 96 h	103.54	78.00	Present study
Mucor subtilissimus	1% OECW, 5% MWPO and 5% CSL	Static fermentation, 28°C, 96 h	41.90	75.00	Present study
Lichtheimiahyalospora (UCP 1266)	6% wastewater and 4% CSL	Submerged fermentation, 28°C, 120 h, 150 rpm	45,03	83.00	De Souza et al., (2020)
Cunnhighamela elegans SIS 41	9,43% CSL and 42,5% papaya peel	Submerged fermentation, 28°C, 96 h, 150 rpm	37,25	86.00	Berger et al., (2018)
Syncephalastrumracemosum (UCP 1302)	8% CSL and 2% sugarcane bagasse	Solid fermentation, 28ºC, 96 h	23.5	80.00	Leite et al., (2015)
*Mucor rouxii (*ATCC 24905)	Soybean meal	Solid fermentation, 25ºC, 144 h	34.4	55.00 – 60.00	Mondala et al., (2015)
Cunninghamella elegans (UCP 542)	7g CSL/ 100 mL	Submerged fermentation, 28ºC, 180 rpm, 72 h,	1.73	81.00	Oliveira et al., (2014)
Cunninghamella elegans (UCP/WFCC 0542)	4% CSL and 10% cassava wastewater	Submerged fermentation, 28ºC, 150 rpm, 72 h	57.82	88.24	Berger et al., (2014a)
Rhizopus arrhizus UCP 1295	Cashewapple (40% (v/v)) andCheese whey (30% (v/v))	Submerged fermentation, 96 h, stirring 150 rpm, 28 °C, 144h	40.88	75.00	Berger et al., (2020)
Syncephalastrumracemosum (UCP/ WFCC 0148)	2% CSL	Submerged fermentation, 25°C, 150 rpm, 120 h	62.44	88.14	Batista et al., (2013)
Rhizopus arrhizus(UCP 402)	6% CSL and 13,24% honey	Submerged fermentation, 28ºC, 150 rpm, 96 h	29.30	86.00	Cardoso et al., (2012)
Mucor circinelloides (UCP 050)	Yam bean (*Pachyrhizuserosus*L. Urban)	Submerged fermentation, 28ºC, 150 rpm, 96 h	64.00	83.00	Fai et al., (2011)

Assays 7 and 8 showed that MWPO and CSL combined at the highest levels also favored biomass production. As for chitosan production, conditions 3, 5 and 7 with concentrations of OECW (1%) were favorable to higher yields, while

The interaction between MWPO and CSL (2by3) had a positive and significant effect on biomass production. The interaction between OECW and CSL (1by3) had a negative effect and was statistically significant. As for chitosan

production, Figure 3B again shows the positive effect of MWPO (2) and CSL (3), and the negative effect of OECW (1), all of which were statistically significant. The increase in the concentration of MWPO (2) and CSL (3), as well as the interaction between them (2by3), and the decrease in the concentration of OECW (1) were favorable for chitosan production.

COMPARATIVE ANALYSIS OF THE INFLUENCE OF CONCENTRATIONS OF INDUSTRIAL WASTES IN BIOMASS AND CHITOSAN PRODUCTION IN *Cunninghamella bertholletiae* URM 7658, *Rhizopusarrhizus* URM 7651AND *Mucorsubtilissimus* URM 7659

The analysis of the influence of different concentrations of OECW, MWPO and CSL was carried out comparatively in this topic. The highest biomass and chitosan yield by *C. bertholletiae*URM 7658 (25.95 g/L; 69.05 mg/g) and *M. subtilissimus* URM 7659 (8.74 g/L; 41.90 mg/g) were obtained in assay 7 (1% OECW, 5% MWPO and 5% CSL, while the highest biomass production by *R. arrhizus*URM 7651 (12.28 g/L) was obtained in assay 8 (5% OECW, 5% MWPO and 5% CSL). These results showed that under the tested conditions the concentration at the maximum level of MWPO and CSL improved biomass production by all isolates and higher chitosan production by *C. bertholletiae* URM 7658 and *M. subtilissimus* URM 7659. On the other hand, among the three isolates, the highest chitosan production (103.54 mg/g) was obtained by *R. arrhizus* URM 7651 in assay 5 (1% OECW, 1% MWPO and 5% CSL), where CSL concentration was higher than OECW and MWPO. Therefore, the regulation of CSL concentration at the highest level was favorable to the increase of chitosan production by *R. arrhizus* URM 7651, being the independent variable that showed more significance for the production of this response variable. In contrast, the OECW concentrations showed an isolated effect and negative interaction for the tested response variables.

The positive influence of CSL on Mucorales growth and chitosan production has also been reported in other studies (Cardoso *et al.*, 2012; Batista *et al.*, 2013; Leite *et al.*, 2015; Berger *et al.*, 2018; De Souza *et al.*, 2020). Santos *et al.* (2013) suggest that the increase in biomass is favored by the presence of large number of amino acids and vitamins present in the CSL, being essential for the primary metabolism of the microorganism. Although waste post-frying oil has been found to be satisfactory as a nutritional source for Mucoralean fungi growth and chitosan production in this work, there are still few studies exploring this type of industrial waste as an alternative cultivation medium for fungal chitosan production. However, several authors have shown the use of post-frying oils as a nutritional medium for growth and production of different value-added compounds by Mucorales strains (Andrade Silva *et al.*, 2014; Souza *et al.*, 2016a; Hasanizadeh *et al.*, 2017; Pele *et al.*, 2018). The use of agro-industrial waste as a low-cost raw material for microorganism growth and production of compounds of industrial interest has been widely explored (Panesar; Panesar, 2015; Ravindran *et al.*, 2018; De Souza *et al.*, 2020). Bioprocesses are dependent on appropriate conditions and favorable to the maintenance of microorganisms to express their biotechnological potential, changes in culture conditions may influence cell wall synthesis in fungi and consequently improve chitosan yield (Pareek *et al.*, 2011; Antunes *et al.*, 2012; Zhang *et al.*, 2014). Therefore, substrate specific concentrations are an important factor in the

formulation of culture media, which together with other culture conditions, favor the growth of microorganisms and obtaining the product of interest (Berger *et al.*, 2011; Berger *et al.*, 2014b; Zhang *et al.*, 2014; Ghormade; Pathan; Deshpande, 2017). Table 4 shows the chitosan production values obtained in this study compared with production values by other Mucoralean fungi, confirming that the chitosan production depends on the fermentation time, culture medium composition and cultivation conditions.

Fermentation process under non-sterile conditions: In this study, the amount of inoculum (10 discs with fungal growth), initial pH of the medium (4,5), and incubation temperature 28°C were pre-established to perform biomass and chitosan production in low-cost medium. non-sterile conditions. The results showed that all isolates showed abundant growth until the end of the incubation time (96h), with chitosan production at 28°C. In addition, no significant cell growth was detected at this temperature and initial pH of the medium. Based on these results, it was concluded that the studied fungi were able to grow abundantly and stand out against possible contaminants during the process of natural competition for nutrients present in the medium. In recent decades there has been a growing demand to reduce the huge cost of production in various industrial sectors. Therefore, alternative fermentation conditions are developed in the laboratory to reduce production cost and save time and money (Tasar *et al.*, 2016). The use of industrial waste or by-products as substrate provides an economical production process. Sterile culture conditions are used for microbial fermentation processes in most studies (Canli; Tasar; Taskin; 2013; Tasar; Erdal; Algur; 2015). On the other hand, the use of non-sterile conditions may be preferable rather than sterile conditions to reduce the cost of production and labor (Pattra *et al.*, 2011; Jiang *et al.*, 2013). Taskin *et al.* (2013) reported that some disadvantages occur under non-sterile cultivation conditions, such as undesirable microbial contamination and yield reduction. However, these situations can be avoided or reduced by adjusting cultivation conditions in the non-sterile fermentation process (SantaMauro *et al.*, 2014; Tasar *et al* 2016). Fermentations performed at low temperatures may prevent or limit undesirable mesophilic contaminants (Margesin *et al.*, 2003). Low pH values (4.5) were favorable for ethanol production under non-sterile fermentation (Tao *et al.*, 2005). Other studies also show lower pH values in inhibiting contamination caused by weak acidic, nesophilic or alkalophilic microorganisms under non-sterile conditions (Santamauro *et al.*, 2014). Combinations of variables such as inoculum quantity, low temperatures, and pH under non-sterile culture conditions for production of biomolecules of industrial interest may be possible (Taskin *et al.*, 2015).

Chitosan Characterization

Infrared vibrational spectroscopy: Infrared vibrational spectroscopy allows us to observe some bands related to the vibration characteristics of the functional groups present in the structure of the chitosan molecule (Poon, Wilson and Headley, 2014; Raghavendra *et al.*, 2016). Infrared spectra were obtained from chitosan samples only from the best assay according to 2^3 FFD for *C. bertholletiae* URM 7658 (assay 7), *R. arrhizus* URM 7651 (assay 5) and *M. subtilissimus* URM 7659 (assay 7) and are shown in Figure 4. The obtained spectra show characteristic wide bands in the OH stretch region between (3410 - 3408 cm^{-1}), which appear overlapping

the group axial deformation band of the NH amino group, as also observed by Raghavendra *et al*., (2016). Narrow bands in the range of 2918 to 2916 cm^{-1} were observed in the spectra corresponding to chitosan of *C. bertholletiae* URM 7658 and *M. subtilissimus* URM 7659, representing the C-H aliphatic stretch. The presence of an amide group is shown by the characteristic bands in the range from 1635 to 1662 cm^{-1}, which represents the C=O stretch of the amide group I of chitin. The presence of these bands indicates that chitosans are not fully deacetylated (Kumar-Krishanan *et al*., 2015; Xiao *et al*., 2015). The peaks in the region near 1535 cm^{-1} correspond to the combination of N-H deformations of primary amides II, asymmetric stretching of C-N in amide as well as in amide II. The peaks near 1408 cm^{-1} represent the C-O stretch of the primary alcohol group (-CH2-OH) C-O. Peaks in the range 1016 to 1035 cm^{-1} indicate vibrational stretching of the C-O-C bond of the glycosidic ring. IV is a technique used to certify the hydrolysis of acetyl groups present in chitin, when deacetylation occurs there is a decrease in the C=O region of amide I and an increase in axial deformation of the amine group (Kumari; Rath, 2014). The presence of characteristic peaks found in this study is similar to those observed in the literature (Cardoso *et al*., 2013; Mondala *et al*., 2015; Berger *et al*., 2018).
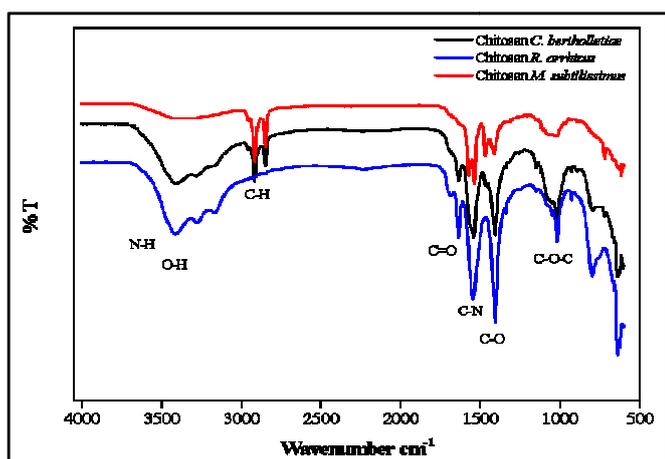

Figure 4. Infrared spectra of chitosan produced by *C. bertholletiae* URM 7658, *R. arrhizus* URM 7651 and *M. subtilissimus* URM 7659 using OECW, MWPO and CSL

Degree of deacetylation (DD, %): Infrared spectrum is one of the analytical methods used to define the degree of deacetylation of chitosan. The DD% calculated based on the spectra of chitosan produced by *C. bertholletiae* URM 7658 (assay 7), *R. arrhizus* URM 7651 (assay 5) and *M. subtilissimus* URM 7659 (assay 7) are shown in Table 5.

Table 5. Characterization of chitosans produced by the fungal isolates using industrial wastes

Chitosan	Degree of deacetylation (DD, %)	Crystallinity index (%)	Viscosity (cP)
Cunninghamella elegans URM 7658	87	26	3.7
Rhizopus arrhizus URM 7651	78	19	5.4
Mucor subtilissimus URM 7659	75	21	2.6

The DD% is an important parameter that determines the physicochemical properties of chitosan because it is linked to its cationic properties (Fai *et al*., 2011; Mondala *et al*., 2015). The DD% value is directly proportional to the positive charge density in the molecule that gives chitosan a unique and greater capacity for industrial, medical or pharmaceutical applications (Ebrahimzadeh*et al*., 2013; Omogbai *et al*., 2013). The deacetylation values of fungal chitosans obtained in this study are comparable with the literature (Fai *et al*., 2011; Cardoso *et al*., 2012; Berger *et al*., 2018).

X-ray Diffraction (XRD): Figure 5 is the X-ray diffractogram of chitosan extracted from *R. arrhizus* URM 7651 (assay 5). It is possible to observe two regions, one of lower intensity 2Θ = 9 to 10°, attributed to the amorphous region, and another region in 2Θ = 19 to 20°, more intense and predominant, attributed to the crystalline region. The crystallinity indices were determined from the dispersion intensity in both regions. The characteristic presence of both regions shows a partial crystallinity of the chitosan sample (Kaczmarek *et al*., 2010, Gámiz-Gonzales *et al*., 2015). The diffraction pattern shows intense refractions at the angles of 20.0° and 9.0°, which are two characteristic peaks of chitosan (Fai *et al*., 2011; Kumirska *et al*., 2011). According to Silva *et al*. (2014), a crystalline organization of chitosan is possible through intra and intermolecular interactions formed between the amino, hydroxyl and amide groups.
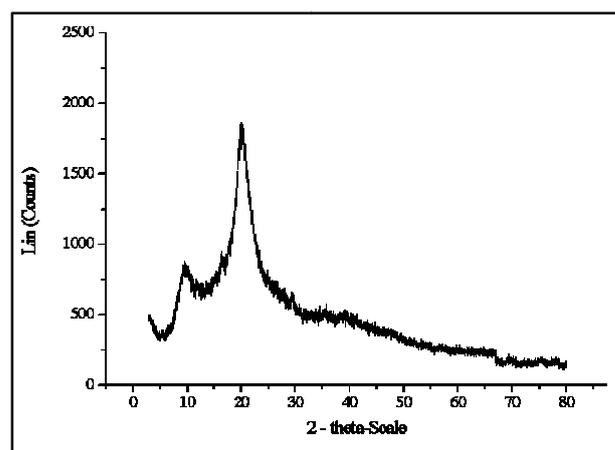

Figure 5. X-ray diffractogram of chitosan produced by *R. arrhizus* URM 7651 (assay 5) using OECW, MWPO and CSL

Viscosity: The viscosity of chitosans produced by the Mucoralean isolates are shown in Table 5. The viscosity of chitosans produced by three isolates were considered low. Some authors report that chitosan of fungal origin presents low viscosity (between 1.0 to 7.2 cP) when compared with chitosan extracted from crustaceans (between 20 and 500 cP) (Khalaf *et al*., 2004; Omogbai; Ikenebomeh, 2013; Yang *et al*., 2017). This result is in agreement with other fungal chitosans (Tayel *et al*., 2010; Omogbai; Ebrahimzadeh *et al*., 2013). The viscosity of chitosan is closely related to its molecular weight. High molecular weight chitosan has high viscosity compared to low molecular weight chitosan; thus, fungal chitosan has low viscosity and low molecular weight (Omogbai; Ikenebomeh, 2013). Also, chitosan viscosity is an important factor determining its commercial applications. Chitosans with low viscosity and high degree of deacetylation are promising for specific applications, such as pharmaceutical and food products, mainly due to their high solubility and higher charge

density (Rong; Horng-Dar, 1996; Omogbai; Ikenebomeh, 2013).

Conclusions

*C. bertholletiae*URM 7658, *R. arrhizus*URM 7651and *M. subtilissimus*URM 7659 were able to use culture media containing industrial byproducts (OECW, MWPO and CSL) as low-cost substrates for growth and fermentative chitosan production, making the process feasible, economical and environmentally friendly. Mucoralean isolates were able to grow abundantly and to stand out for possible contaminants during the non-sterile culture process, eliminating the step of media sterilizing. Considering the selection of chitosan as one of the most important factors for its industrial application, the results of the physicochemical analysis of chitosan produced by the three isolates showed desirable characteristics, such as high degree of deacetylation, low crystalline index and low viscosity, showing similarity with samples of chitosan of fungal origin reported in the literature. Among the isolates tested in this study, *R. arrhizus* URM 7651 showed higher chitosan production and deacetylation degree of approximately 80%, showing great potential in future applications.

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