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Rhizopus arrhizus ucp1295 como fonte econômica para produção de biopolímeros funcionais quitina e quitosana utilizando substratos renováveis

Rhizopus arrhizus ucp1295 as economic source for production of functional biopolymers chitin and chitosan using renewable substrates

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RESUMO
Neste trabalho foi investigada a produção de quitina e quitosana por *Rhizopus arrhizus* UCP 1295 isolado do solo da Caatinga do Estado de Pernambuco, Brasil, utilizando o efluente industrial de doces e milhocina como substratos de baixo custo, considerando a versatilidade de aplicação das biomoléculas. O micro-organismo foi cultivado em diferentes concentrações dos substratos efluente da indústria de doces e milhocina (CSL) em diferentes valores de pH, de acordo com um planejamento fatorial completo 2^3. Após 96 h de fermentação, a biomassa produzida foi liofilizada e submetida ao tratamento com álcali- ácido-. Os polissacarídeos extraídos foram caracterizados por espectroscopia por transformada de Fourier (FTIR) na região do infravermelho. A maior produção de biomassa (14,11 g/L) foi obtida na condição 6 (8% de efluente industrial de doces, 5% de milhocina e pH 5), enquanto os maiores rendimentos de quitina (169,3 mg/g) e quitosana (239,1 mg/g) foram obtidos em meio contendo 4% de efluente da indústria de doces, sem milhocina, nas condições 3 (pH 7) e 1 (pH 5), respectivamente. A quitina apresentou grau de acetilação de 71,4% e a quitosana de 86,0%, de desacetilação, respectivamente. Além disso, foi demonstrado que o efluente industrial de balas e milhocina são substratos renováveis e alternativos na formulação de novos meios de produção de quitina e quitosana. A versatilidade das biomoléculas deve-se as suas propriedades bioquímicas únicas, como biocompatibilidade, biodegradabilidade, não toxicidade, capacidade de formar filmes e aplicações industriais promissoras.

Palavras-chave: Fungo Mucorales, efluente industrial, co-polímeros, biomoléculas versáteis.

ABCTRACT
This work was investigated the production of chitin and chitosan the production of chitin and chitosan by *Rhizopus arrhizus* UCP 1295 isolated from Caatinga soil of Pernambuco state, Brazil, using the candy industrial effluent and corn steep liquor as low-cost substrates, considering the versatility of application of the biomolecules. The microorganism was cultured in different concentrations of the wastes in different pH values, according to a 2^3 full factorial design. After 96 h of fermentation, the biomass produced was lyophilized and subjected to alkali-acid treatment. The extracted polysaccharides were characterized by Fourier transform spectroscopy (FTIR) in the infrared region. The highest production of biomass (14.11 g/L) was obtained in the condition 6 (8% candy industrial effluent, 5% corn steep liquor and pH 5), while the highest yields of chitin (169.3 mg/g) and chitosan (239.1 mg/g) were obtained in media containing 4% of candy industry effluent, without corn steep liquor, in the conditions 3 (pH 7) and 1 (pH 5), respectively. Chitin showed degree of acetylation of 71.4% and chitosan 86%, of deacetylation, respectively. In addition, it has been demonstrated that candy industrial effluent and corn steep liquor are renewables and alternatives substrates in the formulation of new media for production of chitin and chitosan. The

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versatility of the biomolecules is due to owing to their unique biochemical properties such as biocompatibility, biodegradability, non-toxicity, ability to form films, and promising industrial applications.

Keywords: Mucoralean fungus, industrial effluent, co-polymers, versatile biomolecules.

1 INTRODUCTION

Chitin is constituted by (β-(1–4) -poly-N-acetyl-D-glucosamine is widely distributed in nature and is the second most abundant polysaccharide after cellulose. Chitin, which occurs in nature as ordered macrofibrils, is the major structural component in the exoskeletons of the crustaceans, crabs and shrimps, as well as the cell walls of fungi (BARIKANI et al., 2014; CASTELEIJN et al., 2018).

It is insoluble in water, organic solvents, dilute acids and alkalis, and is in the form of a crystalline or amorphous solid. Chitosan, a copolymer of (1-4) -L-amino-2-deoxy-β-D-glucone (D-glucosamine), is the deacetylated form of chitin. In addition, chitosan is soluble in organic acids, which is one of the main characteristics that distinguishes it from chitin (SYNOWIECKI; AL-KHATEEB,2003; ALJAWISH et al., 2015). Thus, the derivatives of chitin obtained at a degree of deacetylation higher than or equal to 50% are known as chitosan. relative proportions of these units generate different structural features, such as the degree of deacetylation and the molecular mass, which are related to the physico-chemical and biological properties of the polymer (CRUZ et al., 2016).

Rhizopus arrhizus is a filamentous fungus belonging to the order Mucorales that have numerous biotechnological applications due to the production of secondary metabolites of industrial interest (DOLATABADI et al., 2015; KODAL; AKSU, 2017). However, it is one of the species that has been little explored for the production of biopolymers such as chitin and chitosan (BERGER et al., 2014). These functional biopolymers are biocompatible, non-toxic and highly biodegradable. They have a wide variety of applications, such as food, biomedicine, agriculture and veterinary. In addition, they have been widely used in the treatment of effluents, when used as chelating agents of metals, flocculants, adsorbents of dyes and metallic anions, antimicrobial activity, nanomedicine, and other applications (DIAS et al., 2013; GHADI et al., 2014; MACIEL et al.,2015; GHORMADE et al., 2017; AÇIKEL;GÖZE,2017; RAZAK et al., 2018; QUIN; LI, 2020).

Chitosan extracted from fungal cell wall presents several advantages when compared to chitosan extracted from crustaceans, mainly because it does not have the residues of proteins that are responsible for allergic reactions in humans (BATISTA et al., 2013; TAYEL et al., 2014; MAHATA et al., 2014). The production of chitin and chitosan from fungi of the order Mucorales is

an economically viable process due to the possibility of using renewable substrates (DIAS et al., 2013; OLIVEIRA et al.,2014). Industrial wastes have been successfully used as renewable sources in biotechnological processes, considering the presence of high concentrations of nutrients rich in carbon and nitrogen sources, vitamins and minerals. On the other hand, these wastes generated by the industries have a great pollutant load, considering the presence of organic substances. Therefore, appropriate treatment and/or reuse are necessary in order to minimize the environmental impact and thus obtain high value added products contributing to sustainability (ZARGAR et al., 2015; NAMBOODIRI;PAKSHIRAJAN, 2020). Thus, the use of industrial waste to produce functional biopolymers of industrial interest is a sustainable and promising alternative due to the minimization of environmental problems caused by the inappropriate disposal of several wastes (Berger et al., 2014; Razak et al., 2018).

To our knowledge, only a limited work has been reported concerning conversion of renewable substrates (CIE and CSL) to chitin and chitosan production by *Rhizopus arrhizus* UCP 1295 in submerged fermentaion.

2 OBJECTIVE

Production of chitin and chitosan by *Rhizopus* arrhrizus UCP1295 using renewable substrates.

3 MATERIAL AND METHODS

3.1 MICROORGANISM

The microorganism used was *Rhizopus arrhizus* UCP 1295, kindly provided by the Culture Collection of the Catholic University of Pernambuco-UCP, and registered at the World Federation for Culture Collection (WFCC) and the microorganism was maintained on potato dextrose agar at 5 °C.

3.2 SUBSTRATES

Candy industrial effluent (CIE), is described as rich carbon total and soluble (Ozgun et al., 2012) and corn steep liquor (CSL) residue obtained from the processing of corn and the both substrates were kindly provided by Arcor S.A. and Corn Products Ltd, Cabo, Pernambuco, Brazil, respectively, were used as substrates.

3.3 CULTURAL CONDITIONS

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R. arrhizus was grown in Petri dish (9 cm in diameter) containing Yeast Malt Agar (YMA) of composition (g/L): agar 20 g, malt extract 3 g, yeast extract 3 g, peptone 5 g, glucose 10 g, 1000 mL distilled water and pH 5.8. After spore's inoculation Petri plates were then incubated at 28 °C for 120 h until sporulation occurred. Spores were collected and transferred to Erlenmeyer flasks containing 50 mL of 0.9% buffered saline [0.45 g monobasic sodium phosphate, dibasic sodium phosphate 5.8125 g, 2.25 g sodium chloride] obtaining 10^7 spores/mL. Then, 5 ml of the suspension was transferred to Petri dishes containing YMA medium incubated at 28 °C for 24 h. Subsequently, 40 discs of 6mm of diameter containing the young mycelium were used as pre-inoculum in the production media.

3.4 PRODUCTION OF BIOMASS

The production of biomass by *R. arrhizus* was carried out in Erlenmeyer flasks containing 100 ml of production media composed by candy industry effluent and CSL following concentration levels established by the 2^3 FFD with the initial pH adjustment. The cultivation was carried out for 96 h, at 150 rpm in orbital shaker at temperature of 28 °C. The mycelial biomass was removed by vacuum filtration, washed with ice distilled water and lyophilized afterwards. The biomass was kept in desiccator until reaching constant weight, and was estimated by gravimetry.

3.5 FULL FATORIAL DESIGN

A 2^3 full factorial design (FFD), consisting of 8 conditions and 4 replications at the central point: CIE (% w/v) 5.0-3.5-0; pH: 7-6.5 and CSL(% w/v): 8.0-6.0 and 4.0. The experiments were carried out to investigate the effects and interactions of the independent variables on the yield of biomass, chitin and chitosan yields as response variables.

3.6 EXTRACTION OF CHITIN AND CHITOSAN

The process of extraction of chitin and chitosan was performed according to Hu et al. (1999). The procedure involved the deproteinization of lyophilized biomass with 1M NaOH in the ratio of 1:40 (w/v), followed by autoclaving (121°C, 15 min), centrifugation (4000 g, 15 min). The supernatant was discarted and the residue was subjected to acid hydrolysis using 2% (v/v) acetic acid, autoclaving (100°C, 10 min) and centrifugation (4000 g, 15 min). The residue corresponded to chitin was washed with ice-cold distilled water until the pH was near neutrality. The pH of the supernatant was adjusted to 10-12 and the chitosan precipitation occurred at 5^0C overnight.

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Thereafter, this biopolymer was subjected to successive washes with ice-cold distilled water to near neutrality and dry at room temperature.

3.7 DETERMINATION OF PH

The pH of the different conditions of FFD was determined by potentiometry.

3.8 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR) MEASUREMENTS

Two milligrams of chitin and chitosan samples were dried overnight at 60°C under reduced pressure and completely homogenized with 100 mg KBr. The KBr discs prepared were dried for 24 h at 110°C under reduced pressure. Infrared spectroscopy was performed using a Fourier transform (FTIR) spectrometer, BRUKER Mod. IFS. KBr discs will be used as reference. The intensity of the maximum absorption bands range of 4000–500 cm^{-1} was determined by the baseline method.

3.9 DETERMINATION OF THE DEGREE OF DEACETYLATION (DD%)

The degree of acetylation of chitin and deacetylation of chitosan were determined using infrared spectroscopy -FTIR, and calculated according to Roberts (1992), equation 1:

A (%) = (A1655/A3450) x 100 / 1.33 [1]

3.10 RESULTS AND DISCUSSION

The knowledge of *R.arrhizus* metabolism is of great importance in biotechnological processes, allowing the optimization of resources and promoting the reduction of the costs of biomass and biomolecules of interest production. In this study, the components of the production medium (CIE and CSL) probably have supplied the nutritional requirements for the growth of *R. arrhizus* and promoted the production of biomass. Table 1 presents the decoded matrix of the 2^3 full-factorial design used for the production of biomass by R. *arrhizus*, reaching 14.11 g/L in the condition 6 (5% CIE, 8% CSL and pH 5.0). The results suggested that the cultivation in a higher concentration of the agroindustrial CSL residues favored the growth of R. arrhizus. The results reported by Cardoso et al.(2012) using CSL and honney, and Chatterjee et al.,(2019) using waste whey and molasses to biomass to R. arrhizus, and R.oryzae, respectively were obtained lower biomass then the yield with CSL and CIE to *R.arrhizus*.

The yields of biomass, chitin and chitosan produced by *Rhizopus arrhizus* using alternative carbon and nitrogen soures are confirmed to others filamentous fungi as described Syanowiecki & Al-kahateeb (2003), Berger et al., (2014a,b); Chatterjee et al. (2019) and Namboodiri et al.,(2020).

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The results of the production of mycelial biomass obtained in this study were superior to those reported by Oliveira et al., (2014) (7.54 g/L), Berger et al.,(2014) (5.67 g/L) using *Cunninghamella elegans*. Lower results of biomass from fungi were also found by Ebrahimzadeh et al. (2013), Jun et al. (2013) Kaczmareck et al., (2019) and Elsoud &ElKady (2020).

Table 1. Decoded matrix of the 2^3 full-factorial design as factors (CIE, CSL and pH) and response variables are productions of biomass, chitin and chitosan

Assays	CSL (%)	pH	CIE (%)	Biomass (g/L)	Chitin		Chitosan	
					(%)	(mg/g)	(%)	(mg/g)
1	4.0	5.0	0.0	7.08	15.22	152.2	23.91*	239.1*
2	8.0	5.0	0.0	7.42	10.00	100.0	3.61	36.1
3	4.0	7.0	0.0	6.84	16.93*	169.3*	7.48	74.8
4	8.0	7.0	0.0	7.26	9.65	96.5	15.10	151.0
5	4.0	5.0	5.0	13.55	7.09	70.9	3.27	32.7
6	8.0	5.0	5.0	14.11*	9.53	95.3	3.93	39.3
7	4.0	7.0	5.0	11.51	8.53	85.3	1.54	15.4
8	8.0	7.0	5.0	13.31	9.48	94.8	1.23	12.3
9	6.0	6	2.5	11.34	8.73	87.3	2.63	26.3
10	6.0	6	2.5	11.11	10.12	101.2	3.00	30.0
11	6.0	6	2.5	11.09	11.74	117.4	2.67	26.7
12	6.0	6	2.5	11.28	10.09	100.9	1.87	18.7

Candy industrial efluent=CIE **Corn steep liquor=CSL**

Contrary to the biomass production, CSL do not influence the production of chitin and chitosan. Opposed results were reported by Oliveira et al. (2014), Batista et al., (2013) and Tayel et al. (2014), who obtained higher yield of chitosan by *C. elegans* and *Syncephalastrum racemosum*, respectively, in the condition of higher concentration of CSL. However, Berger et al.(2014a), obtained higher yield of chitosan in the condition with lower concentration of CSL. Figure 1 shows the Pareto diagram and the cube plot of the mean effects caused by the independent variables (candy industry effluent, CSL and pH), as well as their associations, in the biomass production by *R. arrhizus.* According to the results obtained in the Pareto diagram (Fig. 1a), CSL (3) and candy industry effluent (1) were the independent variables that showed a positive. The influence of industrial residues indicating that higher concentrations of these substrates (CIE and CSL) in the medium allows a greater production of fungal biomass. Similar results in the production of biomass by Mucorales fungi in medium containing CSL were reported by Berger et al. (2014a) in the growth of *R. arrhizus* and *C. elegans*, as well as by Lima et al. (2017) after cultivation of *C. echinulata*. Thus, the results obtained in this study confirm the potential of CSL as an alternative and low-cost substrate.

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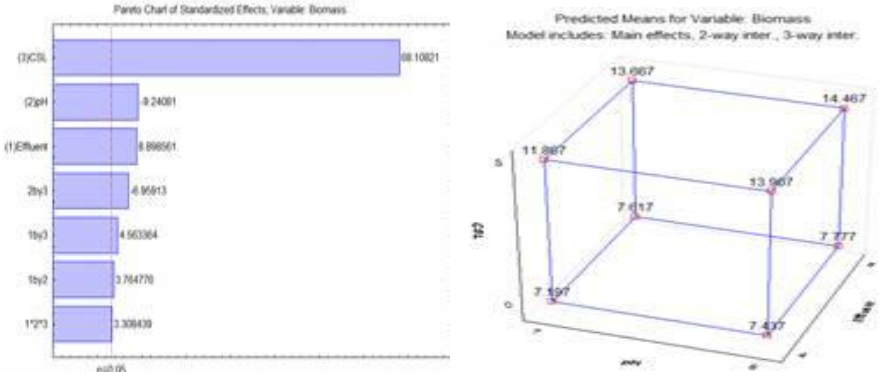
Figure 1. Pareto diagram (a) and cubic plot of the predicted effects (b) of independent variables for production of biomass by *Rhizopus arrhizus*

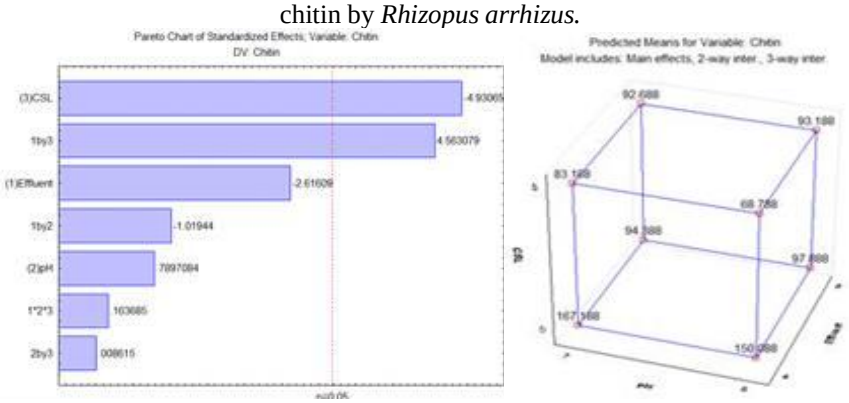


(a) (b)

Another independent variable that was statistically significant was pH (2). However, the effect was negative on biomass production which suggests that an increase in pH of the medium affects fungus growth. Figure 1b confirms the information obtained by the Pareto diagram demonstrating that the interaction between the independent variables pH (2) and CSL (3) were antagonistic, since it shows that higher concentrations of CSL and lower pH values influence the production of biomass. Similarly, the antagonistic interaction between candy industry effluent (1) and pH (2) variables was evidenced, as well as the interaction among all the independent variables studied, on biomass production.

On the other hand, the effects of the independent variables candy industry effluent (1), pH (2) and CSL (3) on chitin production by *R. arrhizus* are presented in Figure 2. According to the Pareto diagram (Fig. 2a), CSL (3) and the association between candy industry effluent (1) and CSL (3) were those that had a significant effect on chitin production. Figure 2b shows that the interaction between the residues (1 and 3) had a synergistic negative effect, in which lower concentrations of the substrates favor the production of chitin.

Figure 2. Pareto diagram (a) and cubic plot of the predicted effects (b) of independent variables for production of chitin by *Rhizopus arrhizus.*



(a) (b)

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The production of chitosan by *R. arrhizus* evaluated by the Pareto diagram (Fig. 3a) shows that all the independent variables, as well as their associations, were statistically significant except for the interaction of pH (2) and CSL (3). CSL was the most significant variable, but it had a negative effect on chitosan production. Similarly, CIE (1) and pH (2) had a negative effect. However, the associations between CIE and pH (1by2) and CSL (1 and 3) had a positive effect on chitosan production, favoring its maximum production. The results on the interaction of the variables can be confirmed by the cube plot of predicted effects (Fig. 3b), which indicates the highest production of chitosan in the medium containing the lowest effluent concentrations and pH 5, as evidenced in Table 1.

Figure 3. Pareto diagram (a) and cubic plot of the predicted effects (b) of independent variables for production of chitosan by *Rhizopus arrhizus.*

(a) **(b)**

The infrared spectrum of chitin showed the presence of characteristic bands such as peaks 1473 cm^{-1}, 1560 cm^{-1} and 1648 cm^{-1}, corresponding to the stretching of the CN bond plus bulging of CH_3(Figure 4a). As well as the N-H deformation in the CONH plane, including the amide II, and elongation of the carbonyl group, C=O (amide I) but less intense. According to Ebrahimzadeh et al.,(2013) and Berger et al.(2014ab), the intensity of the acetyl group in the amine group (stretch, C=O, amide I) corresponds to the degree of deacetylation of chitosan(Figure 4b).

Figure 4. Infrared absorption spectra of the microbiological polymers obtained from *Rhizopus arrhizus* biomass as: (a) chitin and (b) chitosan.

(a) **(b)**

As with the infrared spectra of chitin, chitosan showed amide bands between 1480 cm^{-1}, 1562 cm^{-1} and 1633 cm^{-1}, but with less intensity than chitin, especially the 1650 cm cm^{-1} peak. According to Ebrahimzadeh et al. (2013), during the chitosan production process, the acetyl group is removed by hydrolysis, and the carbonyl group is then removed in chitosan. However, the presence of an acetyl group on the amino group proves that the chitosan is not totally deacetylated. Similar results were observed by Berger et al. (2014b). The infrared spectra of chitin and chitosan obtained by *R. arrhizus* from condition 1 of 71.4% of chitin acetylation degree, and 86% of deacetylation of chitosan, respectively.

4 CONCLUSION

The formulation media for production chitin and chitosan containing CIE and CSL as carbon and nitrogen sources proved to be an alternative and low-cost media for the production of fungal biomass. *R. arrhizus* presents high biotechnological potential in the production of chitin and chitosan in the alternative medium. The quantity and/or quality of chitin and chitosan in the fungal cell wall may change due to environmental and nutritional conditions mediated by CIE and CSL. Variations of pH values significantly influenced the production of chitosan, considering the importance in the deacetylation of chitin to chitosan by the enzyme chitin deacetylase. These biopolymers obtained by *R. arrhizus* with quality offer wide range of the possibilities of applications.

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