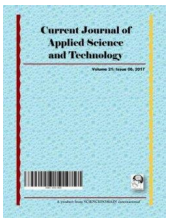

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Production of *Agaricus bisporus* (JE Lange) Imbach Biomass under Submerged Cultivation by Using *Citrus reticulata* Blanco Waste as an Alternative Carbon Source

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TMS and MH designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors ARL and VAC performed the statistical analysis. Authors APM, JJ, YGK, GGS and RCSS managed the analyses of the study. Authors TMS, LBB and MH managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the influence of different parameters in the biomass production of *Agaricus bisporus* by submerged cultivation using *Citrus reticulata* B. waste as an alternative carbon source.
Place and Duration of Study: University of Santa Cruz do Sul.

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Methodology: Fruit juice was extracted from the *C. reticulata* B. waste in a manual juicer. To determine the initial available amount of glucose and fructose in the liquid extract, high-performance liquid chromatography analysis was used. Temperature, pH and fermentation time were selected for the study of response surface methodology in a central composite design. Pectinolytic activity, reducing sugars and total proteins were used for bioprocess monitoring, and visible molecular absorption spectroscopy was used as the monitoring method.
Results: The highest amount of biomass was (47.06 g/L) obtained at 28°C, a pH of 6 and 8 days of culture. The application FT-IR spectroscopy associated with partial least squares regression enabled simultaneous quantification of the parameters of submerged cultivation, with a root mean square error prediction and a coefficient of determination of 0.0012 U/L and 0.9760, 0.23 mmol/L and 0.9956, and 0.03 g/d/L and 0.9957, respectively.
Conclusion: This is the first work that has monitored the biomass production of *A. bisporus* through FT-IR using the response surface methodology. The data showed that the liquid extract from *C. reticulata* B. waste is an excellent source of carbon for the mycelial production of *A. bisporus* and this may be an interesting alternative use of this residue.

Keywords: Mandarin; edible mushroom; mycelium; response surface methodology; bioconversion; FT-IR.

1. INTRODUCTION

Millions of tons of *Citrus* sp. waste are generated annually worldwide [1]. The production of juices generates waste in the form of peels, pulp and seeds, which represent 50% of the initial mass of the fruit [2]. Another example of generating such waste comes from the extraction of essential oils from the fruit peel of the green mandarin (*Citrus reticulata*); after this process, the entire fruit is practically discarded. The most commonly used method for the recovery of waste from *Citrus* sp. in the agro-industry is to transform it into pelletized meal for animal feed [1,2]. However, the drying cost is a limiting factor in these processes, and the amount of untreated waste released into the environment poses a serious environmental problem [3,4].

One of the main characteristics of this waste is the high content of soluble and insoluble carbohydrates and moisture, making it a potential energy source for biological conversion into higher value-added products—for example, as microbial biomass [1,5]. Several biopolymers, such as enzymes (invertase, glucosidases), nucleotides, proteins (mannoproteins) and especially polysaccharides (glucans, mannans, galactans), as well as lipids such as phospholipids and ergosterol [6] can be extracted from fungal biomass.

The most common method of obtaining microbial biomass is through submerged fermentation (SmF) or submerged cultivation (SmC) because it is a faster alternative to the cultivation of filamentous fungi [7-9]. Using agro-industrial waste in SmC has been successfully employed for the cultivation of *A. bisporus* mycelium. This species has nutraceutical potential for the production of food for human and animal consumption because its biomass contains bioactive substances with antimicrobial, anti-tumor, anti-carcinogenic, and antioxidant properties as well as immune system activators and liver protectors, making it a functional food [10-12].

The study of microbial behavior on the substrate and growth conditions is critical to achieve optimum conditions to obtain the desired bioproduct. In this context, response surface methodology (RSM) has been increasingly used to understand and optimize fermentation processes [13,14]. It is a powerful technique to test multiple variables because fewer experimental trials are needed compared to the study of one variable at a time. In addition, this technique can identify and quantify interactions among variables [15].

Among the various classes pertaining to the RSM, planning by Central Composite Design (CCD) is the most accepted because it offers a great deal of information about the model studied, revealing the experimental error of the model with a relatively small number of experiments [16]. This methodology has enabled the optimization of macromycete bioproducts for industrial use, such as *A. blazei* and *A. bisporus* [15-17].

The monitoring of the bioconversion is essential to control and ensure maximum efficiency of the fermentation process. The search for sensitive analytical techniques that are efficient and have a low environmental impact, in this context, has been achieved by Fourier Transform Infrared Spectroscopy (FT-IR), wherein compound identification and analysis of mixtures is based on vibrational movements [13]. This analytical technique is constantly developing because it provides fast analysis with a high yield without destruction of the sample [18,19]. Moreover, its use in the fermentation processes is being increasingly exploited because it allows for the monitoring of complex biological processes in real time [20-22].

The purpose of this research study was to use the liquid waste of *C. reticulata B.* to obtain a biomass of *A. bisporus* by employing the RSM and to monitor its efficiency with diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS).

2. MATERIALS AND METHODS

The microorganism used in this study was a commercial strain of *A. bisporus*, and the mycelium was maintained on potato dextrose agar (PDA) with subculturing. Samples of *C. reticulata* B. waste were kindly provided by the cooperative of Ecological Citrus Growers of the Vale do Caí - Ecocitrus, located in southern Brazil.

2.1 Inoculum Preparation

The inoculum was obtained according to the methodology proposed by Carvajal and Koehnlein [23]. Samples of five, 5-mm disks of mycelial agar BDA (7 days, 28°C) were transferred to Erlenmeyer flasks (250 mL) containing 50 mL of sterile broth (121°C, 15 min) composed of the following: 40 g/L glucose, 3 g/L yeast extract, 3 g/L bacteriological peptone, 0.5 g/L K_2HPO_4, 0.3 g/L $MgSO_4.7H_2O$, and 1 L of deionized water. Incubation was performed with stirring at 150 rpm in a shaking/rotating incubator, at a temperature of 28°C, for a period of seven days.

2.2 *C. reticulata* Waste as a Carbon Source

Fruit juice was extracted from the *C. reticulata* waste in a manual juicer. To determine the initial available amount of glucose and fructose in the liquid extract, high-performance liquid chromatography analysis was used. The analysis was conducted in Shimadzu equipment with an isocratic pump, model LC-20AT, and a diode array detector, DAD - M 20A, with a 30 cm Rezex ™ RHM monosaccharide H+ column, at a temperature of 85°C, a flow of 0.6 mL/min of mobile phase milli-Q water, and an injected sample volume of 20 µL.

2.3 Submerged Cultivation (SmC)

The means for SmC preparation were similar to the stock preparation for preparing the inoculum but without the addition of glucose, and the liquid extract of *C. reticulata* was used in place of water. Erlenmeyer flasks (250 mL, triplicate) containing 50 ml of this sterile broth (121°C, 15 min) were inoculated with the mycelial suspension (inoculum) to 5% (v/v). The incubation was performed under continuous stirring at 150 rpm. The control was carried out without the addition of inoculum.

After the incubation period, the mycelium was dried at 45°C and was weighed to a constant weight, thus determining whether it reached biomass yield (g/L). A fraction of the liquid medium was analyzed for the final concentration of glucose and fructose through HPLC. Another liquid fraction was withdrawn and kept cool for subsequent monitoring analysis.

2.4 Experimental Design

The study of the optimization of the most significant variables of SmC was carried out using a statistical design (Table 1). Using Minitab software (version 17), a 23 factorial design was elaborated using the Central Composite Design (CCD) with eight factorial points, six axial points and six central points, totaling 20 trials.

The main effects of the variables and their interactions as well as data on the Analysis of Variance (ANOVA) were calculated. Response surface methodology was used to infer the best SmC conditions and provide an appropriate mathematical model for the biomass production process.

2.5 Monitoring

2.5.1 Determination of the content of reducing sugars (RS)

The method for determining the concentration of reducing sugars used in this study was adapted

from the method proposed by Miller [24], which uses DNS reagent (3,5-dinitrosalicylic acid) with a sensitivity range of 0.1 to 1.0 g/L for reducing sugars expressed as glucose.

2.5.2 Determination of the pectinolytic activity

For the determination of enzyme activities, the study used 100 µL of each sample suspended in 900 µL of a solution containing pectin extract at 5 mg/mL in a McIlvaine buffer with a pH level of 5.0, and the samples were incubated for 4 hours at 37°C for later analysis [25]. The enzymatic activities were determined as the amount of reducing sugar released, and a dinitrosalicylic acid method (DNS) was used for the analysis.

A pectinase unit (U) is defined as the number of micromoles of reducing sugars released per minute per ml of the sample under test conditions (U =µmol RS/min).

2.5.3 Determination of total protein

The determination of total protein in the submerged cultivation was carried out by the Biuret method (Labtest Diagnostica SA, Lagoa Santa, Minas Gerais, Brazil).

2.5.4 Monitoring by FT-IR

Triplicate 10 µL samples were diluted (at a 1:5 ratio of gross substrate: deionized water), added to 10 µL of potassium ferricyanide 10 g/L (internal standard) and scattered in stainless steel probes to uniformly cover the entire surface. Next, the samples were dried (60-65°C, 15 min) and then analyzed by DRIFTS in the range of 4000-450 cm^{-1} using 8 scan pulses and 4 cm-1 of resolution. An infrared Spectrum spectrophotometer 400 FT-IR/FT-NIR Spectrometer Perkin Elmer with a HeNe, 633-nm laser equipped with accessory diffuse reflectance (Pike Technologies, Madison USA) was used. The spectra were normalized by the amplitude of the vCN band, pre-processed by the sample via multiplicative light scattering and by variable via centering means in the medium and analyzed by partial least squares regression (PLS) together with the respective RS, PA and TP values, with cross-validation of one-by-one mutual exclusion using Pirouette 4.0 software.

3. RESULTS AND DISCUSSION

3.1 *C. reticulata* Waste as Carbon Source

The initial glucose concentration in the control sample for SmC was 31.69 g/L. For fructose, the value was 37.33 g/L. The glucose value at the beginning of the process was close to that used by Carvajal and Koehnlein [23] in the cultivation of *Agaricus brasiliense* (40 g/L). In general, there was preferential utilization of glucose during SmC, which is consumed in its entirety in almost

Table 1. Observed and predicted responses obtained for the central composite design

Run	X_1	X_2	X_3	Biomass (g/L)	
	temperature (C°)	pH	time (days)	Actual value	Predict value
1	33	7.5	4	5.80	3.21
2	23	7.5	4	3.85	2.85
3	28	6	8	34.56	39.52
4	28	6	12	37.19	34.70
5	23	7.5	12	21.71	25.76
6	28	6	8	44.95	39.52
7	23	4.5	4	35.30	31.58
8	28	4.5	8	44.39	47.02
9	33	7.5	12	28.91	26.12
10	33	6	8	31.44	32.22
11	23	4.5	12	29.07	27.06
12	28	6	8	45.85	39.52
13	23	6	8	29.05	31.86
14	33	4.5	4	31.18	31.94
15	33	4.5	12	23.53	27.42
16	28	6	8	34.38	39.52
17	28	6	4	18.15	24.87
18	28	6	8	47.06	39.52
19	28	6	8	40.19	39.52
20	28	7.5	8	28.13	32.01

all experiments. Regarding fructose, the average consumption by *A. bisporus* during the submersion process was 13%.

Glucose is considered the best source of carbohydrate for the production of exopolysaccharides associated with fungal biomass in SmC [26,27]. However, variations in the type of carbohydrates assimilated may occur as the studied microorganism [28].

3.2 Submerged Cultivation (SmC)

The CCD was used to identify the optimum levels and interaction of the selected factors. The results are shown in Table 1. Experiment 18 showed the highest biomass response, with 47.60 g/L. The lowest value was found in experiment 2. Multiple regression analysis was used to analyze the data; thus, the polynomial equation was derived from regression analysis as follows:

$$\text{Biomass (g/L)} = -160.4 + 16.78\ X_1 - 14.15\ X_2 + 4.34\ X_3 - 0.299\ X_1^2 - 0.628\ X_3^2 + 1.143\ X_2X_3 \quad (1)$$

As observed in Table 1, the highest results for fungal biomass production were obtained in experiments related to the central point, which corresponds to 8 days of fermentation at a pH level of 6 and at 28°C. The greatest amount of biomass obtained was 47.06 g/L, and the lowest value was 3.85 g/L. In considering the central points, the average yield of biomass reached was 41.17 ± 5.68 g/L. These values were highest than those reached by Kim et al. [12] who used sugar cane extract as a source of carbohydrate at a concentration of 30 g/L and obtained 15.0 g/L (dry weight) of biomass of *A. bisporus*. This was also higher than the results found by Liu and Wang [15], who evaluated the biomass production of *A. blazei* according to different concentrations of glucose, yeast extract and peptone through response surface methodology. Their best result for biomass was 10.86 g/L, with the consumption of 26.30 g/L of glucose.

Thus, the availability of glucose and fructose in the liquid extract of *C. reticulata* demonstrates the potential use of this waste as a source of carbohydrates because it is easily assimilated for the biological conversion by *A. bispo*rus and does not need pretreatment for providing these sugars.

Using ANOVA, the model for biomass response was verified, and the results are represented in Table 2. The R^2 value closer to unity, significant model F value, insignificant lack-of-fit F value, and a standard deviation less than 10 in all cases implied that the model was significant. The calculated R^2 value of 0.897 for biomass production showed improved correlation between the observed and predicted response. The R^2 value was always between 0 and 1. The closer the R^2 is to 1, the stronger the model and better it predicts the response. The P values represent the significance of the coefficients and are also helpful in understanding the pattern of the mutual interactions between the variables. The value of Prob>F less than 0.05 indicates that the model terms are significant. X_2, X_3, X_3^2 and X_2X_3 are significant models in this case.

In Fig. 1, it is possible to determine the influence of pH versus time in the growth of *A. bisporus* in the liquid extract of *C. reticulata* waste at the time intervals of 4, 8 and 12 days. After four days of the process, the analysis reveals a more

Table 2. Analysis of variance for the response of biomass

Source	Sum of square	DF*	Mean square	F value	Prob>F
Model	2436.35	9	270.70	9.68	0.001
X_1	776.70	1	0.353	0.01	0.913
X_2	563.55	1	563.55	20.15	0.001
X_3	212.80	1	212.79	7.61	0.020
X_1^2	911.39	1	137.65	4.92	0.051
X_2^2	70.05	1	3.09	0.11	0.746
X_3^2	256.09	1	256.08	9.16	0.013
X_1X_2	44.23	1	44.22	1.58	0.237
X_1X_3	1.83	1	1.83	0.07	0.803
X_2X_3	376.07	1	376.06	13.45	0.004
Residual	279.69	10	27.97		
Lack of fit	118.05	5	23.61	0.73	0.631

** Degrees of freedom*

Significant decrease in biomass production as opposed to the increase in pH. The highest values of the biomass are given after 8 days of the process. After 12 days, the influence of pH on the biomass production decreases considerably. This is possibly due to the adaptation of *A. bisporus* to the conditions of the medium and long process time (12 days). However, this condition showed the worst performance in the production of biomass through submerged cultivation.

To produce biomass for subsequent isolation of the chitin synthase enzyme contained in the cell walls of *A. bisporus*, Hänseler et al. [9] employed submerged fermentation for 6 days, corresponding to the log phase of the microorganism at a temperature of 28°C and used a more acidic medium, with a pH level of 5.3. Kim et al. [12] obtained the best growth results of *A. bisporus* in the liquid phase at a temperature of 28°C and at an initial pH of 6.0. However, these authors used *A. bisporus* Suksung, a strain adapted for submerged fermentation that produces a high biomass yield in a specific medium.

The response surfaces for biomass are shown in Fig. 2. These surfaces provide a visual interpretation of the interaction between the two variables being a variable maintained at the central point and facilitating the location of the optimal conditions of the experiment. Fig. 2A shows the effect of temperature and time for producing biomass. It is possible to observe the small and not significant influence of the quadratic effect of the temperature and time on the response surface. Fig. 2B shows the effect of pH and time on biomass production, demonstrating the reduction of biomass with increasing pH. The quadratic and linear effects can be observed simultaneously. Fig. 2C shows the effect of temperature and pH on the biomass response. The interaction of these two factors was not significant, although the response surface shows an increasingly negative effect on biomass production as the pH rises.

Thus, the response surface results once again show that the use of a culture medium with a more acidic initial pH (4.5) favors the production of the biomass of *A. bisporus* under the conditions tested. This information is important because it suggests that amounts of biomass can be obtained without significant losses in a 4-day cultivation period, favoring the applicability of SmC.

3.3 Monitoring of SmC

Fig. 3 shows the means of the two submerged culture FT-IR spectra with potassium ferricyanide ($K_3[Fe(CN)_6]$) as the internal standard. The multivariate model predicts the substrate and biomass correlation of the concentrations based on NIR spectra. The utility of the FT-IR spectroscopic technique in this work was the combination of chemometric methodology in rapidly classifying the liquid extract and confirmation of the measurement of its chemical constitutes, as performed by Giordano, et al. [21] from biomass spectra and chemical constitutent results. These data can be used to optimize the process for an industrial scale according to Liu and Wang [15] and Mao et al. [17] to obtain higher value-added products from the biomass [6].

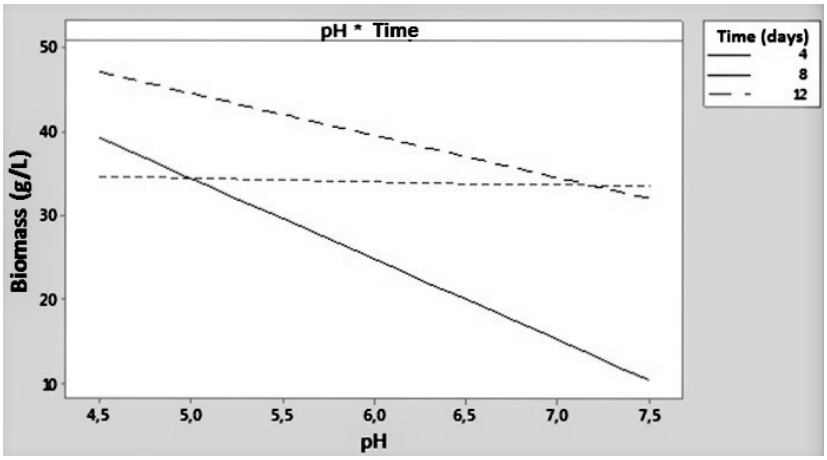

Fig. 1. *A. bisporus* biomass production as function of pH and time

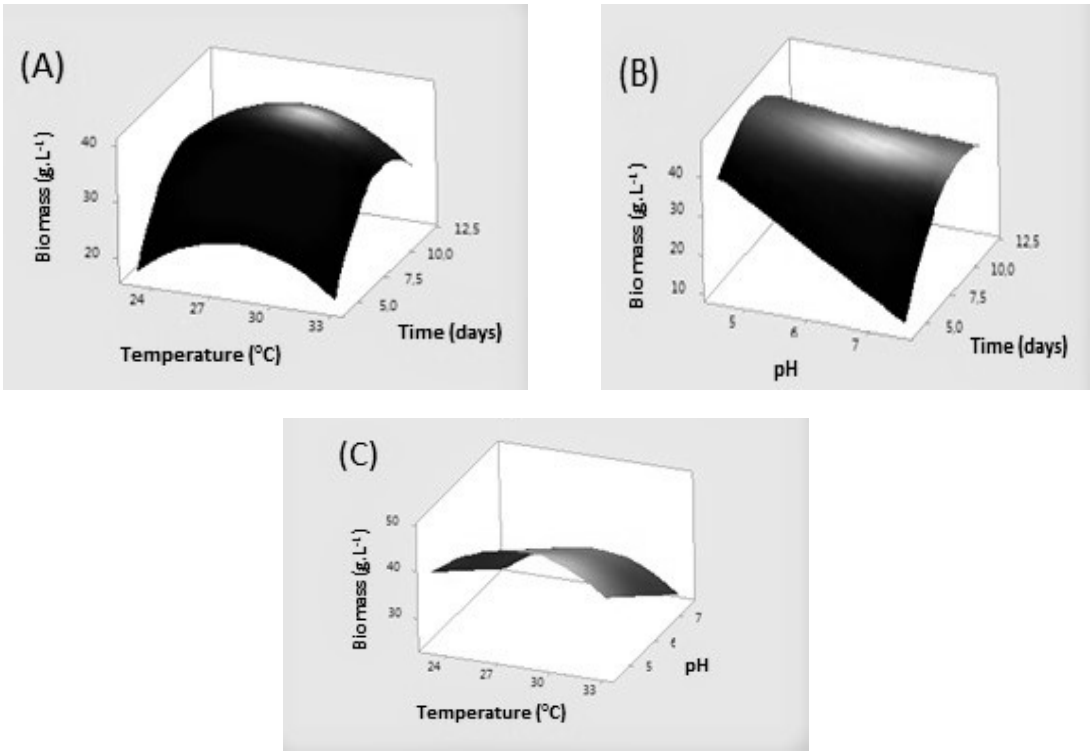

Fig. 2. Response surfaces of biomass through submerged fermentation of *A. bisporus* extract liquid waste of *C. reticulata* (LMW) as a function of (a) temperature and time, (b) pH and time and (c) temperature and pH. The factors that were not included in the axes were set at their optimum level

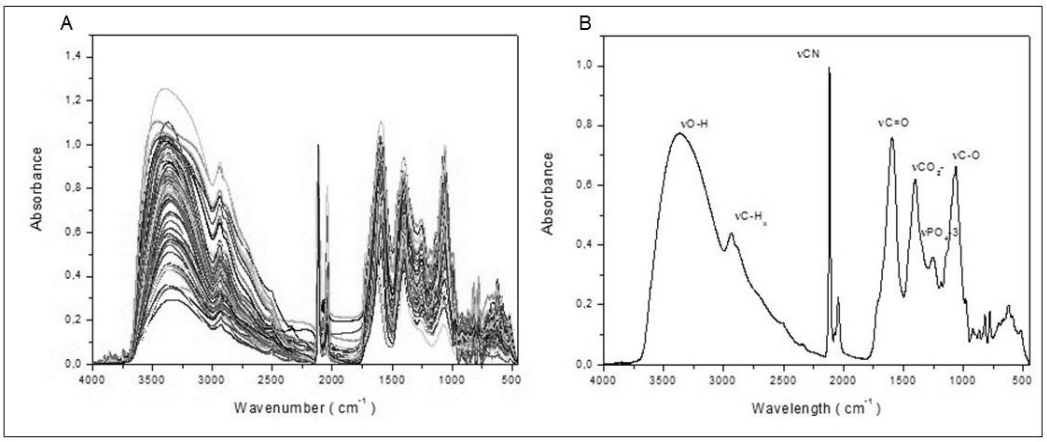

Fig. 3. The normalized spectrum DRIFTS of the submerged cultures containing the set of spectra (a) and the average SmC spectrum (b), both with the internal pattern of potassium ferricyanide in the region of 4000-450 cm^{-1}

The first spectrum shows the set of spectra obtained for SmC. The second spectrum shows the average value of all the analyzed spectra. In Fig. 3A, it is possible to view the set of spectra obtained for SmC. Fig. 3B presents the average value of all these spectra analyzed as well as the identification of the main bands found. The liquid extract spectrum of the submerged cultivation is

characterized by the presence of two protein bands, one at 1650 cm^{-1} (νC=O called band amide I) and another at 1398 cm^{-1} (νC-O, characteristic of peptidic groups in proteins), a phospholipid band at 1250 cm^{-1} (νas P=O), and a polysaccharide band at 1200 cm^{-1} (νC-O-C) since it is possible to determine the presence of sugar bands, which are present in regions between 2500 cm^{-1} and 3300 cm^{-1} (νC-H e νC-OH) [22].

The region between 2000 and 2200 cm^{-1} is assigned to the CN stretch present in potassium ferricyanide, presenting a complex set of bands, two being more intense (2000 and 2200 cm^{-1}). The FT-IR spectra reflect a complex mixture of wavelengths and absorption intensities. It is often difficult to find a single absorption band that can serve as a basis for quantifying a single component.

Based on the spectra, DRIFTS-PLS models were developed for the quantification of pectinolytic atividate (PA), reducing sugars (RS) and total proteins (TP) present in the submerged liquid culture using *C. reticulata* extract. Table 3 shows that it was possible to obtain prediction models for the concentration of PA, RS and TP through FT-IR with good statistical quality. The values obtained for the root mean square error of prediction (RMSEP) and coefficients of determination (R2) for PA, RS and TP were 0.0012 U/mL and 0.9760, 0.23 mmol/L and 0.9956 and 0.03 g/dL and 0.9957, respectively. These values are within the acceptance criteria compared with those obtained in other studies of biological systems.

The liquid extracts had their compositions changed due to the production of biomass. With more biomass produced, there was a greater change in culture medium. This was identified by spectra analysis, confirming the negative influence of pH on the production of biomass. The RMSe indicated the highest biomass yield at eight days of fermentation in the liquid extract. Additionally, a reduction of the substrate can be observed when analyzing the reducing sugars and correlating them with the FT-IR spectra, thus corroborating the results found in the analysis.

These data support the analysis conducted with the liquid extract and demonstrate that the complex composition of the samples analyzed by FT-IR correspond to the compositions shown by chemical methods. Although monitoring the fermentation processes through FT-IR using bacteria or filamentous fungi is analytically more difficult than monitoring unicellular microorganisms, the basis of these processes are subjected to vigorous agitation and aeration and also possess a highly viscous culture medium, with a fluid that presents non-Newtonian rheological behavior, even of those that have been increasingly studied. The analysis of parameters that can be consumed (carbohydrate sources such as glucose, fructose, and lactate) or produced (protein sources, such as ammonia, glutamate, amino acids, and enzymes) during the SmC are used in industrial level bioprocesses, especially when the objective is real-time monitoring [20].

The combination of two well-known techniques leads to improved online applications using FT-IR, even if the two methods show large uncertainty when applied separately [19]. In this sense, the results shown demonstrated an excellent correlation between the spectral data and presence of reducing sugars and total proteins, confirming that the PLS technique aided in the recognition of the substrate composition compared with biomass formation.

Table 3. Model figures of merit PLS / FT-IR submerged culture parameters

Parameter	Range	RMSECV/REP	R^{2**}
PA			
Cal	0.001-0.032	0.0025	0.9122
Val	0.002-0.031	0.0012	0.9760
RS*			
Cal	1.21-18.89	0.11	0.9997
Val	2.48-15.38	0.23	0.9956
TP*			
Cal	0.06-5.65	0.08	0.9979
Val	0.08-1.51	0.03	0.9957

*RMSECV = root mean square error of cross-validation; *with baseline correction; R^2 = calibration coefficient of correlation and cross-validation*

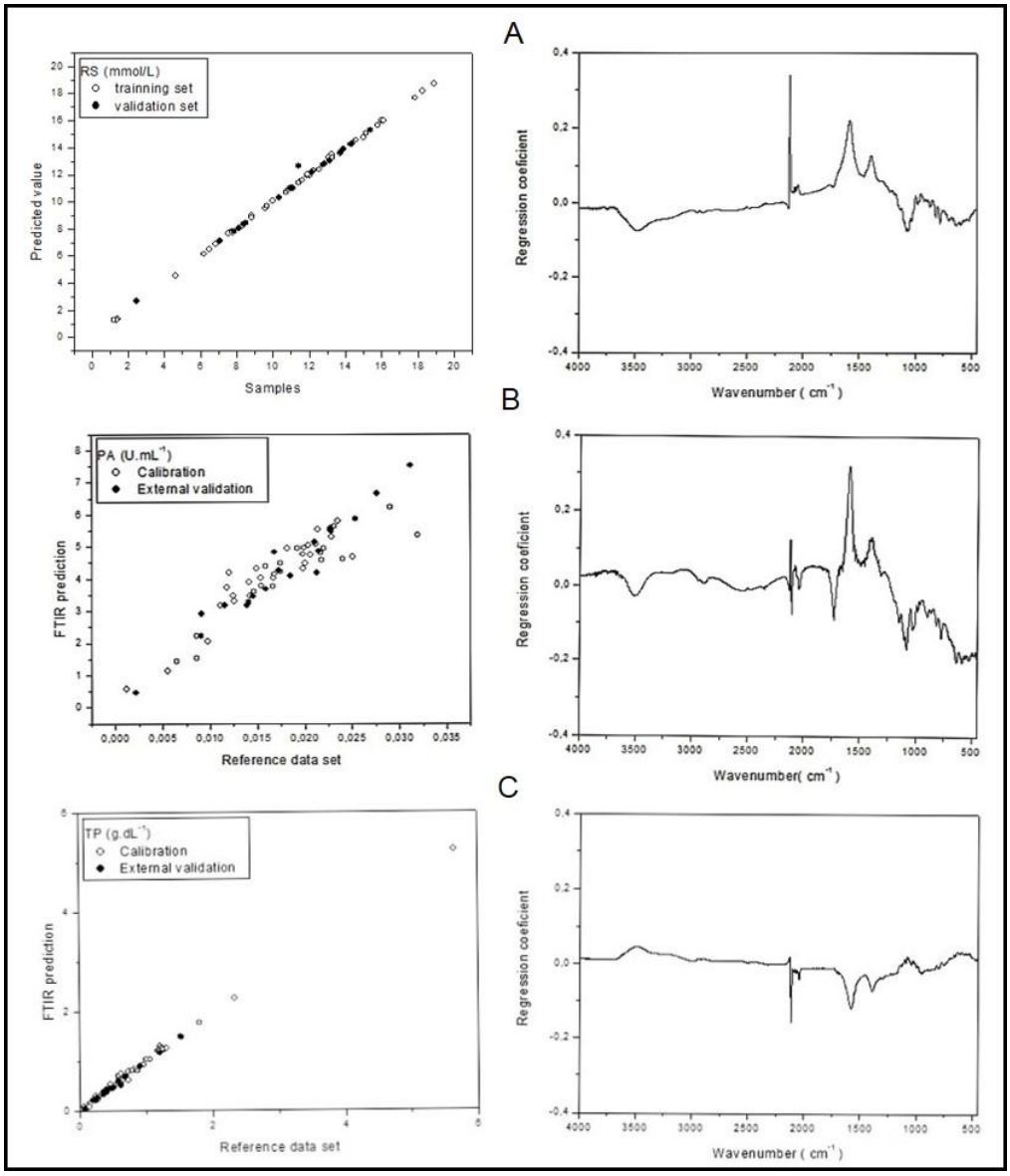

Fig. 4. Shows the correlation curves between the samples for the calibration and validation for RS (Fig. 4A) PA (Fig. 4B) and TP (Fig. 4C) values tabulated with the values predicted by the PLS model, and also with their respective regression vectors

4. CONCLUSION

The use of liquid extract from *C. reticulata* waste proved to be an excellent source of carbon for the biomass production of *A. bisporus*, obtaining the maximum value of 47.06 g/L. SmC optimization using the liquid extract of *C. reticulata* waste was statistically validated for predictive purposes, and response surfaces indicated the interaction between the pH and time, demonstrating that the use of the pH level of 4.5 can produce more biomass than the pH level of 6, and the maximum biomass yield was achieved under the conditions of 28°C, an initial pH of 6 and an 8-day cultivation period. Employing FT-IR for monitoring the bioprocess was effective; however, it is understood that the analysis should be further optimized for this purpose. This opens the opportunity for mycelial production of *A. bisporus* and products derived from the liquid waste of *C. reticulata* as well as faster, eco-friendlier monitoring with FT-IR.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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