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Potential of *Aspergillus niger* Tiegh 8285 in the bioremediation of water contaminated with benzonitrile

Potencial de *Aspergillus niger* Tiegh 8285 na biorremediação de água contaminada com benzonitrila

Potencial de *Aspergillus niger* Tiegh 8285 en biorremediación de agua contaminada con benzonitrila

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Mariana Perin Mituishi
ORCID: https://orcid.org/0000-0002-3620-3999
State University of Santa Cruz, Brazil
E-mail: https://orcid.org/marimituishi@gmail.com
Luiz Henrique Sales de Menezes
ORCID: https://orcid.org/0000-0002-9071-4423
State University of Santa Cruz, Brazil
E-mail: luizmenezes1@gmail.com
Iasnaia Maria de Carvalho Tavares
ORCID: https://orcid.org/0000-0002-0478-7977
State University of Southwest Bahia, Brazil
E-mail: iasnaiamct@gmail.com
Janaina de Silva Freitas
ORCID: https://orcid.org/0000-0002-7691-4701
State University of Southwest Bahia, Brazil
E-mail: silvafreitasbr@yahoo.com.br
Cristina Maria de Souza-Motta
ORCID: https://orcid.org/0000-0002-0964-8271
Federal University of Pernambuco, Brazil
E-mail: cristina.motta@ufpe.br
José Luiz Bezerra
ORCID: https://orcid.org/0000-0002-7917-3400
State University of Santa Cruz, Brazil
E-mail: jlulabezerra@hotmail.com
Andréa Miura da Costa
ORCID: https://orcid.org/0000-0003-2823-1305
State University of Santa Cruz, Brazil
E-mail: amcosta@uesc.br
Ana Paula Trovatti Uetanabaro
ORCID: https://orcid.org/0000-0003-3992-8474
State University of Santa Cruz, Brazil
E-mail: aptuetanabaro@gmail.com
Marcelo Franco
ORCID: https://orcid.org/0000-0002-7827-789X
State University of Santa Cruz, Brazil
E-mail:mfranco@uesc.br
Julieta Rangel de Oliveira
ORCID: https://orcid.org/0000-0001-8571-7294
State University of Santa Cruz, Brazil
E-mail:jroliveira@uesc.br

Abstract
Benzonitrile is a compound found in pesticides. The use of these pesticides can cause environmental contamination, and the search for non-aggressive methods to eliminate these residues is necessary. In this study, fungi *Aspergillus* isolated from cocoa were investigated for their benzonitrile bioremediation potential. The fungi were cultured in a solid medium supplemented with nitrile and glucose (**a**), nitrile (**b**), and glucose (**c**). Independent variables: time, inoculum, and nitrile were optimized using a central composite design to determine the best microbial growth and wet biomass (dependent variable) as a response in the bioremediation process. *A. niger* Tiegh 8285 showed good adaptation, especially in situation **b** in nitrile 5 days, 3 mycelial inoculums and 54 µL of benzonitrile for microbial growth, resulting in 1.83 ± 0.03 g of wet biomass, confirming the efficiency of the selected mathematical model. *A. niger* Tiegh 8285 proved to be a promising bioremediation agent for benzonitrile.
Keywords: Biocatalysis; Central composite design; Microorganism; Optimization.

Resumo

Benzonitrila é um composto encontrado em pesticidas. O uso desses agrotóxicos pode causar contaminação ambiental, exigindo a busca de métodos não agressivos para eliminação desses resíduos. Neste estudo, fungos *Aspergillus* isolados de cacau foram investigados quanto ao seu potencial de biorremediação de benzonitrila. Os fungos foram cultivados em meio sólido suplementado com nitrila e glicose **(a)**, nitrila **(b)** e glicose **(c)**. Variáveis independentes: tempo, inóculo e nitrila foram otimizados usando um planejamento Composto Central para determinar o melhor crescimento microbiano e biomassa úmida (variável dependente) como resposta no processo de biorremediação. *A. niger* Tiegh 8285 apresentou boa adaptação, principalmente na situação **b** em 5 dias, 3 inóculo micelial e 54 µL de benzonitrila para crescimento microbiano, resultando em 1,83 ± 0,03 g de biomassa úmida, confirmando a eficiência do modelo matemático selecionado. *A. niger* Tiegh 8285 provou ser um promissor agente de biorremediação para benzonitrila.

Palavras-chave: Biocatalise; Microrganismos; Otimização; Planejamento composto central.

Resumen

El benzonitrilo es un compuesto que se encuentra en los pesticidas. El uso de estos pesticidas puede causar contaminación ambiental, siendo necesaria la búsqueda de métodos no agresivos para la eliminación de estos resíduos. En este estudio, los hongos *Aspergillus* aislados del cacao fueron investigados por su potencial para la biorremediación del benzonitrilo. Los hongos se cultivaron en medio sólido suplementado con nitrilo y glucosa **(a)**, nitrilo **(b)** y glucosa **(c)**. Variables independientes: tiempo, inóculo y nitrilo fueron optimizaron mediante un diseño de Central Composite para determinar el mejor crecimiento microbiano y biomasa húmeda (variable dependiente) como respuesta en el proceso de biorremediación. *A. niger* Tiegh 8285 mostró buena adaptación, principalmente en situación **b** en 5 días, 3 inóculo micelial y 54 µL de benzonitrilo para crecimiento microbiano, resultando 1.83 ± 0.03 g de biomasa húmeda, confirmando la eficiencia del modelo matemático seleccionado. *A. niger* Tiegh 8285 demostró ser un agente de biorremediación prometedor para el benzonitrilo.

Palabras clave: Biocatálisis; Microorganismos; Mejoramiento; Planificación compuesta central.

1. Introduction

Nitriles are organic compounds with toxic, mutagenic, and carcinogenic properties (Graham et al., 2020). They also cause adverse effects on human health, such as respiratory system inactivation (Heidari & Asoodeh, 2019), loss of hair cells (responsible for balance), neurobehavioral abnormalities, decreased hearing, altered serotonin levels, and corneal opacity (Saldanha-Ruiz, et al., 2012). However, nitriles are widely used as intermediates in organic synthesis reactions (Chmura, et al., 2008; Santos, et al., 2021) such as hydrolysis (Mehtra, et al., 2017; Zhan, et al., 2018) and cycloaddition reactions (Umemoto, et al., 2020). They are also used as ingredients for obtaining plastics (An, et al., 2020), synthetic rubbers (Xiao, et al., 2019), and polymers (Lai et al., 2020), in addition to herbicides and pesticides (An, et al., 2018).

Benzonitrile (C_6H_5CN) is an aromatic nitrile found in the composition of herbicides such as dichlobenil (2,6-dichlorobenzonitrile), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and ioxynil (3,5-diiodo-4-hydroxybenzonitrile) (Pei, et al., 2017). Dichlobenil is commonly used in plant and garden nurseries, while bromoxynil and ioxynil are generally used for weed pest control (Pei, et al., 2017). The metabolites from benzonitrile herbicides are hazardous to human health due to their toxicity and they are considered a risk for soil and groundwater contamination (Pei, et al., 2017).

Any chemical contamination in a natural environment, such as rivers and groundwater, is undesirable in any circumstance. Therefore, the environmental pollution problems associated with social and technological development have spurred the search for different methods of water treatment, the most promising of which is bioremediation (Fu, et al., 2020). Bioremediation is a branch of biotechnology in which microorganisms such as bacteria and fungi (Quintella, et al., 2019) are used to transform contaminating and toxic compounds, such as dioxins (Dao, et al., 2019), agricultural effluents (Neoh, et al., 2016), soil contaminated with oil (Chaudhary, et al., 2019; Li, et al., 2020) or heavy metals and pesticides (Zhang, et al., 2020), and wastewater from the pharmaceutical industry (Shah, et al., 2020) into compounds of low or zero toxicity, while obtaining water and carbon dioxide during the process (Liu, et al., 2017). Moreover, bioremediation has been extensively explored in wastewater treatment (Lu, et al., 2014; Catania, et al., 2020; Hubabillah, et al., 2020; Zhou, et al., 2020) and can be characterized as a renewable process since it uses living organisms. Another advantage is that this process does not require the use of chemical catalysts and the reactions can be carried out at approximate room temperature (Zhang, et al., 2013).

Studies have been carried out involving the bioremediation of several compounds such as *Myceliophthora thermophile* (Salami, et al., 2018) and *Coriolopsis gallica* (Vidal-Limon, et al., 2012) in the bioremediation of environmental contaminants, *Sinanaonta woodiana*, a freshwater mollusk, in the bioremediation of aquaculture effluents (reservoirs for breeding marine species, such as fish and shellfish) (Sicuro, et al., 2020), and *Bacillus velezensis* in the bioremediation of textile dyeing residues (Gowri, et al., 2020).

In bioremediation, each microorganism reacts differently depending on the conditions. Therefore, it is important to provide ideal conditions for the microorganism to increase its bioremediation potential. For this purpose, chemometric techniques are considered useful alternatives. Thus, the aim of this study was to investigate the bioremediation potential of fungi of the genus *Aspergillus*, isolated from the stem and leaves of cocoa in southern Bahia, in the presence of benzonitrile and to optimize the conditions of bioremediation using central composite design (CCD) using the variables time, inoculum, and volume of nitrile.

2. Materials and Methods

2.1 Microorganisms

Aspergillus niger Tiegh. 8068, *A. parasiticus* Speare 7967 and *A. niger* Tiegh. 8285 were isolated from the exterior part of conventional post-harvest and post-fermentation cocoa beans, respectively, in the city of Arataca (Bahia, Brazil), and the endophytic fungi, *Aspergillus* Tiegh. 8066 and *A. niger* Tiegh. 8067 were isolated from stems and leaves at the Matinha Municipal Park (Itapetinga, Bahia, Brazil). The fungal strains are stored in mineral oil, PDA, except *A. parasiticus* Speare 7967, which was stored in mineral oil, Malt, in the URM library of the Mycology Center, Biological Sciences Department at the Federal University of Pernambuco (UFPE, Brazil).

2.2 Screening in a minimal solid mineral medium in the presence of benzonitrile

Screening was carried out in a minimal solid mineral medium [Na2HPO4 (1 g/L); $MgCl_2.7H_2O$ (0.5 g/L); KCl (0.5 g/L); $FeSO_4.7H_2O$ (0.01 g/L); $CoCl_2.6H_2O$ (0.001 g/L); $ZnSO_4.7H_2O$ (0.0067 g/L); agar (15 g/L) supplemented with glucose (15 g/L) and nitrile (200 µL/L) (De Oliveira et al., 2014) in Petri dishes (10x1 cm) and pH 7 in three different situations: glucose and nitrile (**a**), nitrile without glucose (**b**), and glucose without nitrile (**c**). After microbial growth in a BOD incubator (TE-371, Tecnal, Piracicaba, Brazil) at 30ºC for 192 hours, the number of spores was counted using a Neubauer chamber and binocular microscope (BIOVAL L1000).

2.3 Central composite design for optimization of bioremediation of water contaminated with benzonitrile

Aspergillus niger Tiegh 8285, previously grown in a minimum solid mineral medium **a**, was inoculated in a minimum liquid mineral medium (De Oliveira, et al., 2013), situation **a**. An Erlenmeyer flask (250 mL) containing 100 mL of minimal liquid mineral medium supplemented with glucose (1.5 g) was autoclaved (CS Prismatec, Itu, Brazil) at 121°C for 15 min. When it reached room temperature, benzonitrile was added. In parallel, small slices of the minimum solid medium containing the fungus mycelia were cut from the stock culture and inoculated. Then, the experiment was incubated in an orbital shaker (Tecnal, Piracicaba, São Paulo, Brazil) at 30°C at 120 rpm. Finally, the reaction was filtered using a vacuum pump (Prismatec, Itu, Brazil), and the wet biomass was weighed.

The procedure was optimized using a central composite design (CCD) (Dos Santos et al., 2016; Marques et al., 2018) with the independent variables reaction time (t), quantity of inoculum (In), and volume of nitrile (Nit) (Table 1) and the dependent variable wet biomass.

Table 1. Experimental factors and variable levels used in the central composite design (CCD) for optimizing bioremediation of benzonitrile by *Aspergillus niger* Tiegh 8285.

Variables	Unity	Codification	Variable level				
			-α	-1	0	+1	+α
Time	day	t	2.6	4.0	6.0	8.0	9.3
Inoculum	mm	In	1.3	2,0	3.0	4.0	4.7
Nitrile	μL/100 mL	Nit	16.5	30.0	50.0	70.0	83.5

Source: Authors.

The experimental project was modeled and analyzed using Statistica v.12.0 software (Statsoft, USA). The CCD obtained for the maximum biomass production was quadratic, as suggested by the software, and presented in equation 1. In the experimental matrix, the coded values of all parameters vary in five levels (-α, -1, 0, +1, α) (Table 1), totaling 17 experiments (Table 2). At the beginning and end of each reaction, pH was measured only as a reaction indicator.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

Where Y is the predicted value, β0 is constant, β1, β2, β3 are linear coefficients, β12, β13, β23 are coefficients between products, and β11, β22, and β33 are quadratic coefficients. All experiments were performed in triplicate and expressed as average values.

Table 2. Results obtained in the central composite design (CCD), pH measured in the experiments and experimental and predicted results for optimizing bioremediation of benzonitrile by *Aspergillus niger* Tiegh 8285.

Essay	Factors				Damp mass (g)	
	t	In	Nit	pH	Experimental values	Predicted values
1	4.0	2.0	30.0	6.84	1.75	1.70
2	4.0	4.0	70.0	6.91	1.87	1.87
3	8.0	2.0	70.0	6.83	1.73	1.71
4	8.0	4.0	30.0	6.98	1.64	1.66
5	6.0	3.0	50.0	6.52	1.80	1.81
6	4.0	2.0	70.0	6.91	1.82	1.86
7	4.0	4.0	30.0	6.80	1.71	1.78
8	8.0	2.0	30.0	6.88	1.47	1.53
9	8.0	4.0	70.0	7.12	1.65	1.76
10	6.0	3.0	50.0	6.90	1.83	1.81
11	2.6	3.0	50.0	6,83	1.52	1.52
12	9.3	3.0	50.0	7,82	1.37	1.29
13	6.0	1.3	50.0	6.52	1.56	1.57
14	6.0	4.7	50.0	6.53	1.78	1.68
15	6.0	3.0	16.5	6.50	2.11	2.08
16	6.0	3.0	83.5	6.50	2.35	2.30
17	6.0	3.0	50.0	6.52	1.79	1.81

Source: Authors.

2.4 Model adequacy and validation

The adjustment quality of the statistical model was verified by the coefficient of determination (R^2), and the significance of the model was tested by Fisher's test (F-value) through analysis of variance (ANOVA), according to Ferreira et al 2007. Model adequacy was analyzed using the observed vs predicted graph. The interactions between the variables and their influence on the response obtained were analyzed using a Pareto chart, and the optimal point of maximum bioremediation was obtained using equation two and the response surface graph. The selected statistical model was validated by running the experiment under optimized conditions and comparing it with the expected response (Ferreira, et al., 2007; Bezerra, et al., 2020).

3. Results and Discussion

3.1 Screening in a minimal solid medium in the presence of benzonitrile

Fungi of the genus *Aspergillus* from different biomes in the state of Bahia with biocatalytic potential in the presence of aromatic nitriles were selected through screening in a minimal solid mineral medium supplemented with glucose and benzonitrile, according to situations **a**, **b**, and **c**, using quantitative evaluation through the number of spores (Table 3). As observed in Table 3, the fungi showed microbial growth in situations **a**, **b**, and **c** and a better adaptation in media supplemented with benzonitrile (**a** and **b**), indicating the inducing role of nitrile since it is the only source of nitrogen, as well as the possible biocatalytic potential of the respective fungi. These results corroborate data from the literature that stress the importance of nitriles as an inducer in microbial growth (Coady, et al., 2013; De Oliveira, et al., 2013; De Oliveira, et al., 2014), in particular, benzonitrile (Agarwal, et al., 2017; Serra, et al., 2019).

Table 3. Results obtained from *screening* in minimal solid mineral medium for benzonitrile by counting spores using a Neubauer chamber and binocular microscope.

Fungi	Glucose and nitrile (spores x mL^{-1})	Nitrile without glucose (spores x mL^{-1})	Glucose without nitrile (spores x mL^{-1})
A. niger Tiegh. 8068	14.2 x 10^4	19.1 x 10^4	14.3 x 10^4
A. niger Tigeh. 8285	19.4 x 10^4	63.2 x 10^4	1.8 x 10^4
A. parasiticus Speare 7967	12.6 x 10^4	7.73 x 10^4	5.8 x 10^4
A. niger Tiegh. 8066	27.7 x 10^4	21.5 x 10^4	13.0 x 10^4
A. niger Tiegh. 8067	23.6 x 10^4	13.8 x 10^4	7.3 x 10^4

Source: Authors.

According to Table 3, *A. niger* Thiegh 8285 showed a significant increase in spores in the situation that only contained benzonitrile **b** (63.2 x 10^4 spores x mL^{-1}), which represents an increase of approximately 35 times compared to situation **c**. The good adaptation of *A. niger* Thiegh 8285 revealed that the addition of benzonitrile alone in a medium with minimal amounts of nutrients was sufficient to supply the needs of the microorganism.

3.2 Optimization of bioremediation

From the results obtained from screening in minimal solid mineral medium (Table 3), *A. niger* Tiegh 8285 was selected to evaluate its potential in the benzonitrile bioremediation process through the central compound design with 17 experiments (Table 2).

The analysis of variance (ANOVA) calculated model efficiency and adequacy for the experimental design used, as shown in Table 4. The computed *F* value (22.80) for the model was considerably higher than in the Table 4 (3.67), showing that the model was significant.

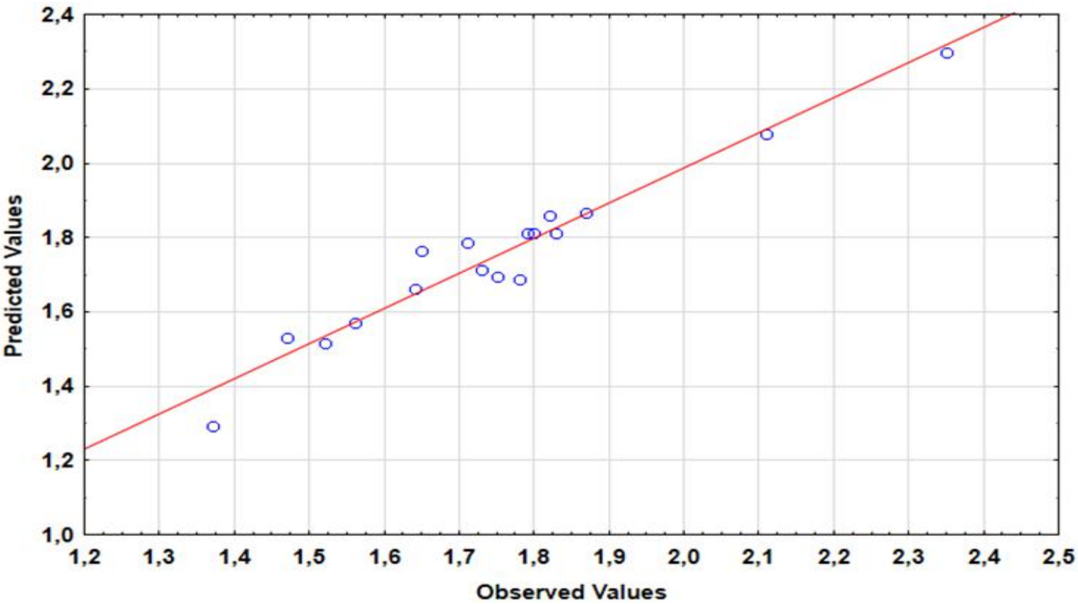
Table 4. Analysis of variance to adjust the quadratic model with a 95% confidence level.

Factor	SS	df	MS	F calc.	F tab.
Model	0.82	9	0.091	22.80	3.67
Residue	0.028	7	0.003985		
Lack of fit	0.027	5	0.005405		
Pure error	0.000867	2	0.000433		
Total	0.845612	16			
R^2	0.97				
R^2-adj	0.92				
SS - Sum of squares; DF= Degree of freedom; MS = Medium square					

Source: Authors.

The model's capacity was assessed using the R^2 determination coefficient, which was calculated to be 1.0. The value found for R^2 (0.97) indicates that the model reported 97% of the experimental data, and there were only 3% of errors, to which noise can be attributed. The observed vs. predicted graph (Figure 1) corroborates the model's adjustability, with experimental values close to the predicted values, indicating the authenticity of the polynomial model.

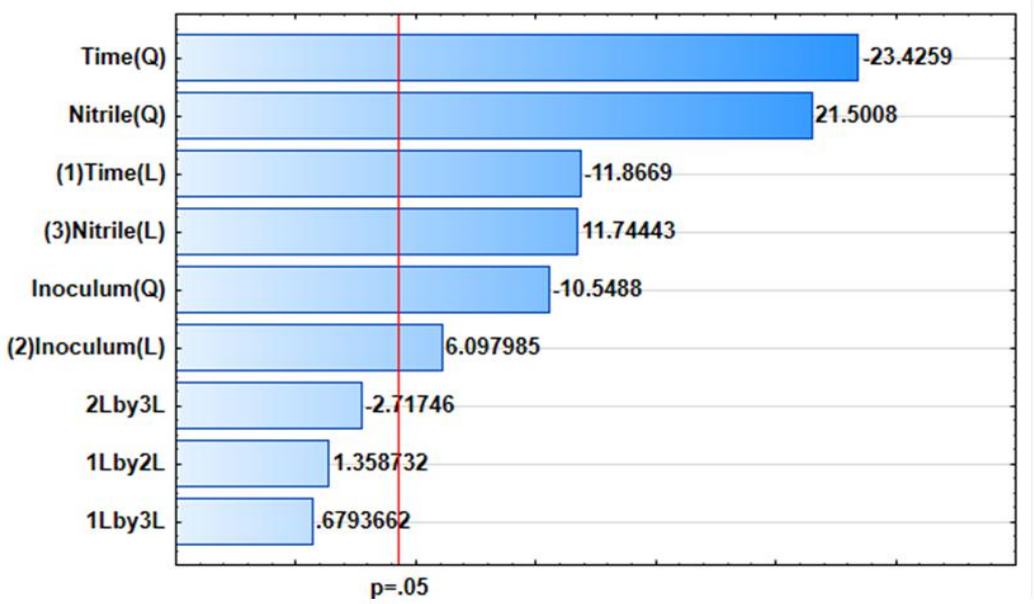
Figure 1. Observed vs. predicted graph showing the approximation between the results obtained experimentally from wet biomass in each of the experiments of the central composite design (CCD) and the theoretically indicated values (red line).



Source: Authors.

According to the analysis of the Pareto chart (Figure 2), the variables that most influence the response (P <0.05) were quadratic effect of time and nitrile. The quadratic variable time was the most significant variable, with a negative correlation, indicating that an increase in time reacts negatively to the response and results in a decrease in wet biomass, revealing that all the nutrient is bioconsumed in a short time; therefore, *A. niger* Thieg 8285, in addition to being resistant, needs little time to bioremediate nitrile. The quadratic variable had a positive correlation, indicating that, as the volume of nitrile increased, the expected response (biomass) also increased since the culture medium is deficient in nutrients and the only source of nitrogen is the benzonitrile itself. This result shows that *A. niger* Thieg 8285 was induced to consume nitrile to survive, thus proving it adapted to the established conditions.

Figure 2. Pareto chart for optimization of bioremediation of water contaminated with nitrile according to independent variables time, inoculum, and volume of nitrile.



Source: Authors.

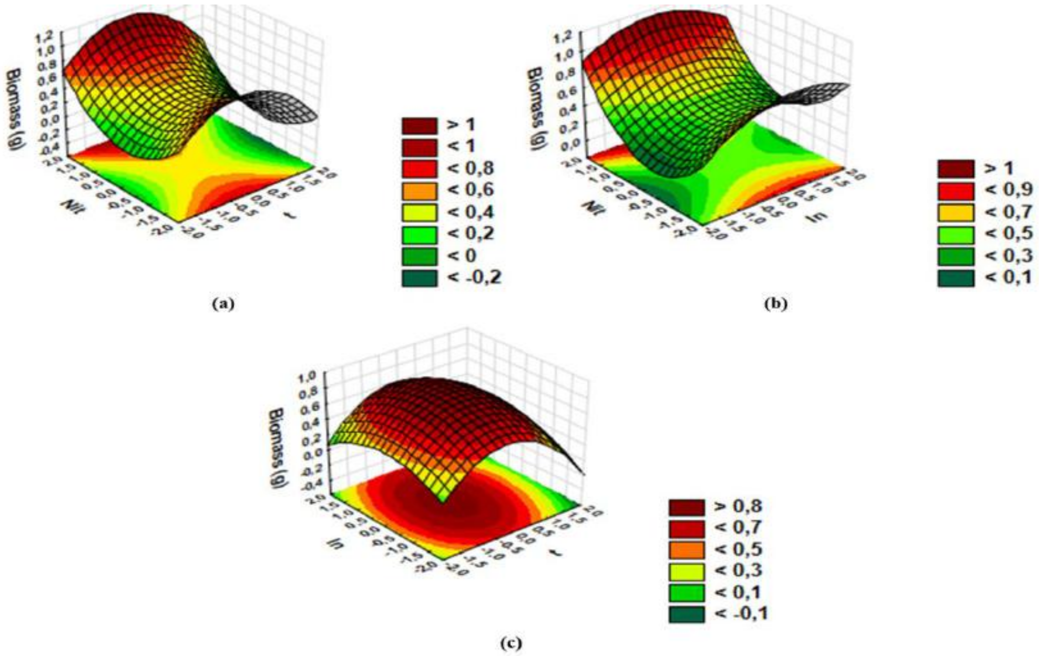
The analysis of the Pareto chart allowed selection of statistically significant terms at 95% significance and removed the non-significant terms (*P* >0.50) from the mathematical model. After being the adequacy of the model approved using ANOVA, response surfaces (Figure 3a, b and c) were obtained, making it possible to observe the influence of combinations between the independent variables.

Polynomial Eq. 2 was used to express the relationship between the coded independent variables and to predict a response:

$$Y = 1.81 - 0.067X_1 + 0.034X_2 + 0.066X_3 - 0.146X_1^2 - 0.066X_2^2 + 0.134X_3^2 + 0.01X_1X_2 + 0.005X_1X_3 - 0.02X_2X_3 \quad (2)$$

Based on equation 2 and the response surface, the maximum biomass production point of reaction was 5 days and 12 hours, inoculum 3.38 mm and 54.31 µL of benzonitrile with theoretical production of 1.82 g of wet biomass. The reaction time obtained (5 days and 12 hours) is within the average time interval for several species of *A. niger*, which is between 5 (Sattar, et al., 2019; Papadaki, et al., 2020; Putri, et al., 2020) and 7 days (Khan, et al., 2019; Aboyeji, et al., 2020). The volume of nitrile obtained from the statistical design used (CCD) revealed that the microorganism studied has resistance to nitrile since microorganisms generally have limited tolerance to high concentrations of nitriles due to their toxicity (Sattar, et al., 2019).

Figure 3. Response surface for the dependent variable (wet biomass) considering the interactions between (a) nitrile volume versus time, (b) nitrile volume versus inoculum and (c) inoculum versus time. The figures were obtained with Statistica v.12.0 software (Statsoft, USA).



Source: Authors.

3.3 Statistical validation of bioremediation

For model validation, experiments at the optimum point were performed in triplicate using the ideal conditions provided by equation 2 and the response surface (Figure 3). The wet biomass value predicted by the model was 1.82 g. By performing the optimal point experiment in triplicate, it was possible to obtain an average of 1.83 ± 0.03 g and a recovery of approximately 100% between the theoretical and the experimental value. These results corroborate those obtained in the central composite planning and validate the use of the proposed method for the bioremediation of water contaminated with benzonitrile by *A. niger* Tiegh 8285.

4. Conclusion

The role of benzonitrile as an inducer was confirmed with fungi of the genus *Aspergillus* of the state of Bahia, particularly *Aspergillus niger* Tiegh 8285, isolated from cocoa beans. This study has a high scientific value since a chemometric tool (central compound design) was used for the statistical optimization of bioremediation of water contaminated with benzonitrile by *A. niger* Thiegh 8285. Moreover, this study is relevant in terms of environmental preservation concerns by presenting a promising method for the treatment of water contaminated with residual nitriles. The result is encouraging for future studies on the bioremediation of aromatic nitriles.

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