

Multivariate analysis of nutritional parameters required for the submerged cultivation of three coprinaceous fungi for biosurfactant production

Análise multivariada dos parâmetros nutricionais necessários ao cultivo submerso de três fungos coprináceos para produção de biosurfactante

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ABSTRACT

Biosurfactants are natural hydrocarbon surfactant compounds that decrease surface tension and have a high emulsifying capacity. Biosurfactants are produced through microbial metabolic processes and have several advantages over chemical surfactants, including low toxicity and high biodegradability. Bioremediation offers the possibility of eliminating or transforming various contaminant compounds present in soil and water. Fungi are effective transforming agents due to their ability to degrade a diverse variety of organic substances. Thus, considering the potential of fungi to produce biosurfactants and the importance of these compounds for various industrial processes, the objective of this work was to verify the effects and interactions of different concentrations of various nutrients on biosurfactant production in three coprinaceous fungi isolates. These isolates were obtained from spent mushroom substrate (SMS) produced during the edible mushroom growth process. All three fungal isolates, termed FS-4.1, FS-4.2, and FS-4.3, were potential sources for biosurfactant production, as evidenced by the emulsifier index of the fermentation medium. However, biosurfactant production was maximum in isolate FS-4.1, yielding an emulsifier index of 73.45%. To optimize the FS-4.1 biosurfactant production process, corn oil, glycerol, and yeast extract must be absent from the fermentation medium (to minimize negative effects on the process) and increase the amounts of glucose and soy oil.

Index terms: Biological surfactant; emulsifier index; *Coprinus*.

RESUMO

Biosurfactantes, são emulsificantes naturais de hidrocarbonetos que diminuem a tensão superficial e têm alta capacidade de emulsão. Os biosurfactantes são produzidos pelo metabolismo de microrganismos e apresentam vantagens sobre o uso de surfactantes químicos, principalmente por sua baixa toxicidade e alta biodegradabilidade. A biorremediação é uma opção para a transformação de vários compostos contaminantes no solo e na água, sendo que os fungos são agentes transformadores eficazes, dada a sua capacidade de degradar uma ampla diversidade de substâncias orgânicas. Assim, considerando o potencial dos fungos na produção de biosurfactantes e a importância desses compostos para vários processos industriais, o objetivo deste trabalho foi verificar o efeito e as interações das concentrações de diferentes nutrientes na produção de biosurfactantes, utilizando três fungos coprináceos isolados do substrato pós-cultivo de cogumelos (*spent mushroom substrate* - SMS) resultante do processo de cultivo de cogumelos comestíveis. Os isolados fúngicos filamentosos codificados FS-4.1, FS-4.2 e FS-4.3 demonstraram potencial de produção de biosurfactantes, evidenciado pelo índice emulsificante no meio de fermentação. No entanto a produção biosurfactante foi máxima no isolado FS-4.1, produzindo um índice de emulsificante de 73,45%. Para otimizar o processo de produção biosurfactante FS-4.1 o óleo de milho, o glicerol e o extrato de levedura devem estar ausentes do meio de fermentação (para eliminar os efeitos negativos no processo) e aumentar as quantidades de glicose e óleo de soja.

Termos para indexação: Surfactante biológico; índice emulsificante; *Coprinus*.

INTRODUCTION

Biosurfactants are biopolymers of microbial origin (bacteria, yeasts, and fungi) that are composed of hydrophobic and hydrophilic moieties. Biosurfactants are a viable alternative to synthetic surfactants, characterized by low toxicity and high biodegradability, and are amenable to low-cost production using agro-industrial raw materials (Mukherjee; Das; Sen, 2006). Biosurfactants comprise a large group of chemical compounds, including glycolipids, lipopeptides, phospholipids, lipoproteins, and lipid-polysaccharide complexes (Van Hamme; Singh; Ward, 2006). Their potential applications include their use as emulsifiers, conditioners, cosmetics, and in food industries (Asgher et al., 2020). Previous studies have mostly described the production of biosurfactants from bacteria (Joshi et al., 2008; Zhao et al., 2017; Rani; Weadge; Jabaji, 2020). In contrast, the production of fungal biosurfactants has rarely been described in the literature, although some studies have reported on the ability of specific fungi, mainly ascomycetous yeasts and filamentous fungi, to produce these compounds (Sen et al., 2017; Sanches et al., 2018; Da Silva et al., 2019). Although biosurfactants are produced by all fungal species, previous studies on biosurfactant production from basidiomycetes are scarce.

Biosurfactants play an important role in the bioremediation of impacted environments, as they facilitate the emulsification and solubilization of hydrophobic compounds, making them available for subsequent biodegradation (Karlapudi et al., 2018). As a consequence of this process, microorganisms can utilize the chemical contaminants in the soil as a source of energy and, through oxidation-reduction reactions, metabolize these compounds. Biosurfactants are synthesized by bacteria and fungi using a variety of substrates, including sugars, glycerol, oils, hydrocarbons, and alternative substrates such as agro-industrial residues (Cooper; Goldenberg, 1987; Khopade et al., 2012; Das; Kumar, 2019).

Within the group of xenobiotic-degrading fungi, lignolytic fungi that act on lignin degradation are exceptional, as they can tolerate pollutants at concentrations toxic to other organisms (Van den Brink et al., 1998). The enzymatic systems of lignolytic fungi is composed of lignin peroxidase, laccase, and manganese-dependent peroxidase, which catalyze the metabolization of lignin-like structures, including polycyclic aromatic hydrocarbons (PAHs) and phenols (Eggen; Sasek, 2002). Although the utilization of fungi for the removal of pollutants has been studied for the last 30 years, it has gained prominence in recent years. In recent decades,

there has been a steady increase in research concerning the production of biosurfactants by filamentous fungi (Soares et al., 2011; Pereira; Duvoisin; Albuquerque, 2017).

Following demonstration of their usefulness for biodegradation and their use of enzymatic systems that biochemically transform organic compounds into a nutrient and energy source, fungi are considered an attractive and promising group of microorganisms for use in the degradation of contaminants and for the recovery of degraded areas. Spent mushroom substrate (SMS) refers to the biomass waste generated from mushroom production (Lin; Ge; Li, 2014). SMS contains a rich microbiota and composition that can be used in a variety of applications, including bioremediation agents, feed supplements, substrates in agriculture, energy, and wastewater treatment (Rinker, 2017; Moraes et al., 2020).

Any study on the biosurfactant production should investigate the factors that influence microbial growth, including the optimum environmental and nutritional conditions necessary for growth (Varjani; Upasani, 2017). Data concerning factors that affect growth, such as requirement for carbon, nitrogen, phosphorus, iron, manganese, and magnesium, as well as pH, temperature, agitation, oxygen availability, and process conduction, are of significant value in any study on production efficiency. Depending on the culture medium composition, microorganisms may produce biosurfactants with variable properties, especially when there are changes in the carbon source (Banat, 1995; Fontes et al., 2008).

Choice of carbon and nitrogen sources based on the classic optimization method (changing an independent variable while keeping others fixed) is both time-consuming and expensive, especially in the case of a large number of variables. In addition, observing the effects of variables and their interactions is important to understand the processes undergoing in a specified system (Pereira-Filho; Poppi; Arruda, 2002). The limitations of the single-factor optimization process can be overcome through the use of multifactor statistical experimental designs, such as the factorial and surface response experimental design (Joshi et al., 2008). According to Peralta-Zamora, Moraes and Nagata (2005), factorial experimental planning allows us to simultaneously evaluate the effect of a large number of variables from a small number of experimental trials.

In this context, the objective of the present study was to verify the effects and interactions of different concentrations of nutrients on biosurfactant production and to study the ideal conditions for biosurfactant production by coprinaceous basidiomycetes isolated from SMS coded as FS-4.1, FS-4.2, and FS-4.3.

MATERIAL AND METHODS

Selection of fungal producers of biosurfactants

Basidiomycetous fungi isolated from SMS were maintained in test tubes containing potato dextrose agar (PDA) at 4°C. To select the biosurfactant-producing fungi, we analyzed the emulsification index of the culture filtrate. Three isolates (FS-4.1, FS-4.2, and FS-4.3) showed a high emulsification index. These three isolates were identified as *Coprinus* sp. by their characteristic macroscopic and microscopic features, as described by Largent, Sultz and Hadley (1986) and Putzke and Putzke (2017).

Experimental design

The fractional factorial design $2^{(6-2)}$ was obtained using the Statistica 8.0 computer program. The fractional factorial design was used to reduce the number of experiments. Based on the systematic sequencing of factorial experiments, it was possible to statistically test the significance of the factors' effects, and develop empirical models that facilitate predicting the effects of specific combinations of factors on the system response variables, according to the testing interval taken into consideration (Montgomery, 1991).

Emulsifying activity was considered as the dependent variable, while soybean oil (X1), corn oil (X2), glycerol (X3), glucose (X4), ammonium sulfate (X5), and yeast extract (X6) were the independent variables studied in the factorial design $2^{(6-2)}$ as described in Table 1.

Inoculum preparation and inoculation

Inoculum were prepared by growing the fungal isolates FS-4.1, FS-4.2, and FS-4.3 in potato dextrose broth supplemented with yeast extract (PDYE) and incubated at 25 °C for 48 h while shaking at 200 rpm. Subsequently, 1% (v/v) aliquots of the inoculum were transferred to

10 experimental Erlenmeyer flasks containing synthetic medium for the submerged fermentation experiment. The synthetic medium (the independent variable) contained: soybean oil (X1); corn oil (X2); glycerol (X3); glucose (X4); ammonium sulfate (X5); and yeast extract (X6). The exact concentrations of each were established by the factorial design determined using the Statistica 8.0 computer program (Table 2). After transferring the inoculum, the flasks were incubated at 25 °C for 144 h while shaking at 200 rpm.

Emulsifying index

The emulsifying index was determined according Cooper and Goldenberg (1987) with slight modifications. The centrifuged fermented medium (2 mL) was transferred to test tubes and 2 mL of kerosene was added to it. The mixture was vortexed for 2 min and the tubes were left undisturbed for 24 h. The percentage emulsifier index was calculated according to Equation 1:

$$\text{Emulsifier index (\%)} = (Hex100)/Ht \quad (1)$$

where,

He = emulsion height (cm);

and, Ht = total height of the solution (cm).

Statistical analyses

The experimental design and statistical analyses were evaluated using Statistica® 8.0 (Weib, 2007).

RESULTS AND DISCUSSION

The coefficient of determination (R^2) of 0.999998 demonstrated an excellent relationship (99.99%) between the experimental data and the curve established by the mathematical model. Therefore, only 0.01% of the variation in the emulsifying index could not be explained by the factors studied.

Table 1: Matrix of experimental design $2^{(6-2)}$ and the levels of the variables studied. Details of the 10 experiments chosen using the Statistica 8.0 computer program.

Variables	Unit	Code	Levels		
			Minimum	Central	Maximum
Soybean oil	mL 100 mL ⁻¹	X1	2.0	11	20.0
Corn oil	mL 100 mL ⁻¹	X2	2.0	11	20.0
Glycerol	mL 100 mL ⁻¹	X3	2.0	11	20.0
Glucose	g 100 mL ⁻¹	X4	1.0	5,5	10.0
Ammonium sulfate	g 100 mL ⁻¹	X5	0.2	1.10	2.0
Yeast extract	g 100 mL ⁻¹	X6	0.2	1.10	2.0

Using the experimental results, biosurfactant production was estimated from the emulsifying index in the fermented medium containing the three isolates (FS-4.1; FS-4.2; FS-4.3) (Table 3).

Table 3 shows the emulsifying index values obtained for each isolate when grown under different experimental conditions. The emulsifying index of isolate FS-4.1 ranged from 6.06% to 73.45%. The emulsifying index of isolate FS-4.2 ranged from 0.26–57.10%. The emulsifying index of isolate FS-4.3 ranged from 7.30–68.82%. The experimental conditions yielding the lowest emulsifying index for each isolate were: isolate FS-4.1, Experiment 08 (6.06%); isolate FS-4.2, Experiment 05 (0.26%); and isolate FS-4.3, Experiment 10 (7.18%). The

highest emulsifier index was obtained using isolate FS-4.1 with Experiment 03 (73.45%) (Table 3). Therefore, optimum growth conditions for producing biosurfactant were achieved using a medium consisting of 2% soybean oil, 20% corn oil, 2% glycerol, 1% glucose, 2% ammonium sulfate, and 0.2% yeast extract. In contrast, treatment with the maximum concentration (Experiment 08) presented an unfavorable condition for biosurfactant production, as evidenced by the low emulsifying index (6.06%).

In Experiment 03, which yielded an emulsifier index of 73.45% for FS-4.1, corn oil and ammonium sulfate were the only components present at their maximum concentrations (Table 3). However, the second best emulsifier index (67.71%) was obtained using

Table 2: Fractional factorial design $2^{(6-2)}$.

Experiments	Soybean oil (mL/100mL)	Corn oil (mL/100 mL)	Glycerol (mL/100 mL)	Glucose (g/100mL)	Ammonium sulfate (g/100 mL)	Yeast extract (g/100mL)
1	2.0	2.0	2.0	10.0	2.0	2.0
2	2.0	2.0	20.0	10.0	0.20	0.20
3	2.0	20.0	2.0	1.0	2.0	0.20
4	2.0	20.0	20.0	1.0	0.20	2.0
5	20.0	2.0	2.0	1.0	0.20	2.0
6	20.0	2.0	20.0	1.0	2.00	0.20
7	20.0	20.0	2.0	10.0	0.20	0.20
8	20.0	20.0	20.0	10.0	2.0	2.0
9	11.0	11.0	11.0	5.5	1.10	1.10
10	11.0	11.0	11.0	5.5	1.10	1.10

Table 3: Percentage emulsifying index of filamentous fungus species.

Experiments	Independent variables						Emulsifying Index (%)		
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	FS-4.1	FS-4.2	FS-4.3
1	2	2	2	10	2	2	58.50	55.45	7.30
2	2	2	20	10	0.2	0.2	67.71	6.94	36.69
3	2	20	2	1	2	0.2	73.45	1.67	44.79
4	2	20	20	1	0.2	2	11.25	10.33	56.25
5	20	2	2	1	0.2	2	54.50	0.26	55.40
6	20	2	20	1	2	0.2	59.76	1.83	45.03
7	20	20	2	10	0.2	0.2	62.50	57.10	68.82
8	20	20	20	10	2	2	6.06	29.34	10.97
9	11	11	11	5.5	1.1	1,1	63.07	36.39	8.51
10	11	11	11	5.5	1.1	1.1	63.55	26.02	7.18

Source: Own.

Experiment 2, with glycerol at 20% and glucose at 10%, while the second worst emulsifier index (11.25%) was obtained using Experiment 4, with corn oil and glycerol both at 20%. The combination of soybean oil with corn oil or glycerol did not reduce the emulsifier index significantly (62.50 and 59.76, respectively). These results indicate that various combinations of culture medium components yield high values of emulsifier index.

The difference in emulsifier index values obtained between Experiment 3 and Experiment 8 (73.45%-6.06%) indicated that the emulsifying index increased by 67.39% when the concentration of these variables varied from their maximum level to their minimum level (while maintaining the concentration of the variables corn oil and ammonium sulfate at their maximum levels). Lack of adequate nutrients may limit high production of biosurfactant (Aguilar et al., 2015). However, the lower percentage of emulsification obtained in Experiment 8 may be related to excess of one or more components, or to the combination of different components at their maximum level.

For the isolates FS-4.2 and FS-4.3, the best results were obtained using Experiment 7, which yielded emulsifier indexes of 57.10% and 68.82%, respectively. For the same Experiment, FS-4.1 showed an emulsifier index of 62.50%, which was higher than the other isolates (although it was not the highest index). Therefore, we may conclude that the best Experiment for FS-4.1 was different from the best Experiment for FS-4.2 and FS-4.3. Likewise, the worst Experiment also differed for each isolate. It is worth noting that FS-4.1 showed an emulsifier index above 50% with eight Experiments, whereas for FS-4.2 and FS-4.3, an emulsifier index above 50% was

obtained only with two and four Experiments, respectively. These results provide evidence of a higher potential for biosurfactant production from the isolate FS-4.1, which demonstrated high biosurfactant production in a wider range of cultivation media.

According to the statistical analyses, the independent variables and their interactions produced different positive and negative effects on the emulsifier index for the three isolates (Table 4).

Glycerol was the only independent variable to exert a negative effect on the emulsifier index for isolate FS-4.2 (-16.5100). However, this effect was not statistically significant at the 95% confidence level ($p \geq 0.05$). Although the interaction between soybean oil and yeast extract ($X_1 * X_6$) produced a negative effect (-21.6250), this was not statistically significant ($p \geq 0.05$). Likewise, while the interaction between soybean oil, corn oil, and glucose ($X_1 * X_2 * X_4$) produced a negative effect (-21.6800), this was not statistically significant at the 95% confidence level ($p \geq 0.05$).

For isolate FS-4.3, the independent variables that had a negative effect on the emulsifier index were glycerol (-6.8401), glucose (-19.4228), ammonium sulfate (-27.2671), and yeast extract (-16.3544). The effects obtained with glucose, ammonium sulfate, and yeast extract were statistically significant at the 95% confidence level ($p \leq 0.05$). Although the interaction between soybean oil and yeast extract ($X_1 * X_6$) produced a negative effect (-7.3856), this was not statistically significant ($p \geq 0.05$). In contrast, the interaction between the variables, soybean oil, corn oil, and glucose ($X_1 * X_2 * X_4$) produced a positive effect (65.6220), which was statistically significant at the 95% confidence level ($p \leq 0.05$).

Table 4: Estimates of the effects of each variable, their interactions, and significance ($p \leq 0.05$) for the emulsifier index.

Variables	Estimates of the effects		
	FS-4.1	FS-4.2	FS-4.3
Soybean oil (X_1)	-7.0218*	3.5350	8.7956*
Corn oil (X_2)	-21.8023*	8.4900	9.1023*
Glycerol (X_3)	-26.0448*	-16.5100	-6.8401
Glucose (X_4)	-1.0492	33.6850	-19.4228*
Ammonium sulfate (X_5)	0.4533	3.4150	-27.2671*
Yeast extract (X_6)	-33.2769*	6.9600	-16.3544*
$X_1 * X_6$	2.4290	-21.6250	-7.3856
$X_1 * X_2 * X_4$	-28.1847*	-21.6800	65.6220*

*indicates statistical significance at the 95% confidence level ($p \leq 0.05$).

Accorsini et al. (2012) studied the production of biosurfactants by yeast and found that soybean oil and glucose are excellent carbon sources for biosurfactant production. The emulsifying indices obtained in this work corroborated the findings of the earlier studies which reported emulsifying indices between 49–64.3% (Dikit et al., 2010), 53.0% (Luz et al., 2011), $62.5\% \pm 0.88$ (Decesaro et al., 2013), 61.00% (Santos; Linard, 2004), and 48.89% (Mollea; Bosco; Ruggeri, 2005).

Figures 1, 2, and 3 show Pareto graphs of the significant effects ($p \leq 0.05$) of isolates FS-4.1, FS-4.2, and FS-4.3, respectively. All values to the right of the dashed line are statistically significant ($p \leq 0.05$). Analyzing the Pareto graph of isolate FS-4.1 (Figure 1), we observed that

yeast extract exerted the most negative effect, while glucose exerted the least relevant negative effect on biosurfactant production. On the contrary, the most significant positive effect on biosurfactant production was caused by the interaction between soybean oil and yeast extract ($X1 * X6$), while ammonium sulfate had the least positive effect.

Analyzing the Pareto graph of isolate FS-4.2 (Figure 2), we observed that glucose had the most positive effect on biosurfactant production, while ammonium sulfate had the least positive effect. The interaction between soybean oil and yeast extract ($X1 * X6$) had the most negative effect on biosurfactant production, while the interaction between soybean oil, corn oil, and glucose ($X1 * X2 * X4$) had the least relevant negative effect.

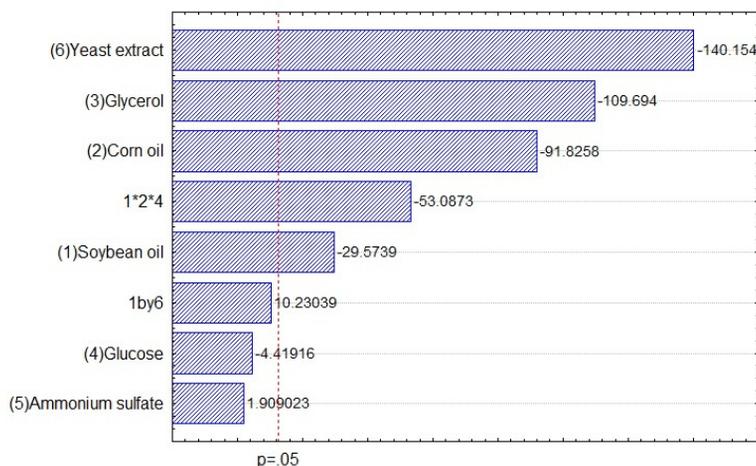


Figure 1: Pareto graph of isolate FS-4.1 with standardized absolute values of the effects of each variable and their interactions.

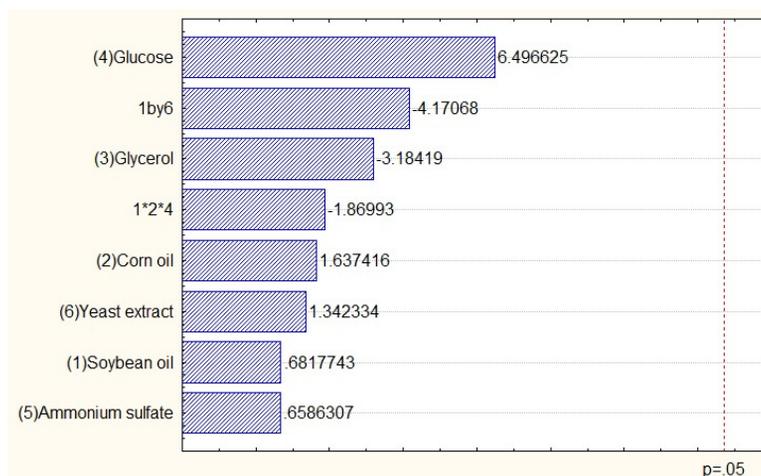


Figure 2: Pareto graph of isolate FS-4.2 with standardized absolute values of the effects of each variable and their interactions.

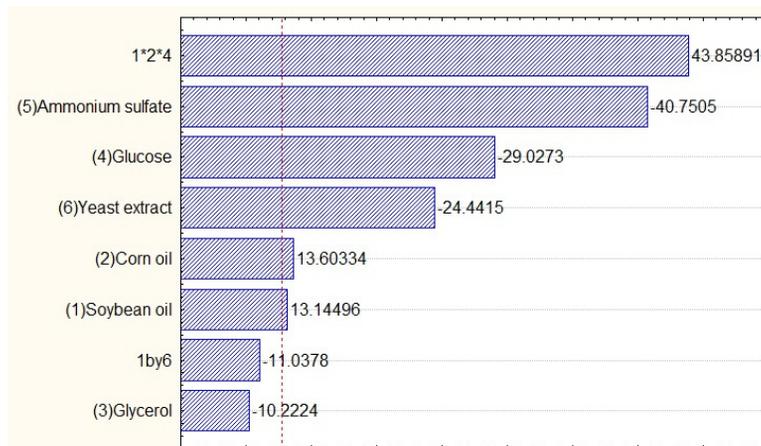


Figure 3: Pareto graph of isolate FS-4.3 with standardized absolute values of the effects of each variable and their interactions.

According to the Pareto graph of isolate FS-4.2 (Figure 3), the interaction between soybean oil, corn oil, and glucose ($X1 * X2 * X4$) had the most positive effect on biosurfactant production, while soybean oil had the least positive effect. Ammonium sulfate had the most negative effect on biosurfactant production, and glycerol had the least relevant negative effect.

Microbial production of biosurfactants may be achieved using different substrates, but the composition and characteristics of the biosurfactants are influenced by the nature of the carbon and nitrogen sources used (Fontes et al., 2008). Therefore, a study of the nutritional requirements and process conditions of microbial production is essential. Decesaro et al. (2013) studied the production of biosurfactants using microorganisms isolated from diesel oil-contaminated soil as a function of nitrogen source and inducer type (soybean oil and diesel oil). The best results were obtained using 1% soybean oil as an inducer, without the addition of ammonium sulfate as a nitrogen source. Considering the results shown in Figure 4, it can be inferred that within the range of conditions studied for isolate FS-4.1, nitrogen source influences the emulsifier index.

Yeast extract exerted a negative influence on biosurfactant production, with a statistical significance at the 95% confidence level ($p \leq 0.05$). In contrast, ammonium sulfate exerted a positive influence on biosurfactant production, although this effect was not statistically significant ($p \geq 0.05$).

In addition, the different carbon sources also influenced the emulsifier index of isolate FS-4.1 (Figure 5). Although glucose had a negative influence

on biosurfactant production, the effect was not statistically significant at the 95% confidence level ($p \geq 0.05$). The combination of water-insoluble substrate (soybean oil) and carbohydrate (glucose) also increased biosurfactant production by isolate FS-4.1. However, glycerol demonstrated a statistically significant negative influence ($p \leq 0.05$) on biosurfactant production as shown in Figure 5 (a). As the glycerol concentration increased ($X3$) and glucose concentration ($X4$) decreased, the emulsifier index decreased. In contrast, the emulsifier index increased when the glycerol concentration decreased ($X3$) and glucose concentration ($X4$) increased.

Several studies have reported the production of biosurfactants as a function of the composition of the culture medium, type of microorganism, and process operational conditions (Barth; Gaillardin, 1997; Casas; Ochoa, 1999; Amezcuaeva et al., 2007; Fontes et al., 2008; Luna; Sarubbo; Campos-Takaki, 2009; Das; Kumar, 2019). According to Napp et al. (2018), several microorganisms can degrade or assimilate hydrocarbons. Among these microorganisms, many are capable of emulsifying these hydrocarbons during substrate degradation.

Biosurfactant production can be spontaneous or it can be induced by the presence of lipophilic compounds, by variations in pH, temperature, aeration, and agitation speed, or by cell growth under stress conditions, such as low nitrogen concentrations (Desai; Banat, 1997). Many additional factors influencing the production of biosurfactants have been explored in previous studies, including the effects of sugars, glycerol, ethanol,

crude oil, alkanes, paraffin, vegetal oils, and agro-industrial waste on different types of substrates (Barth; Gaillardin, 1997; Desai; Banat, 1997; Casas; Ochoa, 1999; Ravelet et al., 2000; Lukondeh; Ashbolt; Rogers, 2003; Amezcuaavega et al., 2007; Luna et al., 2009; Decesaro et al., 2013). In addition to the effects of the type of substrate, nutrient availability, carbon source,

environmental conditions, and other factors, production efficiency is also determined by the genetics of the microorganisms (Lukondeh; Ashbolt; Rogers, 2003). In the present study, large differences in biosurfactant production were observed between different isolates, providing evidence of a significant role played by the genetic difference between the isolates.

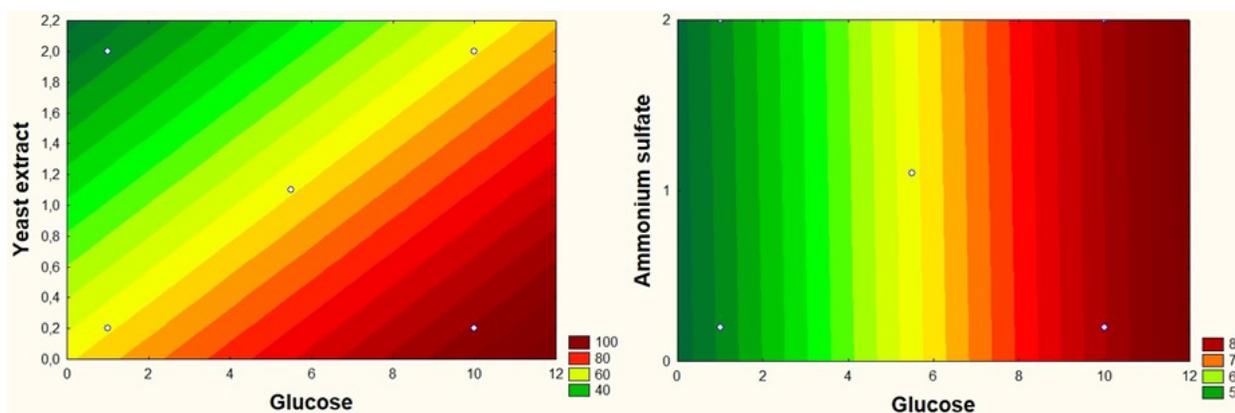


Figure 4: Contour graphs showing the effects of the independent variables on the emulsifier index of isolate FS-4.1. Source: Statistica 8.0 computer program.

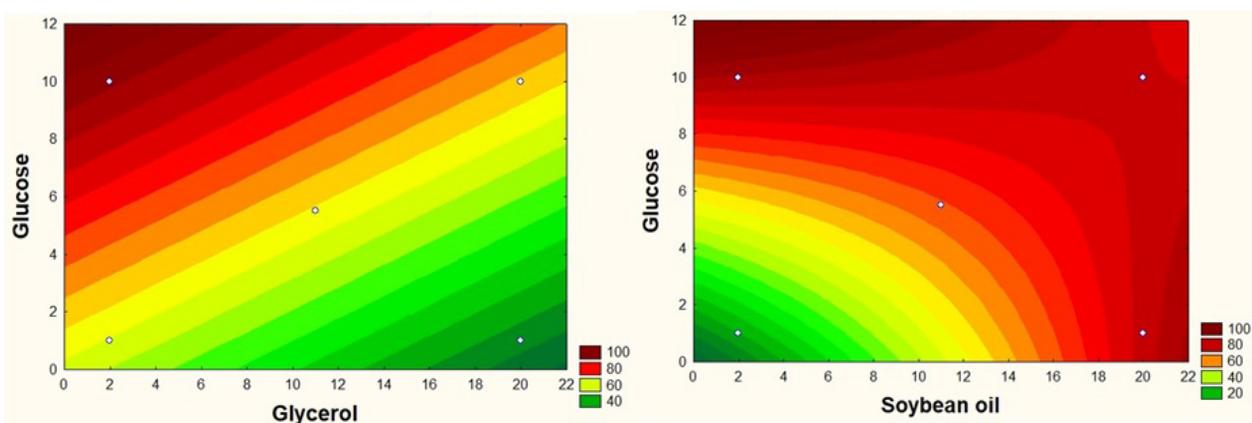


Figure 5: Contour graphs showing the effects of independent variables on the emulsifier index of isolate FS-4.1. Source: Statistica 8.0 computer program.

CONCLUSIONS

It is reported on a new group of basidiomycetes capable of producing biosurfactants. Coprinaceous fungi demonstrate great potential as biosurfactant producers. While all three isolates investigated in the present study demonstrated biosurfactant production capabilities, biosurfactant production by isolate FS-4.1 subjected to Experiment 03 was most notable, yielding an emulsifying index of 73.45%. To maximize the biosurfactant production, it was necessary to adjust the concentration of culture medium components (to eliminate any negative effects on the process) and increase the amounts of glucose and soy oil. Our results can be used to design improved production processes by considering the parameters that positively influenced biosurfactant production.

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