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Optimal glucose and inoculum concentrations for production of bioactive molecules by *Paenibacillus polymyxa* RNC-D

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The production of antimicrobial metabolites by *Paenibacillus polymyxa* RNC-D was assessed. Two process variables, glucose and inoculum concentrations, were evaluated at different levels (5–40 g L^{-1}, and at φ_r = 2.5–5.0 %, respectively), and their effects on biomass formation, minimal inhibitory concentration (MIC) against *Escherichia coli*, and surface tension reduction (STR) were studied. When the fermentation process was carried out under non-optimised conditions, the biomass, MIC, and STR achieved the following values: 0.6 g L^{-1}, 1 g L^{-1}, and 18.4 mN m^{-1}, respectively. The optimum glucose (16 g L^{-1}) and inoculum volume ratio (φ_r = 5.0 %) were defined in order to maximise the biomass formation, with a low value of MIC and high STR of extract. The experiments carried out under optimal conditions showed the following values for the dependent variables: biomass concentration 2.05 g L^{-1}, MIC 31.2 μg mL^{-1}, and STR 10.7 mN m^{-1}, which represented improvement of 241.7 %, 96.9 %, and 41.9 % for the responses of biomass, MIC, and STR, respectively. This is the first recorded study on the optimisation of culture conditions for the production of antimicrobial metabolites of *P. polymyxa* RNC-D, and constitutes an important step in the development of strategies to modulate the production of antimicrobial molecules by this microorganism at elevated levels.
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Introduction

Endophytes are regarded as an outstanding source of bioactive natural products because they occupy a unique biological environment: living plants (Strobel et al., 2004). Plant-associated microorganisms are subjected to constant metabolic and environmental interactions and, as a consequence, these organisms should produce even more secondary metabolites (Schulz, 2002). These molecules are characterised by their diverse chemical structures and may be of

use due to the wide range of their bioactivity against pathogens. *Paenibacillus polymyxa* strains, for example, are recognised for their ability to produce antimicrobial peptides active against a broad range of microorganisms. One group of compounds, bioactive against both Gram-positive and Gram-negative bacteria, includes polymyxins (Katz & Demain, 1977), jolipeptin (Ito & Koyama, 1972a, 1972b), polypeptins (Sogn, 1976), gavaserin, and saltavalin (Pichard et al., 1995). Another group is comprised of molecules responsible for the bioactivity against Gram-positive

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bacteria and fungi, and includes gatavalin (Nakajima et al., 1972) and fusaridicins (Kajimura & Kaneda, 1996, 1997).

Production of secondary metabolites by microorganisms varies qualitatively and quantitatively according to the strain and the cultivation conditions used (Lam et al., 1989; Wang et al., 2010). There are many factors potentially affecting the microbial metabolic processes, including the substrate and its concentration, inoculum concentration, medium composition, supply of air, temperature, pH, and others. These conditions vary from species to species of microorganisms; as a consequence, it is important to know the environmental conditions of the microorganisms for maximum production of the desired metabolite (Gogoi et al., 2008; Adinarayana et al., 2003). The initial substrate and inoculum concentrations are two of the most important factors affecting the product formation by microorganisms, which is why they have been evaluated in many studies. However, it is of considerable interest to evaluate the simultaneous effect of the initial substrate and inoculum concentrations so as to avoid the use of a low substrate concentration per cell, which would yield low product formation, and also to avoid the use of an elevated substrate concentration per cell, which might lead to inhibition of the microbial metabolism.

The selection of the best operating conditions through factorial design and response surface methodology is a practice commonly employed in biotechnological processes, since this methodology permits the study of the effects of several factors influencing the responses by carrying out a limited number of experiments. Several works report on the use of statistical tools for the optimisation of fermentation conditions, such as initial substrate and inoculum concentrations, pH, temperature, and aeration, among others (Santos et al., 2005; Mussatto & Roberto, 2008; Shen et al., 2005).

The present paper is the first study on the optimisation of the culture conditions for the production of antimicrobial metabolites by *Paenibacillus polymyxa* RNC-D. Consequently, taking the above reasons into consideration, it focused on evaluating the effect of two process variables, namely the glucose and inoculum concentrations, on biomass formation, surface tension reduction, and the antimicrobial activity of the extract obtained from the fermentation cultures of endophytic *P. polymyxa* RNC-D.

Experimental

Bacterial strains and culture media

The endophytic bacterium *Paenibacillus polymyxa* RNC-D was isolated from leaves of *Prunus* spp., a Brazilian tropical savannah plant (Ratti et al., 2008). *P. polymyxa* RNC-D was grown in a YPM (yeast extract peptone agar medium) composed of: glucose (20 g L^{-1}), yeast extract (11.25 g L^{-1}), peptone (11.25 g L^{-1}), malt extract (20 g L^{-1}), and agar (15 g L^{-1}). The pH of this medium was adjusted to 7.0 by adding a 5 M NaOH solution. For preparation of the YPM, all the components were sterilised in an autoclave at 121 ℃ for 15 min, except for the glucose solution that was sterilised in an autoclave at 112 ℃ for 15 min, and aseptically mixed with the other components of the medium. For long storage periods, the bacterium was cultivated in YPM broth at 30 ℃ for 16 h, and then kept frozen in glycerol ($\varphi_r = 15$ %, $t = -80$ ℃). The Gram-negative indicator *Escherichia coli* ATCC 25922 was cultivated in Trypticase soy agar (TSA, Himedia, JMGS, Portugal) at 37 ℃, and kept in slant tubes at 4 ℃. Stock cultures of this microorganism were refreshed every three weeks. TSA was sterilised by autoclaving at 121 ℃ for 15 min.

Inoculum and fermentation conditions

An aliquot (50 μL) of stock culture of *P. polymyxa* RNC-D was spread over a Petri dish containing YPM agar and maintained at 30 ℃ for 48 h. The inoculum was then prepared by transferring a single colony from the Petri dish into a 250-mL Erlenmeyer flask containing 100 mL of YPM broth. The flask was incubated in an orbital shaker at 30 ℃ and 180 min^{-1} for 16 h. The cells were recovered by centrifugation (18895g, 15 min) and washed twice in sterile water. Subsequently, the inoculum was prepared by adjusting the optical density (600 nm) to 1.0 and a volume ratio in the range of $\varphi_r = 2.5$–5.0 % was transferred to the fermentation media. Batch fermentations were carried out in 500-mL Erlenmeyer flasks containing 200 mL of YPM broth (pH 7.0) with glucose concentrations varying from 5 g L^{-1} to 40 g L^{-1}. The pH was not monitored in the course of the fermentation. The flasks were incubated at 30 ℃, 180 min^{-1}, for 96 h. After this time, the fermented broth was centrifuged (18895 g, 15 min) and the pellet thus obtained was used to determine the biomass formation. The extract was filtered through an 0.22 μm membrane filter and further used to measure the surface tension and to assess the antimicrobial activity.

Analytical methods

The biomass concentration (g L^{-1}) was determined by measuring the absorbance of the fermented medium, which were then correlated via a calibration curve (dry mass × optical density). A volume of 100 mL of 96-h culture broth was centrifuged (18895g, 15 min) and the pellet was washed twice in distilled water. Next, the cells were re-suspended in 100 mL of distilled water and this cellular suspension was diluted 10-, 20-, 30-, 40-, 50-, 100-, 200-, 500-, and 1000-fold. The optical density at 600 nm of the diluted so-

Table 1. Experimental matrix with real and coded values of independent variables used in glucose fermentation by *Paenibacillus polymyxa* RNC-D, and results obtained for responses of biomass formation, MIC against *E. coli*, and surface tension reduction (STR)

| Assay | Independent variables[a] Real and (coded) values | | Responses (dependent variables) | | |
| | x_1 | x_2 | Biomass | MIC | STR |
	g L^{-1}	%	g L^{-1}	μg mL^{-1}	mN m^{-1}
1	5 (−1)	2.5 (−1)	0.60	1000.0	18.4
2	40 (+1)	2.5 (−1)	2.13	15.6	14.84
3	5 (−1)	5.0 (+1)	0.76	250.0	20.0
4	40 (+1)	5.0 (+1)	4.11	15.6	11.5
5	40 (+1)	3.75 (0)	3.09	31.25	11.6
6	5 (−1)	3.75 (0)	0.69	500.0	16.25
7	22.5 (0)	5.0 (+1)	3.33	62.5	14.1
8	22.5 (0)	2.5 (−1)	2.90	62.5	10.5
9	22.5 (0)	3.75 (0)	3.05	31.25	9.8
10	22.5 (0)	3.75 (0)	3.13	62.5	9.8
11	22.5 (0)	3.75 (0)	3.01	62.5	10.2

a) x_1 = glucose concentration; x_2 = inoculum volume fraction (φ_r).

lutions was measured. Meanwhile, known volumes of the cells suspension prepared in 100 mL of distilled water were dried at 105 °C until they achieved a constant mass. The biomass concentration in each diluted sample was then correlated with the respective value of absorbance. A calibration curve was established for each glucose concentration used in the experiments (OD – optical density; B – biomass concentration), namely 5 g L^{-1} (Eq. (1)), 22.5 g L^{-1} (Eq. (2)), and 40 g L^{-1} (Eq. (3)), and elevated values of the coefficient of determination (R^2) were obtained.

$$OD = 0.0363B + 0.0273; \ R^2 = 0.9862 \tag{1}$$

$$OD = 0.0237B + 0.0259; \ R^2 = 0.9937 \tag{2}$$

$$OD = 0.0436B + 0.0339; \ R^2 = 0.9947 \tag{3}$$

The surface tension of extracts produced by *P. polymyxa* RNC-D was determined by the ring method (Rodrigues et al., 2006) using a KRUSS tensiometer (Kruss, Dias de Sousa, Portugal) equipped with a 1.9 cm De Noüy platinum ring (Kruss, Dias de Sousa, Portugal) at ambient temperature. The surface tension reduction (STR) in the extracts, as compared with the medium, was expressed in mN m^{-1}.

The antimicrobial activity of the extracts was quantified by the micro-dilution technique (National Committee for Clinical Laboratory Standards, 2002) and the minimal inhibitory concentration (MIC) was expressed in μg mL^{-1}. Gram-negative indicator strain was cultivated for 16 h in Tryptic soy broth (TSB) at 37 °C and 120 min^{-1}, being the cells collected by centrifugation (9055*g*, 5 min). The bacterial biomass was re-suspended in Mueller–Hinton broth (MHB, Oxoid, JMGS, Portugal) and the inoculum adjusted to 10^6

CFU per mL. A volume of 100 μL of the bacterial suspension was displayed in each well of a 96-well plate. Similarly, the extracts were twice serially diluted using MHB and 100 μL was added to the wells, in triplicate. Positive and negative controls were made by the following procedures, respectively: 100 μL of YPM plus 100 μL of bacterial inoculum (MHB) and 100 μL of YPM plus 100 μL of MHB. The 96-well microplates were incubated at 37 °C, 120 min^{-1} for 24 h. MIC is defined as the lowest extract concentration in which indicator microorganisms did not demonstrate visible growth.

Experimental design

The influence of the variables: glucose concentration (x_1) and inoculum concentration (x_2) on the production of antimicrobial metabolites by *P. polymyxa* RNC-D was evaluated through a 2^2 central composite design with three coded levels, leading to eleven sets of experiments. For statistical analysis, the variables were coded according to Eq. (4), where each independent variable is represented by x_i (coded value), X_i (real value), X_0 (real value at the centre point), and ΔX_i (step change value). The range and levels of the variables, which were selected based on data from the literature, are given in Table 1.

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \tag{4}$$

Three assays in the centre point were carried out to estimate the experimental error needed for analysis of the variance, as well as to examine the presence of curvature in the response surfaces and to investigate the suitability of the proposed models. The biomass

Table 2. Analysis of variance for effects of glucose and inoculum concentrations on responses of biomass formation, MIC against *E. coli* and surface tension reduction (STR), during glucose fermentation by *Paenibacillus polymyxa* RNC-D

Source of variation	Biomass				MIC				STR			
	SS	DF	F-value	p-value	SS	DF	F-value	p-value	SS	DF	F-value	p-value
(1) Glucose (L)	8.84	1	297.65	0.000[a]	474637.5	1	42.87	0.001[a]	46.54	1	31.41	0.003[a]
Glucose (Q)	3.63	1	122.30	0.000[a]	142097.4	1	12.84	0.016[b]	39.24	1	26.48	0.004[a]
(2) Inoculum (L)	1.10	1	37.04	0.002[a]	93750.0	1	8.47	0.033[b]	0.58	1	0.39	0.560
Inoculum (Q)	0.00	1	0.10	0.769	2878.9	1	0.26	0.632	13.52	1	9.13	0.029[b]
(1) L by (2) L	0.82	1	27.61	0.003[a]	140625.0	1	12.70	0.016[b]	6.10	1	4.12	0.098
Error	0.15	5	–	–	55357.3	5	–	–	7.41	5	–	–
Total	14.76	10	–	–	932057.6	10	–	–	130.65	10	–	–

a) Significant at 99 % confidence level; *b*) significant at 95 % confidence level. SS – sum of squares; DF – degrees of freedom; L – linear term; Q – quadratic term. $R^2 = 0.99$ for biomass, and 0.94 for MIC and STR.

formation, MIC against *E. coli*, and the surface tension reduction (STR) were taken as dependent variables or responses of the design experiments. Analysis of the data by response surface methodology made it possible to define equations to allow calculation of the maximum values to be achieved for each response, individually. The quadratic models for predicting the optimal point to each response were expressed by Eq. (5), where y_i represents the response variable, b_0 is the interception coefficient, b_1 and b_2 the linear terms, b_{11} and b_{22} the quadratic terms, and x_1 and x_2 represent the variables studied.

$$\hat{y}_i = b_0 + b_1 x_1 + b_2 x_2 + b_{12} x_1 x_2 + b_{11} x_1^2 + b_{22} x_2^2 \quad (5)$$

Design expert version 5.0 and Statistica version 5.0 were the software used for regression and graphical analyses of the data. The statistical significance of the regression coefficients was determined by Student's *t*-test. The second-order model equations were determined by Fischer's test, and the proportion of variance explained by the models obtained was given by the multiple coefficient of determination R^2. The optimum values of the variables were obtained by graphical and numerical analyses, based on the criterion of desirability. At a later stage, a condition capable of simultaneously maximising the three responses was established by graphical optimisation, and fermentation experiments were performed in triplicate to validate the model and to calculate the standard error.

Results and discussion

A 2^2 central composite design with three repetitions at the central point was employed to study the effect of glucose and inoculum concentrations on the production of antimicrobial metabolites produced by *P. polymyxa* RNC-D. The experimental matrix with the real and coded levels of the variables, as well as the results of biomass formation, MIC against *E. coli* and STR, is shown in Table 1. As may be seen, the values of the responses varied significantly depending on the glucose and inoculum concentrations used in each

experiment. Biomass formation, for example, had an almost 7-fold increase under the experimental conditions applied. The MIC values also varied considerably from 15.6 µg mL^{-1} to 1000 µg mL^{-1}, while the maximum reduction in the surface tension of extract reached 20.0 mN m^{-1} (assay 3).

Analysis of variance of the data presented in Table 1 revealed significant effects ($p < 0.05$) of both variables, glucose and inoculum concentrations, in all the responses (Table 2). The initial glucose concentration was also shown to have a significant influence in the production of a novel antimicrobial peptide by *Bacillus* sp. fmbJ224 (Shen et al., 2005). According to this study, the optimal concentration of glucose was 8.13 g L^{-1}, which in combination with other variables allowed the content of the peptide to increase from 1,304.21 µg mL^{-1} to 1,487.58 µg mL^{-1}, representing an improvement of 14.05 %. The data presented in this study plus those attained by Shen et al. (2005) confirmed, as stated previously, that the initial substrate and inoculum concentrations were parameters of significant influence on fermentation processes, as also reported by other authors (Adinarayana et al., 2003; Gogoi et al., 2008; Mussatto & Roberto, 2008).

The statistical significance of the quadratic terms (Q) (Table 2) suggests that second-order polynomial equations are more suitable than first-order equations for describing the responses variations as a function of the glucose and inoculum variations. The parameters of the second-order models used to estimate the biomass, MIC and STR variations as a function of the variables variations were then obtained by multiple regression analysis. When possible, the variables or interaction terms not significant at 95 % confidence level were excluded from the models. Mathematical models representing the biomass formation, MIC against *E. coli*, and STR in the experimental region considered in the present study, were expressed by Eqs. (6), (7), and (8), respectively, where the variables (glucose concentration, x_1; inoculum concentration, x_2) assumed their coded values. The models did not show lack-of-fit and presented high coefficients of determination ($R^2 \geq 0.89$), explaining more than 89.0 % of the vari-
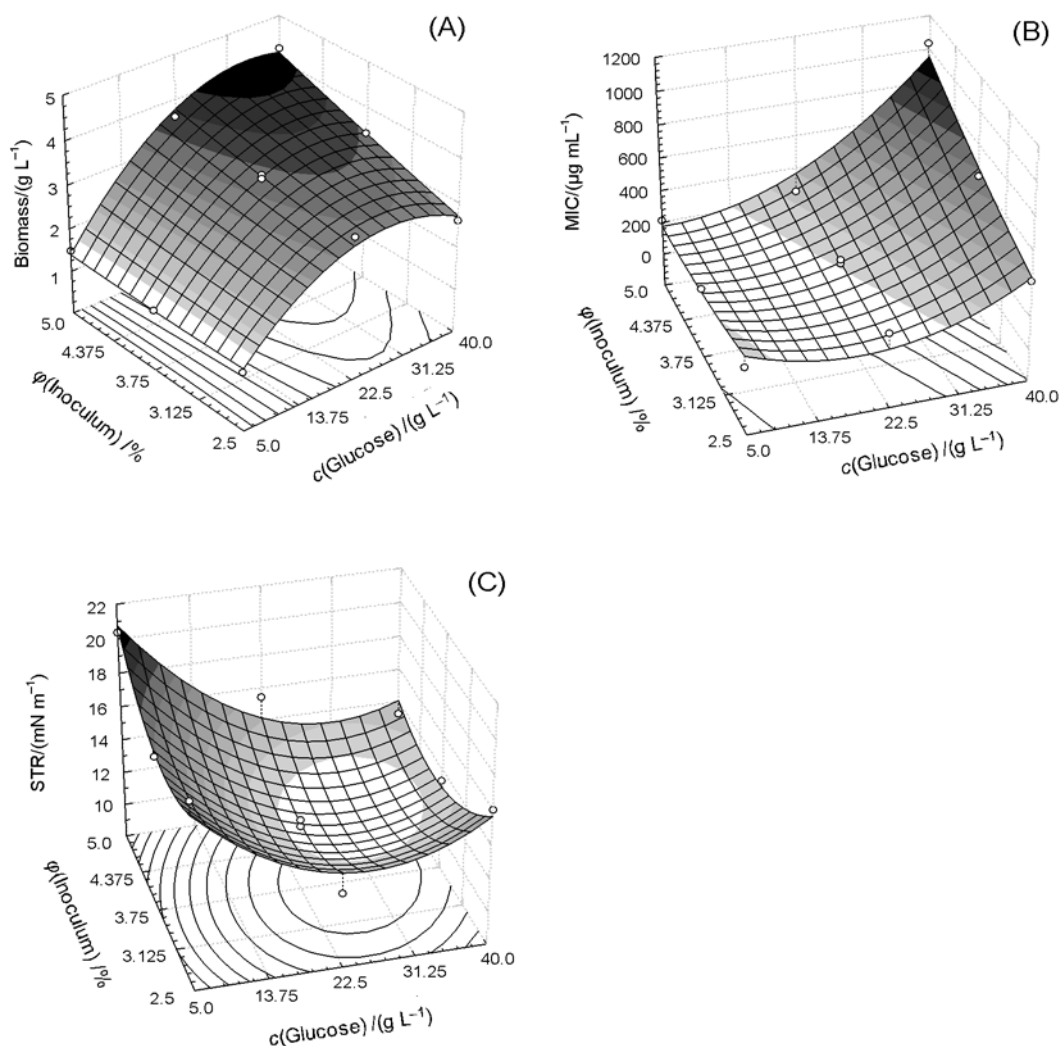
Fig. 1. Response surface described by models representing biomass formation (A), MIC against *E. coli* (B), and surface tension reduction – STR (C) in glucose fermentation by *Paenibacillus polymyxa* RNC-D.

ability in the responses. These models are very useful for rapid prediction of the maximum value to be obtained for each response when varying the glucose and inoculum concentrations in the range of values studied here.

$$\text{Biomass (g L}^{-1}) = 3.08 + 1.21x_1 - 1.19x_1^2 + \\ + 0.43x_2 + 0.45x_1x_2; \ R^2 = 0.99 \quad (6)$$

$$\text{MIC (µg mL}^{-1}) = 56.25 - 281.26x_1 + \\ + 245.83x_1^2 - 125x_2 + 187.5x_1x_2; \ R^2 = 0.94 \quad (7)$$

$$\text{STR (mN m}^{-1}) = 9.96 - 2.79x_1 + 3.94x_1^2 + 2.31x_2^2; \\ R^2 = 0.89 \quad (8)$$

Raza et al. (2010) also used the RSM to evaluate the effect of metal ions Ca^{2+}, Ni^{2+}, Mn^{2+}, Cu^{2+} on the production of antifungal compounds (fusaricidns A, B, C, and D) by *P. polymyxa* SQR-21. The values of responses diameter of inhibition zone (mm)

and growth (OD at 600 nm) were recorded for each experimental condition used in this study and a high degree of correlation between the observed and predicted data of the inhibition zone ($R^2 = 0.74$) and growth ($R^2 = 0.71$) was obtained by these authors. In the present study, higher values of the coefficients of determination were achieved, such as 0.99 for biomass, 0.94 for MIC, and 0.89 for STR. These elevated values represent a better fit for the regression equations to the experimental results.

The three-dimensional response surfaces described by the model equations are represented in Fig. 1. These surfaces clearly show the existence of regions where the response values are maximised or minimised, presenting the relationships between variables and responses. Biomass formation, for example (Fig. 1A), is maximised when using the highest inoculum level and glucose concentration varying between 22.5 g L^{-1} and 40 g L^{-1} (dark region of the graph). Similar values of glucose and inoculum concentration
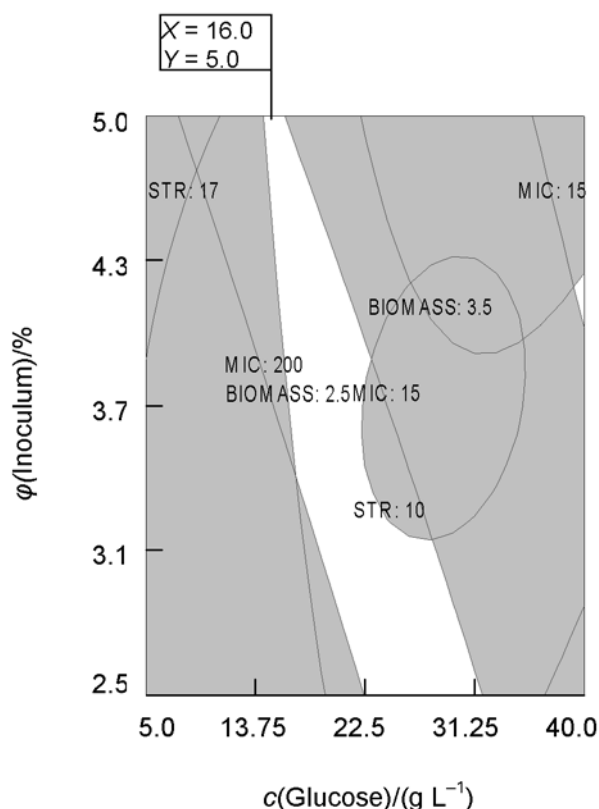
Fig. 2. Optimum region by overlay plots of three responses evaluated (biomass formation, MIC against *E. coli*, and surface tension reduction – STR) as a function of glucose and inoculum concentrations.

were not best suited for obtaining the lowest MIC values (white region in Fig. 1B) or the highest STR (dark region in Fig. 1C). Therefore, a graphical optimisation based on the three proposed models was performed to find out a point or a region where the results of biomass and STR could be as high as possible, whereas the MIC values could be as low as possible.

The graphical optimisation method essentially consisted of overlaying the curves of all the models according to the criteria imposed. In the present study, the criteria adopted were: biomass formation between 2.5 g L^{-1} and 3.5 g L^{-1} (a), MIC values between 15 µg mL^{-1} and 200 µg mL^{-1} (b), and STR between 10 mN m^{-1} and 17 mN m^{-1} (c). The overlaying plot attained (Fig. 2) shows an area where all the imposed criteria were satisfied (white region). A point was assigned in this area as optimum point (marked by the square) which corresponded to an initial glucose concentration of 16 g L^{-1} and inoculum of $\varphi_r = 5$ %. Under these conditions, the model predicts a biomass of 2.76 g L^{-1}, MIC of 15.80 µg mL^{-1} and STR of 14.58 mN m^{-1}. Assays for validation of this model were then performed under the established operating conditions and values were found of 2.05 g L^{-1}, 31.2 µg mL^{-1}, and 10.7 mN m^{-1} for biomass, MIC, and STR, respectively. These values are in agreement with the criteria

adopted for optimisation and confirm the validity of the model.

A similar initial substrate concentration (12.3 g L^{-1}) was optimised for the production of antifungal active substances by *P. polymyxa* Cp-S316 from lactose (Wang & Liu, 2008). In that study, preliminary experiments revealed that the carbon source lactose was presumed to affect antifungal active substances production by *P. polymyxa* Cp-S316. This strain is capable of producing antifungal compounds, polymyxin E, and at least three novel peptide antibiotics when cultivated in a medium with potato, lactose, beef extract, NH$_4$SO$_4$, MgSO$_4$, KH$_2$PO$_4$. By applying an inoculum of $\varphi_r = 2$ % and a lactose concentration of 12.3 g L^{-1}, it was possible to obtain 4.69 µg mL^{-1} of antifungal compounds, representing a 3.05-fold increase when compared with the production obtained in the basal medium. This initial concentration of carbon source is close to that (16 g L^{-1}) predicted and validated in the present study. The adjustment in the concentration of carbon source had an important role in activation of the secondary metabolism of *P. polymyxa* Cp-S316, resulting in increased production of antifungal compounds. The same result was observed in this study, in which the percentage of improvement for MIC against *E. coli* was 96.9 %.

Establishment of the optimum fermentation condition constitutes an important step in the development of strategies to modulate the production of antimicrobial molecules by *P. polymyxa* RNC-D at elevated levels. Data acquired from a previous study revealed that *P. polymyxa* RNC-D produced three antimicrobial molecules when cultivated in YPM medium: fusaricidin A (active against Gram-positive bacteria and fungi), polymyxin E, and a novel antimicrobial peptide (AMP) – both active against Gram-negative bacteria. The AMP is a short depsipeptide with five residues of amino acids, and which includes hydroxyproline in its structure.

Conclusions

Glucose and inoculum concentrations are variables with substantial influence on the production of antimicrobial metabolites by *P. polymyxa* RNC-D. By using the experimental factorial design method and response surface methodology followed by graphical optimisation, it was possible to determine the optimum operating condition in order to achieve the biomass and STR as high as possible, and the MIC values as low as possible. The validity of the proposed model was verified and confirmed. When the fermentation process was carried out under non-optimised conditions, the values of biomass, MIC, and STR were 0.6 g L^{-1}, 1 g L^{-1}, and 18.4 mN m^{-1}, respectively. However, when the experiments were performed under optimised conditions, these values were correspondent to 2.05 g L^{-1}, 31.2 µg mL^{-1}, and 10.7 mN m^{-1}, respectively, repre-

senting a percentage of improvement for each target response of 241.7 %, 96.9 %, and 41.9 %. This study thus constitutes an important step in the development of strategies to modulate the production of antimicrobial molecules by *P. polymyxa* RNC-D at elevated levels. The next stage of this research will spotlight enhancement of the production of the novel antimicrobial peptide by the endophytic strain.

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