



Enhanced production of target bioactive metabolites produced by *Pseudomonas Aeruginosa* LV strain

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ABSTRACT

Pseudomonas aeruginosa LV strain produces phenazine-1-carboxylic acid – PCA, phenazine-1-carboxamide – PCN, 2-carboxy-2-heptano-indol-3-ona – IDC, and fluopcin C – OAC. These molecules from secondary metabolism showed a potential for many biotechnological applications. The concentration of every compound obtained in the culture medium was below 6 mg L⁻¹ and, in order to increase these concentrations a process of statistical optimization was carried out. Under optimum conditions the production of IDC, PCA, PCN and OAC increased 42, 38, 250 and 0.84 folds, respectively. The minimum inhibitory concentration of OAC against *P. aeruginosa* LV strain was 15 mg L⁻¹, indicating that the concentration obtained (11.11 ± 1.05 mg L⁻¹) was very close to the maximum concentration possible in the medium. The optimized conditions presented here enhance the production of IDC, PCA, PCN and OAC by *P. aeruginosa* LV strain and should be implemented in this bacterial culture.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative γ -proteobacterium widely distributed in nature. This species is able to use distinct organic compounds as nutrients and grow in many different environments such as soil, water, plants and animal tissues (Stover et al., 2000). *P. aeruginosa* is known as an opportunistic human pathogen that causes persistent lung infections in patients with cystic fibrosis and immunocompromised patients (Davies, 2002; Johansen et al., 1998). Nevertheless, these bacteria can also be useful in diverse biotechnological applications, such as to produce biosurfactants (Yin et al., 2009), metabolites with antimicrobial activity (Kerbaudy et al., 2016), lipases (Chaudhry et al., 2015), and to remove branched chain alkanes from crude oil-contaminated water (Nie et al., 2016).

P. aeruginosa LV strain was isolated from citrus canker lesions of orange (*Citrus sinensis* cv. Valence) at Astorga, Paraná state, Brazil (Rampazo, 2004). This strain produces different types of phenazines, including phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN), secondary metabolites with potential antibiotic activity against bacteria, fungi and nematodes (Wen et al., 2016). PCA is a compound produced by plant growth-promoting rhizosphere (PGPR) pseudomonads and can be used for the biological control of pathogens

(Jain and Pandey, 2016; Upadhyay and Srivastava, 2011; Xu et al., 2015). Furthermore, PCA is a precursor of PCN regulated by *phzH* gene (Jin et al., 2015) and, whilst both compounds present antibiotic properties, PCN has an increased antifungal activity when compared to PCA (Chin-A-Woeng et al., 1998).

Additionally, *P. aeruginosa* LV strain produces 2-carboxy-2-heptano-indol-3-ona (IDC) and fluopcin C (OAC), both with high antimicrobial activity (Kerbaudy et al., 2016). The IDC showed antitumoral activity under in vitro conditions and the ability to promote plant growth (unpublished data) and the antibiotic activity of OAC was observed against *Xanthomonas citri* cv. *citri* (Oliveira et al., 2011), *Klebsiella pneumoniae* Carbapenemase (KPC)-producing (Kerbaudy et al., 2016) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Cardozo et al., 2013). Recently, Gionco (2016) reported the inhibitory capacity of OAC on five multiresistant isolates of *Acinetobacter baumannii*, an important multiresistant pathogen related with severe hospital infections.

The secondary metabolites production process of *P. aeruginosa* LV strain was established and patented (Andrade, 2008), but the compounds are produced and obtained in low concentrations. This fact limits the economic viability of their application under field conditions, by increasing production costs. Therefore, the objective of this study

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was to determine the optimum culture conditions and enhance the production of the metabolites of interest, produced by *P. aeruginosa* LV strain, using the response surface methodology (RSM).

2. Materials and methods

2.1. Bacterial strain

P. aeruginosa LV strain is deposited in the Culture Collection of the Microbial Ecology Laboratory, Londrina State University, Brazil. The strain is kept in glycerol stocks (40%) and stored at -20°C .

2.2. Preparation of standards

2.2.1. Growth condition and metabolites production

The secondary metabolites were produced according to the patented procedure PI0803350-1 (Andrade, 2008). The *P. aeruginosa* LV strain was activated on Petri dishes in nutrient agar plus copper chloride (% w/v): peptone, 0.5; meat extract, 0.3; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; and agar, 1.5; pH 7.0, and incubated at 28°C per 24 h. Aliquots of 50 μL of cell suspension in log phase ($\text{O.D.}_{590\text{nm}} = 0.09 = 10^8 \text{ CFU mL}^{-1}$) was inoculated into fermentation vessels (Thermo Scientific Nalgene polycarbonate carboy, 10 L) containing 5.0 L of nutrient broth named as basic culture medium (BCM) (%w/v): peptone 0.2; meat extract 0.12; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.0005; with pH 6.8 and sterilized (121°C , 20 psi, 20 min). The fermentation vessels were kept in a room at 28°C for 10 days, with a continuous supply of micro-filtered air. The culture was centrifuged ($4500 \times g$, 20 min, 4°C) and the cell-free supernatant was concentrated in a rotary evaporator at 70°C (Rotavapor R 215, Büchi). Aliquots (250 mL) of concentrated supernatant were used to extract and purify the metabolites of interest (Oliveira et al., 2016).

2.2.2. Calibration curves for HPLC analysis

A two-fold serial acetonitrile solution was prepared (100, 50, 25, 12.5, 6.25 and 3.125 mg L^{-1}), samples were filtered with $0.22 \mu\text{m}$ membranes (Macherey-Nagel, reference number 729022, pore $0.22 \mu\text{m}$, diameter 15 mm) and the HPLC analysis was carried out using an Agilent 1260 Infinity with an online vacuum degasser, a QuatPump, an automated injection valve, a UV-VIS detector, and Agilent ChemStation software (Agilent Technologies, USA). The analysis was performed using a C18 reversed-phase column ($250 \text{ mm} \times 4.6 \text{ mm i.d.}$, $5 \mu\text{m}$, Agilent, USA) with the modification of a previously described chromatographic method for phenazine derivatives (Dietrich et al., 2006). A $10 \mu\text{L}$ aliquot of each acetonitrile solution was used for HPLC analysis, eluted in a mix of 0.1% acetic acid: pure water (A) to acetonitrile (B) at flow rate of 1 mL min^{-1} under the following conditions: 0–2 min, 1–15% B; 2–22 min, 15–83% B; 22–24 min, 83–1% B. The column was stabilized for 3 min after the run (until 27 min). The detection of OAC, PCA and PCN was best set at $\lambda = 250 \text{ nm}$ and IDC at $\lambda = 230 \text{ nm}$. The runs were monitored at 215, 230, 250, 262, 280, 290, 316 and 360 nm with a bandwidth of 4 nm in all cases. Retention times for IDC, PCA, PCN, and OAC averaged (min) 22.4, 19.84, 17.63, and 14.46, respectively. Two analyses were utilized for each point on the calibration curves and all curves had $R^2 > 0.99$. The detection limits were (mg L^{-1}): PCA, 1.01; PCN, 1.40; OAC 1.61; and IDC, 1.47.

2.3. Fermentation profiles in the current production system (CPS)

IDC, PCA, PCN and OAC were produced in 10 L fermentation vessels, as described above, and in 500 mL shake flasks containing 100 mL of BCM incubated at 28°C with an orbital shaker (170 rpm) for 10 days. Aliquots of 2 mL of culture medium were taken every 24 h and stored at -20°C . These samples were used to measure the concentrations of the secondary metabolites.

2.4. Preparation of samples for HPLC analysis and metabolite quantification

The culture was centrifuged ($8000 \times g$, 4°C) and free-cell supernatant was acidified with 1 M HCL to pH 4.0 and centrifuged again under the same conditions. The metabolites were extracted three times from 2.0 mL of acidified cell-free supernatant using two volumes of dichloromethane (DM). Finally, the DM was removed by a rotary evaporator as described above. The dried solid was solubilized with 0.5 mL of acetonitrile and filtered ($0.2 \mu\text{m}$, Macherey-Nagel, reference number 729022). A $10 \mu\text{L}$ aliquot was taken for HPLC analysis as described above and samples were diluted with acetonitrile when necessary.

2.5. Optimization process

A two-step process was designed to increase the final concentration of the IDC, PCA, PCN and OAC. Initially, unique experimental factors and Box-Behnken design were used to optimize the concentration of the nutrients used in the patented process (Andrade, 2008). After that, a four-step statistical approach was used to evaluate the effect of nine different components of culture medium and two physical parameters of compounds production.

2.5.1. Optimization of the CPS

2.5.1.1. One-factor-at-a-time (OFAT). The effects of the inoculum size (% v/v: 0.01, 1, 2, and 4), peptone (g L^{-1} : 1, 2, 5, 10 and 20), meat extract (in g L^{-1} : 0.6, 1.2, 2.4 and 5) and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (mg L^{-1} : 0, 5, 15, 30 and 50), on the concentration of IDC, PCA, PCN, and OAC were determined. The LV strain was cultured in 500 mL erlenmeyer containing 100 mL of culture medium as described above, and cultivated in rotary shaker (Lab Companion IS-971R) per 8 days at 28°C and 170 rpm. The treatments were performed in triplicate. To determine the significative difference among treatments ($p < 0.05$), one-way ANOVA followed by Tukey's multiple comparison test was performed (Statgraphics Centurion version XVI).

2.5.1.2. Optimization by Box-Behnken design (BBD). A three-level BBD was employed (Box and Behnken, 1960). The ranges of values of peptone (g L^{-1} : 2, 13 and 24), meat extract (g L^{-1} : 1, 4 and 7) and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (mg L^{-1} : 0, 15 and 30) were selected based on OFAT experiments. A 15 run BBD, including three replicates for the center point, was applied. Statgraphics Centurion version XVI was used to predict the optimal concentrations of each variable for maximum IDC, PCA, PCN, and OAC production. A second-order polynomial model was fitted to correlate relationship between independent variables and response (Eq. (1)). The responses function (Y_i) for the three factors were divided into the linear, quadratic and interactive terms below:

$$Y_i = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \sum_{j>i}^k \beta_{ij} X_i X_j + \sum_{i=1}^k \beta_{ii} X_i^2 \quad (1)$$

Where Y_i is the predicted response, X_i and X_j represents coded independent variables, β_0 is the population value of the average of all the response values, β_i is the i th linear coefficient, β_{ii} is the i th quadratic coefficient, and β_{ij} is the ij th interaction coefficient. Subscripts i and j takes values from 1 to the number of variables.

The relation between the coded level and the actual values of each independent variable was described as:

$$x_i = \frac{X_i - X_0}{\delta X} \quad (2)$$

Where x_i is the coded value and X_i the actual value of an independent variable, X_0 is the value of X_i at the center point and δX is the step change of the variable. All experiments were performed in duplicate. Regression analysis and analysis of variance (ANOVA) was used for

fitting the models represented by Eq. (1) and also to examine the statistical significance of the model terms. The adequacy of the models were determined using model analysis and R^2 (coefficient of determination) analysis. F-Value was determined to check the significance of all the fitted equation at 5% level of significance.

2.5.1.3. Experimental validation. Four optimum formulations were selected. The resultant experimental values of the responses were quantitatively validated with the predicted values. Additionally, the optimized variables values that yielded the maximum responses were experimentally compared by culturing LV strain in optimized and BCM. The production, extraction and HPLC analysis of IDC, PCA, PCN and OAC was carried out as described above. All treatments were performed in triplicate and expressed as means $\pm$ standard deviation.

2.5.1.4. Minimum inhibitory concentrations (MIC). The MIC of OAC against LV strain was determined using a two-fold serial dilution method ($120\text{--}0.234\text{ mg L}^{-1}$) in a 96 wells microplate where $50\text{ }\mu\text{L}$ of LV strain cells suspension in log phase (10^6 CFU mL^{-1}) cultured in nutrient broth were mixed with $50\text{ }\mu\text{L}$ of OAC. The effect of the initial pH (6.8, 7.8 and 8.8) on the MIC value was also evaluated. A cellular suspension of LV, with non-addition of OAC, and non-inoculated nutrient broth were considered as positive and negative controls, respectively. The microplates were incubated at 28°C for 72 h and the bacterial growth was evaluated each 24 h by measuring the optical density (OD) at $\lambda = 590\text{ nm}$. To reveal, an aliquot of $50\text{ }\mu\text{L}$ of 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) solution [2 mg mL^{-1}] was added to each well and, after 2 h, the color development was observed, indicating that cells were still alive. The MIC was obtained by mean values of four independent experiments and the lowest concentration that completely inhibited bacterial growth was recorded.

2.5.2. Establishment and optimization of new production conditions

2.5.2.1. Growth condition and metabolites production. The *P. aeruginosa* LV strain was inoculated in Petri dishes containing (%w/v): peptone, 0.5; meat extract, 0.3 and agar, 1.5; pH 7.0 and incubated at 28°C for 48 h. For the inoculum production, a single colony of *P. aeruginosa* LV strain in log phase was inoculated in a 500 mL shake flask containing 100 mL of BCM, pH 6.8, and incubated at 28°C for 18 h in an orbital shaker (170 rpm). An initial inoculum size of 1% was used for each experiment related to screening and optimization.

2.5.2.2. Identifying the significant variables – first screening. Eleven variables were selected based on data from published studies (Chin-A-Woeng et al., 2001; Chincholkar et al., 2013; Huang et al., 2009; Spaepen and Vanderleyden J, 2011; Su et al., 2010; van Rij et al., 2004; Yuan et al., 2008) and previous studies of our research group (unpublished data). All the variables were investigated at two levels: high (+) and low (–) (Table S1. Supplemental data). A PBD with 12 assays (carried out in duplicate) was designed and five center points were added for estimating the experimental error (Plackett and Burman, 1946). The experimental sequence of the design was randomized in order to minimize the effects of the uncertainty of systematic errors. The effects of each independent variable on the final concentrations of IDC, PCA and PCN were determined as the difference between the average value of the response for the experiments at the high level (+) and the average value for the experiments at the low level (–), using the Yates algorithm (Cavalitto and Mignone, 2007).

$$E_i = \frac{\sum R_+}{n} - \frac{\sum R_-}{n} \quad (3)$$

Where E_i is the effect of the independent variable “i”, R_+ and R_- are responses at the high (+) and low (–) levels, respectively, and “n” is the number of experiments at each level. Subsequently, the experimental error (σ) was estimated from data at central point by using Eq. (4).

$$\sigma_{cp} = \sqrt{\frac{SSE_{cp}}{a}} \quad (4)$$

Where, SSE_{cp} is the sum of the squared differences, and “a” is the total number of central points. Finally, the minimum significant factor effect (MIN) was calculated by multiplying “ σ ” with the student t-value at an alpha level of 0.2. An effect was considered as significant when the absolute value of E_i was larger than MIN value.

2.5.2.3. Identifying the significant variables – second screening. A second PBD for seven variables with 12 randomized assays was designed (Table S2. Supplemental data). The effects of each independent variable on the final concentrations of IDC, PCA and PCN were calculated by the Eq. (3). Experimental error was estimated by calculating the variance among the dummy variables (V_{Eff}) as (Prajapati et al., 2014):

$$V_{Eff} = \frac{\sum E_d^2}{n} \quad (5)$$

Where E_d is the effect for the dummy variables and “n” is the number of dummy variables used in the experiment. The standard error (σ_d) of dummy variables, that is the square root of V_{Eff} , was used to determine the significance level of each effect as mentioned above.

2.5.2.4. Optimization by central composite design (CCD). Soybean protein, glucose, yeast extract, and KH_2PO_4 concentrations were chosen to design, model and optimize the production of IDC, PCA and PCN by RSM (Table S3. Supplemental data). RSM study was performed at Statgraphics Centurion Software Version XVI, and a five-level four-factor central composite design (CCD) was used to investigate the significance of the effect of each independent variable (Box and Wilson, 1951). Thirty experiments were proposed by experimental design including 12 axial points, 12 face centered points and 6 replications at center point. The standard error was estimated by calculating the experimental error among the center points (code 0). The data were used to develop a mathematical model of second degree correlating each answer with the independent variables by the Eq. (1).

2.5.2.5. Validation. Two optimum formulations were selected and validated as described above.

2.5.2.6. Three-level full factorial design. In several studies, temperature, pH and their interactions are highlighted as key factors affecting not only biomass productivity but also the synthesis of metabolites by microorganisms (Akkermans et al., 2017; Gonçalves et al., 2017; Lee et al., 2014; Wu et al., 2017). Therefore, a three-level full factorial design was selected, in order to obtain a clearer view of the interactions between these parameters. Temperature (28 , 32 , and 37°C) and initial pH (6.8 , 7.4 and 8.0) values were chosen based on the results found in screening experiments. All treatments were carried out in triplicate and expressed as means $\pm$ standard deviation.

3. Results

3.1. Optimization of the CPS

3.1.1. Fermentation profiles in the CPS

The fermentation profiles in the CPS for both shake flask and fermentation vessels are shown in Fig. S1 (Supplemental data). The OAC was observed in both culture conditions in a late stationary phase. The maximum OAC concentrations in shake flasks ($6.02 \pm 0.18\text{ mg L}^{-1}$, OD_{590} of 0.15) and in fermentation vessels ($4.25 \pm 0.35\text{ mg L}^{-1}$, OD_{590} of 0.10) were recorded after 240 h. The PCA was detected after 24 h in the final log phase, significative differences among the final concentrations obtained in shake flask ($0.96 \pm 0.08\text{ mg L}^{-1}$, OD_{590} of 0.14) and fermentation vessels ($2.97 \pm 0.69\text{ mg L}^{-1}$, OD_{590} of 0.17) were observed.

In shake flasks, the PCN concentration was always lower than 0.35 mg L^{-1} (estimated value based on detection limit) and the maximum IDC concentration was $2.51 \pm 0.10 \text{ mg L}^{-1}$, after 240 h. Instead, in fermentation vessels the IDC concentrations were always lower than 0.37 mg L^{-1} (estimated value based on detection limit) and the PCN concentrations ranged between (mg L^{-1}) 0.63 ± 0.25 (72 h) and 0.81 ± 0.17 (240 h).

3.1.2. OFAT experiments

OFAT experiments were performed to evaluate the effects of inoculum size, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, peptone, and meat extract concentrations on the metabolite production in 500 mL shake flasks with 100 mL of culture medium (Fig. S2. Supplemental data). The inoculum size of 1%v/v increased PCA and PCN production and the presence of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ in the culture medium did not affect IDC and PCN production, and decreased PCA level. The OAC was not detected in culture medium without $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and their production was statistically the same at 50 mg L^{-1} than 30 mg L^{-1} . Peptone and meat extract showed significant effect on IDC, PCA and PCN production, but OAC was not detected when the peptone concentration was higher than 2 g L^{-1} and meat extract higher than 1.2 g L^{-1} .

3.1.3. IDC, PCA and PCN optimization by RSM

In order to optimize the IDC, PCA and PCN production, a BBD with three factors and three levels was performed. The design consisted of 12 factorial points and three central points in a random run order (Table 1). The central points (runs 6, 9, 12) were used to calculate the pure error sum of squares. Neglecting the statistically insignificant terms ($p > 0.05$), the following quadratic regression equation was obtained to describe the IDC (Eq. (6).), PCA (Eq. (7).) and PCN (Eq. (8).) production:

$$Y_1 = 8.495 + 2.4662X_1 - 0.8368X_2 - 4.3538X_1^2 - 0.38X_1X_2 + 0.4512X_2X_3 \quad (6)$$

$$Y_2 = 6.5034 - 1.0575X_1 - 0.92X_2 - 0.595X_3 + 4.5819X_1^2 - 0.6919X_2^2 + 0.44X_2X_3 \quad (7)$$

$$Y_3 = 18.3133 + 3.78X_1 - 0.9012X_2 + 1.8788X_3 - 7.6654X_1^2 - 3.24X_1X_2 - 1.8829X_2^2 + 0.0011X_1 - 1.3979X_3^2 - 0.8725X_2X_3 \quad (8)$$

Where Y_1 , Y_2 and Y_3 are IDC, PCA and PCN concentrations expressed in mg L^{-1} ; X_1 , X_2 and X_3 represent the coded variables (Eq. (2)) of

peptone, meat extract and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, respectively.

The analysis of variance (ANOVA) was carried to test model and the independent variables (data not shown). The adjusted R^2 was 0.98, 0.92 and 0.90 to IDC, PCA and PCN respectively. These values indicate that the response could be explained by the predicted models (Box and Drapper, 1987). The quadratic regression models (Eqs. 6, 7 and 8) were significant to predict the IDC, PCA and PCN concentrations. The results from the regression models and the actual and predicted values of each dependent variable, based on BBD experiment, are shown in Table 1.

Different combinations of the three factors yielded IDC, PCA and PCN concentrations ranging from (mg L^{-1}) 0.98–9.23; “not detected” to 8.53; and “not detected” to 18.67, respectively, and 3D response surface curves were assembled based on these regression models (Fig. S3. Supplemental data). The optimal concentrations of the independent variables and the maximal concentrations predicted of the dependents variables were determined, thus, the following medium culture compositions were obtained (%w/v): peptone 17 and meat extract 1.0 for IDC production; peptone 11.7 and meat extract 1.2 for PCA production; and peptone 16.7, meat extract 2.8 and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.0064 for PCN production.

Further experiments were performed to validate the models and modifications in specific culture medium for production of IDC (IDCM), PCA (PCAM), and PCN (PCNM) were prepared. The concentrations of the independent variables were established at the optimal levels predicted by the models (Fig. 1A, B, C and D). The results were compared to both the predicted values and actual values obtained in BCM (Table S4. Supplemental data). The concentrations of IDC ($9.27 \pm 0.69 \text{ mg L}^{-1}$, OD_{590} of 0.28), PCA ($6.74 \pm 0.26 \text{ mg L}^{-1}$, OD_{590} of 0.26) and PCN ($18.85 \pm 0.65 \text{ mg L}^{-1}$, OD_{590} of 0.26) obtained in shake flasks maintained in optimal conditions represented (in %) 91, 87, and 96 of the predicted values and confirmed the validation of the response model. Additionally, the concentrations of IDC, PCA and PCN increased in 2.7, 6.0 and 52.9 fold, when compared to BCM, respectively.

According to the results obtained in the experimental validation, the final concentration of IDC did not differ in IDCM, PCAM and PCNM culture media (Fig. 1A). Also, PCN concentrations were not significantly different in PCAM and PCNM (Fig. 1C). Then, PCAM was selected as the final optimized culture medium, not only for PCA production (Fig. 1B), but also for PCN and IDC production. The fermentation profiles for both PCAM and OACM are presented in Fig. 2A and B,

Table 1

Box-Behnken design (BBD) with the values of the independent variables in natural and coded units in culture of studied *P. aeruginosa* LV strain. X_1 = peptone (g L^{-1}), X_2 = meat extract (g L^{-1}), X_3 = $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (mg L^{-1}), Y_1 = IDC, Y_2 = PCA, Y_3 = PCN, Y_4 = OAC.

Standard	Run	Independent variables			Dependent variables (mg L^{-1})							
		X_1	X_2	X_3	Y_1^a	Y_1^p	Y_2^a	Y_2^p	Y_3^a	Y_3^b	Y_4^a	Y_4^b
6	1	24 (+1)	4 (0)	0 (−1)	7.17	6.86	1.11	1.13	14.00	15.08	ND	−
9	2	13 (0)	1 (−1)	0 (−1)	9.20	9.68	8.53	7.66	18.54	16.94	ND	−
10	3	13 (0)	7 (+1)	0 (−1)	6.84	7.10	4.15	4.94	15.32	16.88	ND	−
8	4	24 (+1)	4 (0)	30 (+1)	6.09	6.51	0.44	0.37	9.93	10.98	ND	−
4	5	24 (+1)	7 (+1)	15 (0)	5.26	5.31	ND	ND	11.05	8.40	ND	−
13	6	13 (0)	4 (0)	15 (0)	8.21	8.51	6.64	6.66	18.67	18.30	ND	−
11	7	13 (0)	1 (−1)	30 (+1)	9.23	8.97	6.37	5.59	16.49	14.93	ND	−
12	8	13 (0)	7 (+1)	30 (+1)	8.67	8.19	3.75	4.62	9.78	11.38	ND	−
14	9	13 (0)	4 (0)	15 (0)	8.73	8.51	6.41	6.66	17.92	18.30	ND	−
5	10	2 (−1)	4 (0)	0 (−1)	1.81	1.39	3.61	3.68	8.22	7.17	ND	−
3	11	2 (−1)	7 (+1)	15 (0)	0.98	1.14	2.51	1.66	7.84	7.32	2.61	−
15	12	13 (0)	4 (0)	15 (0)	8.59	8.51	6.92	6.66	18.30	18.30	ND	−
1	13	2 (−1)	1 (−1)	15 (0)	2.11	2.06	2.34	3.15	ND	2.64	5.43	−
7	14	2 (−1)	4 (0)	30 (+1)	1.80	2.12	2.08	2.06	4.85	3.77	4.72	−
2	15	24 (+1)	1 (−1)	15 (0)	7.91	7.75	0.53	1.38	16.17	16.69	ND	−

ND.: not detected.

^a Actual value.

^b Predicted value.

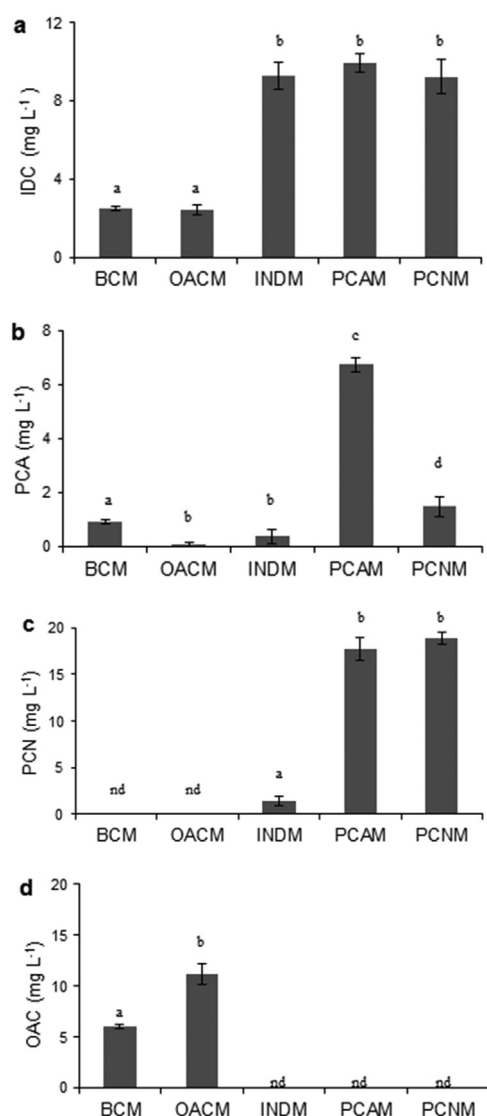


Fig. 1. Validation of the experimental design. BCM (%w/v: peptone, 0.2, meat extract, 0.12, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.0005), IDCM (%w/v: peptone, 1.69 and meat extract, 0.1), OACM (%w/v: peptone, 0.23, meat extract, 0.1 and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.003), PCAM (%w/v: peptone, 1.17 and meat extract, 0.12).

respectively. The ideal time required for the production of each compound was established as eight days for IDC and OAC production, one day for PCA and three days for PCN production.

3.2. OAC production and minimal inhibitory concentration (MIC)

OAC was not detected in most RMS optimization experiments (Table 1) and the highest OAC concentration ($11.11 \pm 1.05 \text{ mg L}^{-1}$, OD_{590} of 0.12) was obtained in OFAT experiments using BCM plus 25 mg L^{-1} of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (Fig. S2 and Table S4. Supplemental data). The reduction of nutrient concentration should increase the OAC production, and two additional culture medium (g L^{-1} : peptone, 0.6, meat extract, 0.36; and peptone, 1.2, meat extract, 0.72) plus 30 mg L^{-1} of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ were tested under the same culture conditions. The final obtained concentrations of OAC (1.67 and 2.52 mg L^{-1} , OD_{590} of 0.10 and 0.11, respectively) were lower than those obtained using BCM supplemented with 25 mg L^{-1} of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ($11.11 \pm 1.05 \text{ mg L}^{-1}$, OD_{590} of 0.12). The results suggested that the low level of OAC produced could be related to the inhibitory effect against *P. aeruginosa* LV strain, once the OAC has a high antibiotic effect against many Gram-

negative bacteria (Munhoz et al., 2017; Murate et al., 2015; Oliveira et al., 2016; Silva et al., 2014).

The MIC of OAC for *P. aeruginosa* LV strain was determined in different initial pH (6.8, 7.8 and 8.8), due to the fact that pH increases quickly from 6.8 to 8.8 during the first days of culture (Fig. 2B), and it has been demonstrated that pH may affect the antibacterial activity of natural compounds, and hence MIC values (Li et al., 2014; Olaimat and Holley, 2013). At an initial pH value of 8.8, a 50% decline in the MIC value was observed at 24 and 48 h, when compared to the initial pH values of 6.8 and 7.8. This can be explained by the fact that the growth rate of *P. aeruginosa* LV strain (expressed as O.D. versus incubation time for positive control samples) was considerably reduced at initial pH 8.8 (Table S5. Supplemental data).

No difference was observed when comparing the MIC in different pH levels during the experiment. The MIC values changed during the time, increasing from 7.5 to 15 mg L^{-1} in 48 h and 72 h of incubation in any pH. The antibiotic-producing bacteria are innately resistant to the antibiotics produced by itself (Opal and Pop-Vicas, 2015) and *P. aeruginosa* LV strain needs to increase its resistance to the new environmental stresses generated by OAC. In Gram-negative bacteria, the entrance to the stationary phase is a highly regulated process governed by RpoS, involving production of cell envelopes for an effective protection against many stressors, such as oxidative stress, heat shock, osmotic stress, antibiotics presence and pH changes (Navarro et al., 2010). During the process, the inner membrane has a highly ordered structure to reduce fluidity (Nystrom, 2004), contributing to an intrinsic resistance and increasing the antibiotic resistance (Alvarez-Ortega et al., 2011; Wang et al., 2016).

3.3. Establishment and optimization of new production conditions

3.3.1. Identifying the significant variables – first and second screening

The PBD was used for screening because it was proved a powerful and useful mathematical tool to reduce the number of input variables in the optimization processes, thus conducted by a smaller number of experimental trials. The matrix applied in this first screening, the interpretation of the coded levels and the results are shown in Table 2. A considerable fluctuation in results was observed, where a total IDC, PCA and PCN concentrations ranged from “not detected” to 16.77 , 73.02 and 64.49 mg L^{-1} , respectively. Improvement of 0.8 (IDC), 9.8 (PCA) and 2.4 (PCN) times were found, comparing to PCNM (Table 2). The results confirm that other factors were required to increase the production of these compounds by *P. aeruginosa* LV strain. The main effects of each independent variables on each response, and their statistical significance ($p < 0.2$), were calculated by using the Eqs. 1 and 2, respectively, and the results were represented by Pareto charts (Fig. 2A, B and C).

Tryptophan has been reported as main precursor in the biosynthesis of indole-3-acetic acid (IAA) (Spaepen and Vanderleyden, 2011) and their biosynthesis starts with chorismate in the shikimate pathway, a common pathway of the biosynthesis of phenazines (Guttenberger et al., 2017). The addition of tryptophan in the culture medium could induce the production of both phenazines and IDC. However, when tryptophan was added in the culture media, this has no positive effects on the IDC, PCA and PCN production (Fig. 2A, B and C). Similarly, the increase in the concentration of meat extract had no effects on IDC, PCA and PCN concentrations. The concentrations of both culture medium components were fixed at low levels in further experiments. On the other hand, peptone concentration and addition of salts in the culture medium showed significant positive effects only on IDC and PCN concentrations, respectively. Because of this restricted effect, for these components being expensive and for the complexity of salt addition to the culture media, turning their manufacturing process more laborious and less desirable, these items were also kept at low levels in further experiments.

The ethanol showed a negative effect on PCN, enhanced PCA and

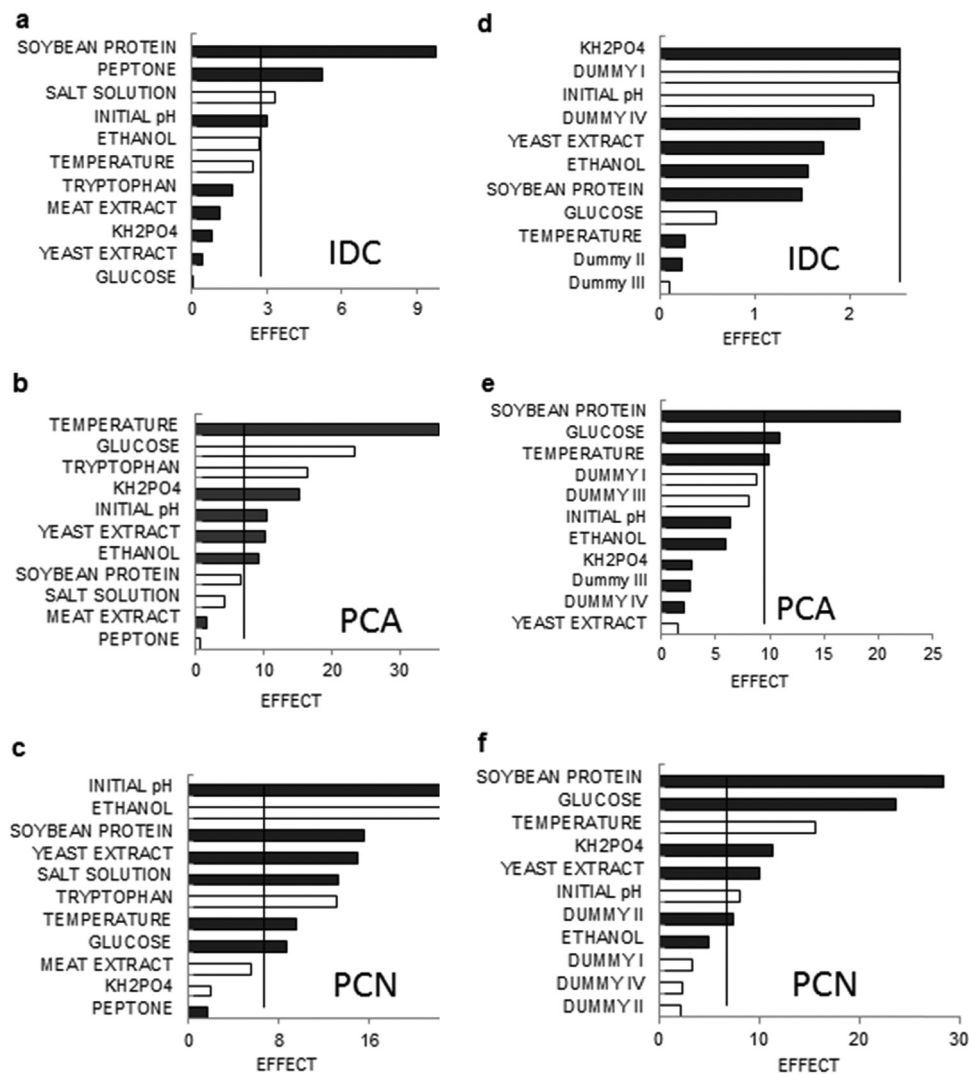


Fig. 2. Pareto charts representing the estimated effects of the independent variables on the final concentrations in the responses. The vertical line on the plot judges the effects that are statistically significant ($p < 0.20$). The bars extending beyond the line correspond to the effects that are statistically significant. At first screening, the critical values of minimum significant factor effects were 2.77 (A), 12.33 (B), and 6.81 (C), while at second screening these values were 2.52 (D), 16.70 (E), and 6.72 (F) for IDC, PCA and PCN, respectively. Positive and negative effects are represented by black and white color bars, respectively.

Table 2

Plackett-Burman design: first screening. A: peptone (g L^{-1}); B: meat extract (g L^{-1}); C: soybean protein (g L^{-1}); D: yeast extract (g L^{-1}); E: glucose (g L^{-1}); F: ethanol (mL L^{-1}); G: Salt solution (mL L^{-1}); H: KH_2PO_4 (g L^{-1}); I: tryptophan (g L^{-1}); J: temperature ($^{\circ}\text{C}$); K: initial pH.

Run	Independent variables											Dependent variables		
	A	B	C	D	E	F	G	H	I	J	K	IDC (mg L^{-1})	PCA (mg L^{-1})	PCN (mg L^{-1})
1	2(+1)	0.5(−1)	10(+1)	0(−1)	2(−1)	2(−1)	1(+1)	0.5(+1)	0.05(+1)	26(−1)	8(+1)	12.46	3.84	42.78
2	2(+1)	1(+1)	2(−1)	1(+1)	2(−1)	2(−1)	0(−1)	0.5(+1)	0.05(+1)	30(+1)	6(−1)	7.04	51.22	9.66
3	1(−1)	1(+1)	10(+1)	0(−1)	10(+1)	2(−1)	0(−1)	0(−1)	0.05(+1)	30(+1)	8(+1)	13.33	7.60	42.44
4	2(+1)	0.5(−1)	10(+1)	1(+1)	2(−1)	10(+1)	0(−1)	0(−1)	0(−1)	30(+1)	8(+1)	16.77	63.37	46.18
5	2(+1)	1(+1)	2(−1)	1(+1)	10(+1)	2(−1)	1(+1)	0(−1)	0(−1)	26(−1)	8(+1)	5.06	0.28	60.59
6	2(+1)	1(+1)	10(+1)	0(−1)	10(+1)	10(+1)	0(−1)	0.5(+1)	0(−1)	26(−1)	6(−1)	12.85	1.51	ND
7	1(−1)	1(+1)	10(+1)	1(+1)	2(−1)	10(+1)	1(+1)	0(−1)	0.05(+1)	26(−1)	6(−1)	2.25	ND	6.23
8	1(−1)	0.5(−1)	10(+1)	1(+1)	10(+1)	2(−1)	1(+1)	0.5(+1)	0(−1)	30(+1)	6(−1)	8.74	32.60	64.49
9	1(−1)	0.5(−1)	2(−1)	1(+1)	10(+1)	10(+1)	0(−1)	0.5(+1)	0.05(+1)	26(−1)	8(+1)	ND	11.42	12.89
10	2(+1)	0.5(−1)	2(−1)	0(−1)	10(+1)	10(+1)	1(+1)	0(−1)	0.05(+1)	30(+1)	6(−1)	ND	6.49	1.33
11	1(−1)	1(+1)	2(−1)	0(−1)	2(−1)	10(+1)	1(+1)	0.5(+1)	0(−1)	30(+1)	8(+1)	ND	73.02	19.46
12	1(−1)	0.5(−1)	2(−1)	0(−1)	2(−1)	2(−1)	0(−1)	0(−1)	0(−1)	26(−1)	6(−1)	ND	6.00	4.13
13–17	1.5(0)	0.75(0)	6(0)	0.5(0)	6(0)	6(0)	0.5(0)	0.25(0)	0.025(0)	29.5(0)	7(0)	11.74 ± 2.10	52.80 ± 4.77	79.69 ± 5.16

Table 3

Plackett–Burman design: second screening. A: KH_2PO_4 (g L^{-1}); B: soybean protein (g L^{-1}); C: yeast extract (g L^{-1}); D: glucose (g L^{-1}); E: ethanol (mL L^{-1}); F: temperature ($^{\circ}\text{C}$); G: pH initial; H: dummy I; I: dummy II; J: dummy III; K: dummy IV.

Run	Independent variables											Dependent variables		
	A	B	C	D	E	F	G	H	I	J	K	IDC	PCA	PCN
1	0.5(+1)	10(+1)	0.5(−1)	6(+1)	6(+1)	32(+1)	7(−1)	−1	−1	+1	−1	10.57	53.63	51.04
2	0.5(+1)	2(−1)	1.5(+1)	6(+1)	6(+1)	28(−1)	7(−1)	−1	+1	−1	+1	12.96	32.90	55.36
3	0(−1)	10(+1)	1.5(+1)	6(+1)	0(−1)	28(−1)	7(−1)	+1	−1	+1	+1	7.52	26.63	54.60
4	0.5(+1)	10(+1)	1.5(+1)	0(−1)	0(−1)	28(−1)	8(+1)	−1	+1	+1	−1	9.04	34.29	47.40
5	0.5(+1)	10(+1)	0.5(−1)	0(−1)	0(−1)	32(+1)	7(−1)	+1	+1	−1	+1	9.52	40.86	26.44
6	0.5(+1)	2(−1)	0.5(−1)	0(−1)	6(+1)	28(−1)	8(+1)	+1	−1	+1	+1	6.76	10.33	0.77
7	0(−1)	2(−1)	0.5(−1)	6(+1)	0(−1)	32(+1)	8(+1)	−1	+1	+1	+1	5.08	33.88	3.25
8	0(−1)	2(−1)	1.5(+1)	0(−1)	6(+1)	32(+1)	7(−1)	+1	+1	+1	−1	6.59	10.24	1.68
9	0(−1)	10(+1)	0.5(−1)	6(+1)	6(+1)	28(−1)	8(+1)	+1	+1	−1	−1	3.34	49.29	53.38
10	0.5(+1)	2(−1)	1.5(+1)	6(+1)	0(−1)	32(+1)	8(+1)	+1	−1	−1	−1	4.58	29.71	18.21
11	0(−1)	10(+1)	1.5(+1)	0(−1)	6(+1)	32(+1)	8(+1)	−1	−1	−1	+1	10.31	54.90	17.72
12	0(−1)	2(−1)	0.5(−1)	0(−1)	0(−1)	28(−1)	7(−1)	−1	−1	−1	−1	5.41	10.05	0.33

did not interfere on IDC production. On the other hand, glucose had positive, negative, and no effect on PCN, PCA, and IDC productions, respectively. Hence, in order to achieve a better understanding about the effects of these two parameters on metabolite production, their intervals were resized from 2% to 10% in the first screening and from 0% to 6% in second. The temperature and yeast extract enhanced PCA and PCN, and no effect was detected on IDC. When the initial pH increased, the three compounds also enhanced. Therefore, higher values of these three parameters were explored. The addition of KH_2PO_4 increased PCA, but did not affect IDC nor PCN concentrations. Finally, soybean protein enhanced IDC and PCN, but little effect was observed on PCA. In the second experiment, the factor ranges were selected based on the analysis of significant effects ($p < 0.2$) found in the first screening.

The experimental design matrix and output responses are presented in Table 3. Four dummy factors were introduced to measure the experimental error and to identify variables that may affect the system (Prajapati et al., 2014). In the seven real factors, only ethanol did not show effect on all response variables (Fig. 2D, E and F). Additionally, IDC and PCN concentrations were increased when KH_2PO_4 was added (Fig. 2D, F) and no significant effects on the PCA production was noticed. Soybean protein and glucose enhanced PCA (Fig. 2E) and PCN (Fig. 2F), and temperature increased PCA, but not PCN.

The maximum concentrations of IDC, PCA and PCN decreased by (in %) 22.7, 26.1 and 14.2, respectively, with regard to the results obtained in the first screening. The reductions were related to some changes made in the amount of peptone and glucose, which enhanced the biosynthesis of compounds in the first screening. Surprisingly, there was an increase in all the minimum concentrations (Tables 2, 3). This could indicate that those variables that were eliminated (e.g. salt solution) or resized (e.g. ethanol, pH and temperature), from the first to the second screening, were limiting the synthesis of these compounds.

When comparing the results between first and second screenings, contradictory effects were observed in specific responses. For instance, glucose concentration decreased PCA production in the first screening (Fig. 2B) and increased in the second (Fig. 2E). Temperature and initial pH enhanced PCN in the first PBD (Fig. 2C), and decreased in the second (Fig. 2F). Such contradictions may be present in two-level factorial designs, since the main effects can be heavily confounded with two-factor interactions (Cavazzuti, 2013). It could also explain the positive effect of dummy II on PCN concentrations (Fig. 2F), indicating that, possibly, the known variables interacted in such way that this interaction affected the dummy response. In this case, two-level factorial designs are only useful to identify the key input variables affecting the process, but not to determine specific amounts in the culture media composition.

The ethanol was discarded in the subsequent experiment and four

nutritional requirements (soybean protein, glucose, yeast extract and KH_2PO_4) were analyzed and optimized by RSM. The physical factors, temperature and initial pH, were fixed at low levels and studied by three-level full factorial design in further experiments.

3.3.2. Optimization by central composite design (CCD)

To maximize the production of IDC, PCA and PCN compounds by *P. aeruginosa* LV strain, the levels of soybean protein, glucose, yeast extract and KH_2PO_4 were determined. Multiple regression analyses were performed in order to fit the experimental concentrations (Table 4) by the Eq. (1). Based on the repeated experiments at central point (runs 3, 9, 13, 20, 25 and 30), the coefficients of variation to IDC, PCA and PCN were calculated in (in %) 4.3, 5.9 and 4.0, respectively, and these low values indicated the high reproducibility of the experiments. The F-values were 4.98, 5.44 and 31.96 to IDC, PCA and PCN, respectively. Models were greater than F-value of 2.42 indicating that all regression analyses were significative at 95% confidence. The good concordance between model predictions and detected values, as shown in Fig. S5 (Supplemental data), validated the proposed model.

The culture media composition obtained for IDC, PCA and PCN production had the same amount of meat extract and peptone, with changes in soybean protein, glucose, and yeast extract (Fig. S6. Supplemental data). The soybean protein highly influenced the biosynthesis of the evaluated compounds.

3.3.3. Validation

The validation experiment was performed to confirm the applicability of the models (Fig. 3). The concentrations were (mg L^{-1}): 13.67 ± 0.39 for IDC, 44.56 ± 3.65 for PCA and 60.98 ± 2.92 for PCN. The values corresponded to 101%, 104% and 88% of the predicted concentrations, which once more proved the validity of each proposed model. Concentrations of IDC, PCA and PCN did not present differences in all media tested, once their composition was very close and small variations did not show any influence on the response (Fig. 3). In fact, the highest expected variations on the optimum values were 14, 5.6 and 6.1 (%) for IDC, PCA and PCN concentrations, respectively. According to these results, two adjusted culture media (media A and B) were selected, with changes only in KH_2PO_4 concentration.

3.4. Effect of temperature and initial pH on the production of IDC, PCA and PCN

The medium B was employed to evaluate the effect of temperature and initial pH on production of IDC, PCA and PCN by a 3^2 full factorial design (Fig. 4A, B and C). The two factors were evaluated independently, because of the interaction between these factors, which

Table 4

Central Composite Design (CCD) matrix with the values of the independent variables in natural units. (In g L^{-1}): X_1 = soybean protein, X_2 = glucose, X_3 = Yeast Extract, X_4 = KH_2PO_4 . (In mg L^{-1}): Y_1 = IDC, Y_2 = PCN, Y_3 = PCA.

Run	Independent Variables				Dependent Variables					
	X_1	X_2	X_3	X_4	Y_1^a	Y_1^b	Y_2^a	Y_2^b	Y_3^a	Y_3^b
1	15	15	0.3	0.3	11.36	11.66	26.67	31.49	49.30	49.31
2	15	5	0.3	0.9	11.41	10.05	25.39	25.60	49.69	54.03
3	10	10	0.6	0.6	12.22	12.24	34.51	37.06	58.60	58.74
4	10	5	0.9	0.3	12.28	12.66	30.73	30.39	43.90	41.02
5	15	5	0.9	0.9	11.65	10.07	15.57	20.41	35.72	33.62
6	5	15	0.9	0.9	7.59	6.97	7.73	15.23	20.45	23.76
7	15	15	0.3	0.9	11.30	9.94	21.06	23.05	39.00	37.24
8	5	5	0.3	0.9	5.80	4.74	6.74	5.50	31.50	29.49
9	10	10	0.6	0.6	12.57	12.24	36.10	37.06	55.35	58.74
10	10	10	0.6	0.0	14.90	13.35	42.47	34.34	52.00	52.49
11	5	15	0.9	0.3	8.09	8.59	10.58	15.82	27.95	25.70
12	10	10	0	0.6	10.40	11.53	32.82	30.53	44.70	43.68
13	10	10	0.6	0.6	11.35	12.24	37.75	37.06	58.68	58.74
14	10	10	1.2	0.6	10.49	11.51	34.15	24.01	24.95	23.38
15	10	20	0.6	0.6	6.86	7.55	32.06	19.77	31.80	28.45
16	0	10	0.6	0.6	3.12	3.18	1.68	− 6.72	12.83	7.94
17	5	5	0.9	0.9	6.96	5.80	1.78	2.40	17.30	19.39
18	20	10	0.6	0.6	8.64	10.49	30.00	25.62	45.70	45.88
19	10	0	0.6	0.6	7.01	8.47	17.58	17.44	42.55	43.32
20	10	10	0.6	0.6	12.01	12.24	38.56	37.06	62.44	58.74
21	5	5	0.3	0.3	7.73	8.91	10.30	14.81	36.15	39.70
22	15	15	0.9	0.3	10.82	10.59	19.86	28.07	36.60	39.11
23	5	15	0.3	0.9	7.50	6.45	10.17	15.79	14.31	17.84
24	15	15	0.9	0.9	10.80	9.43	18.94	20.40	31.50	32.85
25	10	10	0.6	0.6	12.14	12.24	40.12	37.06	58.27	58.74
26	5	5	0.9	0.3	9.52	9.40	5.78	10.94	20.10	23.79
27	10	5	0.3	0.3	14.04	12.69	36.36	35.30	67.70	62.08
28	10	10	0.6	1.2	4.32	8.02	21.65	17.35	39.10	36.02
29	5	15	0.3	0.3	8.35	8.64	15.02	17.15	23.00	25.60
30	10	10	0.6	0.6	12.90	12.24	34.96	37.06	57.00	58.74

^a Actual value.

^b Predicted value.

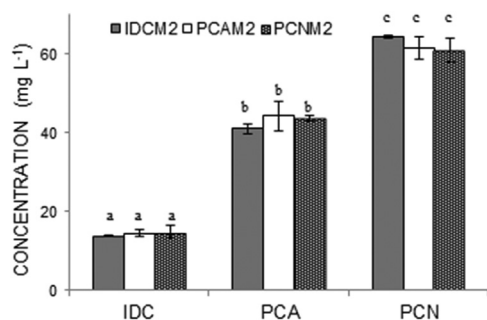


Fig. 3. Validation of the experimental design. Bars with the same letter are not significantly different ($\alpha = 0.05$). A. IDC2 (%w/v: soybean protein, 1.4, glucose, 0.66 and yeast extract, 0.026), B. PCAM2 (% w/v: soybean protein, 1.45; glucose, 0.72; yeast extract, 0.043; and KH_2PO_4 , 0.022), C. PCNM2 (%w/v: soybean protein, 1.44; glucose, 0.60; yeast extract, 0.032; and KH_2PO_4 , 0.031). All the culture medium also contained (%w/v: meat extract, 0.05 and peptone, 0.15). The experiments were conducted for 8 days at 28 °C, initial pH 6.8 and 170 rpm.

can affect the biomass production and metabolites biosynthesis of microorganisms (Dinarvand et al., 2017), and the same conjunct effects were previously observed in the production of IDC, PCA and PCN (Fig. 2A, B, C, E and F). The concentrations at 28 °C and initial pH 6.8 were (mg L^{-1}) 13.19 ± 1.14 for IDC, 42.21 ± 3.06 for PCA and 58.32 ± 1.81 for PCN, with reduction of 3.5%, 2.1% and 4.4% on the maximum concentrations obtained in the validation phase (Eqs. 5, 6 and 7). No differences ($p > 0.05$) were found between these values.

The IDC concentration ranged from 11.44 ± 0.88 (37 °C, initial pH 7.4) to 14.83 ± 1.40 (mg L^{-1} (28 °C, initial pH 7.4), indicating that the

temperature decreased IDC production (Fig. 4A) and no difference was observed when these results were compared to 28 °C and initial pH 6.8. PCA production was strongly influenced by temperature, initial pH and the interaction between these factors (Fig. 4B). At 32 °C and initial pH 6.8, the maximum concentration of PCA was 1.8-fold the optimum value (81.76 ± 2.55 mg L^{-1}) in the validate phase. On the other hand, the PCN concentration was not influenced by initial pH and decreased with changes in the temperature (Fig. 4C).

3.5. Definition of culture media and fermentation time

The pH 6.8 level was chosen to determine the fermentation time of media A and B in two different temperature conditions (28 °C and 32 °C). The synthesis of IDC at 28 °C was detected after five days of incubation (Fig. 5A and B) and, at 32 °C, it was detected just after 3 days of incubation (Fig. 5C and D). The maximum concentration was observed after eight days and no significative differences were observed between all incubation times. As expected, PCA and PCN production was influenced by temperature and the higher concentrations of PCA were recorded at 32 °C, while PCN was better at 28 °C. Also, the addition of KH_2PO_4 in the culture medium reduced PCA and increased PCN level.

The optimum concentrations obtained in this study are summarized in Table 5. Medium A at 28 °C was selected for IDC production with an expected concentration of 15.50 mg L^{-1} at 8 days of fermentation (Fig. 5A), while medium A at 32 °C was selected for PCA production, with an expected concentration of 113 mg L^{-1} at 3 days of fermentation (Fig. 5C). The Medium B at 28 °C was selected for PCN production, with an expected concentration of 177 mg L^{-1} at 5 days of fermentation (Fig. 5B). In shake flasks, the concentration increased more than 5 times (IDC), 117 times (PCA) and 506 times (PCN) when compared to the

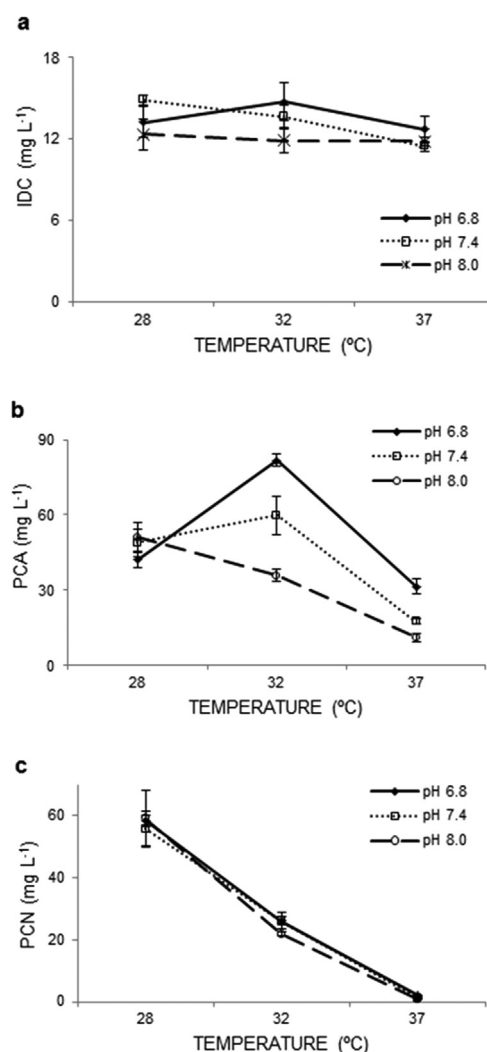


Fig. 4. Effect of temperature and initial pH on the final concentration of IDC (A), PCA (B) and PCN (C). Medium B (% w/v: soybean protein, 1.4; glucose, 0.60; yeast extract, 0.04; KH₂PO₄, 0.03; meat extract, 0.05 and peptone, 0.15) was used. The samples were taken after 8 d.

final concentrations in BCM. The increments were greater than 42 (IDC), 38 (PCA) and 250 (PCN) fold when compared to the current system production in 10 L fermentation vessels.

4. Discussion

Differences in the concentration of IDC, PCA, PCN and OAC were found when comparing their production in shake flasks to their production in fermentation vessels, using both BCM and optimized culture medium (Fig. S1 and Fig. S4. Supplemental data). In fermentation vessels, although the OAC, PCA and PCN concentrations increased when BCM was being used, the results were much lower than those obtained in shake flasks with optimized culture medium (Table S4. Supplemental data). This could be explained by the fact that it is harder to control the temperature, air flow and agitation in the fermentation vessels, which are crucial factors in the scale-up of fermentation processes. It is known that poor mixing conditions favor the creation of limited oxygen regions (dead zones), directly affecting metabolite production (Cuello et al., 2016). In previous studies, it was reported that an increase of 20% of dissolved oxygen (DO), with optimized aeration and agitation conditions, increased the PCA concentration produced by *Pseudomonas* sp. M18G (Li et al., 2010). Moreover, van Rij et al. (2004) reported an increase of PCN levels at 1% of DO by *P.*

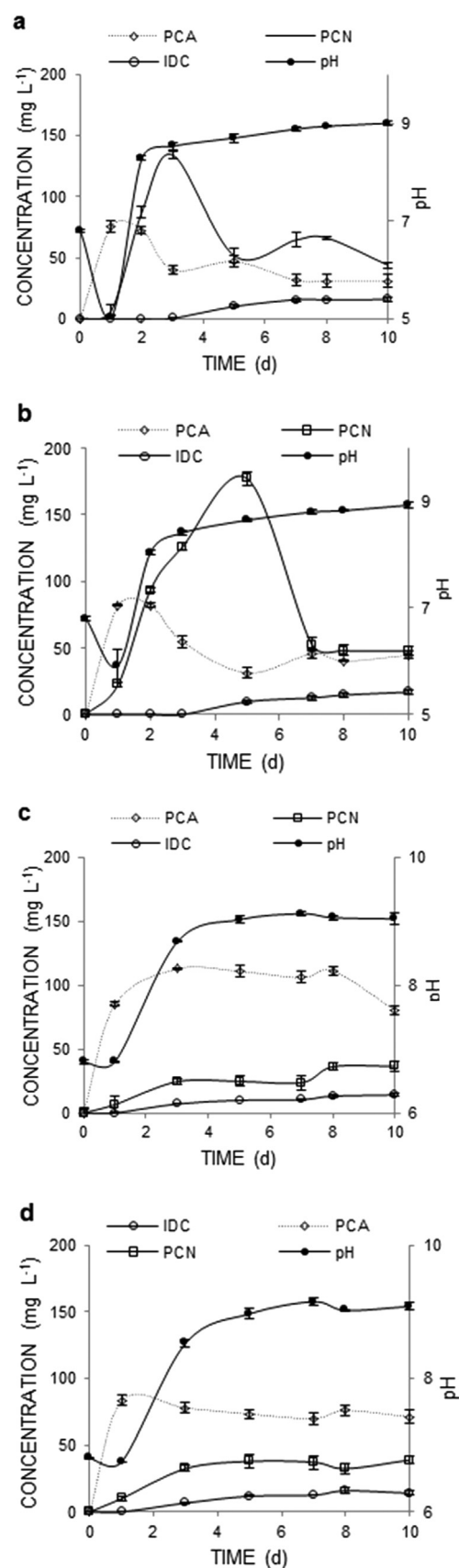


Fig. 5. Fermentation profiles. Medium A at 28 °C (A); Medium B at 28 °C (B); Medium A at 32 °C (C); Medium B at 32 °C (D). Medium A (% w/v: soybean protein, 1.4; glucose, 0.60; yeast extract, 0.04; meat extract, 0.05 and peptone, 0.15). Medium B same conditions as in Fig. 4.

Table 5

IDC, PCA and PCN concentrations in current production system and optimized production protocols obtained in the present study. SF (shake flasks). FV (Fermentation vessel).

Compound.	Concentrations (mg L ⁻¹)				Variation (%)	
	Basic culture medium		Optimized culture medium			
	SF (100 mL)	FV (5 L)		SF (100 mL)	FV (5 L)	
IDC	2.51 ± 0.10	< 0.37	15.61 ± 1.26	522	4119	
PCA	0.96 ± 0.08	2.97 ± 2.09	112.89 ± 2.87	11659	3701	
PCN	< 0.35	0.71 ± 1.01	177.31 ± 3.99	> 52860	24873	

chlororaphis PCL1391.

The temperature also showed to affect the ratio between PCA and PCN (Fig. 4). It is proven that the gene expression pattern involved in the conversion of PCA to others phenazines is regulated by a temperature-dependent and strain-specific manner at different temperatures. Huang et al. (2009) demonstrated that the quantitative ratio of PCA to pyocyanin (PYC) (1-hydroxy-5-methylphenazine) reached 105:1 at 28 °C and was 5:1 at 37 °C in *P. aeruginosa* M18, while was only 1:2 at 37 °C in *P. aeruginosa* PAO1. In addition, previous studies reported that a pH decrease from 7 to 6, or a decrease in growth temperature from 21° to 16°C, drastically affected the production of PCN by PCL1391 (van Rij et al., 2004).

On the other hand, the results suggested that OAC synthesis by *P. aeruginosa* LV strain is likely to be triggered by starvation conditions (Fig. 1D, Table 1). It is known that the cells respond to different stress conditions, such as low levels of carbon, nitrogen or phosphorus source, including amino acid starvation, triggering the alternative sigma factor RpoS synthesis, the primary regulator of stationary phase genes (Navarro et al., 2010). In Gram-negative bacteria, RpoS controls genes that prepare the cell for survival in crude settings, affecting the production of exotoxin A, pyocyanin and pyoverdine from *P. aeruginosa* (Suh et al., 1999). The maximum MIC value of OAC ranged between 7.5 and 15 mg L⁻¹ and the highest concentration observed in the cultures was 11.11 ± 1.05 mg L⁻¹, in OACM, which was the same of MIC. The results suggested that, for increasing the OAC production, different ways should be explored, like chemical synthesis, random mutation and screening or metabolic engineering tools, which are fundamental for the production of many natural compounds (Nielsen and Keasling, 2016; Tamano, 2014).

In contrast with OAC, the production of IDC, PCA and PCN by *P. aeruginosa* LV strain was favored by higher nutrient concentrations (Table 1) and the presence of KH₂PO₄ in the culture media improved the production of PCN (Fig. 5). Earlier studies reported that the production of phenazines by *Pseudomonas* strains was significantly influenced by phosphate concentration. Ingledew and Campbell (1969), and Turner (1986) found that phosphate deficiency enhanced PYC synthesis using *P. aeruginosa*. On the other hand, van Rij et al. (2004) observed that the production of PCL1391, PYC and PCN by *P. aeruginosa* and *P. chlororaphis* increased in a medium containing intermediate level of phosphate. Different effects of physical and chemical factors on *Pseudomonas* strains were observed, indicating that environmental conditions influence metabolite production (Bhattacharyya and Jha, 2012). Recinos et al. (2012) found that the expression of both *phz1* and *phz2* operons, which are involved in the biosynthesis of PCA and derivatives by *P. aeruginosa* (Blankenfelt and Parsons, 2014; Cui et al., 2016), was environment-dependent and played differential roles in the pathogenicity of *P. aeruginosa* PA14.

Several studies have been carried out in order to increase phenazines concentrations, using fermentation-based production from wild-type and mutant bacteria strains. PCN production by *P. chlororaphis* strain PCL1391 yielded less than 40 mg L⁻¹ in shake flasks with

modified Vogel Bonner medium (MVB1)-glucose (van Rij et al., 2004), whilst PCN concentrations greater than 900 mg mL⁻¹, produced by *P. chlororaphis* strain P3, were achieved by using optimized fermentation conditions, and even greater yields (over 2800 mg mL⁻¹) achieved by mutation breeding of *P. chlororaphis* P3 strain (Tan et al., 2016). For their counterpart, PCA production by *P. aeruginosa* strain M18 yielded 20 mg L⁻¹ in shake flask using PPM medium and greater than 4000 mg mL⁻¹ was obtained with optimized conditions in fed-batch culture, after being subjected to many processes of genetic manipulation (current industrial strain) (Du et al., 2014; Ge et al., 2006; Li et al., 2008, 2010; Su et al., 2010; Zhou et al., 2010). Similarly, Jin et al. (2015) modified the central biosynthetic and secondary metabolic pathways in *P. aeruginosa* strain PA1201 and reported a PCA concentration of 9882 mg L⁻¹ in fed-batch fermentation.

5. Conclusions

Two culture conditions were optimized to produce IDC, PCA and PCN compounds by *P. aeruginosa* LV strain using screening experiments and RSM methodology. Under optimized conditions, the production of IDC, PCA and PCN increased by 42, 38 and 250 times, respectively, when compared to the CPS in fermentation vessels. In addition, the harvesting time for PCA and PCN was decreased. Consequently, the production of metabolites was increased, which should help to improve the supernatant downstream process under laboratory conditions (concentration, extraction and purification) and to decrease the costs of production. The OAC concentration did not increase more than MIC and, in order to increase the OAC production, further studies are necessary.

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Compliance with ethical standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bcab.2019.01.025.

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