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Factorial design and response surface optimization of crude violacein for *Chromobacterium violaceum* production

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Abstract

Initially an eleven variable, sixteen assay 2^{15-11} fractional factorial design, was used to determine the key factors in the production of violacein produced by *Chromobacterium violaceum*, CCT 3496. Subsequently five and three factor central composite designs were executed to determine response surfaces with the aim of optimizing cellular mass and crude violacein production. The 7.5 g l^{-1} dry cell mass and 0.17 g l^{-1} crude violacein productions obtained with our initial culture medium were increased to 21 g l^{-1} and 0.43 g l^{-1}, respectively, for a medium investigated in the three factor design.

Introduction

Violacein is a pigment produced by bacterium *Chromobacterium violaceum* with bactericidal (Lichstein & Vandesand 1945), trypanocidal (Durán *et al*. 1994), tumoricidal (Melo *et al.* 2000), mycobactericidal (Souza *et al.* 1999) and antioxidant properties (De Azevedo *et al.* 2000), whose drug development stage has yet to enter the clinical phase.

The nutritional requirements of *C. violaceum* necessary to increase violacein production have been studied since 1913 using classical univariate techniques. Since then a large number of nutritional factors and physical parameters have been reported as being important for violacein production, such as glucose (Kimmel & Maier 1969, Duran *et al.* 1994), yeast extract, DL-methionine, vitamin B_{12}, peptone (DeMoss & Happel 1959), Zn^{2+} (Hoshino & Yamamoto 1997), L-tryptophan (DeMoss & Evans 1959, Kimmel & Maier 1969), temperature, agitation and pH (DeMoss & Evans 1959, Riveros *et al.* 1989) among others. Besides physical and nutritional factors, violacein production by *C. violaceum* depends on the strain used and only a few reports give the volumetric production (nmol ml^{-1} h^{-1}) of this pigment. The ATCC 553 strain used by DeMoss & Happel (1959) has a 0.47 nmol ml^{-1} h^{-1} rate of violacein production. Riveros *et al.* (1989) used a BB-78 strain and obtained a production rate of 4 nmol ml^{-1} h^{-1}. The CCT 3496 strain used here had a 13.75 nmol ml^{-1} h^{-1} production rate before optimization. These values can be increased, through the determination of optimized factor levels by standard univariate techniques is not always feasible for such a complex system.

The aim of this work was to determine optimized values of these factors by applying groups of statistically planned experiments to increase violacein production and cellular mass. These multivariate ex-

periments are designed to reduce the number of experiments necessary in the optimization process and to produce more precise results than those obtainable by univariate strategies (Barros Neto *et al.* 1996, Box *et al.* 1978). Statistical designs result in successful optimizations even if a fundamental understanding of the mechanisms involved in the process being investigated is lacking.

The first stage of this work involved a fractional design ideal for simultaneously screening up to 15 factors. Central composite designs for the five factors found to be important in the first stage are applied in the next stage of optimization. Based on the most promising factor levels found for this design a final central composite design in three factors is used.

Contrary to the work recently published by Gouveia *et al. (*2001), in which a univariate screening of important factors, like nitrogen sources, was employed for subsequent multivariate optimization, our work uses multivariate experimental designs for both the screening and optimization phases. This minimizes the possibility of excluding factors that are important for optimization from investigation.

Materials and methods

Microorganism

Starting from a culture of *C. violaceum* CCT 3496, from the collection of the Fundação Tropical André Tosello, Campinas, S.P., Brazil, a colony was isolated, after culture by the streak plate method. This pure colony of *C. violaceum* was used in all the fermentations described in this work. Maintenance was done in liquid medium of nutrient broth (D-glucose 5 g l^{-1}; bacteriological peptone 5 g l^{-1}, yeast extract 2 g l^{-1}, in distilled water), on a rotatory shaker (150 rpm) at 30 °C. The inoculum was prepared using this culture medium and the same fermentation conditions after a 15-h period.

Cellular growth and crude violacein dosage

The determination of cellular growth was made after 36 h fermentation by the collection of an aliquot of broth (dilution: 250 μl broth + 2.75 ml distilled water) and reading its absorbance at 720 nm. Cellular concentration was determined constructing a regression curve of cellular concentration (dry cell mass g l^{-1}) *vs.* absorbance (Tortora *et al.* 1998). The determination of crude violacein (violacein + deoxyviolacein) was carried out by the collection of an aliquot of 1 ml of broth after 36 h fermentation, followed by centrifugation at 10 000 *g* for 10 min. The supernatant was eliminated and 5 ml absolute ethanol was added to the pellets of the cells to extract pigment. Later it was centrifuged at 10 000 *g* for 10 min for cell removal. The supernatant, crude alcoholic extract of violacein, was collected and the amount of crude violacein (dilution: 500 μl crude alcoholic extract of violacein + 2.5 ml ethanol) was determined by absorbance measurement at 575 nm (molar extinction coefficient of pure violacein = 0.05601 ml μg^{-1} cm^{-1} in ethanol at 575 nm).

Experimental design

For all experimental designs fermentation was realized in 50 ml Erlenmeyer flasks containing 20 ml of culture medium. Experiments were conducted in a rotatory shaker. As a control, three flasks with initial culture medium (anhydrous D-glucose 5 g l^{-1}, bacteriological peptone 5 g l^{-1}, yeast extract 2 g l^{-1}, L-tryptophan 0.3 g l^{-1} in distilled water) were used in all experiments.

In the initial stage 11 factors were studied by conducting 16 experiments in duplicate with the culture medium formulations and fermentation conditions shown in Table 1. Statistical designs are always expressed in coded values for convenience. High and low levels for each factor, represented by (+) and (−) signs, respectively, in this stage were chosen based on our previous laboratory experience and on information published in the scientific literature about this system. The effects on cellular mass and crude violacein production owing to changes of each factor from its low to its high level are calculated by the equation, Effect = $[(\sum R_+/8) - (\sum R_-/8)]$, where R_+ and R_- are values of the experiments at their high (+) and low (−) levels.

In the second phase of the work a central composite design involving five factors (D-glucose, yeast extract, L-tryptophan, peptone and zinc sulfate), chosen based on the results of the fractional factorial design, was performed. This design consists of a 2^5 factorial and star design plus a replicated central point to determine error and also help judge lack of fit of entertained models. Both linear and quadratic models can be used in attempts to fit the experimental data (Barros Neto *et al.* 1996). Real factor values for the coded $-\alpha$, -1, 0 $+1$, and $+\alpha$ levels are presented below for each factor. The temperature, agitation rate and inoculum used

Table 1. Fractional factorial design results, 2^{15-11}, for cellular mass (CM) and crude violacein (V) productions.

Exp.	Coded value of real factors[a]											Virtual factors				Responses	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	CM (g l^{-1})[b]	V (g l^{-1})[c]
1	−	−	−	−	+	+	+	+	+	+	−	−	−	−	+	3.56	0.21
2	+	−	−	−	−	−	−	+	+	+	+	+	+	−	−	7.34	0.1
3	−	+	−	−	−	+	+	−	−	+	+	+	−	+	−	3.69	0.14
4	+	+	−	−	+	−	−	−	−	+	−	−	+	+	+	4.32	0.07
5	−	−	+	−	+	−	+	−	+	−	+	−	+	+	−	5.33	0.2
6	+	−	+	−	−	+	−	−	+	−	−	+	−	+	+	6.84	0.08
7	−	+	+	−	−	−	+	+	−	−	−	+	+	−	+	6.71	0.17
8	+	+	+	−	+	+	−	+	−	−	+	−	−	−	−	0.78	0
9	−	−	−	+	+	+	−	+	−	−	−	+	+	+	−	3.05	0.05
10	+	−	−	+	−	−	+	+	−	−	+	−	−	+	+	0	0
11	−	+	−	+	−	+	−	−	+	−	+	−	+	−	+	5.96	0.13
12	+	+	−	+	+	−	+	−	+	−	−	+	−	−	−	11.63	0.14
13	−	−	+	+	+	−	−	−	−	+	+	+	−	−	+	4.06	0.09
14	+	−	+	+	−	+	+	−	−	+	−	−	+	−	−	6.33	0.07
15	−	+	+	+	−	−	−	+	+	+	−	−	−	+	−	5.2	0.14
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8.23	0.17

[a]The coded values correspond to for D-glucose (1): −1 (2.5 g l^{-1}), +1 (10 g l^{-1}). For yeast extract (2): −1 (1 g l^{-1}), +1 (6 g l^{-1}). For DL-methionine (3): −1 (0.01 g l^{-1}), +1 (0.1 g l^{-1}). For vitamin B_{12} (4): −1 (0.012 ng ml^{-1}), +1 (0.1 μg ml^{-1}). For L-tryptophan (5): −1 (0.2 g l^{-1}), +1 (1 g l^{-1}). For agitation (6): −1 (150 rpm), +1 (250 rpm). For temperature (7): −1 (28 °C), +1 (33 °C). For pH (8): −1 (6.8), +1 (7.8). For peptone (9): −1 (2 g l^{-1}), +1 (8 g l^{-1}). For inoculum (10): −1 (0.05 ml), +1 (0.25 ml). For zinc (11): −1 (0.05 mM), +1 (0.1 mM).

[b,c]These columns were used to calculate the standard errors in Table 2.

[b]Dry cell mass.

[c]Crude violacein.

Table 2. Statistically significant contrasts at the 95% confidence level for cellular mass (CM) (g l^{-1}) and crude violacein (V) (g l^{-1}) productions obtained from the data in Table 1.

Factors	Effects	
	MC	V
Peptone	3.13	0.066
Temperature	0.86	0.042
Glucose	0.99	−0.056
Inoculum	0.3	0.034
Vitamin B_{12}	0.74	−0.027
Zinc	−1.53	−0.019
L-tryptophan	–	0.017
Yeast extract	1.25	0.013
DL-methionine	0.5	0.014
Agitation	−0.76	−0.012
pH	−1.62	–

were 33 °C, 300 g and 0.25 ml, respectively. The culture medium compositions used corresponding to the codified values are: for D-glucose: $-\alpha$ (1 g l^{-1}), −1 (3 g l^{-1}), 0 (5 g l^{-1}), +1 (7 g l^{-1}), $+\alpha$ (9 g l^{-1}); for yeast extract: $-\alpha$ (2 g l^{-1}), −1 (4 g l^{-1}), 0 (6 g l^{-1}), +1 (8 g l^{-1}), $+\alpha$ (10 g l^{-1}); for peptone: $-\alpha$ (1 g l^{-1}), −1 (3 g l^{-1}), 0 (5 g l^{-1}), +1 (7 g l^{-1}), $+\alpha$ (9 g l^{-1}); for L-tryptophan: $-\alpha$ (0.2 g l^{-1}), −1 (0.4 g l^{-1}), 0 (0.6 g l^{-1}), +1 (0.8 g l^{-1}), $+\alpha$ (1 g l^{-1}); for Zn^{2+}: $-\alpha$ (0.025 mM), −1 (0.0375 mM), 0 (0.05 mM), +1 (0.0625 mM), $+\alpha$ (0.075 mM); central point ($\alpha = 2$).

In the final part of this work three factors, D-glucose, yeast extract and L-tryptophan were studied using a three-factor central composite design. Factor levels used for this design were chosen based on the results of the five factor design. The compositions of the culture media utilized are shown in Table 3 where temperature, agitation rate, inoculum, peptone and zinc sulfate were maintained at 33 °C, 300 g, 0.25 ml, 1 g l^{-1} and 0.0625 mM, respectively. Data analysis was carried out using programs written in our laboratories (Barros Neto *et al.* 1996).

Table 3. Three factor central composite design results for crude violacein (V) and cellular mass productions (CM).

Exp.	Coded value[a]			Responses	
	D-glucose	Yeast	L-tryptophan	CM (g l^{-1})[b]	V (g l^{-1})[c]
1	−1	−1	−1	15.42	0.35
2	1	−1	−1	15.29	0.35
3	−1	1	−1	17.56	0.39
4	1	1	−1	18.82	0.41
5	−1	−1	1	16.05	0.37
6	1	−1	1	14.91	0.31
7	−1	1	1	17.94	0.44
8	1	1	1	17.81	0.35
9	$-\alpha$	0	0	12.89	0.37
10	α	0	0	16.30	0.32
11	0	$-\alpha$	0	10.88	0.28
12	0	α	0	21.09	0.43
13	0	0	$-\alpha$	17.43	0.33
14	0	0	α	17.18	0.36
15[d]	0	0	0	16.80	0.32
16[d]	0	0	0	18.06	0.38
17[d]	0	0	0	17.56	0.37
18[d]	0	0	0	16.55	0.35

[a]The coded values correspond to (in g l^{-1}) for D-glucose: $-\alpha$ (5), −1 (8.4), 0 (10.4), +1 (12.4), + α (15.8). For yeast extract (yeast): $-\alpha$ (6), −1 (9.4), 0 (11.4), +1 (13.4), +α (16.8). For L-tryptophan: $-\alpha$ (0.6), −1 (0.94), 0 (1.14), +1 (1.34), +α (1.68).
[b]Dry cell mass.
[c]Crude violacein.
[d]Central point ($\alpha = 1.68$).

Results and discussion

The results for the 2^{15-11} fractional factorial design are presented in Tables 1 and 2.

The factors presenting significant (95% confidence level) effect values for both responses simultaneously were chosen for study in the second stage of the optimization process. The other factors were fixed at convenient levels. Peptone had the largest effect on cellular mass (3.13) and crude violacein production (0.066). An increase in the glucose level increased the cellular mass but also decreased crude violacein production. This could suggest the existence of genetic repression mechanisms on the production of violacein by glucose (Drew & Demain 1977). Zinc sulfate and yeast extract showed more pronounced effects on cellular mass production (−1.53 and 1.25) than on crude violacein production (−0.019 and 0.013). Although L-tryptophan had a weak but significant effect on crude violacein production (0.017) this factor was maintained in the next design since violacein biosynthesis is produced by the condensation of two modified tryptophan molecules and its presence in the design experiments is necessary (Ruhul Momen & Hoshino 2000). The motive for its small effect value in this factorial design is that the choice of the low level (0.2 g l^{-1}) is close to its optimum value. Temperature and inoculum exerted positive effects, principally relative to crude violacein production (0.042 and 0.034) and for this reason were fixed at their high values (33 °C and 0.25 ml). The agitation rate effect, for the investigated levels, was not significant for crude violacein and only slightly important for cellular mass production. The other factors were less important, like DL-methionine and vitamin B_{12}, or they presented significant effects for only one of the responses, such as pH (phosphate buffer). These were eliminated from the next stage permitting reduction of the statistical design to a more manageable size. However their study in future experimental optimization work would be warranted, specially pH.

The results for the five factor design showed, in general, an increase in cellular mass and crude violacein productions with average values of 7.47 and 0.23 g l^{-1} compared to 5.19 and 0.11 g l^{-1} values for the fractional factorial design. In contrast with the results of Table 1 the results of the five factor composite design have a higher correlation between cellular mass and crude violacein productions.

Linear models proved to be more significant than quadratic ones for both responses. An analysis of the variance for each regression was performed and model parameters significant at the 95% confidence level were chosen. All coefficients of second-order terms (such as G^2, T^2, GT, etc.) are not significant. The regression equations for the linear models for cellular mass (CM) and crude violacein (V) productions in g l^{-1} are given by Equations (1) and (2), respectively:

$$CM = 7.473^* + 2.428G^* + 0.862E^* - 0.473P^* + 0.284T + 0.095Z \quad (1)$$

$$V = 0.230^* + 0.027G^* + 0.020E^* - 0.004P + 0.0212T^* + 0.007Z, \quad (2)$$

where G, E, T, P and Z represent codified values for glucose, yeast extract, L-tryptophan, peptone and zinc sulfate, respectively, and the asterisks represent significant 95% confidence level terms.

The experiments of this design showed that the relation between the nitrogen sources used, yeast extract and peptone, should be varied inversely to obtain the response maximum. In this way the use of high yeast extract levels and lower peptone levels is expected to increase cellular mass and crude violacein production. Peptone changes are insignificant for crude violacein production (see Equation (2)) and shows a low negative effect for cellular mass production (see Equation (1)). Since yeast extract was a better nitrogen source, peptone was fixed at its low level in the next stage of this investigation. The zinc concentration had no significant effects and can be maintained at any convenient level. Here 0.0625 mM was used.

The models for cellular mass and crude violacein have significant and positive terms for glucose and yeast extract. They predict that an increase in these factor levels will result in large increases in cellular mass and crude violacein. For this reason their factor levels were changed from 1–9 g l^{-1} and 2–10 g l^{-1} in the five factor design to 5–15.8 g l^{-1} and 6–16.8 g l^{-1}, respectively, in the final optimization stage involving a three factor central composite design.

Even though L-tryptophan has an insignificant effect on cellular mass it does have a positive and significant effect for crude violacein. As such its levels were increased from 0.2–1 g l^{-1} to 0.6–1.68 g l^{-1} in the three factor design.

The results of the three factor central composite design are presented in Table 3. The average production of the experiments in this design is 0.36 g l^{-1} crude violacein, 50% higher than the average result of the five factor central composite design. The average in Table 3, 16.59 g l^{-1} cell mass, is more than double the one of the five factor central composite design, 7.47 g l^{-1} cellular mass. Linear models for cellular mass and crude violacein are also statistically more significant than quadratic models for these design results. All coefficients of second order terms (such as G^2, T^2, GT, etc.) are not significant at the 95% confidence level. The linear model regression equations for cellular mass (CM) and crude violacein production (V) are:

$$CM = 16.586^* + 0.410G + 2.025E^* - 0.059T, \quad (3)$$

$$V = 0.360^* - 0.016G + 0.033E^* + 0.001T. \quad (4)$$

The models have very significant yeast extract terms. All other terms are not significant except the glucose one in the crude violacein model that is marginally significant. Contrary to what is observed for glucose, increases in yeast extract levels do not repress crude violacein production as shown by experiments 11 and 12 in Table 3. Further increases in the yeast extract level could result in even increased crude violacein (Figure 1) and cellular mass production. The planar response surfaces show a much larger rate of ascent along the yeast extract axis than along the glucose one. The response contour lines are almost perpendicular to the yeast extract axis showing this high rate of increased production.

One carbon (glucose) and two nitrogen sources (peptone and yeast extract) were used in this work. The factorial designs permitted selection of the best nitrogen source for cellular growth and crude violacein production and the best relative quantities of the carbon/nitrogen sources. In the same way one could use factorial designs to determine the best carbon source among those of rapid consumption (e.g., glucose) and those of slower consumption (e.g., sucrose, lactose). It is known that for some microorganisms carbon sources with rapid metabolisms result in an increase in cellular mass and a decrease of secondary metabolic production. However the use of carbon sources of

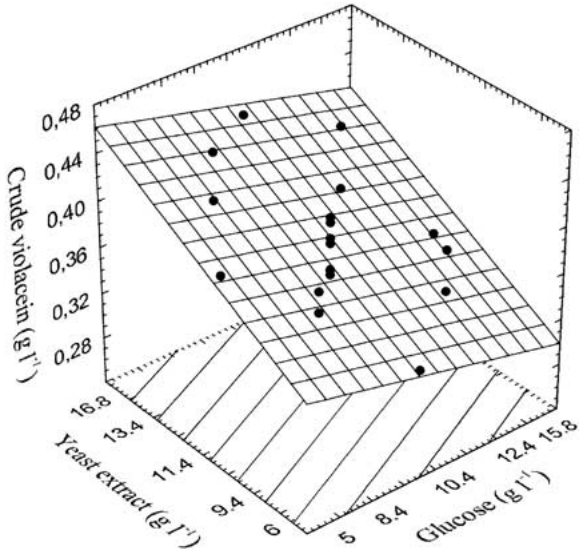

Fig. 1. Response surface for the production of crude violacein. The planar response shows a much larger rate of ascendent along the yeast extract axis than along the glucose one.

slower consumption results in a decrease of cellular growth and an increase in secondary metabolic production (Drew & Demain 1977, Vicik *et al.* 1990). In this case factorial designs can be used to determine the relation between carbon sources of fast and slow consumptions, called diauxic growth, with the objective of obtaining maximum cellular growth with a rapidly consumed carbon source and maximum secondary metabolic production with a slowly consumed carbon source.

Summary

Factorial design and response surface experiments resulted in an increased 21 g l^{-1} cellular mass and 0.43 g l^{-1} (=34.79 nmol ml^{-1} h^{-1}) production of crude violacein compared with 7.5 g l^{-1} and 0.17 g l^{-1} (=13.17 nmol ml^{-1} h^{-1}), respectively, for the initial culture medium. Even higher cellular masses and violacein productions might be obtained by making further adjustments of glucose, yeast and pH levels or by even introducing other carbon sources than glucose, with the objective of obtaining optimization of systems described by a diauxic growth model.

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