Modeling bikaverin production by *Fusarium oxysporum* CCT7620 in shake flask cultures

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**Abstract**

Bikaverin is a fungal red pigment that presents antimicrobial and antitumor activities. Therefore, this substance could be used as an alternative additive in the food and pharmaceutical industries. The aim of this work was to use response surface methodology to optimize the fermentation conditions and maximize the production of bikaverin in shake flasks. The variables investigated were agitation speed (71–289 rpm), temperature (21–35 °C), and substrate (rice) concentration in the culture medium (16.4–83.6 g/L). The agitation speed had a positive effect on red pigment production, while substrate concentration and temperature had the opposite effect. Maximum bikaverin production was predicted to occur using 289 rpm, 24.3 °C, and 16.4 g/L rice concentration. Experimental validation using 289 rpm, 28 °C, and 20 g/L rice concentration was 6.2% higher than predicted by the model. The present investigation was important for defining the best conditions for the production of bikaverin.

**Keywords:** *Fusarium oxysporum*, Bikaverin, Fungal pigment, Submerged cultures, Red pigment

**Introduction**

Natural pigments have been increasingly used in the pharmaceutical, textile, and food industries, due to their environmental friendly nature and the fact that they are considered less toxic than their chemical counterparts, resulting in higher market appeal (Ogbonna 2016). Some synthetic dyes are possible carcinogens, highly toxic, and can induce allergic dermatitis, skin irritation, and mutation in humans (Srivastava et al. 2004; Sinha et al. 2012). They can contaminate the environment, leading to negative ecotoxicalogical effects and bioaccumulation in wildlife (Saha et al. 2010). Therefore, there is increasing preference for natural colorants, particularly in food products, where they can act as functional ingredients (Dufossé et al. 2005; Yolmeh et al. 2014).

Microorganisms are considered a promising source of natural pigments, mainly because they can potentially be produced uninterruptedly, under controlled and optimizable conditions. Therefore, the biotechnological production of pigments has attracted the interest of industry. Some examples are already commercially available, such as β-carotene from microalgae, phycocyanins from *Spirulina*, and *Monascus* pigments (Dufossé et al. 2005; Kongruang 2011).

Polypeptides are a family of chemicals that are formed by condensation of successive acetate units, as a result of the action of multifunctional polypeptide synthase (PKS I) enzymes (Smith and Tsai 2007). The biological activities of these compounds mean that they have significant pharmaceutical importance (Hertweck 2009; Lale and Gadre 2016). Bikaverin is a red polypeptide produced by fungi of the *Fusarium* genus, such as *F. verticillioides*, *F. lycopersici*, and *F. oxysporum* (Balan et al. 1970; Linnemannstöns et al. 2002). The structure of bikaverin (6,11-dihydroxy-3,8-dimethoxy-1-methyl-benzoxanthioin-7,10,12-trion, C$_{20}$H$_{14}$O$_{8}$, molecular mass of 382.068 Da) (Fig. 1) was determined by its chemical synthesis followed by X-ray crystallography (Cornforth et al. 1971).

Feed experiments with acetate isotopes [1,2-13C] demonstrated that bikaverin could be completely biosynthesized by the condensation of nine acetate units in a single polypeptide chain, which was then folded and...
cross-linked to form the backbone of the molecule (McInnes et al. 1976). Further studies revealed that the \textit{bik1} gene, responsible for the synthesis of bikaverin, has five adjacent genes that participate in this process: \textit{bik2, bik3, bik4, bik5}, and \textit{bik6} (Wiemann et al. 2009). The transcription of these genes is affected by pH, nitrogen concentration (Wiemann et al. 2009), and carbon source. Studies found that the highest production of bikaverin was achieved in acid medium, under nitrogen limitation, with sucrose being a better carbon source than glucose (Brewer and Arsenault 1973; Limón et al. 2010).

Due to internal accumulation and excretion of this polyketide, the culture of bikaverin-producing \textit{Fusarium}, such as \textit{F. fujikuroi}, presents a reddish color (Lale and Gadre 2016), which may vary depending on the fungal growth conditions (Linnemannstöns et al. 2002). In addition to its colorant properties, bikaverin has shown antibiotic properties (Deshmukh et al. 2014) and biological activity against the protozoan \textit{Leishmania brasilienensis} (Balan et al. 1970), the oomycete \textit{Phytophthora infestans} (Son et al. 2008), and the nematode \textit{Bursaphelenchus xylophilus} (Kwon et al. 2007). Bikaverin has also been reported to have inhibitory effects against tumor cells, such as MIA Pa Ca-2 (pancreatic carcinoma) (Zhan et al. 2007) and Ehrlich ascites carcinoma (EAC), for which it exhibited ED\textsubscript{50} of 0.5 μg/mL (Fuska et al. 1975). Recently, bikaverin was tested as an inhibitor of protein kinase CK2. This enzyme is highly expressed and active in several tumor cells, being responsible for suppressing apoptosis and stimulating cell growth. Both cell viability and cell proliferation were drastically reduced after treatment with 10 μM bikaverin for 24 h in MCF7 cells (human breast adenocarcinoma cell line), A427 (human lung carcinoma cell line) and A431 (human epidermoid carcinoma cell line) (Haidar et al. 2019).

Although the bikaverin-producing \textit{Fusarium} species are widespread agricultural phytopathogens, there are no reports of negative effects on human health caused specifically by bikaverin (Limón et al. 2010). In rats, bikaverin at concentrations up to 100 μM showed no genotoxic effect on hepatocyte DNA (Norred et al. 1992). The lack of toxicological data and information about other biological activities of bikaverin motivated our research group to produce this compound for further studies. Considering that bikaverin biosynthesis is affected by different environmental factors such as agitation, aeration, temperature, pH (Giordano and Domenech 1999; Bell et al. 2003; Srivastava et al. 2011), and medium composition, including salt starvation and type of carbon source (Rodríguez-Ortiz et al. 2010), and that the optimal conditions may vary for each microorganism, the aim of the present study was to model the effects of the main parameters on bikaverin production by \textit{F. oxysporum} CCT7620.

Materials and methods
The general experimental steps of this study followed the sequence shown in Fig. 2. In the following sections, a detailed explanation of each procedure may be found.

Bioorganism and biomass (inoculum) production
\textit{Fusarium oxysporum} CCT7620, previously isolated from soil, was deposited in the Tropical Cultures Collection of the André Tozello Institute, in Campinas, Brazil (Silva 2013). The microorganism was maintained on potato dextrose agar (PDA) slants at 4 °C, with periodical replication in PDA agar plates.

A piece of agar (1.5 cm\textsuperscript{2}) containing \textit{F. oxysporum} CCT7620 grown for 72 h in PDA medium, at 30 °C, was transferred to a conical flask containing 200 mL of rice medium, followed by homogenization using an UltraTurrax (16,000 rpm). The rice medium consisted of 50 g of rice flour per liter of water, and the rice flour was obtained by milling commercial white rice (polished) in a knife mill. The flasks were incubated for 72 h, at 30 °C and 200 rpm, after which the biomass (inoculum) was recovered by centrifugation at 9000 rpm for 5 min. The supernatant was discarded, the biomass was fractionated (0.4 g wet weight portions), and the material was stored in a freezer (at −18 °C) for subsequent use as inoculum.

Effect of pH on bikaverin production
Various pH conditions were tested to determine whether pH control would be necessary, considering the effect of this parameter on bikaverin production. Given that an acidic medium is known to induce bikaverin production (Wiemann et al. 2009), the pH of the rice medium (50 g of rice flour per liter of water) was adjusted to different values (2.5, 3.0, 3.5, 4.0, and 4.5), using 0.05 mol L\textsuperscript{−1} citrate buffer, before being inoculated with the biomass produced as described above (“Bioorganism and biomass (inoculum) production” section). A buffer-free medium (initial pH of 6.7) was used as the control. The cultures were incubated at 28 °C and 289 rpm (the supposed
optimal condition), using a rotary shaker. Aliquots of 3 mL were collected at different times (0, 24, 48, 72, and 96 h), to monitor the pigment production kinetics. The samples were extracted and analyzed as described in “Extraction and analysis of the pigment produced” section. The importance of pH for bikaverin biosynthesis was evaluated statistically using one-way ANOVA followed by the Tukey HSD test (α = 0.05).

Kinetics of pigment production under different conditions
The experimental design for bikaverin pigment production in liquid culture considered three independent variables (Table 1), employing a 2³ central composite design (CCD) with 6 axial points and 3 replicates at the center point, resulting in 17 different assays (Table 2). The time course of pigment production (measurements at 0, 24, 48, 72, and 96 h) was determined for each of these different culture conditions, using the extraction and analytical procedure described in “Extraction and analysis of the pigment produced” section.

Extraction and analysis of the pigment produced
Samples (whole fermented broth) were transferred to Falcon tubes and extracted with ethyl acetate (3 mL of solvent for each 3 mL of sample), using a vortex for 60 s, followed by centrifugation at 3600 rpm for 5 min. The recovered organic layer was analyzed using a Beckman Du 640 spectrophotometer, recording the absorbance values at 500 nm. Bikaverin was quantified using a calibration curve (Abs₅₀₀ = 0.003553 [bikaverin, mg/L] − 0.029447, R² = 0.9946), as described in Additional file 1: Fig. S1.

Statistical analysis
Response surface methodology (RSM) using the CCD was applied to model the production of red pigment by F. oxysporum CCT7620 in liquid culture medium, according to Eq. 1:

\[
Y = \beta_0 + \beta_1 T + \beta_2 A + \beta_3 [S] + \beta_{11} T^2 + \beta_{22} A^2 + \beta_{33} [S]^2 + \beta_{12} T A + \beta_{13} T [S] + \beta_{23} A [S]
\]

where Y is the bikaverin concentration (mg/L), T, A, and [S] are the coded values of temperature, agitation speed, and rice concentration, respectively, and βᵦ are the estimated regression coefficients associated with

Table 1 Parameters and levels used in the central composite design (CCD)

| Independent variables | Symbol | Coded levels |
|-----------------------|--------|--------------|
| Temperature (°C)      | T      | 21 24 28 32 35 |
| Agitation speed (rpm) | A      | 71 115 180 245 289 |
| Rice concentration (g/L) | [S] | 16.4 30 50 70 83.6 |
temperature (1), agitation speed (2), or rice (substrate) concentration (3), and their combinations. The data were treated using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, USA). Only the statistically significant parameters (α < 0.05) were used to construct the model (Eq. 1). After analysis of variance (ANOVA), response surfaces were constructed from the fitted models.

Results and discussion

Effect of pH control on bikaverin production

It is known that the metabolic pathway responsible for bikaverin production is affected by pH (Bell et al. 2003; Li and Mira De Orduña 2010), and that acidic pH values tend to increase its production. The bik genes responsible for bikaverin biosynthesis are only expressed in acidic medium (Wiemann et al. 2009), suggesting that low pH values are necessary for its synthesis (Medentsev and Akimenko 1998; Wiemann et al. 2009). However, it was unclear if pH control would be beneficial for the present process.

Therefore, instead of including pH as an independent variable in the CCD, an experiment was performed comparing cultures with pH control (with different acidic pH values obtained using citrate buffer) and without pH control. As shown in Table 3, there were no significant differences in bikaverin production (at 72 and 96 h) when the pH was controlled in the range 2.5–4.0. On the other hand, growth without pH control resulted in significantly higher concentrations of bikaverin after 72 h of fermentation, while control of the pH at 4.5 resulted in significantly lower bikaverin production, compared to the other conditions tested.

Previous studies by our group, using F. oxysporum CCT7620 cultivated in an airlift bioreactor operating with no pH control, indicated that the pH decreased continuously from the initial value (~6.0), reaching a value close to 3.5 after 48 h, which then remained stable during the following 48 h. In this case, maximal pigment production occurred between 48 and 72 h (Silva 2013). Similar results were reported for cultivation of F. decemcellulare to produce naphthoquinone (Medentsev

Table 2 Bikaverin production (mg/L) by F. oxyporum CCT7620 for each experimental condition defined by the central composite rotational design

| Run | Temperature (°C) | Agitation (rpm) | Rice flour (g/L) | Bikaverin production (mg/L) |
|-----|-----------------|-----------------|-----------------|-----------------------------|
|     | Coded | Real | Coded | Real | Coded | Real | 0 h | 24 h | 48 h | 72 h | 96 h |
| 1   | −1    | 24   | −1    | 115  | −1    | 30   | 0   | 0   | 0   | 44.53 | 75.45  |
| 2   | +1    | 32   | −1    | 115  | −1    | 30   | 0   | 0   | 0   | 100.34 | 132.74 |
| 3   | −1    | 24   | +1    | 245  | −1    | 30   | 0   | 0   | 0   | 127.90 | 260.15 |
| 4   | +1    | 32   | +1    | 245  | −1    | 30   | 0   | 0   | 0   | 140.43 |
| 5   | −1    | 21   | −1    | 115  | +1    | 70   | 0   | 0   | 0   | 37.86 |
| 6   | +1    | 32   | −1    | 115  | +1    | 70   | 0   | 0   | 0   | 38.11 |
| 7   | −1    | 24   | +1    | 245  | +1    | 70   | 0   | 0   | 0   | 53.24 |
| 8   | +1    | 32   | +1    | 245  | +1    | 70   | 0   | 0   | 0   | 110.12 |
| 9   | −1.68 | 21   | 0     | 180  | 0     | 50   | 0   | 0   | 0   | 42.33 |
| 10  | +1.68 | 35   | 0     | 180  | 0     | 50   | 0   | 0   | 0   | 32.77 |
| 11  | 0     | 28   | −1.68 | 71   | 0     | 50   | 0   | 0   | 0   | 0   |
| 12  | 0     | 28   | +1.68 | 289  | 0     | 50   | 0   | 0   | 0   | 269.89 |
| 13  | 0     | 28   | 0     | 180  | −1.68 | 16.3 | 0   | 0   | 0   | 247.20 |
| 14  | 0     | 28   | 0     | 180  | +1.68 | 83.6 | 0   | 0   | 0   | 269.90 |
| 15  | 0     | 28   | 0     | 180  | 0     | 50   | 0   | 0   | 0   | 269.90 |
| 16  | 0     | 28   | 0     | 180  | 0     | 50   | 0   | 0   | 0   | 269.90 |
| 17  | 0     | 28   | 0     | 180  | 0     | 50   | 0   | 0   | 0   | 269.90 |

Table 3 Influence of culture pH on bikaverin production

| pH   | Mean concentration (mg/L) |
|------|---------------------------|
|      | 72 h | 96 h |
| 2.5  | 185.1** ± 30.5 | 234.5* ± 26.8 |
| 3.0  | 177.6** ± 29.8 | 241.4* ± 52.86 |
| 3.5  | 192.3** ± 26.5 | 229.5* ± 21.5 |
| 4.0  | 196.5** ± 13.5 | 237.7 ± 10.3 |
| 4.5  | 27.0*** ± 21.9 | 24.5** ± 18.0 |
| Control | 352.6* ± 30.4 | 302.5 ± 38.6 |

* Means followed by different asterisks in the same column differ from each other, according to the Tukey test at 5% significance (p < 0.05)
et al. 2005). In another study, the maximum production of bikaverin by *F. fujikuroi* occurred when the initial pH of 4.5 decreased to 3.7 at the end of the fermentation, suggesting that the pH decrease during growth favored the metabolic pathway for formation of this pigment (Giordano et al. 1999). These results suggested that the natural acidification of the medium was sufficient to induce bikaverin production. Therefore, this experiment was important, since it could be concluded that the CCD should not include pH as an independent variable and that pH control would not be indicated to maximize bikaverin production.

**Modeling of bikaverin pigment production**

Based on the experimental design, 17 assays were carried out under different conditions (Table 2). Regardless of the condition tested, maximal red pigment production occurred at 96 h of cultivation. Therefore, this fermentation time was selected for construction of the model, which only considered reasonably significant parameters (p values close to 0.1 or lower). Consequently, the model included the linear coefficients corresponding to temperature, agitation speed, and rice concentration, the quadratic coefficient for temperature, and the interaction of temperature and agitation speed (Additional file 1: Table S1), resulting in the model shown in Eq. 2:

\[
[Bikaverin, \text{mg/L}] = 146.11 - 22.19 T - 32.55 T^2 + 65.90 A - 56.43[S] - 23.11 T.A
\]

(2)

Table 4 presents the ANOVA results for this model (Eq. 2). The ratio between the mean square of regression and the mean square of the residuals (MS_R/MS_E), or \( F_{cal} \), was 18.3, which was close to six times higher than the critical value at 95% significance (\( F_{tab} = 3.20 \)). The ratio between the mean square of the lack of fit and the mean square of the pure error (MS_LF/MS_PE) was 6.3, which was three times lower than the critical value at 95% significance (\( F_{tab} = 19.38 \)). The coefficient of determination (R^2) was satisfactory (0.893) and the p value for the regression was < 0.0001. Therefore, this model was considered suitable for the prediction of bikaverin pigment production after 96 h of cultivation of *F. oxysporum* CCT7620 in rice-based medium. The graphical representation of this model (Eq. 2) could then be constructed (Fig. 3).

The analysis of Fig. 3a–c indicated that the agitation speed had a positive effect on bikaverin production.

![Graphical representation of the model](image)

**Fig. 3** Interactive effect of (a) temperature and agitation speed; (b) temperature and rice concentration; and (c) agitation speed and rice concentration on bikaverin pigment production (mg/L). Rice concentration, agitation speed, and temperature were kept at 50 g/L, 180 rpm, and 28 °C in Fig. 3a–c, respectively.
was expected, since agitation increases mass transfer and gas exchange, resulting in increased growth and metabolite production (Ahmad et al. 1994). It is known that some enzymatic reactions depend on the concentration of oxygen, such as pre-bikaverin C6 and C7 hydroxylation by monoxygenase (Wiemann et al. 2009). Elsewhere, decreased biomass and bikaverin production were observed when a low air/medium ratio was employed for the cultivation of *F. fujikuroi* (Giordano and Domenech 1999).

In contrast, the substrate (rice) concentration presented the opposite effect (Fig. 3b, c). This was also expected, since nutrient limitation is known to induce pigment production in several microorganisms (Stahmann et al. 2001; Lamers et al. 2008; Akilandeswari and Pradeep 2016). In the case of bikaverin, its synthesis is repressed by high nitrogen concentrations (Linnemannstöns et al. 2002). After depletion of the assimilable nitrogen, the pigment is excreted into the culture medium during the stationary phase (Avalos et al. 1999). Similarly, other researchers found that bikaverin production started when nitrogen was depleted and continued until sufficient carbon substrate was available (Giordano et al. 1999). It has reported that bikaverin production by *F. fujikuroi* continued even under carbon source limitation (Chávez-Parga et al. 2005). Therefore, this might explain why rice medium poor in nitrogen (approximately 1.0%, Additional file 1: Table S2), especially at lower rice concentrations, resulted in increased bikaverin production.

The effect of temperature was nonlinear, with maximum bikaverin production close to 25 °C (Fig. 3a, b). The temperature is an important parameter in fermentations, since it influences the metabolic activity of fungi, consequently affecting their growth and pigment production. Temperatures between 27 and 29 °C were reported as optimal for the biosynthesis of fungal pigments in liquid culture media (Dufossé et al. 2005). For *Fusarium oxysporum*, the optimal growth temperature was 28 °C (Gupta et al. 2010). The present results showed that at 250 rpm and substrate concentration of 20 g/L, maximal bikaverin production (317.6 mg/L) occurred at 25.1 °C, although variation of temperature in the range 21.1–29.1 °C led to less than 10% variation of pigment production \( (> 285 \text{ mg/L}) \), indicating that the bioprocess was quite robust, in terms of temperature requirement. However, no pigment production was observed at 35 °C, since this temperature is not ideal for fungal growth and constitutes a limiting factor for the synthesis of naphthoquinones, such as bikaverin (Medentsvev and Akimenko 1998).

Therefore, according to the proposed model (Eq. 2), the conditions for maximal bikaverin production (380.2 mg/L) would be cultivation for 96 h at 24.3 °C and 289 rpm, with 16.4 g/L of rice in the medium. A validation experiment was performed, in triplicate, under the conditions usually employed for other bioprocesses in our laboratory (28 °C, 250 rpm, and rice concentration of 20 g/L), to evaluate the reliability of the model. This resulted in a bikaverin concentration of 320.5 ± 30 mg/L after 96 h, which was 6.2% higher than the predicted value for these conditions (301.7 mg/L). This indicated that the model was able to satisfactorily predict bikaverin production. Furthermore, production equivalent to 84% of the predicted maximum was obtained under these conditions, which were therefore considered most suitable for bikaverin production. These parameter values were similar to the ideal conditions for *Fusarium* fermentation reported in previous studies. For example, the optimum production of *F. moniliforme* pigment was achieved using 2% potato dextrose broth, 2% glucose, 1% peptone, and 0.5% methionine, at pH 5.5, with 8 days of incubation at 28 °C (Pradeep and Pradeep 2013). The highest production of gibberellin (preceding bikaverin) by *F. fujikuroi* in a bioreactor was achieved using 30 °C, C:N ratio of 36.8, and pH of 5.0 (Escamilla-Silva et al. 2001).

**Conclusion**

Response surface methodology was useful for modeling the effects of temperature, agitation speed, and rice concentration on bikaverin production by *F. oxysporum*, enabling identification of the optimal conditions. The ideal conditions for this process consisted of growing *F. oxysporum* CCT7620 in a rice medium (20 g rice flour per liter of water), with incubation for 96 h at 28 °C and 250 rpm, without pH control. This resulted in bikaverin production of 320.5 mg/L. It should be noted that the experimental bikaverin concentration could be underestimated, because the pigment extraction process was not exhaustive and the calibration curve did not consider the matrix effect. The present study represents an important step toward further investigations of this molecule. Current work by our group aims to define the best conditions for extraction of this compound from the culture broth, as well as for purification of the extract.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s40643-020-0301-5.

Additional file 1: Fig. S1. Calibration curve for bikaverin. Table S1. Significant regression coefficients for bikaverin production after 96 h of fermentation. Table S2. Centesimal composition of the culture medium centrifuged at 9000 rpm for 5 min, before inoculation.
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