**Recovery and purification of bikaverin produced by *Fusarium oxysporum* CCT7620**

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**ABSTRACT**

Microbial pigments have a distinguished potential for applications in food and pharmaceutical industries, stimulating the research in this field. The present study evaluated the ideal conditions for extracting bikaverin (red pigment) from the biomass of *Fusarium oxysporum* CCT7620. Among the solvents tested, ethyl acetate extraction resulted in the highest bikaverin concentration and the kinetic study revealed a saturation in bikaverin concentration from 256 min on. Based on a preliminary economic study, three sequential extractions with ethyl acetate was considered the ideal protocol to recover bikaverin. After extraction, chromatographic methods were tested to purify bikaverin. The use of silica gel or Sephadex (open column) could not successfully purify bikaverin, but the semi-preparative HPLC resulted in a bikaverin-enriched fraction with a purity degree equivalent to the commercial analytical standard. This work provides relevant information regarding the extraction and purification of bikaverin, which may be of use for other downstream processes.

**ARTICLE INFO**

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1. Introduction

The interest on microbial pigments has increased in the past few years since they may present relevant biological activities, besides their coloring properties (Lebeau, Petit, Dufossé, & Caro, 2019). Therefore, apart from their use in food and cosmetic sectors, these molecules can find novel applications in the pharmaceutical industry. Many examples can be cited in this sense, one of the most well established being the Monascus pigments. These compounds have been traditionally used in Asian countries as colorants, but their potential applications are now moving towards their antimutagenic, anticancer, antimicrobial and anti-inflammatory activities (Cheng, Wu, Yuan, Su, & Yanai, 2012; Feng, Shao, & Chen, 2012). Moreover, polyketides are secondary metabolites produced by several fungi as a defense mechanism (Medentsev, Arinbasarova, & Akimenko, 2005). Besides manifesting colors (dos Santos & Bicas, 2021), some of these molecules may present a wide spectrum of biological activities (Hertweck, 2009), such as tetracycline, erythromycin, rapamycin, and lovastatin clinically used as antibiotics, immunosuppressants and anticholesterolemics (Shen, 2003). Another promising candidate to increase the list of bioactive polyketide pigments is bikaverin.

Bikaverin (see structure in Fig. 1B) is a red polyketide produced by fungi, especially *Fusarium fujikuroi, F. verticillioides, F. proliferatum*, and *F. oxysporum* (Chelkowski, Zajkowski, & Visconti, 1992). Although originally red, the fungal biomass containing bikaverin can become blue after heat treatment (Brazilian patent BR 10 2013 015305-2 A2). This is an important feature to expand the options of natural blue colors for industrial applications (dos Santos & Bicas, 2021). The biological activities reported for bikaverin include the antibiotic activity against the protozoan *Leishmania braziliensis*, the oomycete *Phytophthora infestans* and the nematoid *Bursaphelenchus xylophilus* (Limón, Rodriguez-Ortiz, & Avalos, 2010). This molecule can also inhibit tumor cell lines, such as human breast adenocarcinoma (MCF7), human lung carcinoma (A427) and human epidermoid carcinoma (A431) (Haidar et al., 2019). More recently, bikaverin was reported as a promising candidate against the coronavirus disease (COVID-19) based on a molecular docking approach (Singh & Florez, 2020). However, bikaverin is commercially available only as a chemical standard (from Sigma-Aldrich, costs c.a. US$ 215 per
mg in Brazilian market). Therefore, investigations involving its production, recovery and purification are encouraged to supply the demand for further application studies.

However, as far as we know, no systematic study on the extraction of fungal bikaverin has been conducted. Regarding the purification of bikaverin, few studies have been reported on open column or preparative chromatographic methods (Deshmukh, Mathew, & Purohit, 2014; Kundu, Saha, Walia, & Dutta, 2016). Therefore, the present study aimed to determine the ideal conditions for the extraction of bikaverin (solvent selection, extraction kinetics, repeated extractions) followed by its purification (open column chromatography and semi-preparative HPLC). The study also included a preliminary economic evaluation of the production and extraction of bikaverin, providing an important estimate on the economic viability of the biotechnological production and extraction of this molecule.

2. Materials and methods

2.1. Bikaverin production

Bikaverin was produced by Fusarium oxysporum CCT7620 (Tropical Culture Collection, Andre Tosello Foundation – http://fat.org.br/) during growth in rice broth (50 g of milled white rice per liter of water). The inoculum was produced by homogenizing with Ultraturrax (Ika®) a piece (1.5 cm³) of Potato Dextrose Agar containing a 72 h-old culture of F. oxysporum CCT7620 in 200 mL of rice broth. Flasks were incubated in rotary incubator operated at 30 °C and 200 rpm for 72 h. One milliliter of this culture (inoculum) was transferred to 200 mL of a fresh rice broth, following incubation at 30 °C and 200 rpm for 96 h (dos Santos, da Silva, da Silva, Cerri, & de Ribeiro, 2020). After this period, the fungal biomass was recovered by centrifugation at 6,000 rpm for 5 min. The resulting red biomass containing bikaverin was used in the following trials. Standard bikaverin (≥98% in HPLC) from Fusarium subglutinans (Sigma-Aldrich, CAS number 33390-21-5, product number SML0724) was also employed for comparison in some analyses.

2.2. Extraction procedure

The basic extraction procedure consisted of mixing wet biomass (obtained as described in Section 2.1) and organic solvent (1:1, m/v) in orbital shaker at 25 °C and 200 rpm. The organic solvent was then recovered by centrifugation at 3,200 rpm for 5 min and the absorbance at 500 nm (Abs₅₀₀) of this solution was recorded to determine the amount of extracted bikaverin (Section 2.4.1). Different solvents and extraction times were tested, as described next. All experiments were done at least in triplicate.

To select the ideal extracting solvent, the extraction procedure was done for solvents with different relative polarities, i.e. petroleum ether (no data for polarity), n-hexane (0.009), diethyl ether (0.117), ethyl acetate (0.228), chloroform (0.259), dichloromethane (0.309), 1-butanol (0.586), ethanol (0.654), and methanol (0.762) (Reichardt & Welton, 2011). In this case, a fixed extraction time of 60 s in vortex was used. The solvent with the highest extraction performance of bikaverin (ethyl acetate) was employed in the further experiments.

In order to identify the extraction time to achieve maximum bikaverin recovery, an extraction kinetic study was done. In this case, samples were periodically taken (1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 min) from the extraction mixture (50 g wet biomass and 50 mL solvent incubated in rotary shaker at 25 °C/200 rpm) and the organic solvent (in this case, ethyl acetate) were recovered for monitoring Abs₅₀₀ (Section 2.4.1).

Subsequently, repeated extractions were done to evaluate the number of sequential extractions required for a maximum bikaverin recovery. In this case, the extraction procedure was done for 260 min using 10 g wet biomass and 10 mL ethyl acetate in rotary shaker (25 °C/200 rpm). After each extraction cycle, the biomass was recovered and re-extracted with fresh solvent. A total of seven sequential extractions were done. Each ethyl acetate extract was independently analyzed in terms of Abs₅₀₀ (Section 2.4.1).

Finally, a preliminary economic evaluation was done to estimate the bikaverin extraction costs in different scenarios and, thus, to identify the ideal number of sequential extractions. This economic study considered the usage time of the main equipment, their depreciation (10 years), the electricity expenses, as well as the costs of the materials and chemicals, and the manpower needed to produce, extract and concentrate bikaverin. The unity costs for each of these variables as well as the costs associated with each batch (inoculum production, bikaverin production, bikaverin extraction and extract concentration) are presented in the Supplementary Material (see spreadsheet). As this is a lab scale study, we considered the conditions found at our laboratory and batch sizes of 200 mL, 1 L and 80:80 mL:g for inoculum production, biomass production and bikaverin extraction, respectively.

2.3. Purification procedures

The bikaverin purification trials were done starting from a bikaverin extract obtained at the ideal conditions determined in the former experiments. This extract was concentrated in a rotary evaporator (Tecnal TE-211) at 45 °C under vacuum and the resulting material, called crude extract, was fractionated in different systems, as detailed next.

2.3.1. Open column chromatography

Two open column chromatographic systems were tested in the present study to purify the bikaverin present in the crude extract. In the first, the concentrated crude extract resuspended in 0.5 mL chloroform was transferred to a glass column (18 cm height, 2.5 cm length, 0.8 cm width) filled with silica gel type H (20–80 μm particle size; code 815330.05 – Sigma), which was percolated with an eluting system consisted of a chloroform:methanol:acetic acid mixture (94:1.5:5. v/v/v), as described by Giordano (1999). Five mL fractions were collected for further analysis (Section 4.4.2). In the second case, the concentrated crude extract resuspended in 0.5 mL methanol was transferred to a glass column (18 cm height, 2.5 cm length, 0.8 cm width) filled with silica gel type H (20–80 μm particle size; code 815330.05 – Sigma), which was percolated with an eluting system consisted of a chloroform:methanol:acetic acid mixture (94:1.5:5. v/v/v), as described by Giordano (1999). Five mL fractions were collected for further analysis (Section 4.4.2).
3.3.2. Semi-preparative high performance liquid chromatography (HPLC)

The crude extract (50 mg) was resuspended in 200 µL of acetone/triter and the resulting solution was injected in a semi-preparative HPLC system equipped with a binary Waters 1525 HPLC pump, a Phenomenex Luna® 5 µm phenyl-hexyl 100 Å LC column 250 × 10 mm (part N° 00G-4257-N0), a Waters 2998 Diode Array Detector and a Waters III fraction collector using a wavelength of 254 nm. The mobile phases, ultrapure water acidified with 0.1% (v/v) formic acid (A) and acetone/triter (B), were fed at a rate of 4.7 mL/min using the following eluting profile (A/B): 60/40 for 0–50 min; 50/50 for 50–65 min; 20/80 for 65–70 min; 0/100 for 70–75 min; 60/40 for 75–82 min.

2.3.3. Nanofiltration

The purification of the non-concentrated crude extract (before rotary evaporation) was also tested in a nanofiltration system. The filtration unity consisted in a jacketed hermetic stainless steel chamber and a magnetic stirrer over the membrane, to simulate a cross-flux process. The filtering membrane (1.52·10⁻³ m²) was used was NF90 (aromatic polyamide, molecular weight cut-off of 180 Da) produced by Dow Filmtec. The temperature was kept at 10 °C by chilled water circulation and the internal pressure was kept at 10 MPa by applying pressurized nitrogen into the chamber (Alves, Antunes, Silva, & Forte, 2021).

2.4. Analytical conditions

2.4.1. Spectrophotometric analysis

The extracts obtained in the extraction assays (Section 2.2) were evaluated in terms of absorbance at 500 nm (Abs500 – maximum bikaverin absorption wavelength) in 1 × 1 cm glass cuvettes using a spectrophotometer (DU-640™, Beckman Coulter Inc.). When required, samples were diluted using the same organic solvent present in the extract to remain within the linear range of Abs500 vs. bikaverin concentration.

2.4.2. Analytical HPLC

The fractions obtained in the purification trials (Section 2.3) were monitored by High Performance Liquid Chromatography (HPLC) using a Shimadzu LC-20AT equipment coupled with a SIL-20A automatic sampler, a Phenomenex Luna® 5 µm phenyl-hexyl 100 Å LC column 250 × 4.6 mm (part N° 00G-4257-E0), a communication module CBM-20 and a SPD-M20A Diode Array Detector. The mobile phases, ultrapure water acidified with 0.1% (v/v) formic acid (A) and acetone/triter (B), were fed at a rate of 1.0 mL/min using the following eluting profile (A/B): 60/40 for 0–50 min; 50/50 for 50–60 min; 0/100 for 61–66 min; 60/40 for 67–77 min. Before being injected (20 µL), samples were filtered through a 13 mm filter with a pore size of 0.22 µm. The retention time of standard bikaverin was used as a reference. A calibration curve was done by diluting known amounts of standard bikaverin in acetone/triter, in a range of 0.97 to 500 mg/L, and further injection at the same analytical conditions (wavelength of 254 nm).

2.4.3. Mass spectrometry (MS) analysis

To confirm the presence of bikaverin in the extract, samples were analyzed in an Agilent 1200 HPLC apparatus coupled with a Agilent iFunnel 6550 Q-ToF mass spectrometer. The electrospray ionization was conducted in a positive mode using a spray gas temperature of 290 °C, capillary voltage of 3500 V, nozzle voltage of 320 V, drying gas flux of 12 mL/min, spray gas pressure of 50 psi, auxiliary gas temperature of 350 °C and auxiliary gas flux of 12 mL/min. Time of flight (ToF) analyzer was set to operate at a m/z range of 50–1500. The collision energy (auto MS/MS mode) was 4 V (slope) + (m/z)/100 + 5 V (offset). Samples were injected (2 µL) in a Thermo Scientific Accucore C18 column (2.6 µm, 2.1 mm × 100 mm). The mobile phases, ultrapure water acidified with 0.1% (v/v) formic acid (A) and acetone/triter (B), were fed at a rate of 0.2 mL/min using the following eluting profile (A/B): 95/5 to 2/98 gradient for 0–10 min; 2/98 for 10–15 min; 2/98 to 95/5 gradient for 15–16.2 min; 95/5 for 16.2–20 min. The mass spectra data obtained were analyzed by the open-source software MZMine version 2.53 (http://mzmine.github.io/).

3. Results and discussion

3.1. Bikaverin production

Fusarium oxysporum CCT7620 is a known producer of bikaverin, whose ideal production conditions in shake flasks were recently published (dos Santos & de Mendonça, 2020). In the present study, bikaverin was identified as the major metabolite in the crude extract (with ethyl acetate extraction). The HPLC-DAD analysis revealed that at 254 nm the area of the peak attributed to bikaverin varied from 40.4 to 45.2% (average ± SD of 42.9 ± 2.0% for four samples - Table 1) of the total peaks area, the other main peaks being the solvent remaining in the concentrated crude extract (peak of 2.2 min) and an unidentified compound (probably another fungal metabolite) at 29.7 min (Fig. 1A). Moreover, at 500 nm, bikaverin was virtually the sole analyte detected (peak area 95.7% total area), confirming that the Abs500 could be reliably used to determine the bikaverin concentration, since no other pigments absorbing at this wavelength were present in significant amounts (Fig. 1B).

This study also confirmed that the main peak in the crude extract corresponded to bikaverin. The mass-to-charge ratio (m/z) of 383.0743 (error –0.7 ppm) [M + H]+ and the major MS/MS fragments from the main peak of the extract (m/z of 324, 340, 355, and 219) (Supplementary Fig. S3) were also present in standard bikaverin analysis (Supplementary Fig. S4). Additionally, the mass spectrometry analyses, based on MS1 of MS2 level suggested that other pigments, such enniatins A, A1, B, B1, B2 and B4, beauvericin, rubrofusarin and aurofusarin were not present in significant amounts (Supplementary Table S1), being detected only at noise level (<10⁻⁴), as observed in the Extracted Ion Chromatograms (EIC or XIC) (Supplementary Fig. S5). Some bikaverin precursors (oxo-pre-bikaverin, me-oxo-pre-bikaverin, norbikaversin) and usually co-produced compounds (gibberellin) were also searched on the sample data, being only me-oxo-pre-bikaverin (m/z 352.0577) detected (Supplementary Table S1 and Supplementary Fig. S5), but in a low-intensity level.

3.2. Bikaverin calibration curves

Many published papers on bikaverin production (Giordano, Avalos, Cerdá-Olmedo, & Domenech, 1999; Lale & Gadre, 2016; Limnemannstons et al., 2002; Rodríguez-Ortiz, Mehta, Avalos, & Limón, 2010) calculate bikaverin concentration from spectrophotometric analyses (absorbance) considering the molar extinction coefficient described by Balan, Fuska, Kuhr, & Kuhrová, 1970. However, the calibration curve described in such study was constructed using an unusual cuvette (20 mm path), a different solvent (chloroform) and another chromatograms (EIC or XIC) (Supplementary Fig. S5), but in a low-intensity level.
range of $A_{\text{max}}$ of 0 to 1.35 (0 – 400 mg/mL$^{-1}$). As may be seen, the linear range expected by the Beer-Lambert law goes beyond 1.0, which is usually considered the limit for linearity (Roodyn, 1970). Considering the equation obtained, one can estimate the molar extinction coefficient as 1558.3 L mol$^{-1}$ cm$^{-1}$ (log $\varepsilon$ = 3.13). This coefficient is significantly different from the value previously reported by Balan et al., 1970 (log $\varepsilon$ = 3.95) due to the different analytical conditions. As far as we know, there are no other reports of the bikaverin molar extinction coefficient for comparison. We consider that the calibration curve presented in the present paper is more appropriated for estimating the amount of bikaverin in ethyl acetate at 500 nm.

In this study, we also reported the calibration curve for HPLC-DAD analyses of bikaverin (Supplementary Fig. S2), but the bikaverin quantifications made throughout this text was based on the spectrophotometric analysis (Supplementary Fig. S1) instead, due to its simplicity and reliability.

3.3. Selection of the extracting solvent

Depending on the cultivation conditions, the biomass produced by Fusarium oxysporum can be rich in bikaverin, being part of the pigments excreted by the cell (Lebeau et al., 2019), and conferring an intense red color in the culture medium (Linnemannstons et al., 2002). Understanding the factors that influence extraction is a critical step in optimizing the amount of pigment that will follow for the purification process.

Intracellular pigments of fungal origin can be extracted from the mycelium simply and efficiently with the application of centrifugation and solid/liquid extraction methods. The solid-liquid extraction is based on the preferential dissolution of one or more components of a solid mixture in a liquid solvent. There is no standard method for extracting polyketides from fungal biomass. However, the most commonly used processes involve extraction with ethyl acetate or chloroform (see, for instance, Lale & Gadre, 2016; Lebeau et al., 2019).

In the present study, nine solvents with different polarities were tested to maximize bikaverin recovery. The results revealed that ethyl acetate extract resulted in the highest bikaverin concentration, followed by dichloromethane and 1-butanol (Fig. 2). Ethyl acetate was also considered the best solvent since, besides yielding higher bikaverin extraction, it is also one of the cheapest and less toxic among the solvents tested (Medeiros, Alexandrino, Pastore, & Bicas, 2021).

This observation was expected since ethyl acetate has an intermediate polarity, which is compatible with the predicted LogP of bikaverin (3.77, calculated by MarvinSketch). The polarity index of ethyl acetate (4.4) is also close to the polarity index of the solvent used to achieve the highest bikaverin recovery from the mycelia of Fusarium oxysporum LCP531 grown in either Potato Dextrose Broth (PDB) or Defined Minimal Dextrose broth (DMB) (Lebeau et al., 2019). In such study, different mixtures of water (polarity index of 10), methanol (polarity index of 5.0) and ethanol (polarity index of 4.0) were tested, revealing the highest bikaverin recovery for methanol/ethanol mixture (polarity index of 4.5). In another study, the combination of acetone/methanol and ethyl acetate (1:1) also resulted in greater extraction efficiency for bikaverin (93%), compared to acetone/methanol (45%), methanol (57%) and acetone/methanol (65%) (Busman & Butchko, 2012).

3.4. Extraction kinetics

After determining the ideal solvent to recover bikaverin, a kinetic study was conducted to evaluate the time required to saturate the acetothyllic phase during the extraction procedure at 200 rpm and 25 °C. It is expected that the extraction time will lead to a higher amount of pigment extracted, up to a certain limit. Short extraction times, in turn, can fail to fully recover the pigments (Ilavarasi, Pandiaraj, Mubarakali, Ilyas, & Thajuddin, 2012). Thus, the kinetic study must be done to determine when saturation takes place and also the possible occurrence of unwanted reactions. For instance, the ideal time for extracting the intracellular pigments bacteriochlorophyll and the carotenoid spheroidenone, both produced by Dinoroseobacter shibae and Roseobacter denitrificans, was 15 min, since longer extraction times (30, 60, and 120 min) did not result in a significant increase in the amount extracted, while these pigments were degraded by the solvent (methanol) (Ruivo et al., 2014).

The results of the present study revealed that solvent saturation took place at 256 min of extraction, after an exponential increase in bikaverin concentration between 4 and 256 min (Fig. 3). The curve profile indicated a relatively intense mass transfer limitation. This fact can be explained by the fact that bikaverin is associated with the biomass, which presents a complex barrier separating the intra and extracellular compartments. Indeed, previous work described the accumulation of bikaverin in fatty vacuoles of mycelial filaments grown in submerged medium (Balan et al., 1970). This constitutes another barrier for the diffusion of the pigment. Moreover, pigmented compounds produced by F. oxysporum have been associated with the production of an ergosterol derivative (Lebeau et al., 2019), which implies an increase in the mechanical resistance of the membrane and, consequently, in the time of extraction of the intracellular metabolites. Therefore, the extraction process requires longer periods, especially if there is no pretreatment of the biomass. Thus, the extraction time used in the following experiments was fixed at 4 h 20 min (260 min) to assure that equilibrium was achieved.

It is expected that the intracellular pigment extraction will be more complex than extracellular pigments, since the mechanism involves the
incorporation of the solvent into the cell membrane and its affinity with the pigment to be extracted. The intracellular accumulation of bikaverin and other red pigments, such as nor-bikaverin, was reported for \textit{F. oxysporum} LCP531 grown in Defined Minimal Dextrose broth (DMDB) medium, which is considered a 'minimal' nutrient medium and a stressful environment that promoted pigment production as a protection strategy (Lebeau, Petit, Clerc, Dufosse, & Caro, 2018). It is likely that the relatively nutrient-poor rice-based medium used in the present study also favored the intracellular production of red pigments in \textit{F. oxysporum} as a response to a stressful condition.

Several bikaverin extraction procedures have been reported, but as far as we know none of them was concerned with determining the extraction kinetics of this pigment. Most of these extractive processes use chloroform (Chávez-Parga et al., 2005; Lale & Gadre, 2016), ethyl acetate (Zhao et al., 2020), or a combination of ethyl acetate with isopropanol (Reus, Nielsen, & Frandsen, 2019). Although the extraction kinetics has not been investigated in former studies, the duration of the extraction varied from 20 min under agitation with ethyl acetate (Zhao et al., 2020), to 1 h of ultrasonication with a mixture of ethyl acetate and isopropanol (Reus et al., 2019), up to 6 h of reflux with the chloroform solvent (Lale & Gadre, 2016). Shorter periods of extraction have been achieved by pre-treating the cells (e.g., glass beads and acid treatment) to favor cell wall disruption (Caprio, Altimari, & Pagnanelli, 2020), but this strategy was not considered in the present study.

In the extraction of the \textit{Monascus} intracellular pigments, saturation of the Triton X-100 (TX) solvent was observed after 1 h of agitation at 180 rpm and 30 °C. In this case, solvent saturation was related to mycelial density: a high density of mature cells (20 g/L) significantly reduced pigment extraction even with the use of the maximum tested solvent concentration (160 g/L TX) (Chen, Bei, Shi, Tian, & Wu, 2017). In contrast, the kinetics of extracting lutein and β-carotene with methanol from washed pellet of \textit{Tetraselmis obliquus} showed that 90% of the maximum amount of these pigments could be obtained at an extraction time of 60 min with magnetic stirring (Caprio et al., 2020).

Considering that a sequential extraction process can overcome solvent saturation, therefore increasing the total pigment recovery, a procedure with several extraction cycles was proposed. The results are presented in the following paragraphs.

### 3.5. Sequential extractions

The next step involved the evaluation of the number of sequential 260 min-extraction steps in the amount of bikaverin recovered from biomass. Generally, a single equilibrium stage is not sufficient to achieve the desired level of extraction. To increase the recovery of a given compound, a new extraction procedure, with fresh solvent, might be used to assure a maximal concentration gradient in the matrix-solvent mixture. This is the principle of Soxhlet extraction (de Castro & Priego-Capote, 2012). The results from our experiments indicated that each subsequent extraction could recover c.a. 2/3 of the amount recovered in the previous step. However, even after seven extraction cycles, a minor amount of bikaverin was still recovered, suggesting that extraction was not complete even after this exhaustive procedure (Fig. 4). This could be confirmed by the biomass resulting from the seventh extraction, which was still lightly pale red (results not shown).

As a strategy to increase pigment recovery, other authors have considered pre-treating the cells and using unconventional extraction technique. For example, preliminary treatment of \textit{Chlorella vulgaris} cells with bead milling followed by extraction by supercritical carbon dioxide allowed the recovery of carotenoids and chlorophyll present in chloroplasts (Safi et al., 2014). Another approach consisted of using a non-ionic surfactant to increase cell permeability to achieve a complete extraction of \textit{Monascus} pigments (Chen et al., 2017). However, a significant level of residual pigment was observed in the biomass, even after four sequential extractions of 1 h each, due to solvent saturation (Chen et al., 2017).

Other papers have also performed repeated extractions for recovering bikaverin, such as the five sequential extractions from biomass (Zhao et al., 2020) or the two-stage extraction from supernatant (Deshmukh et al., 2014). However, it is not clear whether these number of extractions exhaust bikaverin recovery from their respective matrices. Another study revealed a 96% recovery of the total pigment (lutein and β-carotene) content in biomass of \textit{Tetraselmis obliquus} after five successive extraction stages of 60 min each (Caprio et al., 2020). In the present assay, Fig. 4 indicates that the first, second and third extraction cycles accounted for, respectively, 32.8 ± 11.8%, 21.3 ± 5.4% and 16.8 ± 4.7% of the combined amount of the seven cycles. Thus, three successive extractions would recover close to 71% of the total amount recovered after seven extraction cycles. Although further extractions could increase the total bikaverin recovery, this strategy would substantially increase the time and materials (mostly solvent) required for this downstream process. In other words, considering a constant demand in terms of time and materials for each batch of inoculum and biomass production, bikaverin extraction and extract concentration, it was clear that the production cost of the crude extract (concentrated bikaverin) was not linearly correlated to the number of extraction cycles. Therefore, in order to define the ideal number of sequential extractions, we proposed a preliminary evaluation to determine the costs involved with production, extraction and concentration of bikaverin as a function of the number of extraction cycles. The results are presented and discussed in the next section.

### 3.6. Economic evaluation

The economical exploitation of fungal pigments has been increasing, but the financial investment necessary to place the product on the market is still an issue. One of the main challenges to the successful commercialization of food-grade biotechnological pigments include the high investments required for fermentation installations (Dufosse, 2006). In terms of the extraction processes of bikaverin and norbikaverin, mycelial pigments (such as in the present study) are likely cheaper to extract than the extracellular pigments present in the liquid broth, since in the latter case there is a need to treat large volumes of broth, being recommended nanofiltration procedures or acid precipitation of pigments to concentrate the target compounds (Lebeau et al., 2018). The present study involved only a preliminary economic study, considering a small-scale process and our laboratory conditions. Although it cannot be extrapolated to commercial-scale processes, this section gives valuable insights on the estimation of the bikaverin recovery costs at a lab-scale operation.

For this preliminary economic study, we considered the amount (mg) of bikaverin recovered after each extraction cycle, the bikaverin concentration in the combined extract (assembled fractions) (both obtained...
from data presented in Fig. 4), as well as the costs (depreciation, material, labor and electric expenses) for producing, extracting and concentrating bikaverin from 80 g of biomass at our laboratory conditions (see details in the spreadsheet presented in Supplementary Material). As a result, the unity costs for producing the crude extract (concentrated bikaverin) could be estimated when one or more extraction cycles were employed to recover bikaverin. Thus, Fig. 5 presents the cumulative amount of bikaverin, the bikaverin concentration in the combined extract, as well as the costs for crude extract production when different number of cycles of sequential extractions are employed.

The results indicate that although the total amount of bikaverin increases after each extraction cycle, the bikaverin concentration in the combined extract decreases, as a result of the solvent diluting effect. Assuming a fixed cost for producing the biomass and a variable cost for multiple extractions and concentrations of bikaverin (considered linearly correlated with the number of extraction cycles), the total cost for obtaining a crude extract decreases from one to three sequential extractions, but it rises again from the fourth to the seventh extraction cycle. This behavior can be explained by the fact that the extraction and concentration production costs are not compensated by a proportional increase in the total amount of recovered bikaverin (Fig. 5). As a result, we opted to apply three sequential extractions with ethyl acetate at 25 °C/200 rpm for 4 h 20 min as a standard procedure for crude extract production. At these conditions, we estimate that the costs for obtaining the crude extract would be R$5.43 (close to US$ 1, at the current currency) per mg of bikaverin, not including the purification costs (usually much higher).

The next sections will present the purification procedures tested to purify the bikaverin from the crude extract obtained as previously mentioned.

3.7. Bikaverin purification in open column chromatography

Although not confirmed in the present work (see Section 3.1), it is likely that other pigments may be present in the ethyl acetate extract of Fusarium cultures. Norbikaverin and, to a lesser extent, oxo-pre-bikaverin and me-oxo-pre-bikaverin (see structures in Supplementary Fig. S6), are naphthoquinones that are possibly present in the red extracts of F. oxysporum using medium polarity solvent (Lebeau et al., 2019). Due to the probable diversity of pigments, the crude extract purification procedures followed in open column and semi-preparative HPLC system, aiming at the isolation of bikaverin (Sections 3.7 and 3.8).

The crude extract was applied in Open Column Chromatography systems to check the possibility of purifying bikaverin by two different mechanisms, i.e. adsorption and exclusion. In the first case, the crude extract was fractionated in four visual bands (orange, pink, red and purple) (data not shown). The red fraction was then analyzed by HPLC-DAD. The results obtained suggest that bikaverin was concentrated in this fraction, but surprisingly its estimated purity (% of peak area) decreased, as other analytes were also concentrated in this fraction (Table 1). The same behavior was obtained for crude extract fractionation in Sephadex™ – G-50 fine, although with a final estimated purity similar to the extract before this purification procedure (Table 1). As a result, none of these two systems were considered suitable for purifying bikaverin from the crude extract.

In former studies, bikaverin was isolated and purified by silica gel column chromatography and preparative Thin Layer Chromatography (TLC). The concentrated extract of F. oxysporum was first fractionated in a column filled with silica gel (particle size 100–200 mesh, pre-activated at 110 °C) and percolated with different hexane-ethyl acetate mixtures (7:3, 6:4, 1:1, 4:6, 3:7, 2:8, 0:1, v/v) as eluent. The eluted fractions were refractinated by preparative TLC (silica gel as stationary phase), resulting in bikaverin and other secondary metabolites (3-O-methyl-8-O-methyl fusarubin, 8-O-methyl fusarubin, anhydrofusarubin and fusarubin) (Kundu et al., 2016). The purification of bikaverin was also successful in preparative TLC presenting silica gel (40%) and calcium sulfate (8%) as stationary phase and a mixture of chloroform, methanol and acetic acid (94:1:5, respectively) as mobile phase (Deshmukh et al., 2014).

3.8. Bikaverin purification by nanofiltration

As an alternative to chromatographic methods, this study also considered the concentration of bikaverin, to possibly obtain a purified fraction, by means of nanofiltration (NF). The membrane separation process is based on the capacity of a semi-permeable membrane to control the permeation of different molecules with different sizes and shapes, and is controlled by size selection and/or diffusional aspects. Thus, the permeants are separated by a pressure flow through the pores of the membrane. This simple physical separation can produce high value-added products.

Various types of extracts and natural substances were concentrated by nanofiltration, including, for instance, lacuca extract (Borah, Gogoi, Das, & Hazarika, 2020), blueberry pomace extract (Avram et al., 2017), among others. Regarding pigment separation, it was demonstrated that the NF4 membrane concentrated polyphenol pigments from sugarcane molasses, while removing sugars and salts. After removal of sugar and salts by diafiltration, the phenolic pigments were obtained in the retentate at about 60 mg/g (rutin) (Qiang et al., 2019). Anthocyanins were also efficiently separated from the ethanol extract of jussara using NF (Vieira et al., 2018).

In the present study, however, an efficient separation was not possible using NF. Although it was expected that bikaverin (382.3 Da) would be retained by the nanofiltration membrane tested (NP90), the permeate obtained was red, while no apparent bikaverin (red color) retention was visually observed. The analysis of the retentate did not reveal any peak of bikaverin (data not shown), while the analysis of the permeate revealed new peaks, decreasing the relative peak area of bikaverin after this procedure (Table 1), as also as observed for the Open Column Chromatography on silica gel.

| Sample                              | % Area* |
|-------------------------------------|---------|
| Extracts before purification procedures | 40.4–45.2 |
| After purification                   |         |
| OCC** on silica gel                  | 45.9    |
| OCC** on Sephadex G-50               | 27.5    |
| Nanofiltration (NP90)                | 96.6    |
| Semi-preparative HPLC               |         |

* Percentage of the bikaverin peak area (at 254 nm); **OCC: Open Column Chromatography.
4. Conclusion

This work aimed to describe efficient methods for recovering and purifying biotechnologically-produced bikaverin, which is necessary, for example, to provide this molecule in amounts large enough for application and toxicological studies. This study identified the ideal solvent (ethyl acetate), extraction time (260 min) and the number of repeated extraction cycles (three) to recover bikaverin from the biomass of *F. oxysporum* CCT7620. The purification of this molecule, however, still remains a bottleneck. Among all the tested methods, only the semi-preparative HPLC resulted in satisfactory results. Therefore, an economic method for purifying bikaverin is still necessary. As a suggestion, a promising alternative would be the extraction of biomass with solvents of different polarities, which removes a little amount of bikaverin (for example,hexane) but can eliminate undesirable contaminants, particularly the most apolar ones.

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4.2. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2021.100136.

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