Identification of azaphilone derivatives of Monascus colorants from Talaromyces amestolkiae and their halochromic properties

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ABSTRACT
Currently, the ability to produce several kinds of water-soluble red natural colorants makes the genus Talaromyces particularly important to the dye industry, which can be an alternative to the use of harmful synthetic colorants. In this study, colored compounds produced by Talaromyces amestolkiae were extracted, characterized chemically and the color stability of the fermented broth without any extraction procedure was further evaluated over pH variation. Five azaphilones compounds were detected by Ultrahigh Performance Liquid Chromatography-Mass Spectrometry system, all being complexes of the fatty acid amino-hexanedioic acid and azaphilone Monascus colorants. The color of the fermented broth was stable at a wide range of pH (3-9). Furthermore, T. amestolkiae colorants precipitated through hydrolysis of key chemical groups at extremely acidic (pH 1) and lose red color in extremely basic (pH 13) medium, showing negative halochromism. Nevertheless, these findings enhance the industrial relevance of azaphilone colorants produced by biotechnological process.

1. Introduction

The market for industrial colorants is primarily occupied by synthetic/non-renewable colorants. Azo dyes, a class of common synthetic colors have the advantage of providing high coloring strength, attractive hues, and good stability (Scotter, 2011). However, the bulk of chemically synthesized colors are derived from aniline, a petroleum-derivative product, often linked to environmental toxicity and health concerns (Sen, Barrow, & Deshmukh, 2019). Thus, this led industry to replace artificial colorant by colorant from natural sources, which are less toxic and environmentally safe. However, natural colorants have been the target of much criticism, such as their instability against light, heat, or adverse pH and low water solubility (Ghidouche, Rey, Michel, & Galaffu, 2013; Sen et al., 2019). Instability problems arise from the fact that natural colorants, particularly food ones are not as pure as synthetic counterparts, as they can often contain other components like proteins and sugars, just to mention a few (Ghidouche et al., 2013). Although the problems of instability of natural colorants, industries face other big concerns related to the scarcity of natural red colorants. Up to now, the alternatives of red colorants for food are the plant-originated anthocyanins and the cochineal carmine extract, with well-known restrictions for use at certain values of pH (Chen, Lin, Chen, & Chiang, 2019; Müller-Maatsch, 2016). Thus, the search for new water-soluble red colorants is extremely important for the food industries, driven thus the efforts of academia to work in alternative ways of producing these colorants, being the use of filamentous fungi one of the alternatives (Chen et al., 2017; Mapari et al., 2005).

Ascomycetous fungi are known to produce a significant range of natural polyketide colorants (Dufossé, Fouillaud, Caro, Mapari, & Sutthiwong, 2014; Mapari et al., 2005). Azaphilones are an interesting set of fungal polyketides comprised of a group of pyrano-quinones structures with a high electron acceptor tension determining the sensitivity of
oxygen in the primary ring and a chiral center. This yield γ-pyridones, exhibiting chromophore properties in which colors depend on their chemical structure (Bijinu, Suh, Park, & Kwon, 2014; Chen et al., 2017). To this reason, azaphilones colorants find application in the food industry not only as food colorants but also as stabilizers, emulsifiers, antimicrobials, antioxidants, anti-inflammatory, and cytotoxic agents (Meruvu & Santos, 2021). The use of *Monascus* sp. to produce azaphilone compounds is not new (Chen et al., 2017; Dufossé et al., 2014). In literature, six major *Monascus* colorants are reported: rubropunctamine and monascorubramine (red); rubropunctatin and monascorubrin (orange); monascin and ankaflavin (yellow) (Fig. 1) (Mapari, Thrane, & Meyer, 2010).

Studies focusing on the biosynthetic pathway of *Monascus* azaphilone colorants have been improved. However, it has been widely accepted that biosynthesis follows a polyketide pathway, in which polyketide synthase (PKS) and fatty acid synthase (FAS) have been proposed to play essential roles in the formation of orange colorants through trans-esterification of a polyketide chromophore and a

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**Fig 1.** Classical *Monascus* azaphilone colorants’ structure: yellow (monascin and ankaflavin), orange (rubropunctatin and monascorubrin) and red colorants (rubropunctamine and monascorubramine) and their derivatives. ChemDraw professional (). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) adapted from Blanc et al., 1994; Chen et al., 2017
β-ketoacid, being the yellow and red colorants derived from the orange ones (Yang et al., 2015). According to the molecular structure, the orange colorants could be transformed into yellow colorants by chemical hydrogenation catalyzed by related enzymes (Chen et al., 2017; Yang et al., 2015). As the pyrano-quinone bicyclic core of the orange and yellow azaphilones is often highly oxygenated, the oxygen of the primary ring can react with amines by exchanging the pyran oxygen with nitrogen (aminophilic reaction) leading to a color shift from yellow/orange to red (Chen et al., 2017; Shi et al., 2015). Alternatively, the intracyclic NH-group of the red colorant monascorubramine can react with the amino acids through an amide bond to produce water-soluble red complexes of Monascus colorants (Hajjaj, François, Goma, & Blanc, 2012).

These six classical Monascus azaphilone colorants are hydrophobic and majorly accumulated intracellularly in conventional submerged aqueous culture. The ratio of the orange/yellow/red components is strongly dependent on the nutrients (such as nitrogen source) in the culture medium as well as culture condition (such as pH) (Hajjaj et al., 2012; Shi et al., 2015). Besides the six main components, complexes of Monascus colorants were also identified (Hajjaj et al., 1997; Ogihara, Kato, Oishi, & Yoshinori, 2001) and have gained attention due to water solubility property, such as the sequoiamonascin C, a novel hydrophilic yellow colorant with a carboxyl group; and the hydrophilic colorants N-glutaryl-monascorubramine and N-glutaryl-rubropunctamine produced extracellularly in Monascus sp. culture (Fig. 1) (Hajjaj et al., 1997; Mapari et al., 2010).

Some other non-Monascus species like Talaromyces sp., Fusarium graminearum, Penicillum sp. also produce the typical Monascus colorants as well as Monascus-like colorants, being available for replacing the synthetic colorants (Kalra, Conlan, & Goel, 2020; Venkatatchalam, Zelena, Cacciola, Ceslova, & Girard-Valenciennes, 2018). Furthermore, recent literature has abundantly reported the interest in Talaromyces genus (often known under their previous Penicillum taxonomy) regarding the production of new molecules and, mainly novel colorants and, more specifically, Monascus-like azaphilone colorants (Frisvad et al., 2013; Morales-Oyervides et al., 2020).

Penicillum purpurogenum, for example, was found to be efficient in the production of N-glutaryl-monascorubramine and N-glutaryl-rubropunctamine, both water-soluble Monascus azaphilone colorants, which were discovered in the extracellular colorant extract obtained from the fermented broth (Mapari, Meyer, Thrane, & Frisvad, 2009). The filamentous fungus Talaromyces atroroseus (previously P. purpurogenum) has been shown to secrete large amounts of colored compounds belonging to the Monascus-like azaphilone colorants (Frisvad et al., 2013; Mapari et al., 2005; Mapari, Meyer, & Thrane, 2006). Some of the other colorants, such as PP-O, PP-V, PP-Y, PP-R and monascorubramine were also produced by Talaromyces and Penicillum genus (Fig. 1), however most of the produced colorants have their chemical structures unknown (Leb- eau, Venkatatchalam, Fouillaud, Petit, Vinale, Duflosse, Caro Production, & Method, 2017; Ogihara et al., 2001; Venkatatchalam et al., 2018). The colorants produced by T. amestolkiae DPUA 1275 (previously reported as P. purpurogenum DPUA 1275) have been the target of our research group. Studies in orbital shaker (Santos-Ebinuma, Roberto, Simas Teixeira, & Pessoa, 2013a; Santos-Ebinuma, Simas Teixeira, & Pessoa, 2013b; Santos-Ebinuma, Roberto, Simas Teixeira, & Pessoa, 2014) and stirred tank bioreactor were performed (de Oliveira, Ferrarte, Baptista Neto, Teixeira, & Santos-Ebinuma, 2020b). It is important to emphasize that at the same time, the extracellular colorants were partially maintained into the fermented broth with part of them remaining intracellularly and studies of cell disruption was also performed (de Oliveira, Hirai, Teixeira, Pereira, & Santos-Ebinuma, 2021). However, further optimization studies allowed to a biochemical shift from colorants production by a careful adjustment of nitrogen source and the culture media composition, specifically the use of monosodium glutamate (de Oliveira, Pedrolle, Teixeira, & Santos-Ebinuma, 2019; de Oliveira et al., 2020b), achieving a complete export of the red colorants to the extracellular culture broth. These extracellular colorants were characterized in the present work. Moreover, our findings showed that the fermented broth rich in red colorant exhibited low toxicity against fibroblast cells and effective antimicrobial activity against Staphylococcus aureus (Zaccarim et al., 2018).

Despite the crescent interest in the production of natural (red) colorants by fungi as a solution for their scarcity, for them to be of industrial interest, their chemical structures need to be characterized and their stability (yellow, 400–450 nm; orange, 450–490 nm; red, 490–520 nm) and color properties guaranteed (CIELAB color coordinates H′, L′ and C′) (Mapari et al., 2006; Mapari, Meyer, & Thrane, 2009).

In order to fill the gap on the knowledge of the structures of novel fungal colorants, the current study proposes the chemical identification of novel nitrogen-containing azaphilones derivatives of Monascus colorants produced by the fungus T. amestolkiae DPUA 1275. Their chemical identification was performed by ultrahigh performance liquid chromatography-mass spectrometry (UHPLC-DAD-ESI/MS”). Moreover, the color stability of the fermented broth at various pH values (1–13) was studied. This study answering the halochromic properties of T. amestolkiae colorants will thus add valuable information to the restrictions for use as food ingredients at certain values of pH. The colorants produced by T. amestolkiae DPUA 1275 were applied as additives in bio-based films promoting protection against oxidative action (de Oliveira et al., 2020a). However, information about the color stability is missing. In this sense, in the food industry, there is an interest to develop smart packaging films based on natural food colorants as halochromatic indicators to detect food spoilage (Alizadeh-Sani, Mohammadian, Rhim, & Jafari, 2020; Sani, Tavassoli, & Hamishehkar, 2021). In addition, knowing the degradation mechanisms is valuable in looking for strategies to stabilize the red color during processing.

2. Materials and methods

2.1. Chemicals and microorganism

Glucose, MgSO4, FeSO4 and CaCl2 were purchased from LS Chemicals (Ribeirão Preto, SP, Brazil), monosodium glutamate (MSG) was purchased from Dinâmica (Indaiatuba, SP, Brazil). Potato dextrose agar and yeast extract were obtained from Acumedia (Lansing, MI, USA). All the other reagents were of chromatographic grade.

Talaromyces amestolkiae DPUA 1275 was generously provided by the Culture Collection of Federal University of Amazonas, DPUA, AM, Brazil. The microorganism, preserved in deionized water, was reactivated in 39 g.L−1 of potato dextrose agar supplemented with 5 g.L−1 of yeast extract (PDA-YE) and maintained at 30 °C for 168 h. The media pH was adjusted to 7.0 after sterilization at 121 °C for 15 min. Afterwards, the cultures were kept in the refrigerator at 4 °C and defined as a stock culture for the whole work.

2.2. Colorants production by submerged culture

The colorants production was conducted using 15 mycelial agar discs of T. amestolkiae grown on PDA-YE plate per 50 mL of culture medium in 250 mL Erlenmeyer flasks. All the assays were carried out in an orbital shaker incubator at 30 °C, 168 h and 150 rpm. The culture medium composition used is defined by (g.L−1): glucose 10, MSG 25.0, MgSO4 0.012, FeSO4 0.01, CaCl2 0.015. The media pH was adjusted to 5, being sterilized at 121 °C for 15 min (de Oliveira et al., 2019). After the submerged culture, the fermented broth was filtered firstly using a filter paper—80 g.m−2 (Whatman, UK) and later using a 0.45 μm filter acquired from Millipore (Bedford, MA, USA). The clarified fermented broth was used to extract and characterize the azaphilone colorants and to study their color change under different pH values.
2.3. Extraction of extracellular red colorants

The extracellular colorants from fermented broth were extracted at room temperature by using n-hexane followed by ethyl acetate. The liquid-liquid extraction was performed in a 500 mL separation funnel. Initially, n-hexane (1:1, v/v) was added to the fermented broth to remove the nonpolar substances, a step repeated 3 times, and in which no colored compounds were extracted. Subsequently, the pH of the fermented broth was adjusted to 2.5 by adding HCl 5.0 M. A new separation was done in a 500 mL funnel by adding ethyl acetate to the fermented broth (1:1, v/v), a step repeated 3 times. From this separation, a two-phase system was obtained, in which one phase rich in ethyl acetate containing the colorants and a second phase composed of the remaining fermented broth, which was further discarded. All ethyl acetate fraction was placed together in a glass balloon and evaporated on a rotary evaporator (Heidolph Hei-VAP Advantage) at 40 °C, 150 rpm and 250 bar to obtain the dry extract of ethyl acetate containing the colorants. The dried ethyl acetate extract obtained was further processed in a chromatographic column (150 cm) packed with silica gel spherical particle size 75–200 μm (Sigma-Aldrich). The adsorption column was eluted with toluene-ethyl acetate-formic acid (29:61:10, v/v), the eluted fractions were left to dry and then stored at 4 °C for further analysis on UHPLC-DAD-ESI/MS.

2.4. Azaphilone colorants chemical identification through UHPLC-DAD-ESI/MS

For the UHPLC-DAD-ESI/MS analysis, each eluted fraction was dissolved in ethanol and the resulting solutions were filtered through a 0.2 μm nylon membrane (Whatman). This technique was performed using a Thermo Scientific Ultimate 3000RS UPC2 Dionex equipped with a Dionex UltiMate 3000 RS diode array detector and coupled to a mass spectrometer. The column used was a Thermo Scientific Hypersil gold (Part no. 25002-102130; Dim 100 mm x 2.1 mm) with a particle size of 1.9 μm and its temperature was maintained at 30 °C. The mobile phase was composed of (A) 0.1% formic acid in water (v/v) and (B) acetonitrile, both degassed and filtered before use. The flow rate was 0.2 mL min⁻¹. The elution gradient started with 30% (solvent B) and in 6 min was increase to 100% (solvent B). Maintain 100% during 4 min and return to 30% (solvent B) over 7 min. Finally, re-equilibration of the column with 5% of solvent A for 10 min. The injection volume was 2 μL.

UV–vis spectral data were gathered in a range of 250 to 500 nm and the chromatographic profiles were documented at 280 nm and 500 nm. The mass spectrometer used was an LTQ XL linear ion trap 2D equipped with an orthogonal electrospray ion source (ESI). The equipment was operated in positive-ion mode with electrospray ionization source of 5.00 kV and ESI capillarity temperature of 275 °C. The full scan covered a mass range of 50 to 2000 m/z. Collision-induced dissociation experiments (MS/MS and MS³) were simultaneously acquired for precursor ions.

2.5. Stability of red color in various pH

To study the influence of pH in the color stability of the colorants produced by T. amestolkiae, a mineral acid/base solution was added to the fermented broth. So, 10 mL of the fermented broth (16.26 μA/DPUA) was taken in a sterile test tube and NaOH (1.0 M) or HCl (1.0 M) was slowly added to reach the required pH. The pH values evaluated were 1; 3; 7; 9; 11 and 13, and in the calculations, the variation in volume was taken into account. All experiments were performed by incubating the closed tubes in dark in a thermostated bath at 25 °C. The samples were taken at 0, 1, 2, 3, 12 and 24 h of incubation to monitor the color change by spectrophotometric and colorimetric analysis. After 24 h of incubation, the color reversibility was evaluated by slowly adding NaOH (1.0 M) or HCl (1.0 M) until reach pH 5. The stability of the colorants was evaluated according to UV–vis and 3D fluorescence spectra.

The UV–vis spectra of the colorants’ solutions were determined by scanning the samples over a wavelength range between 390 and 600 nm. The yellow, orange and red colorants were estimated by reading the absorbance of supernatant at 410, 470 and 500 nm, respectively (de Oliveira et al., 2019; Venkatachalam et al., 2018). The measurement was performed using the spectrophotometer EnSpire Alpha Plate Reader (PerkinElmer®) and taking the dilution factor of each sample into consideration. The results were expressed in Units of Absorbance (UA).

A full 3D fluorescence spectrum was acquired at 25 °C with a variable wavelength range of excitation (λex) from 220 to 600 nm with a step width of 2 nm and emission wavelengths (λem) of 370–650 nm with a step width of 1 nm using the spectrofluorophotometer RF-6000 SHIMADZU. The 3D fluorescence spectroscopy patterns in the λex or λem maxima for each of the three fluorophores found were assigned to specific colorant based on comparison with standards found in literature. Though some excitation/emission wavelength patterns found could not be assigned to one specific colorant, since their fluorescence patterns have multiple possible identifications. In all studies, ultra-pure water was used as a dilution medium. The results were expressed in terms of Units of Fluorescence (UF).

The colorimetric values of L*, a*, and b* were measured by a Chromameter with the CIELAB color system (HunterLab ColorQuest XE). These values were then used to calculate chroma (C*) and hue angle (H*) values according to Equations (1) and (2), respectively. L* indicates lightness from 0 (black) to 100 (white). Positives and negatives in a* represent red and green, respectively, whereas positives and negatives in b* represent yellow and blue, respectively. Chroma values denote the saturation or purity of the color. Values close to the center at the same L* value indicate dull or gray colors, whereas values near the circumference represent vivid or bright colors. Hue angle values represent 0 for redness, 90 for yellowness, 180 for greenness, and 270 for blueness.

\[
C = \sqrt{a^*^2 + b^*^2} \tag{1}
\]

\[
H^* = \arctan\left(\frac{b^*}{a^*}\right) \tag{2}
\]

3. Results and discussion

3.1. Identification of colorants using UHPLC-DAD-ESI/MS

A series of eluted T. amestolkiae fermented broth fractions were collected after liquid–liquid extraction (LLE) followed by separation by passing through a chromatographic column packed with silica gel. The fractions (I to V) obtained from the silica chromatographic column were analyzed through UHPLC-DAD-ESI/MS. UV–vis spectral data were monitored in a range of 250 to 800 nm and their chromatograms were documented at 280 nm and 500 nm. Their mass spectra were obtained and compared against the spectral library and literature database for identification. All colored metabolites detected in the five eluted fractions extracted from T. amestolkiae fermented broth are reported in Table 1. The typical representative chromatogram of the detected azaphilone colorants (compounds 1–6) in the fractions extracted from T. amestolkiae fermented broth and detected at the wavelengths of 280 nm and 500 nm are shown in Figure S1 from Supplementary Material.

According to the literature, species of Talaromyces sp. produce various polyketides metabolites (Frisvad et al., 2013). The strain T. amestolkiae DPUA 1275 used in this study, is known by the production of extracellular colorants in a chemically well-defined medium with MSG and glucose, respectively, as nitrogen and carbon sources (de Oliveira et al., 2019). It was reported that when MSG is present in the medium, it leads to most of the colorants produced being in the form of a complex colorant-glutamic acid (Blanc et al., 1994; Hajjaj et al., 1997). The complex colorant-glutamic acid could be formed by a nucleophilic reaction between the orange colorant (monascorubrin and/or...
Table 1
Colored compounds detected by UHPLC-DAD-ESI/MS\textsuperscript{a} in the eluted fractions of \textit{T. amestolkiae} fermented broth, considering the chromatograms shown in Fig. S1 from Supplementary Material as reference. The eluted fractions (I-V) were obtained after the ethyl acetate extract obtained from LLE. It was processed in a chromatographic column packed with silica gel. UV-vis spectral data were gathered in the range of 250 to 800 nm and their chromatograms were documented at 280 nm (for fractions I, II and III) and 500 nm (for fractions IV and V). The retention time ($t_{R}$) is given in minutes; UV-vis ($\lambda_{\text{max}}$) data in nm; protonated precursor ion molecular ion ([M + H]$^+$) in $m/z$; main ion fragments (MS\textsuperscript{2}) analysis in $m/z$, and area in percentage (%) of the peak.

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<th>Peak area (%)</th>
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<td>528 *</td>
<td>526</td>
<td>514 ([M + H-C\textsubscript{4}H\textsubscript{4}O\textsubscript{5}]$^+$) 482 ([M + H-C\textsubscript{4}H\textsubscript{4}O\textsubscript{5}]$^+$) 432 ([M + H-C\textsubscript{4}H\textsubscript{4}O\textsubscript{5}]$^+$) 414 ([M + H-C\textsubscript{4}H\textsubscript{4}O\textsubscript{5}]$^+$) 314 ([M + H-C\textsubscript{4}H\textsubscript{4}O\textsubscript{5}]$^+$) 285 ([M-C\textsubscript{4}H\textsubscript{4}O\textsubscript{5}]$^-$)</td>
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<td>7.96</td>
<td>7.8</td>
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</table>

\textsuperscript{a}Similar to the other samples; \textsuperscript{b}The compound 4 may be obtained during the MS analysis through the compound 6 decarboxylation; \textsuperscript{c}This is not a new compound, but it is the compound 3 that possibly, during the MS analysis, undergoes a decarboxylation.

rubropunctatin) and the amino group of glutamic acid; or through an amide bond between the intracyclic NH-group of the red colorant (monascorubramine and/or rubropunctamine) and the glutamic acid (Blanc et al., 1994). More forward, Zhao and collaborators (Zhao, Lu, Zhang, & Wang, 2017) working with excess of MSG as the sole nitrogen source found a multicomponent character of red 	extit{Monascus} colorants production. The authors reported that red 	extit{Monascus} colorants with MSG residue were the major colorants, but other amine compounds were also produced, due to complex MSG metabolism.

Following the hypothesis that red colorants are derived from the orange/yellow ones by oxygen substitution or from the red well-known 	extit{Monascus} colorants through an amide bond, MSG was used as the sole nitrogen source to obtain extracellular colorants produced by \textit{T. amestolkiae} in a submerged culture. In this case, five major-colored
compounds were tentatively identified in the five eluted fractions of *T. amestolkiae* fermented broth, all corresponding to *Monascus*-like azaphilone colorants with a common azaphilone skeleton and nitrogen-containing residues, including derivatives of the orange colorants PP-O ([10Z]-12-carboxyl monascorubrin) and monascorubrin and the yellow colorant ankaflavin (Fig. 2).

Compound 1 has similar core structure of azaphilone to the orange colorant PP-O ([10Z]-12-carboxyl monascorubrin) (Fig. 1) with a carboxyl group as R”, but it is unique by incorporating the 4-amino-6-hydroxyhexanoic acid into the primary ring. The compound 1 seems to be a homologue structurally related with the red PP-V ([10Z]-12-carboxyl monascorubramine) (Fig. 1). Compound 1 was eluted in fractions I, II and III presenting maximum absorptions (\(\lambda_{\text{max}}\)) at 250, 279, 426 and 535 nm. The mass spectrum showed a protonated precursor ion \([M + H]^+\) at \(m/z\) 542, being thus characterized as N-hydroxyhexanoic acid-(10E)-12-carboxyl-monascorubramine.

Compound 2 has similar core structure of azaphilone to the orange colorant monascorubrin (Fig. 1) with a methyl group as R”, but it is unique because of the substitution of the pyronoid oxygen by the 3-amino-hexanediol acid. The compound 2 seems to be a homologue structurally related with the red monascorubramine (Fig. 1), and was eluted in fractions I, IV and V presenting UV–Visible data of \(\lambda_{\text{max}}\) at 248 and 484 nm; ESI-MS protonated precursor ion was observed at \(m/z\) 526 in the positive mode \([M + H]^+\), being thus characterized as N-hexanediol acid-monascorubramine. Both compounds 2 and 3 are acidic. Therefore, what differs from both is only the side chain at the 6’ position, where in compound 2 it has the prop-1-en-1-yl radical and compound 3 the carboxyvinyl radical. The compound 3, another PP-V

**Fig 2.** Chemical structures of the compounds detected by UHPLC-DAD-ESI/MS<sup>a</sup> in the eluted fractions of *T. amestolkiae* fermented broth. Calculated nominal masses and formula is shown for each compound.
homologue, was eluted in fractions I, II and III presenting maximum absorptions ($\lambda_{\text{max}}$) at 251, 273, 431 and 514 nm. The mass spectrum showed a protonated precursor ion at m/z 556, being thus characterized as N-hexanedioic acid-(10E)-12-carboxyl-monascorubramine, another derivative of PP-O.

Compound 5 has a similar core structure of azaphilone to the yellow colorant ankaflavin (Fig. 1) with a methyl group as R$, but it is unique by substituting the pyrroloid oxygen with the 3-amino-hexanedioic chain. The compound 5 was eluted in fractions IV and V (with $\lambda_{\text{max}}$ 231, 257 and 496 nm; protonated precursor ion was observed in mass spectrum at m/z 528), and was characterized as $[3-(9a\text{-methyl}-3\text{-octanoyl}-2,9\text{-dioxo}-6\text{-\ (prop-1-\ en-1-\ yl)}\text{-4,9,9a-tetrahydrofu}[3,2\text{-}\ g]\text{isoquinolinol-(7(2H)-yl)}}]$ hexanedioic acid]. Whereas the compound 6 seems to be the acid form of compound 5, with a carboxyl group as R$. The compound 6 was eluted in fraction IV presenting maximum absorptions ($\lambda_{\text{max}}$) at 245 and 495 nm; Protonated precursor ion was observed in mass spectrum at m/z 558, and was characterized as $[3-(6\text{-\ (2\text{-carboxyvinyl)}\text{-9a\text{-methyl}-3\text{-octanoyl}-2,9\text{-dioxo-4,8,9,9a\text{-tetrahydrofu}[3,2\text{-}\ g]\text{isoquinolinol-(7(2H)-yl)}$-hexanedioic acid].

Although it was not detected the colorant-glutamic acid complex in the T. amestolkiae MSG-glucose medium cultivation, other amine compounds were produced, namely N-hydroxyhexanoic acid-(10E)-12-carboxyl-monascorubramine and N-hexanedioic acid-(10E)-12-carboxyl-monascorubramine, another derivative of PP-O. In this way, these new compounds isolated from T. amestolkiae will enlarge the list of Monascus-like colorants. To date, more than 100 different chemical structures have been identified as colored azaphilones, characterized by the nitrogen-containing compounds of typical Monascus colorants (Chen et al., 2020; Yang et al., 2015). As in T. amestolkiae species, Tolborg et al. (Tolborg, Oxdum, Isbrandt, Larsen, & Workman, 2020) have identified a new series of complexes of Monascus-like azaphilone colorants from T. atroroseus species, named atrorosins. Atrorosins also have a similar core structure of azaphilone to the orange PP-O, with a carboxylic acid group at R$, differing on incorporating amino acids into the core skeleton of the azaphilone. The authors added strategically to the culture medium 19 amino acids resulting in PP-O derivatives named based on the name of the fungus, using the one-letter amino acid abbreviation to denote which nitrogen-containing compound has been incorporated.

For the novel amino-hexanedioic acid derivatives of Monascus colorants formation some hypotheses may also be suggested: the pyran oxygen of the orange colorants monascorubrin or PP-O and the yellow ankaflavin could be directly substituted for the amino-hexanedioic acid to form the amino-hexanedioic acid Monascus-like colorants. Another hypothesis is that the orange and yellow colorants might be modified to the red derivatives monascorubramine and PP-V. These red derivatives could be further modified to complexes of amino-hexanedioic-colorant through ammonia assimilation or amide bond hydrolysis. The complex MSG metabolism could be related to the production of fractions of amino-hexanedioic acid Monascus-like colorant derivatives by producing other amine compounds during glutamic acid metabolism (Zhao et al., 2017). As l-glutamate is considered a shunt of the tricarboxylic acid (TCA) cycle, a decline in the TCA cycle could lead to an accumulation of acetyl-CoA (Chen et al., 2017). Acetyl-CoA is a pivotal element in processes such as central carbon metabolism, fatty acid metabolism, amino acid metabolism, energy metabolism, and protein acetylation (Krivoruchko, Zhang, Siewers, Chen, & Nielsen, 2015). In this way, further analyses may be performed to explain the chronology of the distribution of the different colored compounds in intra- and extracellular extracts.

### 3.2. Stability of red color in various pH

The stability of a biomolecule considering pH is an important parameter for a series of processes. This condition limits not only the downstream process applied on the recovery and purification of the colorant but also in the colorant application (e.g. as food or textile colorant) (Mapari et al., 2009). Moreover, natural colorants are gaining interest as their potential as halochromic indicator response to pH variation, which is attractive for application in several fields such as packaging materials to monitor and record food status throughout storage, water treatment, biology and cosmetics (Sani et al., 2021). In this sense, the pH effect on the color of the fermented broth of the T. amestolkiae was evaluated. This study was performed with crude fermented broth (without any extraction and/or purification) because the purification process is required for only some applications. The pH range was studied from 1 to 13 by adding HCl (1.0 M) or NaOH (1.0 M) as needed. The experimental data are shown in Table S1 of the Supplementary Material, with the red colorants stability over time depicted in Fig. 3.

The red colorants kept the relative absorbance close to 100% in the pH range between 3 and 11 over the time studied. Interestingly, there was an increase in absorbance under basic conditions (pH 9 and 11). However, extremely acidic and/or alkaline conditions, pH 1 and 13, respectively, promoted a decrease in the absorbance of the red colorants over time. As for pH 3 and 7, the colorants were stable, the UV–vis and fluorescence spectra for pH 3 and 7 are shown in Supplementary Material Fig. S2. Furthermore, for pH 9 and 11, an increase in the absorbance occurred, while for pH 1 and 13, the colorants have shown high color instability. The UV–vis spectra of the colorants produced by T. amestolkiae at different pH values and the fluorescence spectra for each condition tested are shown in Fig. 4, with pH 5 representing the control. After 24 h of incubation at the desired pH condition, which varied between pH 1 and 13, acid or base solution was added to return the pH to 5 in order to evaluate color and fluorescence reversibility.

In addition to the UV–vis and 3D fluorescence spectra, the colorimetric analysis provides more information on the colorants’ stability in the fermented broth (Fig. 5). The so-called Commission International de l’Eclairage (CIE) system is based on the fact that the color characteristics of the samples largely depended on the color and the concentration of the dominant colorant in the mixture. Thus, the values of L* indicates lightness. Chroma value (C*) refers to how pure or ‘saturated’ a color is, as high chroma is more vivid than low chroma. In this way, values close to the center at the same L* value indicate dull or gray colors, whereas values near the circumference represent vivid or bright colors. Hue angle (H') represent 0 for redness, 90 for yellowness, 180 for greenness, and 270 for blueness (Mapari et al., 2006; Mapari et al., 2009).

Colorants are composed of a group of atoms responsible for color, called chromophores, as well as electron donor or receptor radicals. These radicals cause or intensify the color of the chromophores and are called auxochromes. Auxochromes can influence the chromophore group in several ways, shifting the absorbed wavelength to a higher value (bathochromic shift) or to a smaller value (hypsochromic shift) or changing the absorption intensity of the wavelengths, in order to increase it (hypochromic effect) or diminish it (hypochromic effect). The most important chromophores to be mentioned are represented by the: azo chromophores (–N=N–), carbonyl (–C=O), methine (–CH=–), nitro (–NO$_2$) and quinoid groups. In addition, auxochromes include: amine (–NH$_3$), carboxyl (–COOH), sulfonate (–SO$_3$H) and hydroxyl (–OH) groups (Christie, 2001; Pina, Melo, Laia, Parola, & Lima, 2012).

From pH 5 to 1 the L*, H’, fluorescence intensity (I) and absorbance intensity representing the 410, 470 and 500 nm have decreased. However, when the pH was reverted to 5, the values of fluorescence intensity, absorbance, L* and H’ returned to values close to the initial data (Figs. 4 and 5). In extreme conditions of acidity (pH 1), the decrease in L* and H’ seem to indicate that the colored compounds suffered reversible precipitation, as the initial values of these parameters are recovered when the pH returns to 5.

Under basic conditions (pH 9 and 11), both the absorbance and fluorescence set of results increased compared to the initial conditions. For pH 11 there was a hypochromic shift in the $\lambda_{\text{max}}$ from 500 nm to 495 nm accompanied by a hyperchromic effect and an increase in L* and H’; For pH 9, the wavelength shift was seen only after returning to pH 5.
For the fluorophore III, which probably corresponds to the red colorants chromophore (de Oliveira et al., 2019), the fluorescence intensity increased significantly in both conditions (pH 9 and 11), showing even greater values after returning to pH 5, which may also be related to the hypsochromic shift accompanied by the hyperchromic effect. At pH 11, the fluorescence intensity after the pH return to 5 has increased more than 5 times.

Under strongly basic conditions (pH 13), the yellow and red colorants (410 and 500 nm, respectively) were probably transformed into orange colorants, which resulted in a shift in the maximum wavelength (λ\text{max} = 480 nm) accompanied by a hyperchromic effect and a color change from red to orange (H+ 46.72) (Fig. 5). Fluorescence intensification is seen only for fluorophores I and II, even after returning to pH 5. Fluorophore III and the H+ underwent irreversible changes, corroborating the loss of red colorants seen in the UV-vis spectrum (Fig. 4).

Moreover, it can be inferred that the OH- and H+ ions in solution influence the charge of the auxochrome groups of the colorants in order to interfere with the intensity and wavelength absorbed, therefore influencing their color. In very acidic pH, precipitation occurring ostensibly through hydrolysis of key chemical groups. In alkaline conditions pH (9 and 11), both methyl and carboxyl residues in R+ might suffered ionization, enhancing the red color. In very alkaline pH medium, the nitrogen group of the pyrano-quinone bicyclic core could suffer from an oxidation process, this would give a pyronoid oxygen, thus characterizing the orange Monascus colorants. Thus, the red colorants produced by T. amestolkiae is negatively halochromic caused by a hypsochromic displacement of the absorption band. In corroboration with our results, Bhardwaj and co-authors (Bhardwaj et al., 2007) also observed a change of the red to orange color of the P. manneffei colorants when the pH was changed from 8 to 13. Red is one of the colors most used in a wide range of products, especially from food sector. The main natural red colorants include anthocyanins, lycopenes, betalains and carmine (Ghidouche et al., 2013). Anthocyanins are flavonoids and are characterized by the basic flavilium nucleus (cation 2-phenylbenzopyri-lum). A variety of colorant molecules are required to cover all applications. For example, anthocyanin-based colorants give red tones at very acidic pH and blue to green at neutral pH (Chen et al., 2019), while carmine is stable at pH 5 and 7, but is unstable and can precipitate at pH 3. Carminic acid is a water-soluble anthraquinone and its color also changes with pH; if in acidic environments it is orange, when acidity decreases it turns to be red and violet in alkaline conditions (Ghidouche et al., 2013). However, the anthocyanins and carminic acid halochromic properties enable the design of natural colorant-rich films to monitor and maintain the quality of packaged food (Sani et al., 2021).

The color of Monascus azaphilone colorants varies from yellow to orange to red. The six classical Monascus azaphilone colorants in solution were reported to be more stable at near-neutral and/or alkaline pH, being the hydrophobic red colorants less stable than the yellow colorants (de Carvalho, Oishi, Pandey, & Soccol, 2005; Mapari et al., 2009). Carvalho et al. (2005) attributed this effect to the acceleration of the interaction between the water molecules and the colorants caused by the H+ ions, such as breaking of an ester linkage in monascorubramine or rubropunctamine (red colorants); this effect was confirmed when a stability analysis was made with colorants in ethanolic solutions and no significant decrease in color was observed. The studied condition was the pH from 4.1 to 7.9 at 100 °C for 3 h. The authors showed that the color decreases more rapidly in low pH values, which may impose a problem for applying Monascus colorants in acid foods. In the same direction, Mapari and collaborators (Mapari et al., 2009) evaluated the photostability of the fungal colorant extracts produced by P. aculeatum IBT 14,263 and Epicoccum nigrum IBT 41,028 in citrate buffer at acidic and neutral pH. The color degradation was higher at pH 5 than pH 7. In contrast, T. amestolkiae water-soluble red colorants showed broad stability in the range between pH 3 to 11, which makes them applicable over a wide pH range without changing the color and making the molecule instable. Corroborating with our results, Broder and Koehler (1980) reported that the water-soluble red complexes of Monascus colorants, like monascorubramine and rubropunctamine derivatives, were considerably stable to pH changes. In the same way, the water-soluble amino-hexanedioic acid complexes of Monascus colorants produced by T. amestolkiae are substantially stable in a broad range of acidic and basic conditions.

However, as T. amestolkiae colorants studied in the present work derived from a different culture medium could give another pH stability profile. In a previous report from our research group, T. amestolkiae, former P. purpureogenum, was grown on yeast extract and sucrose medium and the results obtained from the pH stability study at 25 °C for 24 h showed that the red colorants produced were more stable at neutral and basic conditions (pH 7 and 8) (Santos-Ebinuma et al., 2013a), as found in this study. However, contrarily to the results obtained in this...
Fig 4. Stability of the colorants of *T. amestolkiae* fermented broth according to UV–vis and 3D fluorescence spectra for pH 1; 9; 11 and 13 after 24 h of incubation at 25 °C and color reversibility evaluation to pH 5 after 24 h of incubation at 25 °C. The pH was altered by adding HCl (1.0 M) or NaOH (1.0 M). The values for fluorescence intensity (I) of all chromophores are shown in Supplementary Material Table S1.
Fig 4. (continued).

Fig 5. Color change in the CIELAB color space for the colorants produced by T. amestolkiae for pH 1 (●), control (○), pH 9 (●), pH 11 (●) and pH 13 (●) after 24 h of incubation at 25 °C and color reversibility evaluation to pH 5 after 24 h of incubation at 25 °C. The pH was changed by adding HCl (1.0 M) or NaOH (1.0 M), as needed.
work, it was observed that the red colorants’ color loss was more pronounced at acidic pH (3, 4 and 5). Taking into account all the results reported so far regarding the stability of these colorants, it seems reasonable to hypothesize that as azaphilone colorant biosynthesis is highly dependent on the medium conditions. Moreover, different compounds could be formed when \( T. amestolkiae \) was grown in nitrogen complex and sucrose than in MSG-glucose medium. When MSG is added to the cultivation medium as nitrogen source, it promotes production and excretion of extracellular colorants; in contrast complex nitrogen sources is known to produce cell-bound colorants (Blanc et al., 1994; Hajjj et al., 1997). This could be a reason for the stronger acid-catalyzed degradation of the hydrophobic red colorants presented and described in other study of our research group (Santos-Ebinuma et al., 2013a). The results presented shows that the \( T. amestolkiae \) colorants derived from MSG-glucose medium are stable in relation to color in a wide range of pH values and could be applied in acidic, mear-neutral and slightly basic food products, however they can precipitate or lose the color characteristics related to the chemical structure of the chromophore in very acidic and alkali conditions.

4. Conclusions

In conclusion, \( T. amestolkiae \) is a promising candidate for producing novel natural colorants for industrial applications. In this study, the colorants produced by cultivation with MSG-glucose medium led to the novel natural colorants for industrial applications. In this study, the colorants produced by cultivation with MSG-glucose medium led to the novel natural colorants for industrial applications. In this study, the colorants produced by cultivation with MSG-glucose medium led to the novel natural colorants for industrial applications. In this study, the colorants produced by cultivation with MSG-glucose medium led to the novel natural colorants for industrial applications.

References


