JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Computerized Screening for Novel Producers of *Monascus*-like Food Pigments in *Penicillium* Species

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Monascus pigments have been used as natural food colorants in Asia for centuries. They are not authorized for use in the European Union and the United States mainly due to the risk of coproduction of the mycotoxin citrinin by *Monascus* spp. In the present study, we screened for novel producers of *Monascus*-like pigments from ascomycetous filamentous fungi belonging to *Penicillium* subgenus *Biverticillium* that are not reported to produce citrinin or any other known mycotoxins. The screening was carried out using the X-hitting algorithm as a tool to quickly screen through chromatographic sample data files of 22 different *Penicillium* extracts with 12 *Monascus* pigment extracts as controls. The algorithm searched for the most similar UV−vis spectra of the metabolites (cross hits) present in the pigment extracts to those of the selected reference metabolites viz. monascin, rubropunctatin, rubropunctamine, and citrinin. The cross hits were then manually identified on the basis of their UV−vis and mass spectra. X-hitting was found to be a good tool in the rapid screening of crude pigment extracts. *Monascus* pigments were discovered in the extracts of two closely related species of *Penicillium* that were only distantly related to the genus *Monascus*. Monascorubrin, xanthomonasin A, and threonine derivatives of rubropunctatin were identified in the extract of *Penicillium aculeatum* IBT 14263, and monascorubrin was identified in the extract of *Penicillium pinophilum* IBT 13104. None of the tested *Penicillium* extracts showed the presence of citrinin. Thus, the present study brought out two novel promising sources of yellow, orange, and purple-red *Monascus*-like food pigments in the species of Penicillia that do not produce citrinin and opened the door to look for several more new promising sources of natural food colorants in the species of Penicillia.

KEYWORDS: Computerized screening; *Monascus* pigments; polyketide; citrinin; X-hitting; *Penicillium*

INTRODUCTION

The production of many currently authorized natural food colorants has a number of drawbacks, including a dependence on the supply of raw materials, which are influenced by agro-climatic conditions (*1*). Ascomycetous fungi provide an alternative source of naturally derived food colorants that could easily be produced in high yields because of the availability of cultivation technology and potential use of metabolic engineering tools. For centuries, *Monascus* spp. have been used for the production of red mold rice that has served as a source of a natural food colorant and/or spice in cooking in East Asia, particularly in Japan and China (*2, 3*). The red rice phenomenon is a result of the red pigment production by *Monascus* species; the six main *Monascus* pigments (*2, 3*) are ankaflavin, monascin, monascorubrin, rubropunctatin, rubropunctamine, and monascorubramine (compounds **1**−**6**, **Figure 1**). Other pigments like xanthomonasin A and B (*4, 5*), monankarines, (*6*) and industrially useful polyketide metabolites such as cholesterol-lowering compounds referred to as monacolins (*2, 7*) are also produced by the genus *Monascus*. *Monascus* pigments are originally cell bound and hydrophobic but contain an aminophilic moiety that reacts with amino group-containing compounds in the medium, such as proteins, amino acids, and nucleic acids, to form water-soluble pigments. In this direction, *Monascus* pigments derived with glutamic acid (*8*−*10*), aspartic acid, and alanine (*11*) have been identified and characterized. Jung et al. (*12*) fermentatively produced *Monascus* pigment derivatives using 20 individual amino acids as side chain precursors. The pigment derivatives obtained in this way were found to be more hydrophilic than their counterparts and more light-stable (*13*). Moreover, various red color hues were exhibited by these pigment derivatives, and the hues of most of these were darker red than their counterparts (*12, 13*). This makes these derivatives

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10.1021/jf801817q CCC: $40.75
Published on Web 10/09/2008

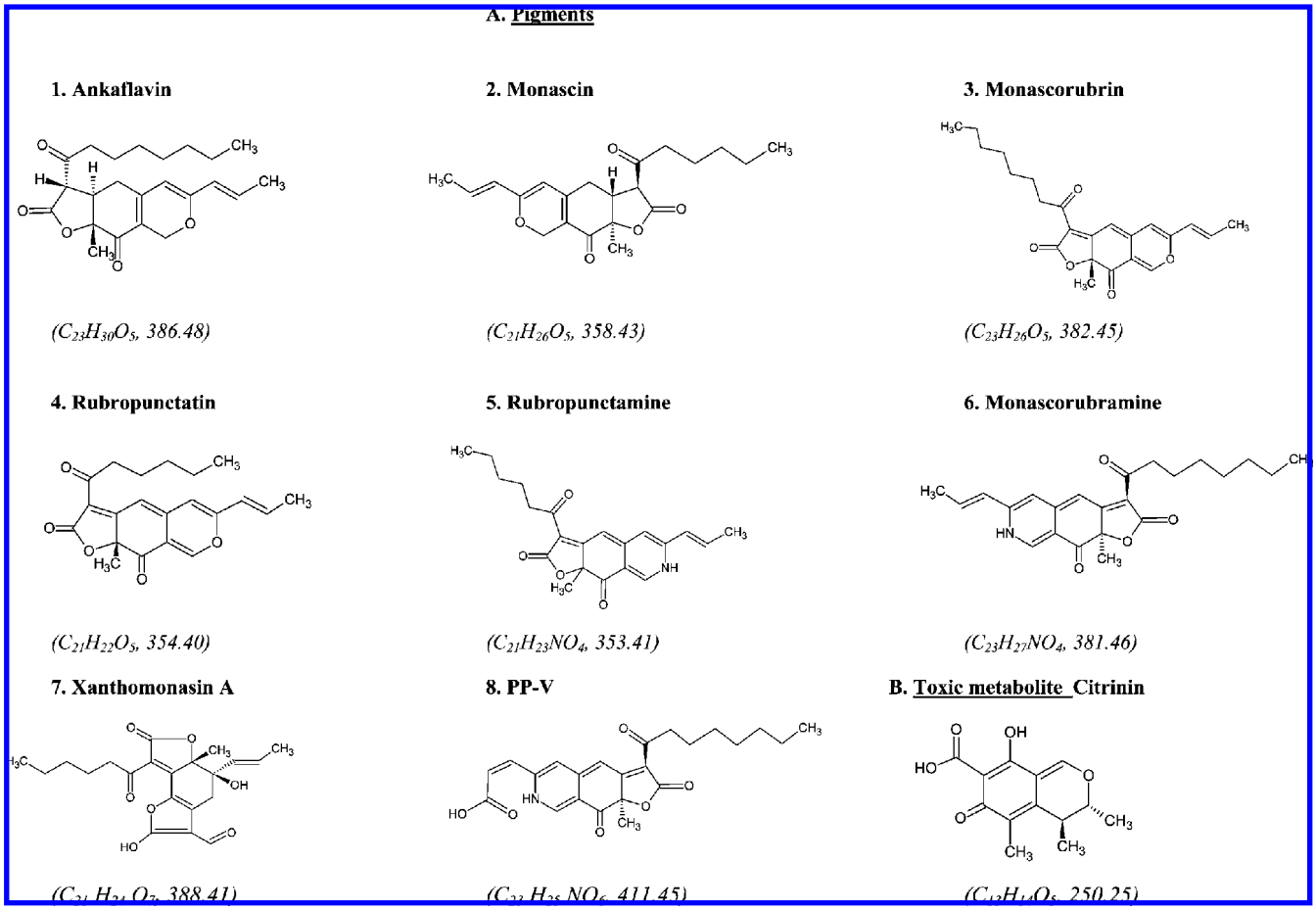

Figure 1. Structures of six characteristic pigments, a pigment, a pigment derivative, and a toxic metabolite of *Monascus* origin detected and identified in the present work (formula and nominal masses in parentheses) (1, 2, and 7, yellow; 3 and 4, orange; and 5, 6, and 8, purple-red).

quite interesting for further exploration as food colorants. However, *Monascus* spp., except a few commercial strains, wild types, and mutants, have also been shown to produce a mycotoxin called citrinin (**Figure 1**) on some media under certain conditions (*14, 15*). Even though both genetic attributes and culture conditions influence citrinin production, there remains a possibility of the presence of this mycotoxin in some batches, posing a critical safety issue in the pigment production process. Citrinin has been detected in some commercial red rice products, which are used as food colorants mainly in Asia (*14*). In addition to citrinin, which has been the major potential threat so far, other potential toxic metabolites of *Monascus* such as monascopyridines (*16*) and their toxic effects (*17, 18*) have also been reported from the red rice recently. This limits the food use of *Monascus* pigments, which are not permitted in Europe or in the United States, and puts a large incentive to screen for other fungi that produce *Monascus* pigments and/or *Monascus*-like pigments but no citrinin and/or other mycotoxins.

It is a well-known fact that the polyketide pathway, leading to the synthesis of the colored azaphilone compounds like *Monascus* pigments and their derivatives, is widely distributed in the fungal world. Also, a number of reports have been published on colored azaphilone compounds produced by fungi other than *Monascus* spp (*19*−*22*). Therefore, we hypothesized that *Monascus* or *Monascus*-like azaphilone pigments are likely to be produced by species in the *Penicillium* subgenus *Biverticillium* that produce copious amounts of red to orange-red pigments. It has also been reported that fungal polyketide-based metabolites like sclerotiorin, rotiorin, and *Monascus* pigments monascin and rubropunctatin form the same biogenic family, which might include such mycotoxins as citrinin (*23*). Therefore, the present work focuses on identifying *Monascus* or *Monascus*-like pigments in the species belonging to *Penicillium* subgenus *Biverticillium.* At the same time, the strains were analyzed for the absence of citrinin. For screening of fungi aimed at biotechnological production of pigments for food use, it is necessary to make sure that the pathogenic and/or toxigenic pigment-producing fungi are pre-eliminated at an early step. In the light of this, we aimed at intelligent screening (*24*), whereby we relied on a priori chemotaxonomic knowledge; there are several species in the *Penicillium* subgenus *Biverticillium* that produce red to orange-red pigments and are neither toxigenic nor pathogenic, and they have been used to preselect some isolates from our IBT Culture Collection at the Technical University of Denmark (DTU). Another effective tool of intelligent screening is a new method for the systematic and automated computer-assisted search of full UV spectra in a large number of data files for new natural products based on the new mathematical algorithm X-hitting (*25, 26*). In the present study, we used X-hitting as a tool to quickly screen through a large number of chromatographic sample data files and came out with the most likely candidates (cross hits) that have similar UV−vis spectra to those of the selected *Monascus* pigments and citrinin, used as the reference compounds. The cross hits were then manually identified on the basis of their UV−vis and mass spectra. To evaluate the citrinin-producing ability of *Monascus* strains on three different media under similar growth conditions to those of Penicillia, *Monascus ruber* and *Monascus purpureus* strains were used as controls.

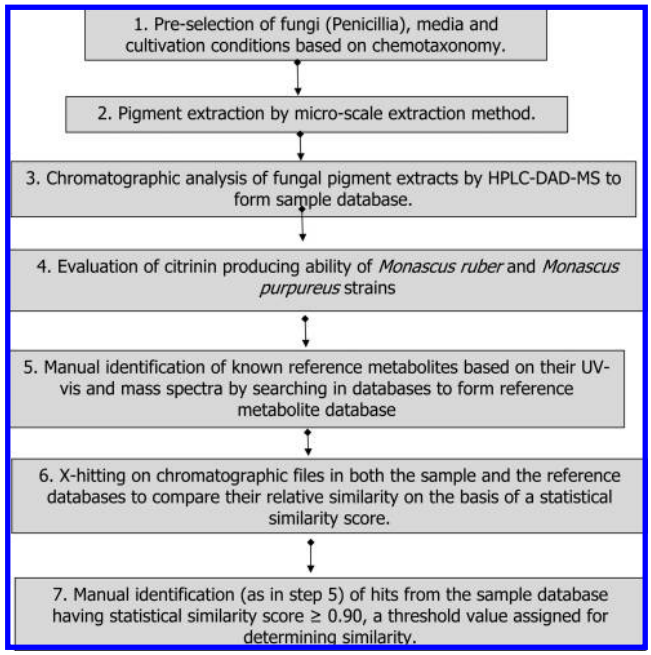

Figure 2. Schematic presentation of the method used in the present study.

MATERIALS AND METHODS

A schematic presentation of the overall methodology used in the present study is shown in **Figure 2**. Specific steps and analytical techniques used in the methodology were as follows.

Preselection of Fungi, Media, and Cultivation Conditions. All fungal isolates used in this study were procured from the IBT Culture Collection at Center for Microbial Biotechnology, Technical University of Denmark (Kgs. Lyngby, Denmark). The fungal isolates were listed by the IBT numbers. All fungi were cultivated on either of the five different solid media, viz. yeast extract sucrose (YES) agar, malt extract agar (MEA), oatmeal (OAT) agar, potato dextrose (PD) agar, and Czapek−Dox yeast autolysate (CYA) agar (*27*), or in specific combinations on which maximum pigment was found to be produced with interesting color hues in the red to yellow spectra. The cultures were incubated in the dark at 25 °C for 7 days. **Table 1** represents the identity of the fungi, the IBT number, and the media used for their incubation.

Extraction of Fungal Pigments. Extraction was carried out by a modified version of the microextraction method (*28*), where 6 mm plugs were extracted in two steps in a 2 mL vial for 30 min, first using 1 mL of ethyl acetate with 0.5% formic acid to break open the cell wall and extract relatively apolar metabolites. The extract so obtained was then transferred to a new 2 mL vial and evaporated in vacuo. The second extraction was performed using 1 mL of isopropanol. By doing so, we could extract the maximum color. The second extract was then added to the vial with the residue from the previous extraction. It was then evaporated in a rotational vacuum concentrator (RVC; Christ Martin, Osterode, Germany). The residue was redissolved in 400 μL of methanol, in an ultrasonic bath (Branson 2510, Kell-Strom, Wethersfield, United States) for 10 min and filtered through a 0.45 μL PTFE syringe filter (SRI, Eatontown, NJ).

Chromatographic Analysis. High-resolution liquid chromatography−diode array detection−mass spectrometry (LC-DAD-MS) was performed on an Agilent HP 1100 LC system with a DAD and a 50 mm × 2 mm i.d., 3 μm, Luna C 18 II column (Phenomenex, Torrance, CA). The LC system was coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, United Kingdom) with a Z-spray electrospray ionization (ESI) source and a LockSpray probe and controlled by the MassLynx 4.0 software. The MS system was operated in the positive ESI mode using a water−acetonitrile gradient system as described by Nielsen et al. (*29*).

Analysis of LC-DAD-MS Data. The presence of metabolites involved in the present study, (**Figure 1**) including both reference metabolites as well as cross hits and new hits was detected in ESI^+ from the first scan function of the reconstructed ion chromatograms. Standard compound citrinin (Sigma-Aldrich) was run with the pigment extracts under study, which was used as a reference for mass validation. The accuracy of mass detection by the instrument was in the acceptable range based on a calibration of the detected monoisotopic masses of the metabolites and the detected monoisotopic mass of standard citrinin. The UV−vis spectrum was obtained from the UV−vis chromatogram after background subtraction. The DAD-MS data for each of these compounds are shown below; however, we show LC-DAD-ESI^+-MS chromatograms of two reference metabolites and three new hits as the representative examples (**Figures 3** and **5**).

Table 1. Identity of the Analyzed Extracts with Their Respective X-Hit Numbers Used in the Present Study

X-hit no.	fungal name	IBT no.	media
1	*Penicillium purpurogenun*	11180	CYA
2			YES
3	*P. pinophilum*	3757	PD
4			OAT
5	*P. pinophilum*	14263	CYA
6			YES
7			MEA
8	*P. pinophilum*	13104	PD
9			YES
10			OAT
11	*P. purpurogenum*	21723	YES
12			CYA
13	*P. funiculosum*	3954	MEA
14			CYA
15	*P. purpurogenum*	3967	CYA
16	*P. purpurogenum*	21347	PD
17			OAT
18	*P. purpurogenum*	23082	MEA
19	*P. aculeatum*	14129	CYA
20	*P. funiculosum*	21276	PD
21	*P. minioluteum*	18368	YES
22	*P. aculeatum*	14259	CYA
23	*Monascus ruber*	98585	MEA
24	*M. ruber*	7904	MEA
25	*M. ruber*	9658	YES
26			PD
27	*M. ruber*	9655	MEA
28			PD
29	*M. purpureus*	9664	PD
30			MEA
31			YES
32	*M. purpureus*	9667	PD
33			MEA
34			YES
35	citrinin standard		

Ankaflavin. Ankaflavin was detected as *m/z* 387.23 $[M + H]^+$ and confirmed by the adduct *m/z* 450.25 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was λ_{max} 232, 283 (shorter peak), and 390.

Monascin. Monascin was detected as *m/z* 359.19 $[M + H]^+$ and confirmed by the adduct *m/z* 422.19 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was λ_{max} 234, 292 (shorter peak), and 394.

Rubropunctatin. Rubropunctatin was detected as *m/z* 355.15 $[M + H]^+$ and confirmed by the adducts *m/z* 377.14 $[M + Na]^+$ and 418.16 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was λ_{max} 246, 288, and 474.

Rubropunctamine. Rubropunctamine was detected as *m/z* 354.19 $[M + H]^+$ and confirmed by the adducts *m/z* 376.20 $[M + Na]^+$ and 417.20 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was λ_{max} 306, 414, and 530 with a shoulder at 256.

Citrinin. Standard citrinin was detected as *m/z* 251.48 $[M + H]^+$ and confirmed by the adduct *m/z* 314.44 $[M + Na + CH_3CN]^+$ and the fragment 233.49 $[M + H - H_2O]^+$. The UV spectrum was λ_{max} 216, 322 with a shoulder at 242.

Monascorubrin. Monascorubrin was detected as *m/z* 383.41 $[M + H]^+$ and confirmed by the adducts *m/z* 405.40 $[M + Na]^+$ and 446.36

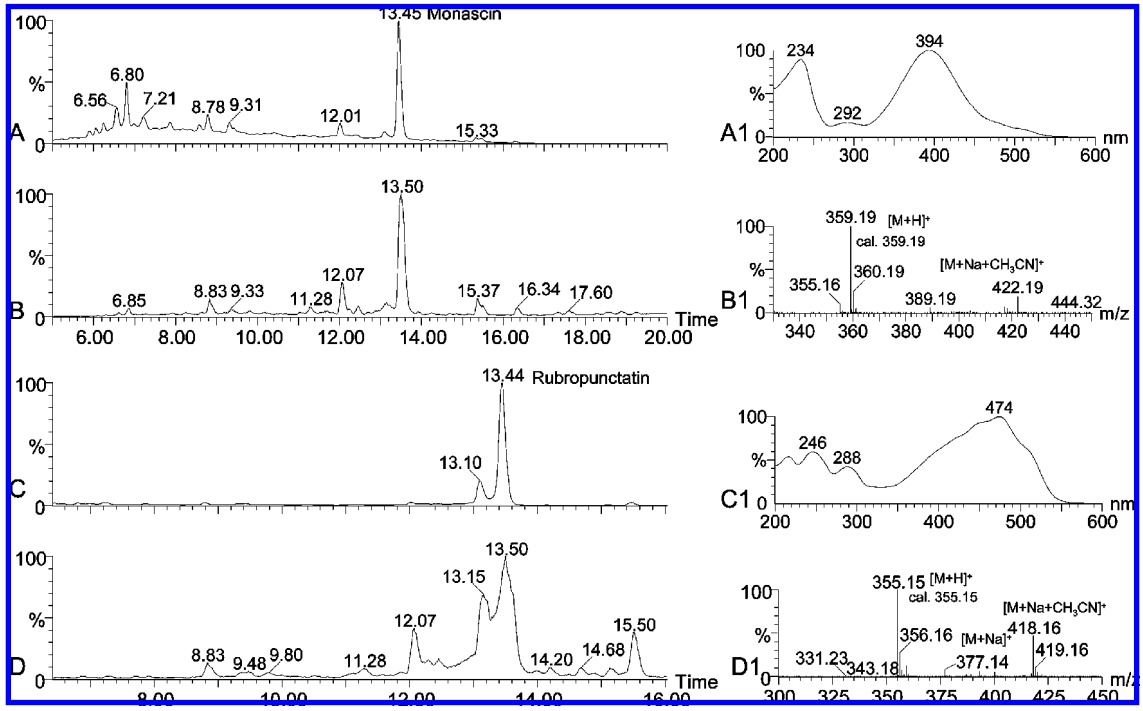

Figure 3. Top panel (**A** and **B**) depicts chromatograms showing monascin in the pigment extract of *Monascus ruber* IBT 9658 on PD (X-hit 26). (**A**) UV−vis chromatogram of 390−700 nm showing colored compounds only. **A1** represents the UV−vis spectrum of monascin. (**B**) Total ion chromatogram (*m*/*z* 100−900) from positive ion electrospray. **B1** represents the mass spectrum of monascin from ESI^+. Bottom panel (**C** and **D**) depicts chromatograms showing rubropunctatin in the pigment extract of *M. ruber* IBT 98585 on MEA medium (X-hit 23). (**C**) UV−vis chromatogram of 390−700 nm showing colored compounds only. **C1** represents the UV−vis spectrum of rubropunctatin. (**D**) Total ion chromatogram (*m*/*z* 100−900) from positive ion electrospray. **D1** represents the mass spectrum of rubropunctatin from ESI^+.

$[M + Na + CH_3CN]^+$ and the fragment 339.51 $[M + H - CO_2]^+$. The UV−vis spectrum was λ_{max} 246, 286, and 478.

Xanthomonasin A. Xanthomonasin A was detected as *m*/*z* 389.44 $[M + H]^+$ and confirmed by the adduct *m*/*z* 452.39 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was λ_{max} 234, 288 (shorter), and 394.

PP-V. PP-V was detected as *m*/*z* 412.21 $[M + H]^+$ and confirmed by the adducts *m*/*z* 434.20 $[M + Na]^+$ and 475.22 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was λ_{max} 204, 300, 418, and 522 with a smaller peak at 252.

Threonine Derivative of Rubropunctatin. The threonine derivative of rubropunctatin was detected as *m*/*z* 456.36 $[M + H]^+$ and confirmed by the adducts *m*/*z* 478.31 $[M + Na]^+$ and 519.28 $[M + Na + CH_3CN]^+$. The UV−vis spectrum was λ_{max} 204, 282, 424, and 524.

Monascorubramine was not detected in the pigment extracts under study.

X-Hitting. *Selection of Reference Metabolites.* The fungus *Monascus* typically produces six major pigments (**Figure 1**): the yellow pigments ankaflavin and monascin, the orange pigments rubropunctatin and monascorubrin, and the purple-red pigments rubropunctamine and monascorubramine. We selected one of the pigments from each color viz. monascin (yellow), rubropunctatin (orange), and rubropunctamine (purple-red) that also represented the three different chromophores present in the azaphilone structures of six major *Monascus* pigments. This selection was based on the fact that the x-hitting would hit the UV−vis spectra of pigments having similar chromophores but different side chains almost equally well as the UV−vis spectra are relatively independent of the length of the side chain. The reference metabolites were manually identified in the three *Monascus* pigment extracts used as standards (X-hit 23, 24, and 26 in **Table 1**) and were used as reference metabolites. In addition to pigments, a standard of citrinin (Sigma-Aldrich) was also used as a reference metabolite.

Metabolite and Sample Database and Their Interrelationship. Two databases viz. metabolite and sample database, both of which were based on UV−vis spectra extracted from the DAD scan function of LC-MS analyses of standards and pigment extracts, were created. The metabolite database contained the information about the reference spectra from samples where the presence of known target metabolite was identified. In the present study, three pigments together with citrinin formed our reference metabolite database. The sample database contained information about the unknown spectra obtained from the analysis of the pigment extracts. The spectra of the reference metabolites were used as the fingerprint spectra, and a match factor was calculated and evaluated between the spectra at given retention times for each reference spectrum in the compound database (**Figure 2**). The spectra were evaluated based on a value describing its retention time (UV scan number). The interrelationship between the two databases was determined on the basis of this match factor, and a statistical similarity score was given.

Matching Algorithm. The algorithm behind X-hitting is described elsewhere (*25*).

RESULTS AND DISCUSSION

Identification of Metabolites Used in the Present Study. *Reference Metabolites and/or Cross Hits.* Because commercial standards of reference metabolites were not available except for the standard citrinin, the reference metabolites used in the present study were detected and identified in the selected control *Monascus* pigment extracts (**Figure 3**). Identification was based on both the UV−vis spectra and the accurate masses; monoisotopic masses are given in the database such as Antibase (Wiley-VCH, Weinheim, Germany) as well as in the available literature references. The UV−vis and mass spectral data of the three reference metabolites viz. monascin, rubropunctatin, and rubropunctamine matched well with the data given by Teng et al. (*30*) and Su et al. (*31*). It must be noted that there are some discrepancies in the available literature with regards to identification of the six characteristic *Monascus* pigments on the basis of the UV−vis and mass spectra. For instance, Akihisa et al. (*5*) reported the spectra of the characteristic yellow pigments ankaflavin and monascin in the visible range to be λ_{max} at 460 nm, and a λ_{max} of 530 nm was shown for the orange pigments

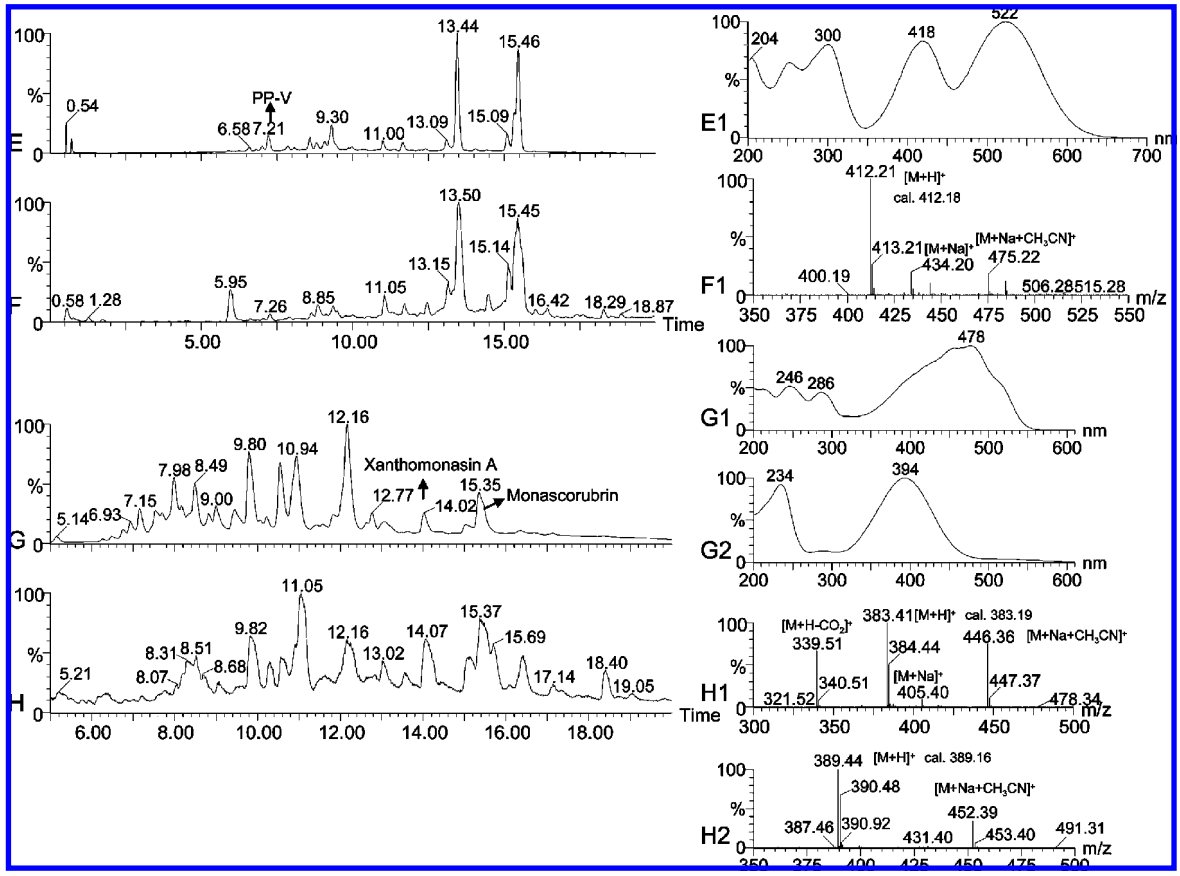

Figure 4. Top panel (**E** and **F**) depicts chromatograms showing new hit PP-V in the pigment extract of *Monascus purpureus* IBT 9664 on PD (X-hit 29). (**E**) UV−vis chromatogram of 390−700 nm showing colored compounds only. **E1** represents the UV−vis spectrum of PP-V. (**F**) Total ion chromatogram (*m*/*z* 100−900) from positive ion electrospray. **F1** represents the mass spectrum of PP-V from ESI^+. Bottom panel (**G** and **H**) depicts chromatograms showing new hits monascorubrin and xanthomonasin A in the pigment extract of *Penicillium aculeatum* IBT 14263 on YES medium (X-hit 5). (**G**) UV−vis chromatogram of 390−700 nm showing colored compounds only. **G1** represents the UV−vis spectrum of monascorubrin, and **G2** represents the UV−vis spectrum of xanthomonasin A. (**H**) Total ion chromatogram (*m*/*z* 100−900) from positive ion electrospray. **H1** represents the mass spectrum of monascorubrin, and **H2** represents the mass spectrum of xanthomonasin A from ESI^+.

monascorubrin and rubropunctatin. Moreover, the molecular weight for rubropunctatin was shown to be 353, and 355 − $[M + H]^+$ was shown for rubropunctamine. These results are neither in agreement with the previously reported (*30*−*33*) values nor with our own results. Thus, utmost care should be taken when comparing the UV−vis and/or mass spectral data to the data available in the literature for the identification of colored compounds, as this is an important step toward the discovery of new compounds.

New Hits. The identification of the new hits was carried out in the same way as for the reference metabolites and/or cross hits (**Figure 4**), and the data analyses are explained in the Materials and Methods section. The UV−vis and mass spectral data of ankaflavin matched well with the data given by Teng et al. (*30*) and Su et al. (*31*). The UV−vis and MS data for PP-V and threonine derivative of rubropunctatin were also well-supported by Ogihara et al. (*36*−*38*) and Jung et al. (*12*), respectively. The MS data for xanthomonasin A matched well with the data given by Akihisa et al. (*5*); however, the UV−vis spectrum for xanthomonasin A described by them is more toward orange (λ_{max} 460nm) side of the visible spectrum. Our results indicated that the λ_{max} was 394 nm, confirming its yellow nature.

Citrinin-Producing Ability of the *Monascus* Cultures. Before proceeding with the X-hitting experiment, the citrinin-producing ability of *Monascus* cultures was demonstrated under similar growth conditions to those of Penicillia in our laboratory on three different media. For this purpose, four *Monascus* cultures, two different strains from two preferably used species of *Monascus*, viz. *M. ruber* and *M. purpureus* [only the pigments of which are authorized for food use in Japan (*9*)], were selected. *M. ruber* IBT 9658 could produce citrinin (C+) only in YES media (**Figure 5A**) and *M. ruber* IBT 9655 did not produce any citrinin (C−) in the three tested media (**Figure 5B**), while *M. purpureus* cultures produced citrinin in all three tested media (**Figure 5C**,**D**). *M. purpureus* cultures produced both pigments and citrinin on YES, MEA, and PD media, while the pattern of citrinin and also pigments varied with respect to the media in case of *M. ruber* IBT 9658. IBT 9658 produced only pigments but no citrinin in PD and neither pigments nor citrinin in MEA and both citrinin and meager pigments in YES media (**Figure 5A**). *M. ruber* IBT 9655 in MEA produced pigments but no citrinin, and in PD medium, scanty pigmentation with no citrinin was found to be produced (**Figure 5B**). Thus, it can be inferred that the pigment and citrinin biosyntheses are independent of each other but the factors by which they are triggered are not clear in the case of the fungal strains belonging to the genus *Monascus.* This was also shown by Pisareva et al. (*34*) and Wang et al. (*15*). Even if the citrinin was not found to be produced by two strains on the media under consideration in the present study, it could very well be produced on other media like rice-based media, which is popularly used for the industrial production of these pigments. As it was shown in an important study (*35*) in China, where 35 *Monascus* strains used in food

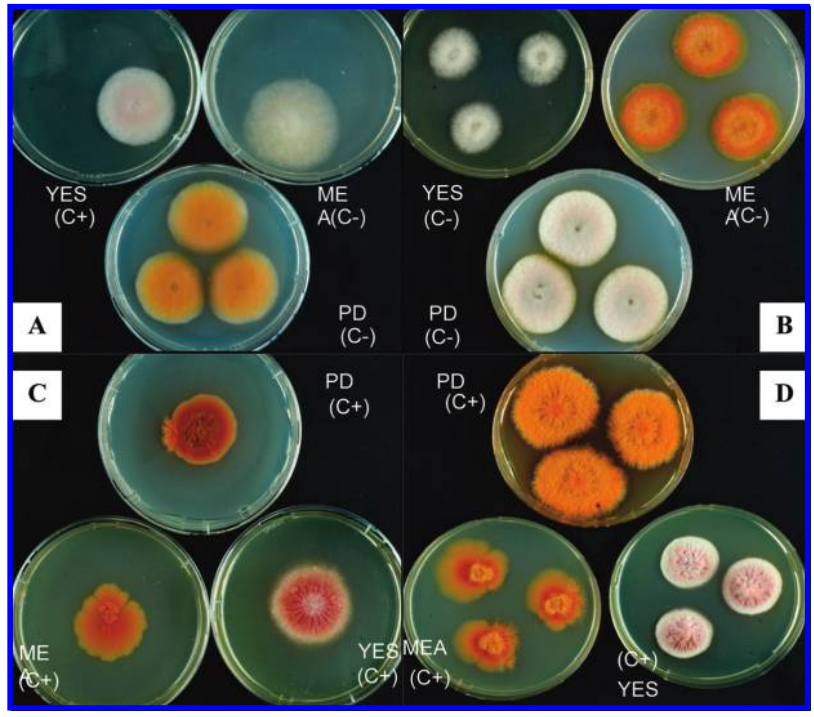

Figure 5. Citrinin and pigment-producing ability of *Monascus ruber* and *M. purpureus* strains in three different media. (**A**) *Monascus ruber* IBT 9658, (**B**) *M. ruber* IBT 9655, (**C**) *M. purpureus* IBT 9667, and (**D**) *M. purpureus* IBT 9664. MEA, PD, and YES represent the respective media, and C+ or C− in parentheses represent their ability to produce citrinin.

industry were selected to investigate the citrinin-producing ability in rice, all strains produced citrinin and thus posed a major safety issue. Another thorough investigation of 23 *Monascus* type cultures representing eight species for their citrinin-producing ability has revealed that citrinin was found to be produced in YES medium by all of them barring a couple of industrial strains (*15*). For the X-hitting experiment, extracts from pigment-producing citrinin positive or negative *Monascus* strains were used as positive or negative controls.

X-Hitting. Thirteen *Penicillium* strains from different species of subgenus *Biverticillium* grown on specific media that could elicit intense pigmentation were included in the present study. Some strains were also tested on two or more media for their ability to produce pigments in extractable amounts. Thus, 22 such combinations in addition to pigment-producing citrinin positive or negative *Monascus* strains (as mentioned earlier) were included, forming a total of 35 data files. These data files were given arbitrary numbers from one to 35 called as X-hit numbers (**Table 1**). Statistical similarity scores were obtained (as described in the Materials and Methods section Metabolite and Sample Database and Their Interrelationship) in gradation starting from the highest value (maximum 1) of that of the reference metabolite. A subjective threshold value of 0.900 of the statistical similarity score was set for all of the hits against each of the reference metabolites. Only those hits having a score value greater than or equal to the threshold value were manually identified in the LC-MS data files. It must be noted that the algorithm only uses the DAD scan function of the ESI^+ of LC-MS system as it is based on the UV−vis spectral similarity; however, in the future, we do plan to combine the two systems. The manual identification of hits resulted in cross hits and new hits (**Table 2**), which can be explained as follows: Cross hits were metabolites that had the maximum similarity (based on the statistical similarity score) of their UV−vis spectra to reference metabolites. Thus, they were the same as reference metabolites but were found in the sample data files other than reference data files. New hits were the other known metabolites that had very similar UV−vis spectra to those of the reference metabolites and were found either in the same or other files (than where reference metabolites were identified) at different retention times in the sample data files. The algorithm X-hitting enabled fast-paced (less than 15 min) and systematic identification of compounds in crude pigment extracts.

Monascin Hits. No cross hits were seen for monascin in the *Penicillium* extracts. All of the three cross hits (CH1, CH2, and CH3 in **Table 2**) for monascin were found to be present in the *Monascus* pigment extracts (controls): two from *M. purpureus* species and one from *M. ruber* species (**Tables 1** and **2**). The retention times of the cross hits were found to be almost identical to that of the reference monascin. Among the three new hits (NH1, NH2, and NH3 in **Table 2**), NH1 was found to be present in the *Penicillium* extract, while NH2 and NH3 were seen in the *Monascus* pigment extracts (controls). NH1 was identified by LC-DAD-MS analysis as xanthomonasin A produced by *Penicillium aculeatum* IBT 14263 on YES medium (**Figures 1** and **4**). NH2 and NH3 were identified as ankaflavin (LC-UV-ESI^+-MS chromatogram not shown) produced by *M. purpureus* IBT 9664 on YES medium and IBT 9667 on PD. The retention time for ankaflavin was 15.35 (**Table 2**), indicating its relative apolar nature due to the presence of a longer aliphatic chain (C_7H_{15}) as compared to monascin (C_5H_{11}) (**Figure 1**). The retention time for xanthomonasin A was slightly higher (**Table 2**) than monascin, indicating a relatively similar degree of polarity. The spectral similarity of these compounds can be explained by looking at their structures (**Figure 1**). The fact that ankaflavin, monascin, and xanthomonasin A—the three yellow pigments of *Monascus*—have similar chromophores (**Figure 1**) differing only in the side chains or aliphatic groups results in very similar spectra; hence, similarity scores of these compounds were found to be close to each other (**Table 2**).

Rubropunctatin Hits. No cross hits were seen for rubropunctatin in the *Penicillium* extracts. Four cross hits (CH4, CH5, CH5, and CH7 in **Table 2**) for rubropunctatin were found to be present in the *Monascus* pigment extracts (controls); two

Table 2. Discovery of Known Metabolites Based on the Spectral Similarity to Reference Metabolites by Manual Search in the LC-DAD-MS Data Files

reference metabolites	retention time (min)	statistical similarity score (arbitrary with a maximum of 1.00)	cross hits (CH) or new hits (NH) or references (REF)/ retention time[a]	location of cross hits in terms of X-hit no. as per **Table 1**	identification[b]
monascin	13.45	0.995	REF/13.45	26	monascin
		0.978	CH1/13.46	32	monascin
		0.969	CH2/13.46	28	monascin
		0.946	CH3/13.45	31	monascin
		0.965	NH1/14.02	6[c]	xanthomonasin A
		0.951	NH2/15.33	31	ankaflavin
		0.973	NH3/15.35	32	ankaflavin
rubropunctatin	13.44	0.989	REF/13.44	23	rubropunctatin
		0.965	CH4/13.44	27	rubropunctatin
		0.958	CH5/13.43	24	rubropunctatin
		0.955	CH6/13.44	30	rubropunctatin
		0.923	CH7/13.44	33	rubropunctatin
		0.952	NH4/15.35	6[c]	monascorubrin
		0.936	NH5/15.35	9[c]	monascorubrin
		0.960	NH6/15.45	29	monascorubrin
		0.950	NH7/15.47	30	monascorubrin
		0.952	NH8/15.46	33	monascorubrin
rubropunctamine	9.42	0.975	REF/9.42	24	rubropunctamine
		0.960	CH8/9.47	27	rubropunctamine
		0.952	CH9/9.47	29	rubropunctamine
		0.943	NH9/8.48	5[c]	threonine derivative of rubropunctatin
		0.958	NH10/7.24	27	PP-V
		0.950	NH11/7.21	29	PP-V
		0.966	NH12/7.26	24	PP-V
citrinin	5.83	0.945	REF/5.83	35	citrinin
		0.938	CH10/5.92	25	citrinin
		0.937	CH11/5.92	29	citrinin
		0.929	CH12/5.77	30	citrinin
		0.934	CH13/5.90	32	citrinin

[a] Cross hits were where metabolites were the same as reference metabolites that had the maximum similarity (based on the statistical similarity score) of their UV−vis spectra to reference metabolites and were found in the sample data files other than reference data files. New hits were where the other known metabolites that had very similar UV−vis spectra to those of the reference metabolites and were found either in the same or other files (than where reference metabolites were identified) at different retention times. [b] Identified based on UV−vis and mass spectra as shown in **Figures 3A**,**B** and **5A**,**B**. [c] Indicate their presence in the *Penicillium* extracts.

each from *M. ruber* and *M. purpureus* species, quite interestingly in the same medium MEA with a retention time of 13.43 (**Tables 1** and **2**). Among the five new hits (NH4, NH5, NH6, NH7, and NH8 in **Table 2**), NH4 and NH5 were found to be present in the *Penicillium* extract, while NH6, NH7, and NH8 were seen in the *Monascus* pigment extracts (controls). All of the new hits were identified by LC-DAD-MS analyses as monascorubrin (**Figures 1** and **4**). NH4 and NH5 were produced by *Penicillium* spp.; NH4 was produced by *P. aculeatum* IBT 14263 on YES medium, and NH5 was produced by *P. pinophilum* IBT 13104 on YES medium (**Tables 1** and **2**). NH6, NH7, and NH8 were found to be produced by *Monascus* strains, out of which NH6 and NH7 were found to be produced by *M. purpureus* IBT 9664 on PD and MEA and NH8 by *M. purpureus* IBT 9667 on MEA (**Tables 1** and **2**). The retention time of monascorubrin ranged from 15.35 to 15.47 (**Table 2**). A slight difference in the retention time could be due to the day-to-day variation in the chromatographic analyses as they were performed on two different days using solvents of different batch and purity. A relatively higher retention time of monascorubrin than rubropunctatin can be explained in terms of its higher hydrophobicity because of the presence of longer aliphatic chains as in case of ankaflavin and monascin (**Figure 1**). The spectral similarity of the two compounds also lies in their similar chromophores just like in the case of ankaflavin and monascin as explained earlier.

Rubropunctamine Hits. No cross hits were seen for rubropunctamine in the *Penicillium* extracts. Two cross hits (CH8 and CH9 in **Table 2**) for rubropunctamine were found to be present in the *Monascus* pigment extracts (controls), one each from *M. ruber* and *M. purpureus* species, respectively, with a retention time of 9.47, indicating the more polar water-soluble nature of this pigment (**Tables 1** and **2**). Among the four new hits (NH9, NH10, NH11, and NH12 in **Table 2**), NH9 was identified in the *Penicillium* extract as a threonine derivative of rubropunctatin (**Figure 1**), and it was found to be produced by *P. aculeatum* IBT 14263 on CYA medium. NH10, NH11, and NH12 were identified in the *Monascus* pigment extracts (controls) by LC-DAD-MS analyses as PP-V, 3-(9a-methyl-3-octanoyl-2,9-dioxo-2,7,9,9a-tetrahydro-furo[3,2-g]isoquinolin-6-yl) acrylic acid (**Figures 1** and **4**), a homologue of monascorubramine. NH10 was identified in the pigment extract of *M. ruber* IBT 9655 on MEA, NH11 in the pigment extract of *M. purpureus* IBT 9664 on PD, and NH12 in the pigment extract of *M. ruber* IBT 7904 on MEA (**Tables 1** and **2**). PP-V, a water-soluble purple pigment, was previously reported to be produced by an unidentified species of *Penicillium* (*36*−*38*) but, to the best of our knowledge, never in *Monascus* spp. As PP-V is a homologue of monascorubramine, the spectral similarity of the two compounds is self-explanatory. In a study by Jung et al. (*12*), glycine derivatives of *Monascus* pigments with side chains of C_5H_{11} and C_7H_{15} were formed, and the structure was analyzed

by ^{1}H NMR and ^{13}C NMR. A careful examination of the structure has shown that it has a similar structure as PP-V. The molecular formula of the glycine derivative of *Monascus* pigment with side chain of C_5H_{11} was given as $C_{23}H_{25}NO_6$, which is the same as of PP-V (*36*). The structures of the other amino acid derivatives were thought to be similar to this derivative except for the amino acid moiety. This could very well explain the spectral similarity of threonine derivative of rubropunctatin to either PP-V or rubropunctatamine or monascorubramine, the amine form of rubropunctatin and monascorubrin, respectively. Sato et al. (*11*) also reported alanine and aspartate derivatives in commercial *Monascus* pigments, but this is the first ever report where an amino acid derivative of *Monascus* pigment was found in the pigment extract of *Penicillium* spp.

The earlier work (*39*) carried out on the chemistry of *Monascus* pigments suggests that the orange pigments viz. monascorubrin and rubropunctatin form the red pigments as the yellow pigments are unable to react with NH groups to produce the corresponding amines as seen from their structures (**Figure 1**). Our results indicated that the cross hit CH8 (rubropunctamine) and new hits NH9 (threonine derivative of rubropunctatin), NH11, and NH12 (PP-V) were found in the data files viz. X-hits 27, 5, 29, and 24, respectively (**Tables 1** and **2**). In these data files, except for the X-hit 5, either rubropunctatin or monascorubrin was also detected. This can be explained as the monascorubrin and/or rubropunctatin produced could react with either the free amino acids, as in case of MEA medium, or the amino acids of the mycelial pool in case of PD medium. As only high pH favors such reactions (*39*) and the drop in the pH of both the media at the time of pigment production, only part of monascorubrin or rubropunctatin could react to form substantially low amounts of amine forms such as rubropunctamine or PP-V or the threonine derivative of rubropunctatin (**Figures 1** and **4**).

Citrinin Hits. Four cross hits for citrinin (CH10, CH11, CH12, and CH13 in **Table 2**) were found to be present in the *Monascus* pigment extracts: CH10 from *M. ruber* IBT 9658 on YES medium, CH11, CH12, and CH13 from *M. purpureus* IBT 9664 on PD and MEA, and *M. purpureus* IBT 9667 on PD, respectively. The retention time of citrinin ranged from 5.77 to 5.92 (**Table 2**). The variation found is explained earlier. The citrinin negative controls of *Monascus* pigment extracts were shown as negatives by the algorithm, meaning that the similarity score was quite low for such hits. However, some false negatives were encountered. The reason could be the very low concentration of citrinin in these samples. It is likely that it was below the detectable range of DAD or that a very, very tiny peak was obtained such that its spectra went undetected by the algorithm or considered as noise. In such a case, they could only be detected manually by the mass and UV−vis spectra. Currently, we are working on such issues as this to make the algorithm more robust. Very interestingly, none of the pigment extracts from *Penicillium* cultures were found to contain citrinin by the algorithm. To be sure, a citrinin search was carried out manually in all of the *Penicillium* extracts, and all of them were found to be citrinin negative. This is quite significant as the production of citrinin, the major toxic metabolite of *Monascus*, prevents its food use by the authority both in the United States and the European Union.

New Hits and Their Sources. The fungus *P. aculeatum* IBT 14263 was found to produce two *Monascus* pigments viz. xanthomonasin A (yellow) and monascorubrin (orange) and one pigment derivative (purple-red). The fungus *Penicillium pinophilum* IBT 13104 was found to produce the orange *Monascus* pigment monascorubrin. It must also be mentioned that several unknown (not found in the databases used in the present study) metabolites in the yellow-orange-red spectrum were found in the extracts (data not shown) of other Penicillia (**Table 1**) used in this study. They are most likely to be *Monascus*-like polyketide-based azaphilone pigments, but it needs to be further investigated. *P. aculeatum* and *P. pinophilum* are closely related species; however, they are remotely related to the genus *Monascus*. The presence of polyketide-based *Monascus* pigments in these two fungi without the coproduction of citrinin forms an interesting avenue to be further explored. This is a significant discovery toward biotechnological production of naturally derived fungal food pigments as these are not known to produce any other known mycotoxins when grown in the media and under the laboratory conditions used in this study. Notably, the mycotoxins rubratoxin A and B have been reported to be produced by *P. rubrum* and *P. purpurogenum*, but this is not correct as these mycotoxins are produced by isolates of the species *P. crateriforme* (*40*). Fungus such as *Penicillium marneffei* belongs to the same group and produces copious amounts of red pigment, but it was deselected as it produces a known mycotoxin secalonic acid D and is also a well-known human pathogen as we have reported previously (*1*). This signifies the chemotaxonomic aspect of our screening methodology. We have also shown in a previous article that the color of some of these pigments including *Monascus* pigments provides additional hues in the red spectra, not covered by some of the commercially available colorants (*41*). PP-V was earlier reported to be produced by *Penicillium* spp. but never in *Monascus* spp. Our results have shown it to be produced by *M. ruber* IBT 7904 and 9655 and *M. purpureus* IBT 9664.

In conclusion, we have shown two newer promising sources of red to orange-red natural food colorants in the species of *Penicillium* that do not produce citrinin, with a possibility of finding several more from this group of fungi. These Penicillia are not reported to produce any other known mycotoxins. In addition, X-hitting was found to be good tool in the rapid screening of crude pigment extracts. Further necessary testing of algorithm is in progress to improve its application as a screening tool, and the pigments are being evaluated for their functionality.

ACKNOWLEDGMENT

Prof. Jens Christian Frisvad is deeply acknowledged for his expertise in chemotaxonomy, and Annette Salskov-Iversen and Hans van den Brink are thanked for critically reading the manuscript.

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Received for review June 13, 2008. Revised manuscript received August 28, 2008. Accepted September 4, 2008. We thank Chr Hansen A/S and the Centre for Advanced Food Studies (LMC)—Food Graduate School for financial support of the project.

JF801817Q