

## Biopigment Production by *Fusarium oxysporum* Strain SS2024 PP593456 Isolated from the Forest Region of Uttarakhand, India

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The present study focuses on the isolation and characterization of *Fusarium oxysporum* strain from the forest region of Uttarakhand, India, and explores its potential to produce pigments under submerged conditions. Fungal isolates were obtained from soil samples collected from diverse forest ecosystems and identified through morphological and molecular techniques. Natural pigment synthesis was initiated following the isolation and identification of *F. oxysporum* from the soil sample. Subsequent dilution and pure culture techniques were employed to select a specific strain producing red pigment for further study. Molecular identification using the ITS region confirmed the fungal strain as *Fusarium oxysporum*. The production of red coloured pigment was achieved through submerged fermentation utilizing rice broth as a growth medium. Subsequent pigment extraction using ethyl acetate demonstrated the potential of *F. oxysporum* to produce natural colorants. This research contributes to the exploration of fungal biodiversity in forest ecosystems and underscores the potential of *Fusarium oxysporum* as a promising source of fungal pigments for various industrial sectors.

**Keywords:** Bikaverin, Carotenoids, Fungi, Pigment, Sustainable

### Introduction

Dyes have always played a fundamental role in human existence, be it in daily life, cuisine, or clothing. Across cultures, people have used various pigments for centuries. Initially, colors were extracted from natural sources; however, with growing demand, humans started creating synthetic color shades.<sup>1</sup> Approximately 60,000 tons of waste, solely from synthetic dyes, are reported to be discharged into the environment, causing pollution.<sup>2</sup> The production of synthetic dyes involves raw materials such as benzene derivatives, naphthalene, benzidine, ammonia, surfactants, chelating agents, salts, and other compounds. These substances are subsequently responsible for causing cancer in living systems, inducing toxicity and altering the biological and chemical aspects of water bodies, which also challenge their removal and treatment processes.<sup>2,3</sup> Natural colouring agents have been derived from plants, insects, animals, and mineral sources since ancient times.<sup>4</sup> Therefore, recent studies have emphasized the development of environmentally friendly dye production approaches due to the

detrimental side effects of the majority of synthetic azo and benzidine dyes.<sup>5-7</sup> Natural pigments are typically classified according to their source of extraction. They can be extracted from chlorophylls found in higher plants, bryophytes, pteridophytes, and some photosynthetic microorganisms exhibiting chlorophyll a and chlorophyll b. The primary focus of such pigments lies in their application in the food industry as colorants. Carotenoids, derivatives of isoprenoids, are present in plants in the form of  $\alpha$ -carotene,  $\beta$ -carotene,  $\beta$ -cryptoxanthin, lutein, violaxanthin, and zeaxanthin. Carotenoids are abundant in algae, bacteria, and fungi and have garnered significant attention owing to their beneficial pharmacological effects, including fungistatic activities.<sup>8</sup>

Microorganisms produce a wide range of pigments in response to physiological and environmental stimuli, aiding in their adaptation and survival. Because microbial pigments originate from natural sources, they are of considerable interest. Nevertheless, concerns related to human health and the environment have gradually surfaced regarding synthetic pigments. As a result, individuals have begun focusing more on the use of natural colour, primarily derived from animals, plants or microorganisms.<sup>1,4,6</sup> Microbial pigments, also

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known as bio-pigments, offer an eco-friendly alternative to synthetic dyes. They can be produced in substantial quantities utilising biotechnological methods and pose minimal environmental concerns due to their rapid decomposition. Furthermore, many of these metabolites are acknowledged for their notable biological activities. These attributes underpin their potential application as food colorants and nutraceuticals designed to counteract oxidative stress-related diseases.<sup>9,10</sup> In recent years, the work on microbial pigments has grown significantly because of their eco-friendly nature, and at the same time, people are now more concerned about their health. These pigments find their uses in many different industries owing to their remarkable biological diversity. Also, studies have reported antimicrobial properties of pigments produced by microorganisms. Pigments produced from *Monascus* have been studied to show antibacterial properties against certain Gram-positive bacteria.<sup>11</sup> Fungal and bacterial pigments play significant roles in human health.<sup>3</sup> Many fungi grow rapidly, are relatively easy to cultivate, and can produce a variety of natural pigments. Because of their sustainability advantages, fungal pigments are being explored as potential alternatives to synthetic colourants.<sup>12</sup>

It is well established that fungi may produce a wide range of colors, encompassing metabolites from many classes, including carotenoids, quinones, naphthoquinone, azaphilones, hydroxyanthraquinones, anthraquinones, and melanin.<sup>9,10,12-16</sup> *Fusarium oxysporum*, a ubiquitous fungal species, has garnered significant attention for its potential in producing various secondary metabolites, including pigments with diverse industrial applications. *Fusarium oxysporum* has emerged as a key reference model in fungal carotenogenesis research.<sup>17,18</sup> *Fusarium oxysporum* produces the polyketidic pigments belonging to naphthoquinones and anthraquinones. These metabolites exhibit significant antimicrobial and insecticidal activities.<sup>19,20</sup> The genus *Fusarium* comprises a broad and intricate collection of plant-pathogenic fungi. With its members, *F. oxysporum* stands out as a primary species responsible for crop diseases.<sup>20,21</sup> Even though *Fusarium oxysporum* is primarily known as a plant pathogen, it also has significant biotechnological potential, particularly for biopigment production. The morphology of *F. oxysporum* colonies displays remarkable variability: certain isolates lack pigment production entirely, while others typically demonstrate a range of pigment production from pale purple to dark magenta when cultured on agar media. This variability suggests

promising genetic potential for pigment production.<sup>22-25</sup> Microorganisms are cultivated in a liquid nutrient media during submerged fermentation. Adequately high levels of dissolved oxygen are typically supplied to enhance microbial proliferation and product formation.<sup>26</sup> Research on *Fusarium oxysporum* has primarily focused on a few well-known metabolites. However, studies addressing the characterization and regulatory mechanisms governing the synthesis of other polyketidic pigments under submerged culture remain limited. Previous research on *Fusarium oxysporum* strains has largely concentrated on their major colorless secondary metabolites. Compounds such as fusaric acid and beauvericin have been the primary focus due to their notable biological activities.<sup>20,27-30</sup> In this study, a natural pigment was biosynthesised by a fungal isolate recovered from a soil sample collected in the forests of Uttarakhand. The isolated fungi were assessed for pigment production capabilities in submerged conditions. However, only one specific fungal culture was able to produce a red pigment. The primary objective of this research is to investigate the potential of *F. oxysporum* to produce natural pigments. These bio-pigments are envisioned as promising and sustainable alternatives to synthetic dyes, known for their toxicity and detrimental effects on the environment and living organisms.

## Materials and Methods

### Sample Collection

To isolate *Fusarium oxysporum*, soil samples were collected from the forest region of Uttarakhand, India, at a depth of 10 cm from the ground. The samples were carefully placed into air-tight sample containers and stored at 4°C for subsequent analysis.

### Isolating Pigment-Producing Fungi

A soil suspension was prepared by mixing 1 g of soil in 9 mL of distilled water. The resulting mixture was then subjected to a series of fivefold serial dilutions. An aliquot of 0.1 mL from each dilution was spread evenly onto Potato Dextrose Agar (PDA) plates. The plates were incubated at 28°C for a period of five days. For obtaining the pure culture, samples from the primary culture were quadrant streaked twice onto fresh PDA plates. In each streaking process, the culture was spread evenly across four different plates. The plates were subsequently incubated at 28°C for another 5 days.

### Identification of Isolates

The pigment-producing microbial strain was identified using the Internal Transcribed Spacer (ITS)

region-based molecular method. Genomic DNA was extracted from the fungal culture. The quality and integrity of the isolated DNA were verified using a 1.0% agarose gel. A fragment of the ITS region was amplified using the PCR technique. Subsequently, the amplified PCR product was purified to eliminate residual contaminants and reaction impurities. The PCR-amplified fragment was subjected to forward and reverse sequencing using ITS1 and ITS4 primers. The sequencing reactions were performed with the BDT v3.1 Cycle Sequencing Kit on an ABI 3730xl Genetic Analyzer. The forward and reverse sequencing reads were aligned using sequence alignment software. This alignment produced the consensus sequence of the PCR amplicon. The ITS region sequence was analysed using BLAST against the NCBI GenBank database to determine sequence similarity. Clustal W was used to align the ten sequences showing the highest identity scores. MEGA 10 was employed to develop the distance matrix and phylogenetic tree.<sup>31</sup>

#### Fungal Pigment Production

For pigment production, the procedure described by Mendonça *et al.* was adopted with some modification.<sup>32</sup> Pigment production was carried out utilizing rice broth, comprising 25 g of milled rice per 0.5 L of distilled water. Inoculum preparation involved homogenizing a 0.7 cm<sup>2</sup> fragment of PDA containing a 72-hour-old fungal culture in 100 mL of rice broth. For incubation, the flasks were kept at 28°C and 100 rpm in a rotary shaker for 5 days. Subsequently, 1 mL of the inoculum was introduced into 100 mL of fresh rice broth, after which incubation was carried out at 28°C and 100 rpm. After the incubation period, the fungal biomass was separated through centrifugation at 5000 rpm for 5 minutes.

#### Pigment Extraction

Extraction of pigments from microbial sources typically involves the exploitation of organic solvents. Pigment extraction was performed by mixing the wet biomass with the organic solvent in a 1:1 m/v ratio in an orbital shaker at 28°C and 100 rpm. From the obtained biomass, 50 mL was extracted, transferred to a new 250 mL sterile flask and mixed with an equal volume of ethyl acetate. This flask was then placed in a rotary incubator maintained at 28°C and 100 rpm for a duration of 3 hours. Following incubation, the organic solvent was separated by centrifugation at 3200 rpm for 5 minutes. This centrifugation step was repeated three times to ensure optimal extraction.

## Results and Discussion

#### Isolation of Pigment-Producing Fungi

Fungi require more carbohydrates for their growth compared to bacteria. The dehydrated potato infusion and dextrose present in potato dextrose agar promote optimal fungal growth. The isolated fungi were observed to produce red, green, orange and yellow pigment zones on PDA. The most noticeable colony amongst them was observed on the 10<sup>-2</sup> dilution plate, appearing red in color. To develop a pure culture, inoculum from the 10<sup>-2</sup> dilution was sub-cultured 3–4 times on PDA. The results of pigment-producing fungi isolated on PDA plates from soil samples are shown in Figs 1 & 2.

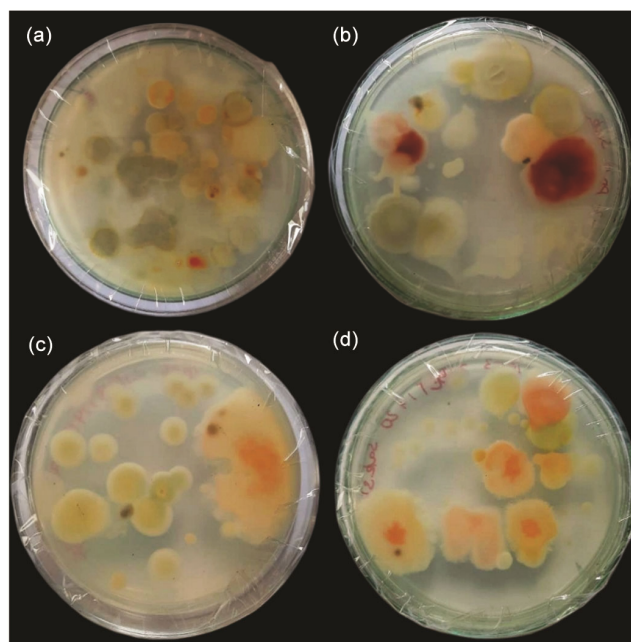


Fig. 1 — PDA plates with the isolated colonies: (a) green and yellow pigment producing fungi in 10<sup>-1</sup> dilution; (b) green, yellow and red pigment producing fungi in 10<sup>-2</sup> dilution; (c) and (d) showing green, orange, and yellow pigment producing fungi

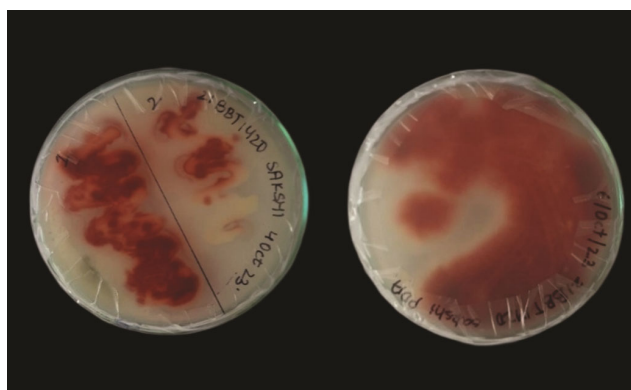


Fig. 2 — Red pigment production by the selected fungi on PDA

Table 1 — BLAST analysis performed using ITS region from NCBI GenBank database

Description	Max score	Total score	Query cover	E-value	Per. Ident	Accession
<i>Fusarium oxysporum</i> cloneSS-2	1009	1009	100%	0.0	100%	MK249867.1
<i>Fusarium oxysporum f.sp.albedinis</i> Isolate FOA34	1009	1009	100%	0.0	100%	OR399533.1
<i>Fusarium oxysporum f.sp.albedinis</i> Isolate FOA8	1009	1009	100%	0.0	100%	OR399526.1
<i>Fusarium oxysporum</i> strain FSD2	1009	1009	100%	0.0	100%	OM876904.1
<i>Fusarium oxysporum f.sp. melonis</i>	1009	1009	100%	0.0	100%	AY188919.1
<i>Fusarium sp.</i> voucher HQUPR06	1007	1007	99%	0.0	100%	MK640560.1
<i>Fusarium oxysporum f.sp.Ciceris</i>	1007	1007	99%	0.0	100%	MK074845.1
<i>Fusarium oxysporum</i> strain JFP9	1007	1007	99%	0.0	100%	MK849925.1
<i>Fusarium oxysporum</i> isolate Acheng18-4	1007	1007	99%	0.0	100%	MK764964.1
<i>Fusarium oxysporum</i> strain MF22472	1007	1007	99%	0.0	100%	MH911412.1

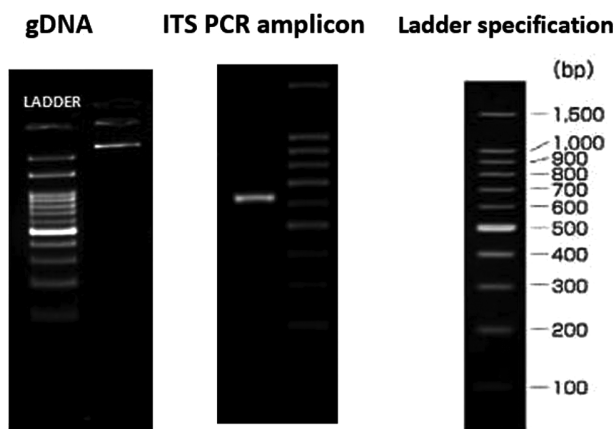
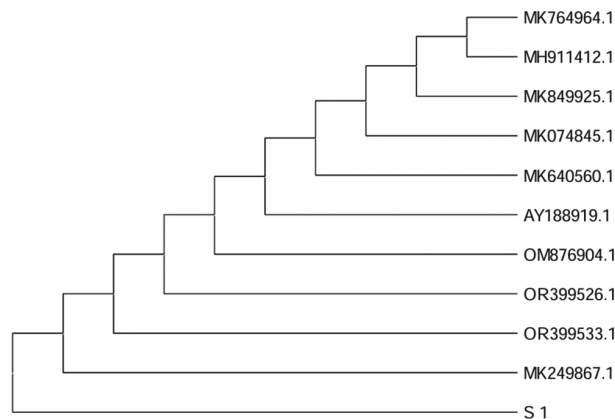


Fig. 3 — ITS PCR amplicon gel visualizing the ITS regions of the isolate

Fig. 1 — Maximum Likelihood-based phylogenetic analysis of *Fusarium* strains

#### Identification of the Red Pigment-Producing Fungi

The microbial strain was identified using a molecular method based on the ITS region. The extracted genomic DNA was checked for quality using a 1.0% agarose gel. A distinct single band indicated the presence of high-molecular-weight DNA. The ITS region fragment was then amplified using PCR. Agarose gel electrophoresis revealed a single, distinct amplicon band of ~600 bp

(Fig. 3). Following gel electrophoresis, purification of the PCR amplicon was conducted to eliminate any impurities. Using the ITS1 and ITS4 primers, forward and reverse sequencing of the PCR amplicon were performed with the BDT v3.1 Cycle Sequencing Kit on an ABI 3730xl Genetic Analyzer. The forward and reverse sequencing data were aligned with the aligner software to obtain the consensus sequence of the PCR amplicon. The ITS region sequence was analyzed using the BLAST tool against the NCBI GenBank database. The results identified the pigment-producing fungal isolate as *Fusarium oxysporum* strain SS2024 (GenBank Accession No. PP593456). This species demonstrated substantial similarity through nucleotide homology and phylogenetic examination. BLAST analysis results have been summarised in Table 1, listing the first ten sequences that showed the greatest identity values. Clustal W, a software program for multiple sequence alignment, was employed to align these sequences. Following this, a distance matrix and phylogenetic tree were generated by employing the MEGA 10 software. The phylogenetic tree representing the first 10 sequences is illustrated in Fig. 4.

#### Pigment Production

Rice broth, abundant in carbohydrates, serves as an optimal growth medium for fungi. The primary objective of employing rice broth for pigment production was to utilize a potential waste product. The broth was inoculated with a 48-hour-old PDA segment, measuring 0.7 cm<sup>2</sup>, containing the fungus culture. The inoculated broth was kept in a rotary incubator at 100 rpm and 28°C for 5 days. A noticeable color change in the medium was observed, indicating pigment production in the rice broth following 5 days of incubation.

#### Pigment Extraction

Pigment extraction from the broth was conducted using ethyl acetate as the solvent after identifying the

microbial strain that produced the red pigment. Following the incubation of an equal ratio of fermented biomass and solvent, the pigment was separated from the medium as it dissolved in the solvent. The extraction from the solvent was accomplished through centrifugation at 3200 rpm for 5 minutes. The centrifugation process resulted in the formation of two distinct layers. The upper layer, which contained the pigment, was collected into a fresh flask and stored for subsequent use (Fig. 5).

### Discussion

It has been documented that *Fusarium oxysporum* can produce pigments in both solid and submerged cultures. Various *Fusarium* species, including *F. oxysporum*, *F. solani* BRM054066, *F. verticillioides*, and *F. graminearum*, isolated from soil, are documented to generate purple, red, and green pigments.<sup>18,33,34</sup> In our study, the isolated fungi were observed to produce orange, green, red, and yellow pigments, with the red pigment zones being particularly noticeable and considered for further analysis. dos Santos *et al.* conducted a study on the production of the red pigment bikaverin from *F. oxysporum* CCT7620 in rice medium, where a production of 320.5 mg/L was observed under incubation conditions of 28°C for 96 hours at 250 rpm.<sup>35</sup> Nagia & Mohamedy isolated *F. oxysporum* from the roots of citrus trees infected with root rot diseases.<sup>36</sup> The isolated fungi were reported to

produce dark red anthraquinone pigments in liquid culture under dark conditions. Mendonça *et al.* investigated the synthesis of bikaverin pigment by *F. oxysporum* CCT7620. The fungi were inoculated in rice broth, resulting in the observation of red biomass after 96 hours of incubation. The extraction of produced pigments was conducted using various polar solvents, including petroleum ether, n-hexane, diethyl ether, ethanol, butanol, and methanol.<sup>32</sup> Pigment biosynthesis in *Fusarium oxysporum* is governed primarily by physiological and environmental parameters such as light, temperature, pH, and carbon source availability rather than geographical location. Under optimized culture conditions, the organism can produce the red carotenoid-type pigment in a wide range of environments. The mechanism behind the production of red pigment in *Fusarium* spp. involves the synthesis of carotenoids, which are derived from geranylgeranyl pyrophosphate (GGPP). This synthesis is regulated by the expression of four genes: *carRA*, *carB*, *carT*, and *carD*. The transcriptional activation of these genes is induced by light. Also, a little amount of  $\beta$ -carotene is also produced and subsequently cleaved by the action of *carX* oxygenase to produce retinal.<sup>17,37,38</sup> The synthesis of bikaverin is however different from that of carotenoids. Bikaverin belongs to the class of polyketides and is distinguished by a benzoxanthone structure. According to Limón *et al.* the synthesis of this compound is initiated by a class I multifunctional polyketide synthase. Subsequent modifications are carried out by monooxygenase and O-methyltransferase enzymes.<sup>39</sup> Additionally, *F. oxysporum* has been studied for its capability to produce purple naphthoquinone pigments through submerged fermentation.<sup>18</sup> These studies align with our research, where *F. oxysporum* was isolated from soil and inoculated in rice broth to produce a red pigment, which was then extracted using ethyl acetate as a polar solvent. This correlation with prior studies suggests that the pigment produced could be either bikaverin or carotenoids, as *F. oxysporum* is known to produce these red color pigments.

Commercial red and reddish-yellow colorants in food and industry are currently obtained from a mix of synthetic dyes (commonly azo and related structures) and natural sources such as plant-derived carotenoids/anthocyanins (e.g., annatto, beet extracts) and animal-derived dyes (cochineal/carmine). Synthetic dyes are generally inexpensive and widely used, but concerns about environmental persistence,

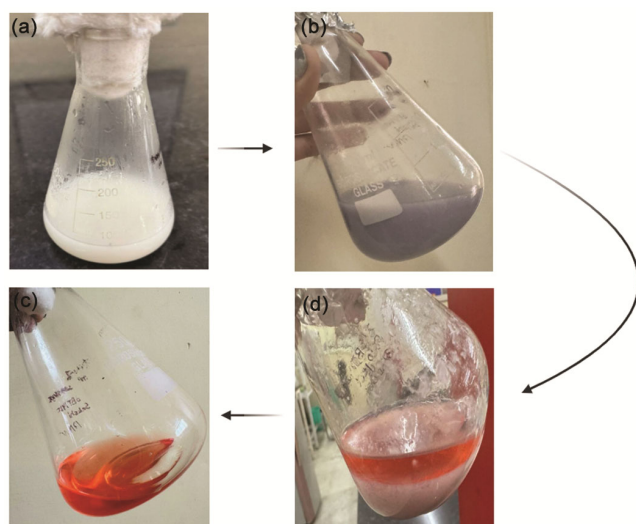


Fig. 5 — Production and extraction of fungal pigment: (a) inoculation of fungi in rice broth medium, (b) change in colour after 5 days of incubation, (c) extraction of pigment by ethyl acetate as a solvent, (d) extracted pigment

wastewater toxicity, and potential human-health effects have driven demand for natural alternatives. Natural plant- and insect-derived colorants are renewable but can suffer from supply variability, seasonal constraints, limited stability in some formulations, or cultural/religious acceptability issues (e.g., cochineal). Microbial pigments including fungal polyketides such as bikaverin and carotenoids from fungi, offer several potential advantages: production under controlled fermentation (independent of season), scalability, the possibility to use inexpensive agro-industrial residues as feedstock, and relatively rapid process cycles. However, challenges remain (pigment stability in final formulations, downstream recovery and purification costs, and regulatory approval for use in foods or cosmetics). To position the pigment from *F. oxysporum* reported in the current study as a competitive alternative, future work should target yield improvement, pigment stability testing in application-specific matrices, and rigorous toxicological profiling.

Although *Fusarium oxysporum* includes plant-pathogenic strains, the isolate employed in this study was handled under controlled laboratory conditions using standard biosafety procedures. Prior to disposal, cultures and residues were autoclaved to prevent any potential release. For future industrial applications, pigment production can be safely achieved through in vitro fermentation systems or closed bioreactors, minimising environmental risk. The use of non-pathogenic, well-characterised strains is recommended to ensure biosafety compliance and prevent adverse effects on agricultural ecosystems. The in vitro (fermentation-based) approach to pigment production by *F. oxysporum* offers several economic advantages compared with plant or insect sources. It allows year-round production, controlled quality, and the use of inexpensive agro-residues as carbon sources.

A practical assessment of commercial feasibility must consider fermentation titer, cost of feedstock, downstream extraction and purification, and regulatory costs. Reported bikaverin production in rice-based cultures by *Fusarium* spp. has been in the range of a few hundred mg per litre (for instance, ~320.5 mg/L under shake-flask conditions in rice medium), which demonstrates proof-of-concept but remains below typical titers required for low-cost industrial colorants. Downstream extraction often using organic solvents, can be a substantial portion of production cost. Studies focusing on recovery and

purification strategies for bikaverin emphasize process development to improve yield and reduce solvent use. To improve economics, the following approaches are promising and should be considered in future work: (i) use of low-cost agro-waste or byproduct substrates (e.g., rice bran, other cereal residues) to lower raw-material costs; (ii) strain improvement and fermentation optimization (bioreactor control, fed-batch/continuous modes) to increase titers toward g·L<sup>-1</sup> levels; (iii) development of greener and more selective downstream processes (e.g., solvent recycling, adsorption/resin-based capture, or alternative extraction methods) to reduce purification costs; and (iv) valorization of co-products or integration into a biorefinery concept to distribute fixed costs. With these measures, fungal pigments could become economically attractive for high-value applications (cosmetics, speciality foods and nutraceuticals).

## Conclusions

The findings of this study align with previous research on *F. oxysporum*, which has shown its capability to produce various pigments, including the red pigment bikaverin and carotenoids. The environmental benefits of using microbial pigments, especially from fungi like *F. oxysporum* lie in their eco-friendly nature, large-scale producibility through biotechnological methods, and suitability for applications in food coloring and nutraceuticals. Therefore, this study contributes to the growing body of research on eco-friendly pigment alternatives, highlighting the potential of *F. oxysporum* as a sustainable source of natural pigments. The results promote further exploration of microbial pigments for their diverse applications in industries, leading towards a greener and healthier alternative to synthetic dyes.

## Conflicts of interest

None

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