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Original Research Article

Characterization of Pigments Produced by *Cercospora personata*

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Abstract: The current study aimed to investigate different pigments obtained from the isolates of *Cercospora personata.* Pigments were developed in the solvent system, which were pale yellow, orange red and deep red fractions. The Rf values of pigments were compared with that of their respective authentic samples. Results from chromatography, spectroscopy, and colour reactions suggested that the pigments produced by *Cercospora Personata* were identified as aveufin, dothistromin, cercosporin.

Keywords: *Cercospora personata*, chromatography, spectroscopy, colour reactions and pigments.

INTRODUCTION

Pigments can be produced naturally or synthesized chemically and they have a chromophorecoloring group. These pigments either directly produces color by light absorption in the visible area wavelength range or transfer energy to color intensifiers called auxochromes [1, 2]. Synthetic colorants pose a risk on human health by contributing to the emergence of mental disease, allergies, and a variety of malignancies [3]. As a result, there is an increasing need for eco-friendly, non-toxic colorants, particularly for applications where human health is at risk, such as coloring of food and the dying of children's clothes and leather clothing [4]. Natural pigments that are derived from a variety of biological sources, including plants, animals, insects, and microorganisms, offer a viable alternative source for coloring [5-8]. Microorganisms have many advantages for pigment production over plants and animals since their cells are much smaller and grow at a faster rate in inexpensive culture medium [9]. In addition, microorganisms have high productivity, and can yield a product throughout the year [10]. Moreover, Various microorganisms, such as algae, bacteria, fungi, and protozoa, produce natural pigments, such as carotenoids, flavins, melanin, quinines, monascin, phycocyanin, or indigo [11]. Fungi can produce the widest range of soluble colors on a variety of substrates and environmental circumstances. These pigments are produced by fungi as secondary metabolites due to nutritional scarcity [12] or to help in improving fungal survival [13]. Many fungal species of the genus Aspergillus and Penicillium have a high potentiality to produce natural pigments [13-17]. The selection of a suitable strain, the fermentation process, as well as the choice of appropriate substrates or media, all play a role in increasing the productivity of fungal pigments [11].

Majority of fungal pigments are used economically as food, feed, and medicine colorants or as important nutrients. The pigment production by microorganisms may enhance the pharmaceutical, food, and feed industial biotechnology. The rapid development lead to the demand of colors in biotechnology related to food, textile, medicine and cosmetics. Microorganisms have potential to produce variety of metabolites which show biological and pharmacological activities. Microbial pigments show great antioxidant, anticancer and antimicrobial activities. Amongst microbes, fungi have great potential to release such metabolites which carry biological and pharmacological activities [16].

MATERIALS AND METHODS

Cercospora Personata was isolated from naturally infected groundnut leaves on PDA medium and maintained on czapek dox agar medium throughout the investigations. The entire objective is based on pigments produced by *Cercospora Personata.* For this purpose ethyl acetate extracts of culture filterates was subjected to dry column of silicagel and TLC and developed in benzene, Ethyl acetate, Formic acid(50:50:1 (V/V).

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Microorganisms

Microorganisms used in the present study are culture of *Cercospora personata* from naturally infected lesions on groundnut leaves of Anantapur district. The cultures were maintained on PDA slants. Prolonged incubations resulted in the production of two variant strains CP3OV and CP3BV of the parent isolate CP3.

Methods used to identify the pigments produced by *cercospora personata.*

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1. Chemical reagents used in identifying the toxic metabolites in spray tests / colour reactions:

A. Test for sugars and carbohydrates

a. Aniline-diphenylamine – phosphoric acid (Stahl, 1969):

- Four gram diphenylamine, 4 ml aniline and 20 ml 85% phosphoric acid were dissolved in 200 ml acetone.
- Chromatograms were sprayed with the above and heated for 10 min at 85^0C carbohydrates turned brown.

b. Aniline pthalate (Stahl, 1969):

- 0.93 g -aniline and 1.66 g O-pthalic acid were dissolved in 100 ml n-butanol saturated with water.
- The reagent was sprayed on the chromatograms which were heated for 10 min at 105^0C. Carbohydrates turned brown.

c. Anthrone:

- 0.2 g -anthrone was dissolved in 100 ml concentrated sulphuric acid.
- 10 ml of the above solution was added to 5 ml of the test solutions carefully along the sides of the tube kept in an ice-bath and the mixture was stirred with a glass rod.
- Then the tube was kept in a boiling water bath for 7.5 min, cooled immediately in an ice-bath and the colour developed was observed.
- A blue colour indicated the presence of carbohydrates.

d. Phenol-sulphuric acid (Wilson, 1959):

- To one ml aqueous solution of test compound 0.5 ml of 80% phenol was added followed by rapid addition of 5 ml concentrated sulphuric acid using a fast delivery pipette.
- After 30 min the optical density was determined at 490 mm. Absorption indicated the presence of carbohydrates.

e. Nelson's reagent (Nelson, 1944):

Reagents:

i) Copper reagent 'A':

In 800 ml of GD water, 25 g of anhydrous Na2Co3, 20 g of sodium potassium tartrate (Rochelle salt), 20 g of NaHCO3 and 20 g of anhydrous Na2SO4 were dissolved and diluted to 1,000 ml with GD water.

ii) Copper reagent 'B': To 100 ml of GD water, 15 g of CuSO4 and 1 or 2 drops of concentrated H2SO4 were added.

iii) Arseno molybdate colour reagent:

To 450 ml of GD water, 25 g of ammonium molybdate, 21 ml of concentrated, H_2SO_4 and 3 g of $Na_2HAsO4.\ 7H_2o$ dissolved in 25 ml of GD water were added and the mixture was kept at 37^0C for 48 h. The reagent was stored in a glass-stoppered brown bottle.

Procedure:

- To 1 ml of the test solution, 1 ml of mixture of reagent 'A' and 'B', prepared by mixing 25 parts of reagent 'A' with 1 part of reagent 'B' was added.
- The mixture was heated for 20 min on boiling water both, cooled in running tap and 1 ml of arsenomolybdate reagent was added.
- The solution was mixed thoroughly and the colour developed was observed. A blue colour indicated the presence of reducing sugars.

B. Tests for phenols and phenolic derivatives

a**. Sulphanilic acid, diazotized (see Stahl, 1969):**

- 4.5 g of sulphanilic acid was dissolved in 45 ml 12 N Hcl by warming and the solution was diluted to 500 ml with water.
- 10 ml of this diluted solution was cooled in ice and 10 ml of cold 4.5% aqueous sodium nitrite solution added.
- The resulting reagent is maintained 15 min and an equal volume of 10% aqueous sodium carbonate was added just before spray.

b. Folin-phenol reagent (see Stahl, 1969):

Chromatograms were sprayed with 20% aqueous sodium carbonate and then with N Folin-ciocaltcan reagent at 1:3 dilution with water.

c. o-nitroaniline, diazotized (see Stahl, 1969):

- **2% p-nitroaniline in 2 N Hcl**
- **10% sodium nitrate**
- **10% sodium acetate**

The above solutions were mixed in the ratio of 1.5 : 1.5 : 5 just before spraying. The plates were again sprayed with 10% NaOH.

C, Tests for amino acids, amino groups and peptides:

a. Biuret reaction (Dittarbrant, 1948):

Procedure:

- To 0.1 ml of test solution, 1.5 ml biuret reagent was added. After mixing, the solution was allowed to stand for 30 min at 37^0C, then read at 555 nm.
- Purple colour and absorption indicated the presence of amino groups.

b. Copper salt micro-method (Spies and Chamber, 1957):

1. 0.05 M copper chloride
2. Sodium borate buffer (pH, 9.1 – 9.2)

40.3 g sodium barate was dissolved in 4 litre of water and filtered. 6 g sodium chloride was dissolved in 100 ml of the barate solution (This NaCl containing solution will be referred to as the buffer solution).

Procedure:

- To 5 ml of buffer solution in a centrifuge tube was added 5 ml of the test solution. 0.1 ml of copper chloride was then added to this mixture, shaken well and let stand for 10 min at room temperature.
- The suspension was centrifuged and the colour solution was decanted and its absorption determined at 230 nm. Transmittance indicates the presence of amino acids or peptides which had formed a complex with copper salt.
- Blank solution was prepared as above using water in place of test solution.

c. Ninhydrin reagent:

- 0.2 g of ninhydrin in 100 ml ethanol formed the ethanolic ninhydrin reagent.
- Plates were sprayed and heated at 1100C for 15 minutes. Aminos and amino groups were indicated by purple-violet colour.

D. Reagents for other groups (see Stahl, 1969)

a. Formaldehyde-sulphuric acid reagent (for polynuclear aromatic compounds):

0.2 ml of 37% formalhyde solution was dissolved in 10 ml of sulphuric acid.

b. Magnesium acetate: (for anthraquinone glycosides and their aglucones):

0.5% methanolic solution of magnesium acetate was sprayed and the plates were heated at 90^0C for 5 min.

c. Potassium permanganate: (for easily oxidized sutstances):

0.05% aqueous potassium permanganate served as the spray reagent.

d. Sulphuric acid: (for carbon compounds):

Sulphuric acid (40%) was sprayed and the plates were heated for 10 min at 150^0C to char the carbon compounds.

e. Folin-phenol reagent for amino groups (Lowry et al., 1951):

Reagents:

Solution a: 6 g sodium hydroxide was dissolved in 25 ml of GD water.3 g sodium carbonate was then added to the solution and dissolved completely.

Solution b: 100 mg sodium potassium tartarate (Rochelle salt) were dissolved completely in 10 ml GD water, followed by addition of 50 mg of cupric sulphate.

Solution c: 50 ml of a and 1 ml of b.

Procedure:

- To 1 ml of the sample were added 5 ml of alkaline copper reagent (solution c) and allowed to stand for 10 min. 0.5 ml of diluted folin-ciocaltcan reagent (reagent: water 1:2) was then added with a blow pipette and mixed thoroughly.
- The tubes were allowed to stand for 30 min for colour development.
- Absorbance / transmittance of the blue colour developed was measured at 500 nm in Bausch and Lomb spectronic 20.

f. Analysis of hydrolysate:

- Monosaccharide components of the toxin released by acid hydrolysis were separated by thin-layer chromatography following the method of Talukdar (1971).
- 200 µl samples of the hydrolysates were spotted on the TLC plates and developed in the following three solvent systems successfully.

1) Ethyl acetate: methanol: butanol: water
(16: 3: 3: 2 v/v)
2) Ethyl acetate: methanol: butanol: propanol: water
(8: 1: 1: 1 v/v)

3) Same as (1).

Chromatograms were sprayed with aniline phthalate and heated at 105^0C for 10 min. Amino acids were resolved two dimensionally on TLC plates as described by Brennur and Neiderwieser (1967).

200 µl of the sample were spotted at a point 2 cm away from either sides and developed in one direction in n-Butanol; acetic acid: water (4: 1:1 v/v). The solvent was evaporated completely and the same chromatogram developed in the second solvent phenol: water (3:1 v/v) in a direction perpendicular to the first run. The phenol free chromatograms were visualized by spraying with ninhydrin reagent.

E. Estimation of amino acid content (Moore and Stein, 1954)

- 25 ml of 4N sodium acetate buffer (pH 5.5) MCTC added to the above solution.
- The reddish reagent was stored under N2 in a dark bottle. To 1.0 ml of test solution, 1.0 ml of ninhydrin solution was added.
- The test solution was brought to a pH between 4-6 with not more than 1 ml of 0.1 N HCL or 0.1 N NaOH.
- After shaking for 10 sec the mixture was heated for 15 min exactly in a covered boiling water bath.
- Aqueous ethanol (1:1) was used as a dilutant and the solution cooled to 30^0C. After shaking for a further 30 sec. in oxidize residual hydrindantin, the absorbance was read at 570 nm.
- The amino acid contents of the samples were determined with the help of a standard curve prepared for glycine.

Preparation of pigment solutions:

- As the pigments were insoluble in water a stock solution of 10 mg in 10 ml ethanol was prepared and started at -10^0C.
- Whenever necessary, a known volume of this concentrated solution was withdrawn and diluted with 0.5 % ethanol to give the required concentration.
- Care was taken to maintain the level of ethanol below 1.0% which was found safe for physiological studies.

Spectral analysis:

- The sprayed chromatograms were viewed under ultraviolet light for absorption.
- Absorption spectra were recorded using appropriate blanks.
- Rooting of detached leaf

Ethylene determination:

- Ethylene production was measured according to the procedure of Ketring and Morgan (1969).
- Each sample for ethylene determination consisted of four leaflets from one tetrafoliate leaf and was replicated thrice.
- The leaflets were separated from petioles and placed on moist filter paper (Whatman No.5) in 100 ml flasks.
- The flasks remained open for about 2 h to allow for wound ethylene production.
- They were flushed with air, sealed and sampled for ethylene, 4 h later. A flask without leaflets was used to determine ethylene production.

Animal Cell culture Media RPMI 1640:

- RPMI 1640 medium was prepared by using re-hydrating RPMI 1640 powder (Sigma USA) in double distilled water and supplemented with 27.3 ml. of 7.5% w/v Sodium bicarbonate solution, 10 units/ml and 100 µg/µl penstrep (pencillin streptomycin) (Sigma, USA).
- The volume was made upto 1 litre and p^H was adjusted to 7.2, with Carbon dioxide (CO_2).
- Medium was sterilized by filtration through 0.45 µm Millipore membrane filters and tested for sterility before use.

(a). Lactophenol mounting fluid (Cappuccino and Sherman, 1996).

Phenol crystals	20 g
Lactic acid	20 ml
Glycerol	40 ml
Distilled water	20 ml
1% cotton blue	5 ml

Lactic acid, glycerol and water were mixed, added to warmed phenol crystals. Cotton blue was added after cooling. This stain was used to prepare permanent slides of fungal cultures.

(b). 0.1 M Phosphate Buffer Saline (PBS)

Sodium chloride (Nacl)	8 g
Potassium Chloride (Kcl)	0.2 g
Di Sodium Hydrogen Phosphate (Na_2HPO_4)	1.15 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g.

The above salts were dissolved in small quantity of distilled water and volume was made upto 1000 ml. PH was adjusted to 7.2 and autoclaved.

(C). Trypan blue solution

400 mg. of Trypan blue was dissolvled in 100 ml PBS buffer and was stored in a brown bottle at room temperature.

(d). Reagents for Gram's staining:

RESULTS AND DISCUSSION

Table 1: Variability of isolates of *Cercospora personata* to produce pigments

Isolate	Pigment
CP_3	Dothistromin Averufin
CP_3OV	Dothistromin Averufin
CP_3BV	Dothistromin Averufin
CP_4	Cercosporin Dothistromin Averufin

Comparison of CP3 pigment with cercosporin by Chemical tests:

- The CP_3 pigment and cercosporin were subjected to chemical tests. Reduction with zinc dust in glacial acetic acid CP_3 pigment became purple and cercosporin gave yellowish green colour.
- With the addition of 0.1 N NaOH, CP_3 pigment gave purple and cercosporin readily dissolved in 0.1 N NaOH yielding clear green colour.
- Cercosporin gave red colour with ferric chloride whereas CP_3 pigment gave reddish brown.
- Addition of concentrated sulfuric acid to CP_3 pigment gave violet colour and cercosporin gave purple colour (Table 2).

Table 2: **Comparison of reactions of CP_3 pigment and cercosporin with chemical reagents**

Test	Reaction with CP_3	Reaction with CP_4 pigment (Cercosporin)
Reduction with zinc dust in glacial acetic acid	Purple	Yellowish green
Addition of 0.1 N NaOH	Purple	Green
Addition of methanolic ferric chloride	Reddish brown	Red
Addition of conc. sulfuric acid	Violet	Purple

Ultraviolet spectra of dothistromin:

- Almost pure dothistromin had max 524, 509, 490, 480, 280 and 233 mm (Fig. 11) and was similar to those reported for anthraquinones with three -hydroxy groups but was different from those with four hydroxyl groups (Thomson, 1971).
- This strongly supports the 1, 4, 5 – tri-hydroxy anthraquinone structure for dothistromin.

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- This strongly supports the 1, 4, 5 – tri-hydroxy anthraquinone structure for dothistromin.

Cercospora personata which causes leaf-spots in groundnut produced more than one phytotoxic metabolite in culture. These are isolated and identified as averaging, Dothistromin, Cercosporin. The factor in the culture filtrate of the fungus having the ability to stimulate respiration in groundnut leaf tissues was found to be thermostable, dialyzable, highly water-soluble and non-ionic and identified as a glycopeptide of molecular weight ca 4483. It was designated as 'personatin'. Production of personatin in culture was associated with the active growth phase of the fungus. Personatin could not be detected in infected leaves. It is suggested that it was produced in rather low concentration in vivo. Cercosporin was not detected in the field infected leaves. However the presence of dothistromin in extracts of naturally infected plants was demonstrated.

To conclude, the chemical procedures what were applied here are helpful in the characterization of individual toxins, obtained from the culture filtrate of *Cercospora personata.* Further investigations are required to understand the immunological and toxic effects of mycotoxins.

CONCLUSION

The current study revealed the potentiality of *Cercospora personata* for natural pigment production on liquid medium. More research is required to identify different fungi and analyze the characteristics of pigment in order to apply in various industries. From the previous results and owing to the different benefits of fungal pigments. We recommend the use of this safe and natural pigment in different industries.

REFERENCES

1. Sajid, S., & Akbar, N. (2018). Applications of fungal pigments in Biotechnology. Pure and Applied Biology, 7, 922-930.
2. Khan, A. A., Alshabi, A. M., Alqahtani, Y. S., Alqahtani, A. M., Bennur, R. S., Shaikh, I. A., ... & Maqbul, M. S. (2021). Extraction and identification of fungal pigment from Penicillium europium using different spectral studies. *Journal of King Saud University-Science*, *33*(4), 101437.
3. Santos-Ebinuma, V. C., Roberto, I. C., Simas Teixeira, M. F., & Pessoa Jr, A. (2013). Improving of red colorants production by a new Penicillium purpurogenum strain in submerged culture and the effect of different parameters in their stability. *Biotechnology progress*, *29*(3), 778-785.
4. Kalra, R., Conlan, X. A., & Goel, M. (2020). Fungi as a potential source of pigments: harnessing filamentous fungi. *Frontiers in Chemistry*, *8*, 369.
5. Vendruscolo, F., Bühler, R. M. M., de Carvalho, J. C., de Oliveira, D., Moritz, D. E., Schmidell, W., & Ninow, J. L. (2016). Monascus: a reality on the production and application of microbial pigments. *Applied Biochemistry and Biotechnology*, *178*, 211-223.
6. Młodzińska, E. (2009). Survey of plant pigments: molecular and environmental determinants of plant colors. *Acta Biologica Cracovienca Series Botanica*, *51*(1), 7-16.
7. McGraw, K. J. (2005). The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants?. *Animal Behaviour*, *69*(4), 757-764.

8. Sugumaran, M., & Barek, H. (2016). Critical analysis of the melanogenic pathway in insects and higher animals. *International journal of molecular sciences*, *17*(10), 1753.
9. Lebeau, J., Venkatachalam, M., Fouillaud, M., Petit, T., Vinale, F., Dufossé, L., & Caro, Y. (2017). Production and new extraction method of polyketide red pigments produced by ascomycetous fungi from terrestrial and marine habitats. *Journal of Fungi*, *3*(3), 34.
10. Sarvamangala, D., & Aparna, S. S. V. (2016). Microbial pigments–a short review. *J Env Sci Toxicol Food Technol*, *10*(8), 1-7.
11. Suwannarach, N., Kumla, J., Watanabe, B., Matsui, K., & Lumyong, S. (2019). Characterization of melanin and optimal conditions for pigment production by an endophytic fungus, Spissiomyces endophytica SDBR-CMU319. *PLoS One*, *14*(9), e0222187.
12. Valenzuela-Gloria, M. S., Balagurusamy, N., Chávez-González, M. L., Aguilar, O., Hernández-Almanza, A., & Aguilar, C. N. (2021). Molecular characterization of fungal pigments. *Journal of Fungi*, *7*(5), 326.
13. Gmoser, R., Ferreira, J. A., Lennartsson, P. R., & Taherzadeh, M. J. (2017). Filamentous ascomycetes fungi as a source of natural pigments. *Fungal biology and biotechnology*, *4*, 1-25.
14. Lagashetti, A. C., Dufossé, L., Singh, S. K., & Singh, P. N. (2019). Fungal pigments and their prospects in different industries. *Microorganisms*, *7*(12), 604.
15. Mapari, S. A., Thrane, U., & Meyer, A. S. (2010). Fungal polyketide azaphilone pigments as future natural food colorants?. *Trends in biotechnology*, *28*(6), 300-307.
16. Venil, C. K., Velmurugan, P., Dufossé, L., Renuka Devi, P., & Veera Ravi, A. (2020). Fungal pigments: Potential coloring compounds for wide ranging applications in textile dyeing. *Journal of fungi*, *6*(2), 68.
17. Sastrawidana, I. D. K., Maryam, S. Y., & Sukarta, I. N. (2016). Natural dyeing of silk and cotton fabric with red pigment from Penicillium purpurogenus which is isolated from goat milk contaminated soil. *J Nat Sci Res*, *6*, 32-37.