Biochemical Profiling of Extracellular Pigment Metabolites of *Aspergillus ustus* Strain KUMBASBT-52

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Abstract

Filamentous fungi are the mesmerizing glory of an extensive variety of pigments. Compared to other microorganisms, they produce pigments of different shades as their secondary metabolites. The study intensified on the cultivation of Aspergillus ustus strain KUMBASBT-52 under submerged fermentation and extraction of extracellular pigment metabolites. The extracted pigment metabolites were analyzed via different analytical techniques. The yield of urochrome-yellow pigment extracted from A. ustus was 2.39 g/L, which is water-soluble in nature. The UV-Vis analysis exhibited absorption peaks at 300 nm and 340 nm. The FT-IR analysis transmits the incidence of different functional groups i.e. 3282cm^{-1} (Hydroxy group; H-bonded OH stretch), 2921cm^{-1} (Methylene; C-H stretch), 2111cm^{-1} (C≡C Terminal alkyne), 1629 cm^{-1} (Secondary amine; >N-H bend), 1390cm^{-1} (Phenol or tertiary alcohol; OH bend), 1233cm^{-1} (Aromatic phosphates; P-O-C stretch), 1016 cm^{-1} (Aliphatic phosphates; P-O-C stretch), 512cm^{-1} (Polysulfides; S-S stretch) and 425cm^{-1} (Aryl disulfides; S-S stretch).

OHR-LC/MS analysis discloses the incidence of 15 bioactive endogenous metabolites, including yellow pigment metabolites 3-(2-methylpropyl)-octahydropyrrolo[1,2-a] pyrazine-1,4-dione and 5-Methoxysterigmatocystin. The A. ustus strain KUMBASBT-52 produces urochrome yellow pigments as their endogenous metabolites, which are more stable than other natural pigments and can be used as a colorant to counter the carcinogenic synthetic azo dyes.

Keywords: *Aspergillus ustus*, Secondary metabolites, Myco-pigment, UV-Vis analysis, FTIR, OHR-LC/MS.

Introduction

Colors are an essential component of our life. The majority of living things on ecosystem exhibit distinct pigmentations as a result of the absorption and refraction of particular light wavelengths. Every pigment has conjugated molecules, called chromophores, which facilitate energy exchanges inside and between cells and enable electronic resonances. It has been demonstrated that the energy absorbed and/or reflected by pigments is engaged in a variety of biological activities including camouflage, mating and pollinator attraction and the use of solar energy for metabolic demands and photo-protection for the maintenance of life[11].

Microbe-derived pigments are superior to that of plant- or animal-derived pigments in several of ways such as reduced environmental impact, viability, profitability and ease of handling before, during and after processing[3]. Among microorganisms of interest, fungi are unique in that they can generate a wide range of soluble pigments on a variety of substrates and circumstances[2]. The fungal pigments are secondary metabolites, which have incredibly varied structures, have been found to exhibit a wide range of bio-efficacies including radical scavenging, immunosuppressive, cholesterol-lowering, anti-malignant, anti-obesity and anti-atherosclerotic and many more biological applications[11]. In the pharmaceutical sector, fungal pigments are a desirable pharmacophore due to their collective advantageous bioactivities for humans.

Many dazzlingly colored pigments, such as anthraquinones, carotenoids, flavins, dihydroxy naphthalenes, indigo pigments, monascins, naphthoquinones, phenazines, melanins and violaceins, are secreted by microorganisms that are isolated from the part of various ecological niches such as forest soil, marine origin, endophytic fungi from terrestrial and marine biome and endo-lichenic fungus etc. Superior coloring capabilities are exhibited by fungal pigments. The desire for natural, organic and environmentally friendly pigments has increased in the modern era due to the many drawbacks of synthetic pigments including their poor degradation, prolonged persistence and potential to induce allergies or cancer[18].

Filamentous fungi are the fascinating source of wide variety of pigments compared to other microorganisms, they produce pigments of different shades as their metabolites. For the biosynthesis of fungal pigments, mainly four pathways are responsible which comprise of polyketide synthetic pathway, shikimate pathway, nitrogen-containing metabolite pathway and terpenoid synthetic pathway. Aromatic amino acids are the precursor of various fungal secondary metabolites such as pigments and vitamins.

Fungal pigments such as melanin, quinones, flavins, ankaflavin and azaphilones have therapeutic applications such as antimicrobial, radical scavenging, anticancer properties[6]. Pigments derived from filamentous fungus are gaining special industrial interest due to their ease of cultivation in the lab and relatively lean downstream procedures that are easily scalable at pilot or plant scales[8].

Fungal pigments are not only eco-friendly but also have many therapeutic applications. *Aspergillus ustus* is a saprophytic fungus generally associated with the humified soil. It produces various extracellular metabolites such as autocystins, austalides, sterigmatocystins, versicolourins and nidulol that possess antibacterial and other biological applications[14].

This study elaborates the cultivation of *Aspergillus ustus* strain KUMBASBT-52 under submerged fermentation and extraction of extracellular pigment metabolites. The extracted pigment metabolites were analyzed via different analytical methods.

Material and Methods

Mass-cultivation of *Aspergillus ustus* strain KUMBASBT-52: The growth medium is required for cultivation of the fungus, *A. ustus* strain KUMBASBT-52, by submerged fermentation (SmF) i.e. potato dextrose broth (PDB) medium supplemented with sucrose (2%) as a carbon source, sodium nitrate (1%) as a nitrogen source, potassium phosphate (0.05%) as a mineral salt source, tyrosine (0.5%) as an amino acid source and pH 7. The starter culture was prepared by inoculating the growth medium with the 7-day-old mycelium of the fungus and incubating at 25 ± 2 ℃ for 15 days. Then the starter culture (1:10 v/v) was transferred into the growth medium and incubated at 25 ± 2 ℃ as a stationary culture for 21–25 days for mass cultivation.

Extraction of extracellular pigment metabolites: The fungus *A. ustus* strain KUMBASBT-52 was harvested by separating the fully grown fungal biomass from the culture broth; the full-fledged broth culture was filtered through Whatmann no. 1 filter paper. The fungal spores in the filtrate were removed by centrifugation at 3000 rpm for 15 minutes and the spore-free, pigmented supernatant was collected. For the pigmented supernatant, 95% methanol was added in a ratio of 1:1 (V/V) and agitated on a rotary shaker for 30 minutes at 150 rpm, then centrifuged at 5000 rpm for 15-20 minutes. The separated pigment phase was retrieved and dried in a lyophilizer to obtain the powdered pigment metabolites. The dried pigment was used for further analysis[20].

Ultraviolet-visible (UV-Vis) spectroscopy analysis: A UV-VIS spectrophotometer (Model: PerkinElmer®-Lambda 950) was used to perform a UV-visible absorption spectral analysis of the extracellular pigment extract. The wavelength of the methanol dissolved pigment extract was measured in the range of 200–800 nm with a 2mm optical path-length and resolution of 0.1 nm)[20].

Fourier transform infrared spectroscopy (FTIR) analysis: FTIR analysis was performed on a PerkinElmer® FT-IR spectrophotometer to detect the functional groups of extracellular pigment metabolites, A 10 mg quantity of pigment sample was ground with 100 mg of potassium bromide and then pressed to form pellets. FT-IR spectra of pigment extract was analyzed in the frequency range of 4000−500 cm^{-1} to observe the spectral bands[10].

Orbitrap high-resolution liquid chromatography and mass spectroscopy (OHR-LC/MS) analysis: The extracellular pigment metabolites were analyzed through a Thermo Fisher Scientific (High resolution Orbitrap Liquid chromatogram equipped with Q extractive plus Mass spectrometer and data acquisition software system Thermo Scientific Xcalibur, Version 4.2.28.14). It has a Hypersil GOLD C18 column of dimension 100×2.1mm-3µ and dual AJS ESI (electro spray ionization) source. The instrument combines the analytical separation of HPLC and powerful detection technique of mass spectrometry and can provide high performance chromatographic separation of compounds with an m/z ratio ranging from 50 to 8000 amu, resolution 280000, scan speed of 12 Hz and mas accuracy less than 1 ppm. The instrument was operated in a stop time mode for 30 minutes, with a gradient elution flow of 0.3 ml/minute and a pressure of 1200 bar. 0.1% formic acid and methanol were used as solvents and 5 µl of sample was injected.

The mass spectra of the compounds were obtained with a scan rate of 1.00 and m/z ratio ranging from 100-1200. The obtained data was processed using the Compound Discoverer 3.2 software system. The obtained data was analyzed based on mzCloud advanced mass spectral database[12].

Results and Discussion

Mass cultivation and extraction of extracellular pigment: Submerged fermentation implies the process of growing microorganisms in broth media for the development and recovery of industrially important commercial products[4]. It accomplishes the voluminous advantages over solid-state fermentation; it is cost effective, produces high yield end products and is preferred for the easy recovery of multifaceted extracellular metabolites[1]. The fungus *A. ustus* strain KUMBASBT-52 (Fig. 1) was mass cultivated in a 2L Erlenmeyer flask containing 1L of growth medium (Fig. 2) and the fungus was harvested on the 25th day. Extracellular urochrome-yellow pigment was extracted with methanol. The dried pigment extract yields 2.39 g/L, which is water-soluble in nature.

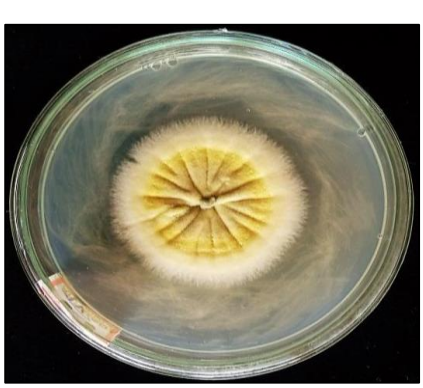

Fig. 1: *A. ustus* strain KUMBASBT-52

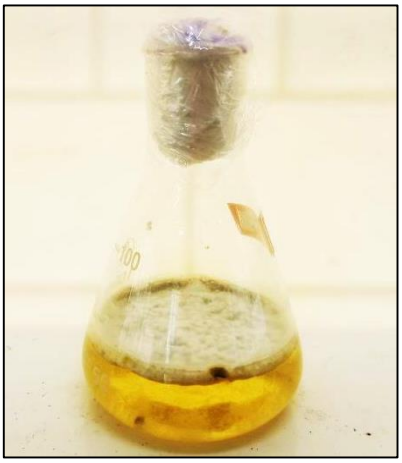

Fig. 2: Cultivation of *A. ustus* in SmF

UV-Visible analysis of extracellular pigment metabolites: UV-visible analysis is an analytical technique used to determine the spectral range of a substance. Nutritional requirements of fungus play a key role in secretion of metabolites. A different fungus produces diverse pigments which have different range of absorption spectrum when analyzed through UV-visible spectrophotometer. It is due to specificity of the metabolites produced by the individual fungus. In the present study, the extracted *A. ustus* strain KUMBASBT-52 pigment metabolites showed absorption peaks at 300nm and 340nm in UV-visible spectroscopy (Fig. 3).

In earlier findings, Zhou et al[20] reported that *A. ustus* (DBFL05) showed absorption peaks at 262nm and 298nm. Khan et al[7] reported that *Penicillium europium* pigment exhibited absorption at 343nm. *Gonatophragmium triuniae* pigment extracted with hexane showed the maximum absorption at 220nm[9]. Toma et al[17] reported the brown pigment of *Aspergillus niger* exhibits the absorption peak at 295nm.

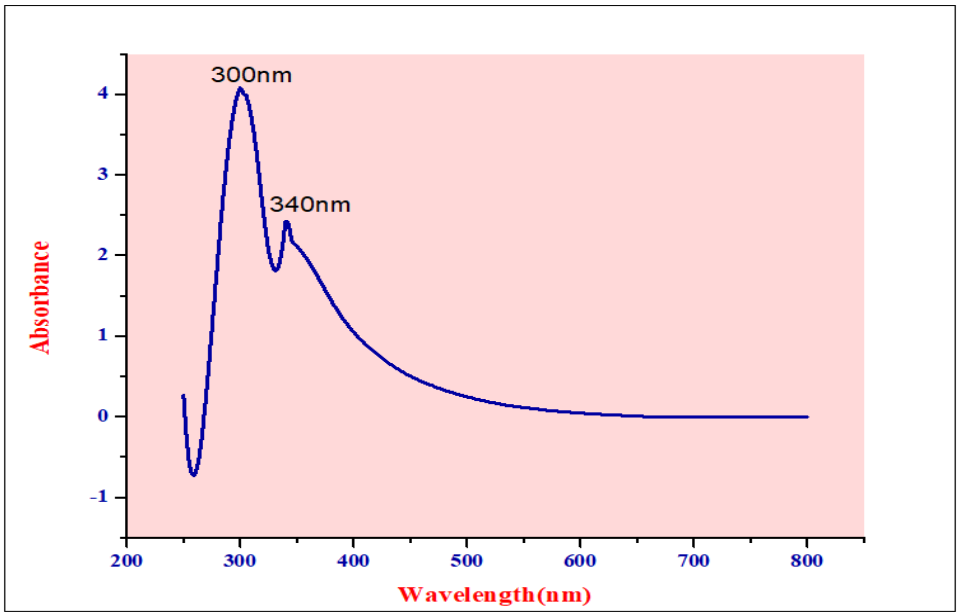

Fig. 3: UV-Visible analysis of *A. ustus* strain KUMBASBT-52 extracellular pigment

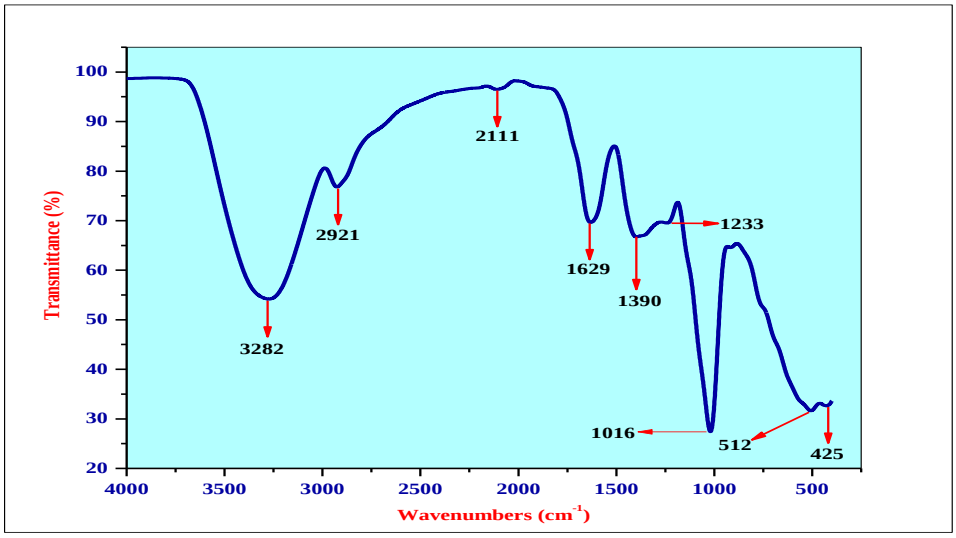

Fig. 4: FT-IR analysis of *A. ustus* strain KUMBASBT-52 pigment metabolite extract

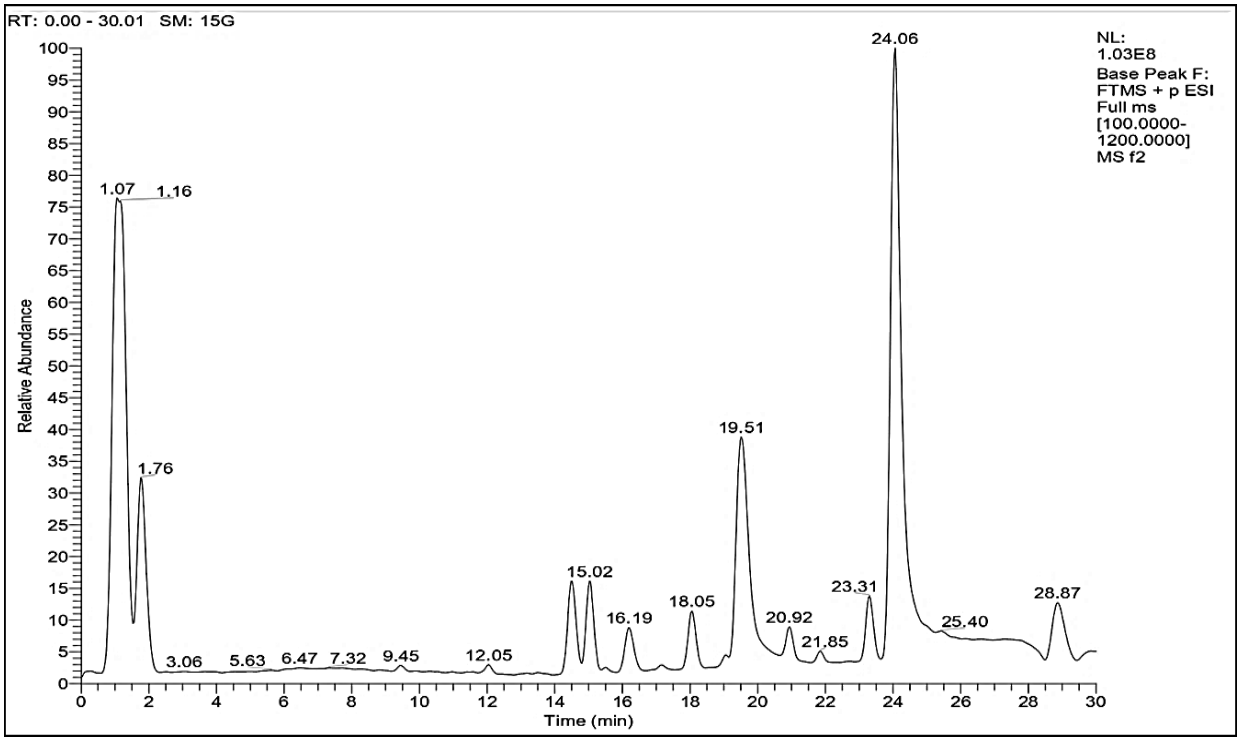

Fig. 5: Orbitrap HR-LC/MS analysis of *A. ustus* strain KUMBASBT-52 pigment metabolite extract

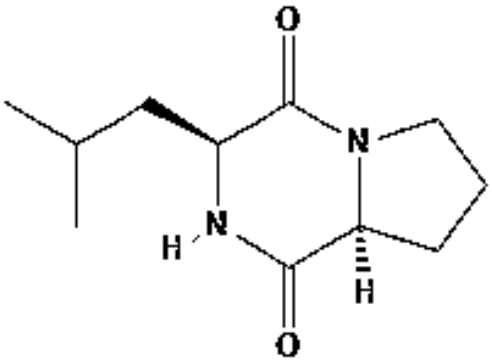

Fig. 6: Structure of 3-(2-methylpropyl) octahydropyrrolo [1,2-a] pyrazine-1,4-dione

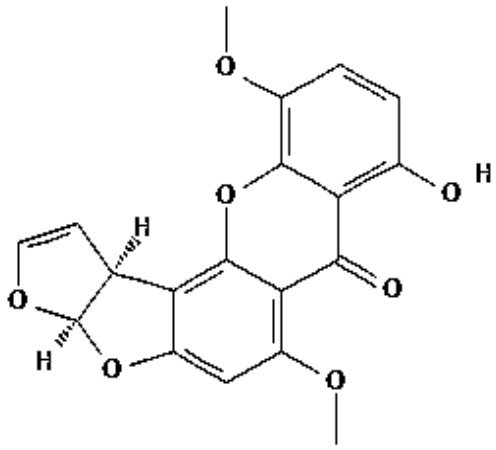

Fig. 7: Methoxysterigmatocystin

FT-IR analysis of extracellular pigment metabolites: FT-IR analysis was accomplished to find the frequency of the functional group of the bonding molecules in the extracted fungal pigment. The distinguished IR absorption spectral bands of *A. ustus* strain KUMBASBT-52 pigment metabolite extract (Fig. 4) comprise of 3282cm^{-1} (Hydroxy group; H-bonded OH stretch), 2921cm^{-1} (Methylene; C-H stretch), 2111cm^{-1} (C≡C Terminal alkyne), 1629 cm^{-1} (Secondary amine; >N-H bend), 1390cm^{-1} (Phenol or tertiary alcohol; OH bend), 1233cm^{-1} (Aromatic phosphates; P-O-C stretch), 1016 cm^{-1} (Aliphatic phosphates; P-O-C stretch), 512cm^{-1} (Polysulfides; S-S stretch) and 425cm^{-1} (Aryl disulfides; S-S stretch), comparatively exemplified in the preceding investigations[7,13,19].

Orbitrap HR-LC/MS analysis of extracellular pigment metabolites: Orbitrap high-resolution liquid chromatography and mass spectroscopy analysis are highly sensitive techniques that detect a wide array of compounds. OHR-LC/MS analysis discloses the presence of 15 metabolite compounds in the pigment extract of *A. ustus strain* KUMBASBT-52. The generated chromatogram is shown in fig. 5 and the compounds in pigment metabolites are listed in table 1. The majority of the detected compounds are pharmacologically important endogenous metabolites that have antimicrobial, antioxidant and anticancer properties. 3-(2-methylpropyl)-octahydropyrrolo[1,2-a] pyrazine-1,4-dione (Fig. 6) and 5-Methoxysterigmatocystin (Fig. 7) are the yellow pigment compounds synthesized by *A. ustus* strain KUMBASBT-52.

Ser et al[15] reported that pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro from *Streptomyces mangrovisoli* is a yellow-colored compound with antioxidant potential.

Table 1
List of compounds in the pigment metabolite extract of *Aspergillus ustus* strain KUMBASBT-52 detected by OHR-LC/MS

S.N.	RT [min]	Compound Name	Molecular Formula	MW (g/mol)
1.	1.07	4-Aminophenol	$C_6 H_7 N O$	109.052
2.	1.16	DL-Malic acid	$C_4 H_6 O_5$	134.021
3.	1.76	5-Hydroxymethyl-2-furaldehyde	$C_6 H_6 O_3$	126.031
4.	5.63	2,3,4,9-Tetrahydro-1H-β-carboline-3-carboxylic acid	$C_{12} H_{12} N_2 O_2$	216.089
5.	9.45	3-(2-methylpropyl)-octahydropyrrolo[1,2-a] pyrazine-1,4-dione	$C_{11} H_{18} N_2 O_2$	210.136
6.	12.05	Azelaic acid	$C_9 H_{16} O_4$	188.104
7.	15.02	Diethyl phthalate	$C_{12} H_{14} O_4$	222.089
8.	16.19	Irbesartan	$C_{25} H_{28} N_6 O$	428.232
9.	18.05	Dibutyl maleate	$C_{12} H_{20} O_4$	228.136
10.	19.51	7-Hydroxycoumarine	$C_9 H_6 O_3$	162.031
11.	20.92	Hydroxyprogesterone caproate	$C_{27} H_{40} O_4$	428.292
12.	21.85	Diisobutylphthalate	$C_{16} H_{22} O_4$	278.151
13.	23.31	5-Methoxysterigmatocystin	$C_{19} H_{14} O_7$	354.381
14.	24.06	NP-019636	$C_9 H_8 O_4$	180.042
15.	28.87	Erucamide	$C_{22} H_{43} N O$	337.333

Cai et al[2] reported that sterigmatocystin derivatives, oxisterigmatocystin A, oxisterigmatocystin B, oxisterigmatocystin C and 5-methoxysterigmatocystin, were isolated from *Aspergillus versicolor*. Dufossé[5] reported that carotenoids, melanin, monascus, riboflavin pigments and other pigments of microbial origin can inhibit various pathogens.

Conclusion

The azodyes are chemically synthesized compounds that are widely used as colorants, but they have an undesirable effect on human health as well as on the ecosystem. Due to the carcinogenicity and non-eco-friendly nature of azodyes, researchers have gained more interest in natural colorants. The filamentous fungi are a fascinating source for the natural colorant with its bioactive metabolites. The output of the study indicates that the fungus *Aspergillus ustus* strain KUMBASBT-52 synthesizes two urochrome-yellow bio-pigments, i.e. 3-(2-methylpropyl)-octahydropyrrolo [1, 2-a] pyrazine-1,4-dione and 5-Methoxysterigmatocystin, including other bioactive endogenous compounds as its extracellular metabolite.

This research consequence is useful for the pharmaceutical, textile and paint industries that are searching for bio-degradable, eco-friendly and feasible non-toxic natural colorants from fungal origin to counter the carcinogenic and hazardous synthetic colorants.

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