Morpho-molecular Characterization and Optimization of *Aspergillus ustus* Strain KUMBASBT-52 isolated from the Litter Soil

Akarsh S., Sowmya H.V. and Thippeswamy B.*
Department of Post Graduate Studies and Research in Microbiology, Bioscience Complex Kuvempu University, Jnanasahyadri, Shankaraghatta-577 451 Shivamogga (Dist.), Karnataka, INDIA
*thippeswamyb272@yahoo.in; thippeswamyb205@gmail.com

Abstract
Western Ghats is the biodiversity hotspot of the world, with its rich flora and fauna. The soil of the forest provides a niche for various microorganisms. Due to the carcinogenicity and non-eco-friendly nature of azodyes, researchers have put a spotlight on natural colorants. Filamentous fungi are a fascinating source of a wide variety of pigments. The present study focused on the isolation and characterization of a pigment-producing fungus and optimized the physico-chemical conditions for its growth and pigment biosynthesis. The fungus was isolated from the forest litter soil collected nearby the location called Sigandooru (14°4'34"N 74°52'20"E). The isolated fungus produces extracellular urochrome-yellow pigment. Based on phenotypic and genotypic characters, the fungus was identified as Aspergillus ustus strain KUMBASBT-52 and its 18s r-RNA gene sequence was deposited at GenBank, NCBI (Accession No. MW130234).

The physico-chemical parameters required for the A. ustus to yield maximum biomass and pigment were recorded as temperature 25 °C, pH 7, carbon source sucrose (2% W/V), nitrogen source sodium nitrate (1% W/V), mineral salt potassium phosphate (0.05% W/V) and amino acid tyrosine (0.5% W/V). The A. ustus strain KUMBASBT-52 can be exploited for bio-pigment production, which can be used as a substitute for hazardous synthetic azo dyes.

Keywords: *Aspergillus ustus*, Molecular characterization, Bio-pigment, Optimization, Physico-chemical parameters, Western Ghats.

Introduction

The Western Ghats is a region of great worldwide importance for the conservation of biological diversity and also feature zones with exceptional standards of geography, ethnicity and aesthetics. This is because they constitute a niche of extraordinary biodiversity and endemism. The chain of mountains that cross the States of Gujarat, Maharashtra, Goa, Karnataka, Kerala and Tamil Nadu, spans an area of 164,280 km² and corresponds to India's west coast.

This Western Ghats mountain range was renowned as a World Heritage site by UNESCO in 2012 because it is well known for being one of the eight "hottest hotspots" of biological diversity in the world[4].

Soil is one of the foremost abiotic factors that sustain life on our planet earth. Soil is the natural medium for the growth of several kinds of saprophytic, parasitic, mutualistic and antagonistic microbes[5]. The litter, which is the topmost humidified organic layer of the soil, plays a vital part in forest ecosystems where microbial diversity is abundant[13]. Western Ghats forest soils are packed with micro and macronutrients due to their biodiversity and soil-associated microorganisms play a key role in nutrient cycling. Saprophytic fungi decompose woody debris and leaf litter in forest ecosystems by producing extracellular enzymes such as ligninase, cellulase, hemicellulose and pectinase, which are capable of breaking down recalcitrant substances[2,14].

Filamentous fungi have been known for their biologically active secondary metabolites including antimicrobials, antioxidants, antitumors etc. Soil from the Western Ghats can be a source of industrially important fungi[16]. Filamentous fungi are a fascinating source of natural pigments, which are an alternative to the hazardous effects of synthetic colorants. Compared to plants and other microbes, filamentous fungi produce a variety of pigments in different shades that are highly stable and water-soluble in nature. Melanins, carotenoids, azaphilones, indigo, quinones, phenazines, quinones, monascin and violacein are some of the pigments that fungi produce in a prolific manner[15].

Fungal pigments are not only eco-friendly but also have many therapeutic applications. *Aspergillus ustus* is a saprobic fungus generally associated with the topsoil. It produces various extracellular metabolites such as autocystins, austalides, sterigmatocystins, versicolourins and nidulol that possess antibacterial and other biological applications[21]. This research study embraces the morphological and molecular characterization of the fungus, optimization of physico-chemical parameters for growth and extracellular pigment metabolite production in submerged fermentation by *Aspergillus ustus* strain KUMBASBT-52, isolated from the litter soil of Western Ghats.

Material and Methods

Collection of litter soil sample: The humified topmost soil (litter) sample in the forest region of Western Ghats was collected aseptically in the sterile container. The sampling

site geographical coordinates was mentioned at the location and the collected litter soil sample was transported into the research laboratory in aseptic condition and further utilized for mycological investigations.

Isolation and screening of pigment producing fungus: The mycoflora of the forest litter soil was isolated by adopting the serial dilution plating method[24]. For isolation, 0.1 mL of inoculum from 10^{-3}, 10^{-4} and 10^{-5} dilutions were inoculated on Potato dextrose agar (PDA) and Czapek Dox agar (CZA) media, amended with 30 mg/L of chloramphenicol to evade bacterial contamination. The plates were incubated at room temperature for 5-7 days. After incubation, plates were visualized for the growth of different fungi[3]. Fungal isolates displaying intense, attractive, bright colors on the agar plates were selected and further transferred to fresh PDA medium for the confirmation of pigment production. The purified colonies of pigment-producing fungal strains were sub-cultured on PDA slants and stored at 4°C in the refrigerator for further studies. For screening of extracellular pigment production by fungus under submerged fermentation, the 5mm of fungal mycelial disc was incorporated into 50 mL of potato dextrose broth medium in 100 mL of Erlenmeyer flask and incubated at room temperature for 14–21 days in static condition. After incubation, the culture broth was filtered and visualized for extracellular pigment production[9,19].

Identification of pigment producing fungal strain KUMBASBT-52: Primary identification of pigment-producing fungus was done based on culture morphological features such as color, texture and shape on PDA plates and confirmed by observing via stereo binocular microscope and microscopic characterization was done by the lactophenol cotton blue (LPCB) stain mounting technique by spotting spore shape, spore size, spore chain arrangement and hyphae arrangement in 40X and 100X magnifications. The fungus was identified by referring to the morphological characters in a manual of the aspergilli[22], practical mycology[12], introductory mycology[1], compendium of soil fungi[10], illustrated genera of imperfect fungi[6] and handbook of soil fungi[19].

Molecular characterization of pigment producing fungal strain KUMBASBT-52: The fungus was grown in PDB under static conditions for seven days at 28 °C. Using the cetyl trimethyl-ammonium bromide (CTAB) technique, genomic DNA was extracted from the freeze-dried fungal mycelium (Ausubel et al., 1999). Using the universal primers ITS-1 (5'-TCCGTAGGTGAACATGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'), the internal transcribed spacer (ITS) region of r-DNA was amplified. The amplicons obtained were gel-purified and sequenced by Sanger's sequencing method. The sequences were assembled using FinchTV software1 and homologies were determined using BLASTn searches in the NCBI GenBank database with nearly allied matches. MEGA (Molecular Evolutionary Genetics Analysis) software (version 7.0) was used to construct the sequence alignments. Sequence alignment was attained using the ClustalW programme. The phylogenetic tree was developed in RAxML (Randomized Axelerated Maximum Likelihood) using the maximum likelihood method to study the evolutionary relationships of the isolated fungus. The phylogeny was analyzed using FigTree software (version v1.4.4) with a p-distance substitution model and 100 bootstrapping replicates[17].

Optimization of physicochemical factors influences growth and pigment synthesis: The optimization of physicochemical factors, including culture media, incubation duration, temperature, pH, carbon, nitrogen, mineral salts and amino acid sources desirable for maximum growth and pigment production by the fungus, was executed by submerged fermentation in triplicate[20] and results are interpreted in mean ± SD (standard deviation).

Optimization of different culture media and incubation duration: 50 mL of six distinct broths, including Potato dextrose broth (PDB), Sabouraud dextrose broth (SDB), Malt extract broth (MEB), Czapek Dox broth (CDB), Carrot broth (CB) and Richard's synthetic broth (RSB) were prepared in 100 mL Erlenmeyer flasks and autoclaved. 5mm of dynamically emerging 7-day-old fungal mycelial discs were plucked by the sterilized cork borer and transferred aseptically into the flasks containing distinct sterile broth media and quickly agitated for a few seconds and incubated at room temperature in static conditions. The synthesis of pigment in distinct media was examined visually and biomass was measured by collecting mycelium after the filtration of fungal broth through Whatman No. 1 filter paper. Mycelium was washed and dried at 55°C until complete dryness or until a consistent weight was attained. The weight of dried mycelium was recorded at intervals of 7, 14, 21 and 28 days and biomass was determined by using the formula:

$$\text{Biomass } (gL^{-1}) = \frac{\text{Weight of dried biomass on the filter paper} - \text{Weight of empty filter paper}}{\text{Volume of culture broth in litre.}}$$

Effect of different temperature and pH on growth and pigment synthesis: All the optimization tests were carried out in PDB. For assessment of the optimum temperature condition required for growth and pigment production, a 5mm fungal disc was inoculated in 50 mL of broth medium and incubated at different temperatures (5, 15, 25 and 35°C). Adjacently, for optimum pH determination, the pH of the medium was adjusted to pH 2, 3, 5, 7, 9 and 11 and the broth was inoculated with a 5 mm fungal disc and incubated at 25 ± 2°C under static conditions. After incubation, pigment synthesis was measured by a spectrophotometer at a wavelength of 480nm and biomass was determined.

Effect of different carbon and nitrogen source on growth and pigment synthesis: The influence of distinct carbon supplements (2% W/V), including glucose, galactose,

fructose, lactose and sucrose and nitrogen supplements (1% W/V) including ammonium chloride, sodium nitrate and sodium nitrite, peptone and yeast extract, on growth and pigment production were determined. 5mm disc of freshly grown pigment producing fungal culture was inoculated into the 50 mL broth medium supplemented with different carbon and nitrogen sources and incubated at optimum temperature in static conditions. Pigment synthesis and biomass were recorded.

Effect of different mineral salt and amino acid on growth and pigment synthesis: A volume of 50 mL of broth was supplemented with 0.05% W/V of different mineral salts including copper sulphate ($CuSO_4$), ferric sulphate $Fe_2(SO_4)_3$, magnesium sulphate ($MgSO_4$), potassium dihydrogen phosphate (KH_2PO_4) and zinc sulphate ($ZnSO_4$) and 0.5% W/V of amino acids such as asparagine (Asn), cysteine (Cys) histidine (His), phenylalanine (Phe) and tyrosine (Tys). A 5-mm, 7-day-old, well-grown fungal disc was inoculated in the mineral salts and amino acids amended medium and incubated at optimum temperature in static conditions. Pigment synthesis and biomass were recorded.

Results and Discussion

Collection of litter soil sample: The forest litter soil sample was collected nearby the location called Sigandooru (14°4′34″N 74°52′20″E), which lies in the Western Ghats region of Sagara taluk of Shivamogga district, Karnataka in India. The sampling spot is enriched by dense flora and fauna. The location of sample collected site is shown in fig. 1.

Isolation and screening of pigment synthesizing fungus: There is a synergistic relationship between the soil and fungi. Soil is the natural medium and comprises of micro and macronutrients that facilitate the growth of different kinds of fungi. The fungus was isolated in a 10^{-4} dilution on the PDA medium and persists as a grayish-tinge mycelium with the urochrome-yellow color pigment production. The isolated fungus was purified by sub-culturing on fresh PDA medium (Fig. 2). The fungus exhibits intensive urochrome-yellow extracellular pigment synthesis in PDB medium (Fig. 3). In the present study, *A. ustus* strain KUMBASBT-52 was isolated from the litter soil by the serial-dilution plating technique, which is preferred for the isolation of soil-borne fungi[11,22].

Characterization of pigment synthesizing fungus: The isolated pigments synthesizing fungal phenotypic characters such as colony culture morphology on different agar mediums are illustrated in table 1 and fig. 4 and microscopic observation comprises of the septate and hyaline hyphae, conidial heads are columnar and biseriate, conidiophores are smooth, vesicles are found to be globose to subglobose; metulae and phialides cover the upper portion of the vesicle. Conidia are globose with rough walls, which confirm the fungus as *Aspergillus* sp. (Fig. 5). The genotypic characterization by molecular 18S r-RNA partial gene sequence confirms the isolated pigment synthesizing fungus as *Aspergillus ustus* strain KUMBASBT-52. The partial gene sequence was deposited in the NCBI GenBank provided with the accession number MW130234. The phylogeny of maximum likelihood was constructed by compiling the data matrix (Fig. 6).

Table 1
Culture morphology of *Aspergillus ustus* strain KUMBASBT-52 on different agar media

S.N.	Culture media	Colony characteristics on agar plate
1.	Potato Dextrose Agar (PDA)	Colony size; 50mm compactly arranged grey colony with thick white margin and reverse side yellow color surrounded by white color margin.
2.	Sabouraud Dextrose Agar (SDA)	Colony size; 45mm buff to grey color colony, compactly arranged mycelium, white margin around the colony and reverse side yellow color surrounded by white color margin.
3.	Malt Extract Agar (MEA)	Colony size; 56mm grey color colony, compactly arranged mycelium, white margin around the colony and reverse side yellow color surrounded by white color margin.
4.	Czapek-Dox Agar (CZA)	Colony size; 42mm white to buff color colony, compact mycelium, irregular margin around the colony and reverse side yellow color surrounded by white color irregular margin.
5.	Carrot Agar (CA)	Colony size; 60mm grey color colony, compactly arranged mycelium, white margin around the colony and reverse side light yellow color surrounded by white color margin.
6.	Rose Bengal Agar (RBA)	Colony size; 55mm buff color colony, transparent to white zonation at margin, thick mycelium and reverse side yellow color surrounded by white color margin.
7.	Richard's Synthetic Agar (RSA)	Colony size; 44mm white to grey color colony, compactly arranged mycelium, white to transparent zonation around the colony and reverse side yellow color surrounded by white color margin.

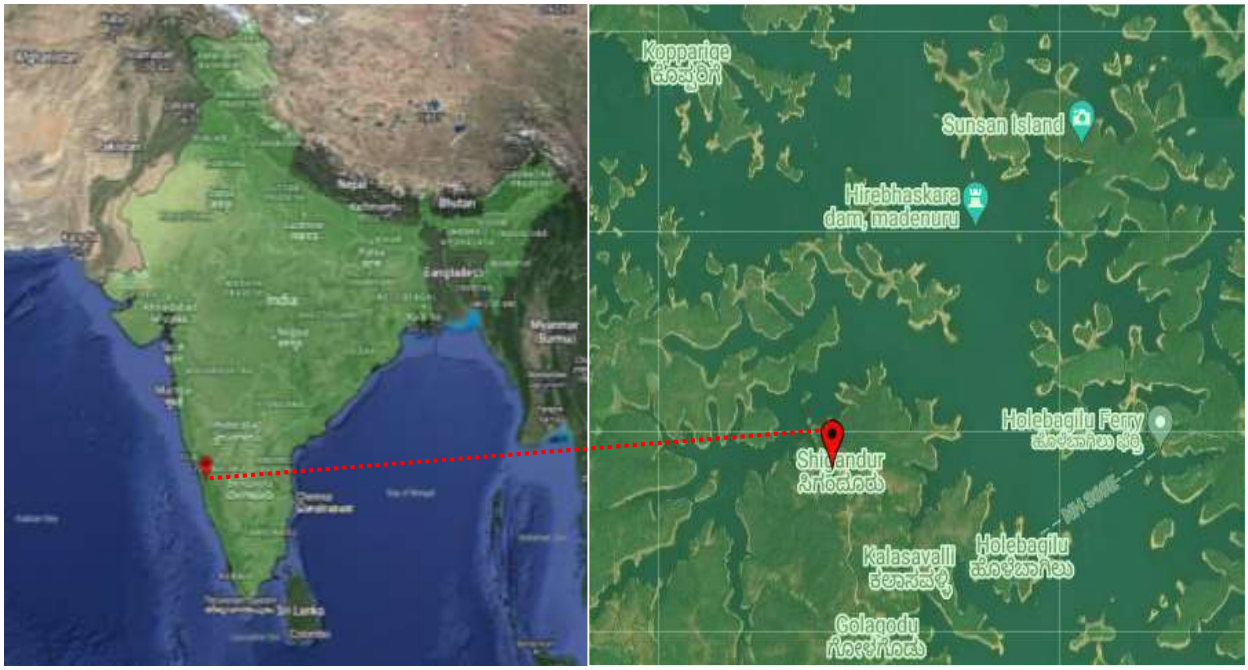

Fig. 1: Location of litter soil sample collected site: Sigandooru (14°4′34″N 74°52′20″E)

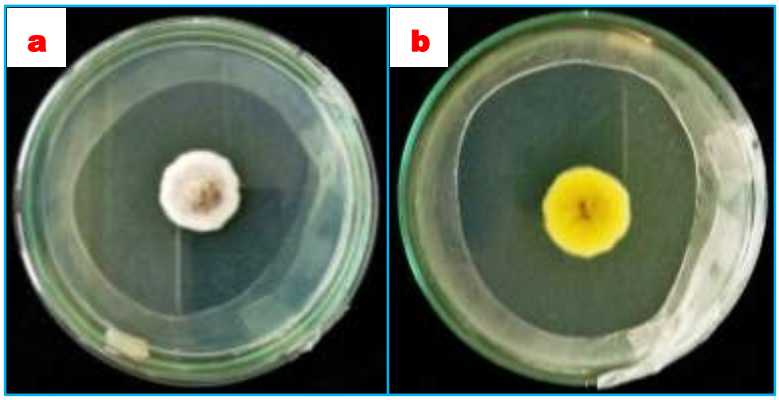

Fig. 2: Culture of *Aspergillus ustus* strain KUMBASBT-52 (a) Anterior view (b) Converse view

Fig. 3: *Aspergillus ustus* strain KUMBASBT-52 in PDB

Optimization, cultivation and extraction of extracellular pigment: Physical factors, namely incubation period, temperature, pH and chemical factors including media, carbon source; nitrogen source, mineral salts; amino acid succors in the nourishment, influence the growth and synthesis of pigments and other metabolites in fungi as well as other microorganisms. The fungus *A. ustus* strain KUMBASBT-52 displayed the maximum growth i.e. 18.64±0.39 g/L and persistent pigment production in PDB medium compared to other media on the 28th day, which was graphically exemplified in fig. 7. Consequently, PDB was adapted for further optimization tests.

The optimum conditions required for maximum biomass and pigment production by the fungus were documented as follows: temperature 25℃ (Biomass 18.55±0.52g/L; OD 0.198±0.008), pH 7 (Biomass 15.33±0.37g/L; OD 0.08±0.002), carbon source sucrose (Biomass 20.72±0.37g/L; OD 0.30±0.09), nitrogen source sodium nitrate (Biomass 16.58±0.38g/L; OD 0.153±0.007), mineral salt potassium phosphate (Biomass 19.65±0.58g/L; OD 0.52±0.007) and amino acid tyrosine (Biomass 20.98±0.58g/L; OD 1.046±0.05) represented graphically in Figures 8a and b, 9a and b, 10a and b, 11a and b, 12a and b, 13a and b respectively.

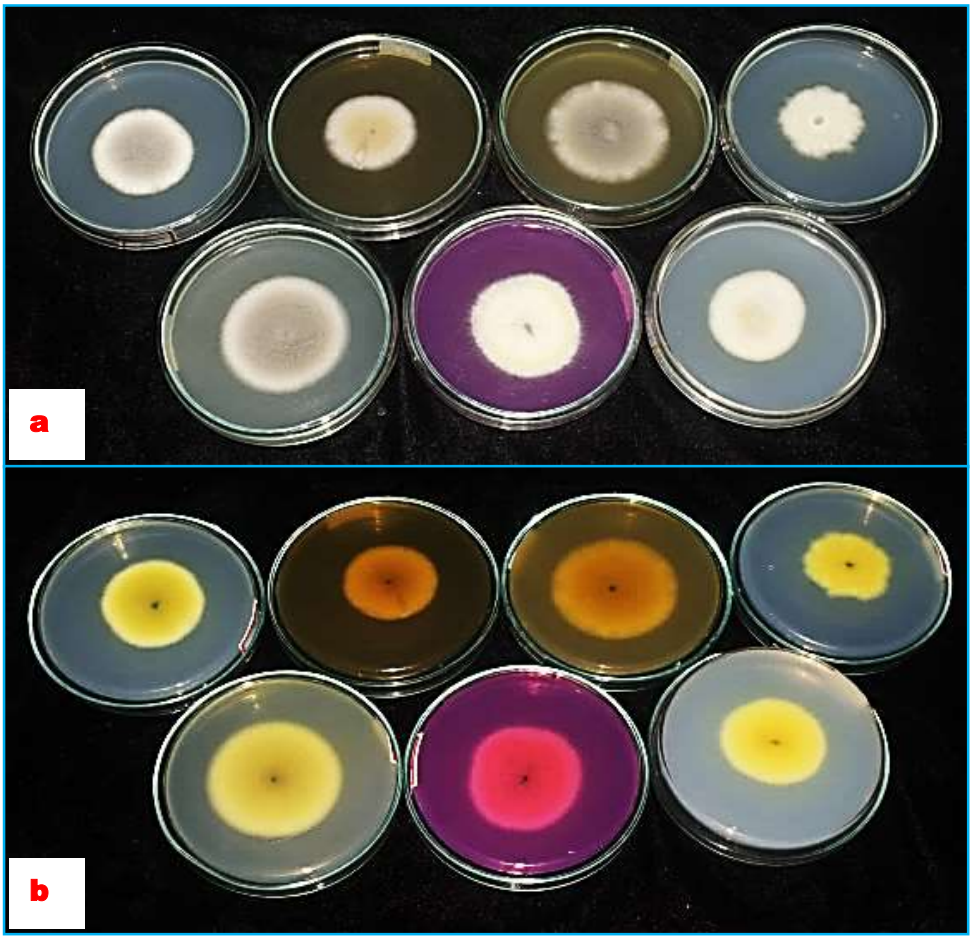

Fig. 4: Culture morphology of *Aspergillus ustus* strain KUMBASBT-52 on different agar media (PDA, SDA, MEA, CZA, CA, RBA and RSA) (a) Anterior view (b) Converse view

Fig. 5: A glimpse *of Aspergillus ustus* strain KUMBASBT-52 under light microscopy

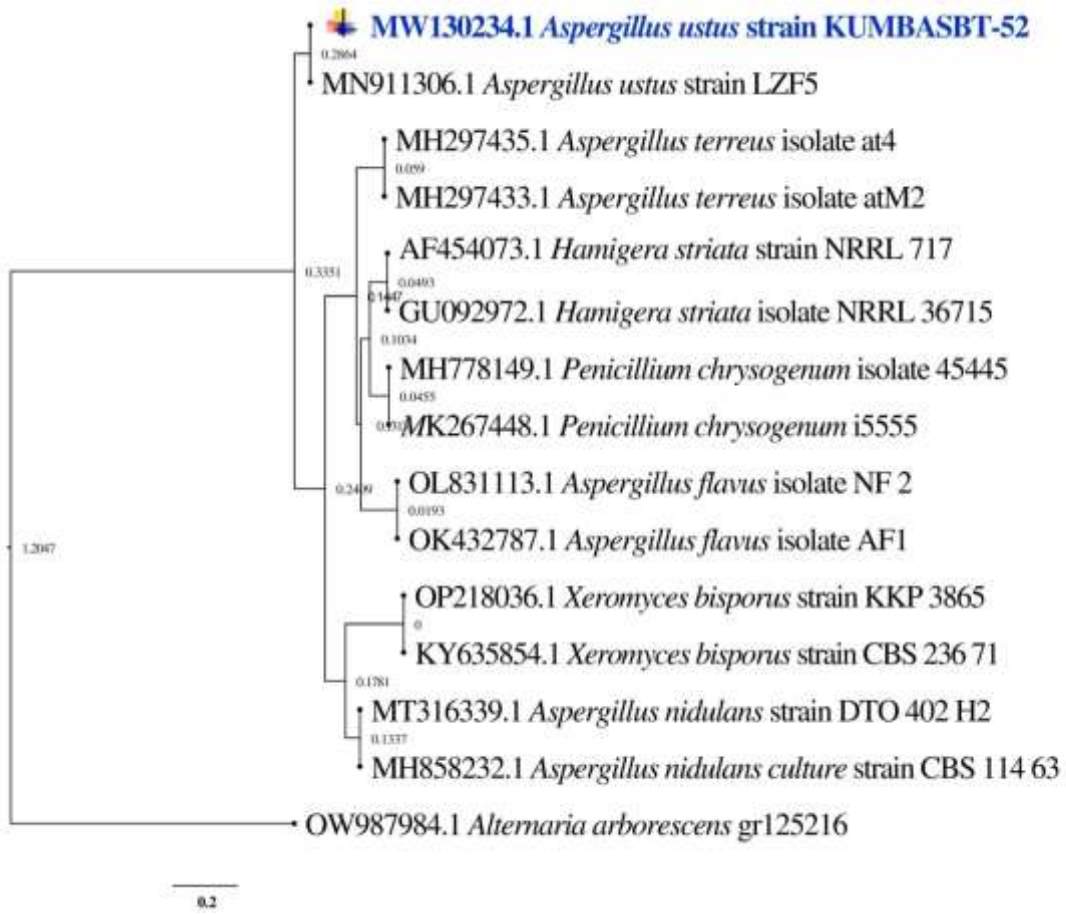

Fig. 6: Phylogenetic tree of *Aspergillus ustus* strain KUMBASBT-52

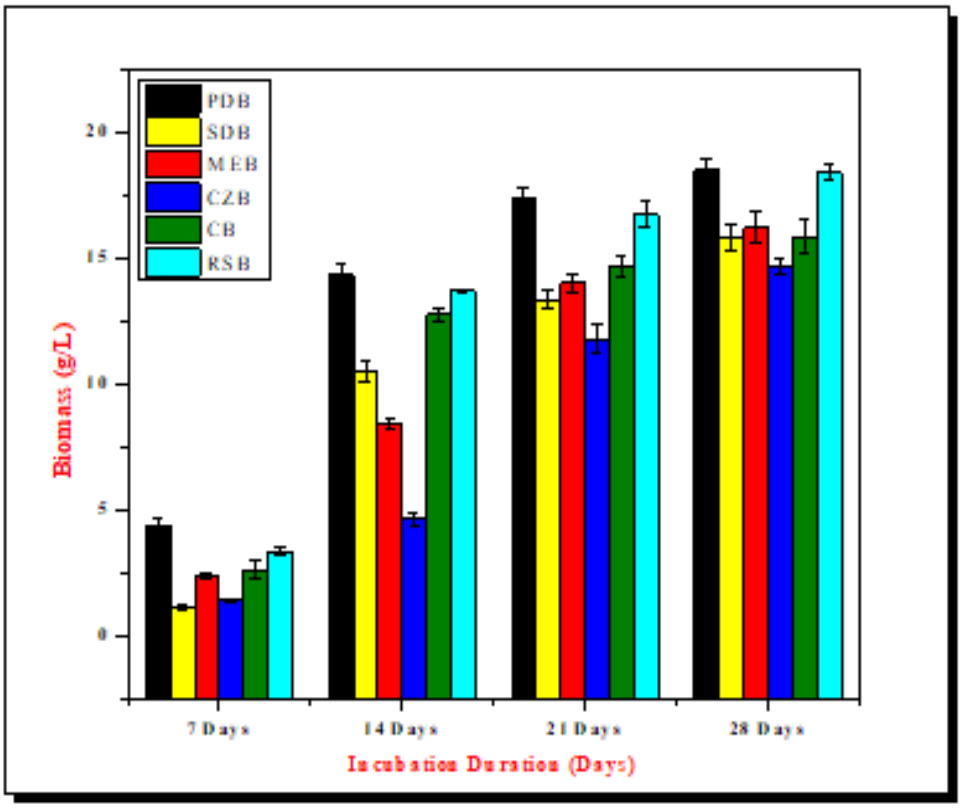

Fig. 7: Effect of different culture media and incubation duration on growth of *Aspergillus ustus* strain KUMBASBT-52

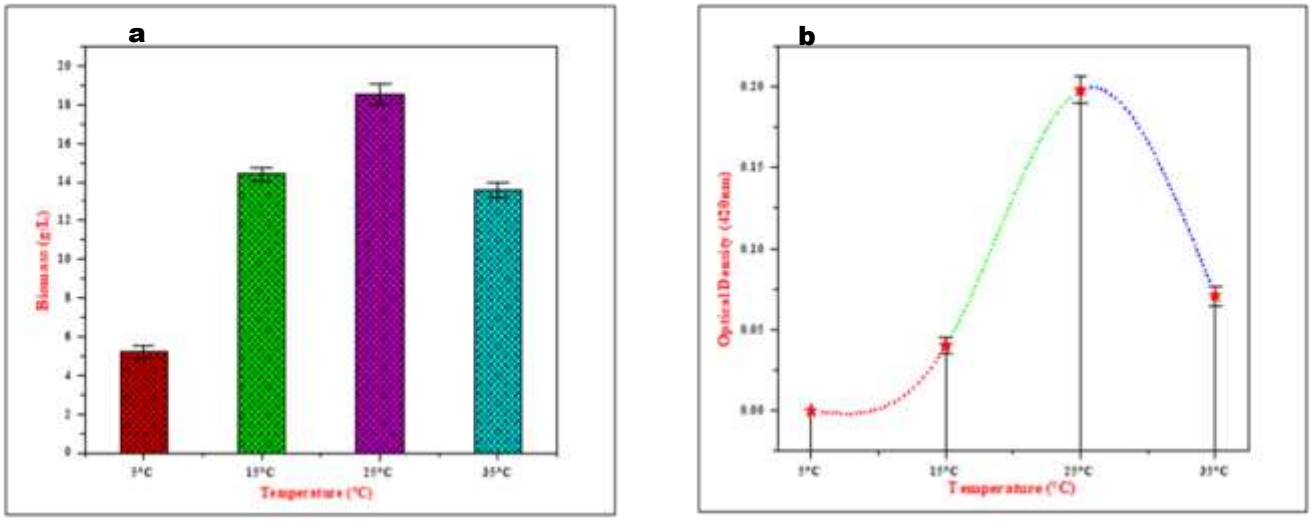

Fig. 8: Effect of different temperature on (a) growth and (b) pigment production of *A. ustus* strain KUMBASBT-52

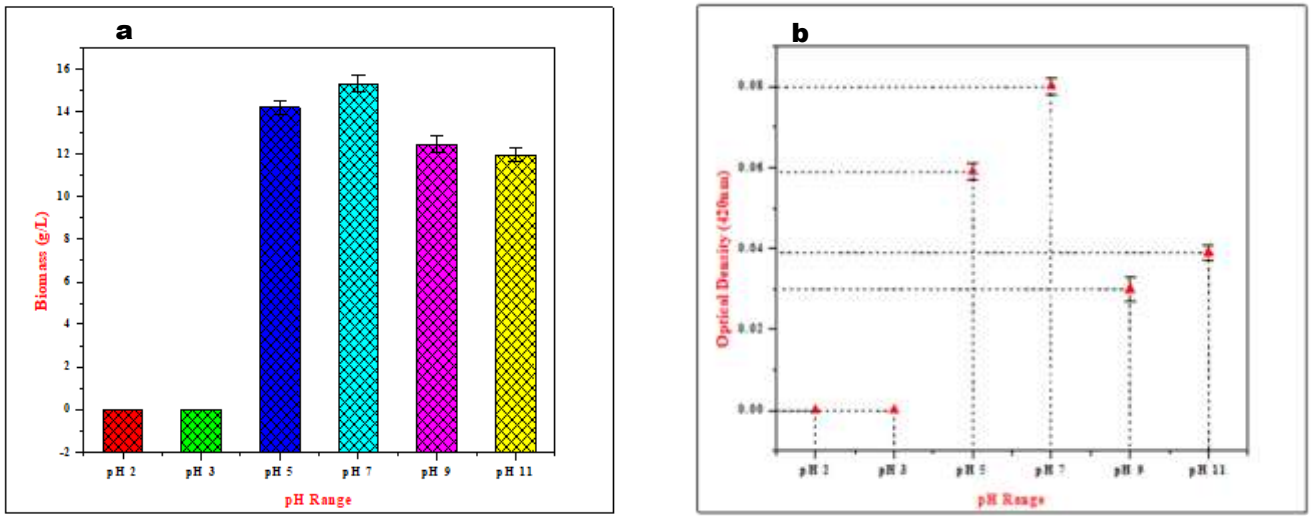

Fig. 9: Effect of different pH on (a) growth and (b) pigment production of *A. ustus* strain KUMBASBT-52

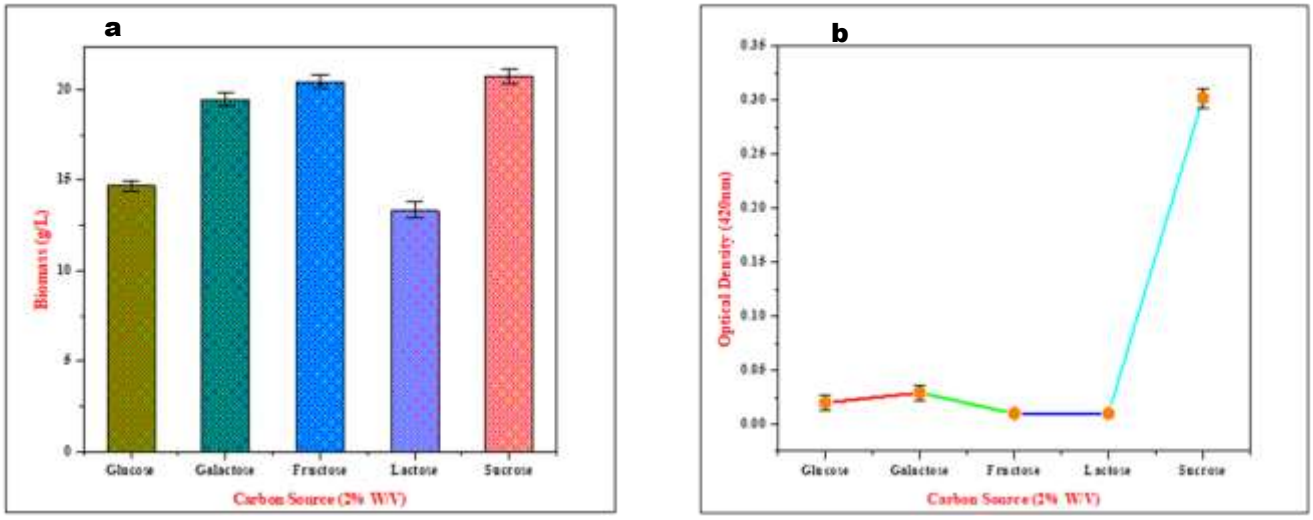

Fig. 10: Effect of different carbon source on (a) growth and (b) pigment production of *A. ustus* strain KUMBASBT-52

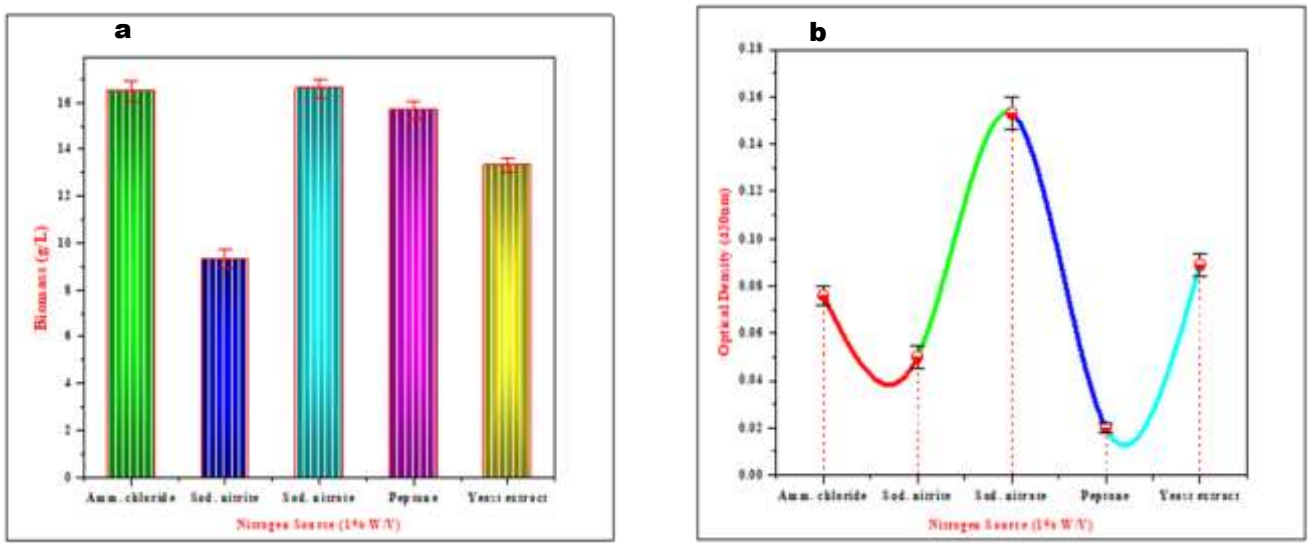

Fig. 11: Effect of different nitrogen source on (a) growth and (b) pigment production of *A. ustus* strain KUMBASBT-52

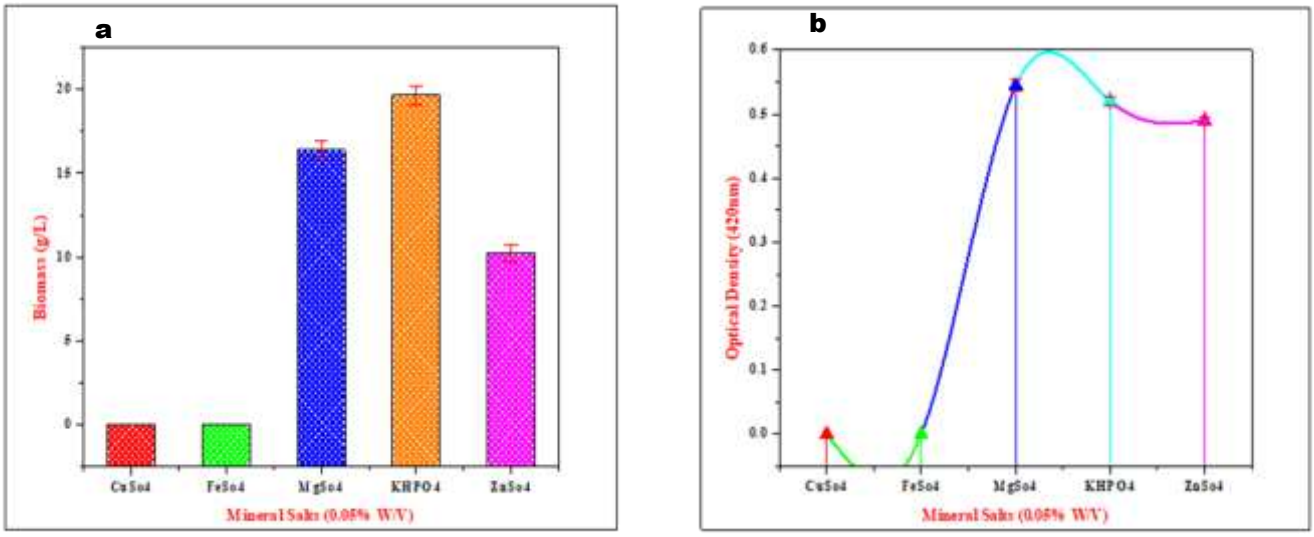

Fig. 12: Effect of different mineral salt on (a) growth and (b) pigment production of *A. ustus* strain KUMBASBT-52

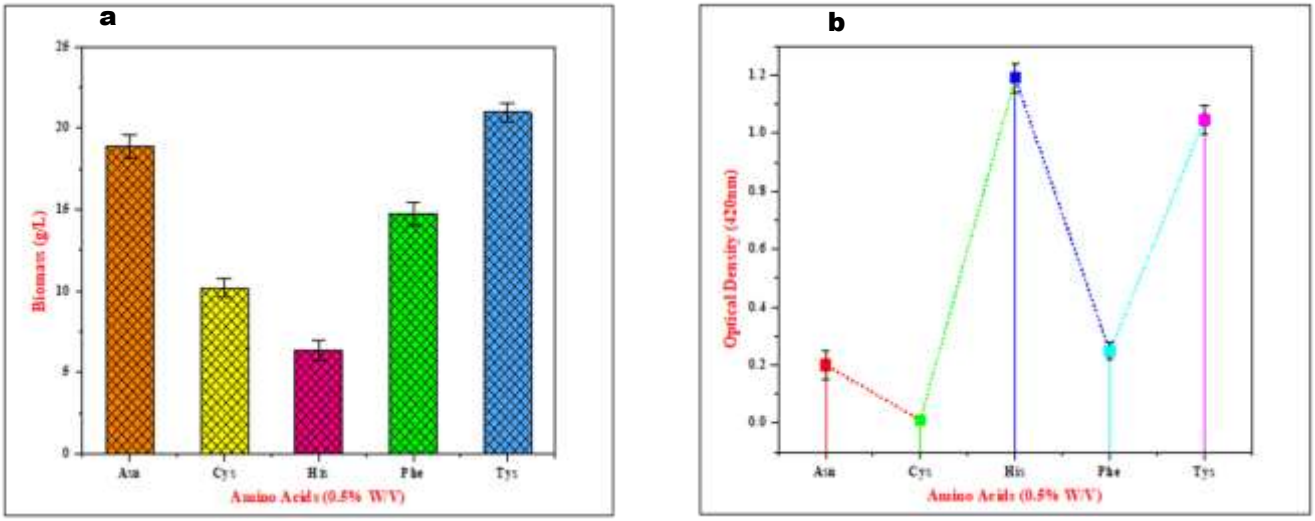

Fig. 13: Effect of different amino acid on (a) growth and (b) pigment production of *A. ustus* strain KUMBASBT-52

A. ustus strain KUMBASBT-52 exhibited maximum growth and pigment synthesis in potato dextrose broth medium supplemented with sucrose as a carbon source, sodium nitrate as a nitrogen source, potassium phosphate as a mineral salt and amino acid tyrosine at pH 7 and temperature 25°C. Temperature and pH play a key role in growth as well as pigment synthesis in fungus, which does not grow in pH 2 and 3 and totally zilch at temperature 5°C. In the previous study, Pandey et al[20] reported that PDB was suitable for maximum growth and pigment production in fungus, but in their study, they reported that maltose and peptone were found to be suitable carbon and nitrogen sources[20]. This may be due to the diversity of filamentous fungal nutritional requirements; it can be a choice for the particular fungus for their nutritional requirements. Submerged fermentation implies the process of growing microorganisms in broth media for the development and recovery of industrially important commercial products[9]. It accomplishes the voluminous advantages over solid-state fermentation, it is cost effective and produces high yield end products and is preferred for the easy recovery of multifaceted extracellular metabolites[7].

Conclusion

The humified litter soil of the Western Ghats is the best source for the isolation of pigment-producing microorganisms because it is packed with lavish nutrients which facilitate the growth of a variety of microorganisms. Azodyes are chemically synthesized compounds that are widely used as colorants, but they have a negative impact on human health as well as on the environment. Due to the carcinogenicity and non-eco-friendly nature of azodyes, researchers have put a spotlight on natural colorants. The isolated fungus *A. ustus* Strain KUMBASBT-52 synthesizes urochrome-yellow bio-pigment as its extracellular metabolite; its biosynthesis is mainly influenced by the physico-chemical parameters.

The optimization assay gives a clear picture of the conditions required for maximum growth as well as pigment synthesis by the fungus. Hence, the findings of this systematic study recommend that *A. ustus* strain KUMBASBT-52 can be exploited for dyeing fabrics, wood varnishing and paintings in the future. Further investigation is needed to know the range of toxicity of *A. ustus* and whether it is suitable for food applications or not. The urochrome-yellow pigment of *A. ustus* strain KUMBASBT-52 is a promising source for combating the negative impact of synthetic azodyes.

Acknowledgement

The authors are grateful to the Microbiome Research Laboratory at Department of Microbiology, Kuvempu University, Karnataka, India., for providing infrastructure to conduct this research work.

References

1. Alexopolus C.J. and Mims C.W., Introductory mycology, 3rd ed., Wiley Eastern Limited, New Delhi **(1979)**

2. Allison S.D. and Vitousek P.M., Extracellular enzyme activities and carbon chemistry as drivers of tropical plant litter decomposition, *Biotropica*, **36(3)**, 285-296 **(2004)**

3. Aneja K.R., Experiments in microbiology, plant pathology and biotechnology, New Age International Publishers, New Delhi, **4**, 157-162 **(2007)**

4. Balasubramanian M. and Sangha K.K., Valuing ecosystem services applying indigenous perspectives from a global biodiversity hotspot, the Western Ghats, India, *Frontiers in Ecology and Evolution*, **11**, 1026793 **(2023)**

5. Banakar S.P., Thippeswamy B., Thirumalesh B.V. and Naveenkumar K.J., Diversity of soil fungi in dry deciduous forest of Bhadra Wildlife sanctuary, western Ghats of southern India, *Journal of Forestry Research*, **23**, 631-640 **(2012)**

6. Barnett H.L. and Hunter B.B., Illustrated genera of imperfect fungi, St Paul Minnesota: The American Phytopathological Society **(1998)**

7. Bharathiraja S., Suriya J., Krishnan M., Manivasagan P. and Kim S.K., Production of enzymes from agricultural wastes and their potential industrial applications, In Advances in food and nutrition research, Academic Press, **80**, 125-148 **(2017)**

8. de Melo Pereira D.Í., Gurgel R.S., de Souza A.T.F., Matias R.R., de Souza Falcão L., Chaves F.C.M., da Silva G.F., Martínez J.G., de Lima Procópio R.E., Fantin C. and Albuquerque P.M., Isolation and Identification of Pigment-Producing Endophytic Fungi from the Amazonian Species *Fridericia chica*, *Journal of Fungi*, **10(1)**, 77 **(2024)**

9. Dhillon G.S. and Kaur S., eds., Agro-industrial wastes as feedstock for enzyme production: apply and exploit the emerging and valuable use options of waste biomass, Academic Press **(2016)**

10. Domsch K.H., Compendium of Soil Fungi, Taxonomically revised by Walter Gams, Eching, Germany, IHW-Verlag **(2007)**

11. dos Reis Celestino J., de Carvalho L.E., da Paz Lima M., Lima A.M., Ogusku M.M. and de Souza J.V., Bioprospecting of Amazon soil fungi with the potential for pigment production, *Process Biochemistry* **49(4)**, 569-75 **(2014)**

12. Funder S., Practical mycology, Manual for identification of fungi, Brggers Boktr, Forlag, Oslo, Norway **(1953)**

13. Geethanjali P. and Jayashankar M., A review on litter decomposition by soil fungal community, *IOSR Journal of Pharmacy and Biological Sciences*, **11(4)**, 1-3 **(2016)**

14. Hyde K.D., Bussaban B., Paulus B., Crous P.W., Lee S., Mckenzie E.H., Photita W. and Lumyong S., Diversity of saprobic microfungi, *Biodiversity and Conservation*, **16**, 7-35 **(2007)**

15. Lagashetti A.C., Dufossé L., Singh S.K. and Singh P.N., Fungal pigments and their prospects in different industries, *Microorganisms*, **7(12)**, 604 **(2019)**

16. Mapari S.A., Thrane U. and Meyer A.S., Fungal polyketide azaphilone pigments as future natural food colorants?, *Trends in Biotechnology*, **28(6)**, 300-307 **(2010)**

17. Mishra R.C., Kalra R., Dilawari R., Deshmukh S.K., Barrow C.J. and Goel M., Characterization of an endophytic strain *Talaromyces assiutensis*, CPEF04 with evaluation of production medium for extracellular red pigments having antimicrobial and anticancer properties, *Frontiers in Microbiology*, **12**, 665702 **(2021)**

18. Nagmani A., Kunwar I.K. and Manoharachary C., Hand book of soil fungi, In International Pvt. Ltd., New Delhi **(2006)**

19. Nirlane da Costa Souza P., Luiza Bim Grigoletto T., Alberto Beraldo de Moraes L., Abreu L.M., Henrique Souza Guimarães L., Santos C., Ribeiro Galvão L. and Gomes Cardoso P., Production and chemical characterization of pigments in filamentous fungi, *Microbiology*, **162(1)**, 12-22 **(2016)**

20. Pandey N., Jain R., Pandey A. and Tamta S., Optimisation and characterisation of the orange pigment produced by a cold adapted strain of *Penicillium* sp.(GBPI_P155) isolated from mountain ecosystem, *Mycology*, **9(2)**, 81-92 **(2018)**

21. Raistrick H and Stickings C.E., Studies in the biochemistry of micro-organisms, 82, Ustic acid, a metabolic product of *Aspergillus ustus* (Bainier) Thom & Church, *Biochemical Journal*, **48(1)**, 53–66 **(1951)**

22. Thom C. and Raper K.B., A Manual of the Aspergilli, *LWW*, **60(4)**, 171-178 **(1945)**

23. Toma M.A., Nazir K.N.H., Mahmud M.M., Mishra P., Ali M.K., Kabir A., Shahid M.A.H., Siddique M.P. and Alim M.A., Isolation and identification of natural colorant producing soil-borne *Aspergillus niger* from Bangladesh and extraction of the pigment, *Foods*, **10(6)**, 1280 **(2021)**

24. Waksman S.A., A method for counting the number of fungi in the soil, *Journal of Bacteriology*, **7(3)**, 339-341 **(1922)**.

(Received 21st August 2024, accepted 23rd October 2024)