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Isolation and screening of pigment producing fungus-*Aspergillus niger* and *Cladosporium sphaerospermum* from petroleum contaminated soil.

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ABSTRACT

The pigments are the most important secondary metabolites formed by microbes. Natural colours are gaining their demand on a daily basis because some synthetic colours have harmful effects. While during the prehistoric times, human beings have already utilized natural colours, the industrial revolution's wake has witnessed excessive utilisation of synthetic colours. Synthetic dyes have numerous drawbacks, for example, negative degradation, more persistence, cancer-causing and allergic properties etc. and therefore the need for natural, organic and biodegradable pigments increased in today's age. Natural pigments are naturally occurring pigments that are synthesized primarily by plants, animals and microorganisms. Our study reveals the hyphae coalesce to create a network of mycelium characteristic of filamentous fungi such as *Aspergillus*, *Penicillium*, or *Fusarium*. Yellow and blue pigments indicate the production of pigment by the fungus resulting from intracellular or extracellular metabolites. Spores or Conidia as dark spots could represent spore formation, indicating the reproductive stage of the fungus. These natural dyes can be commercially manufactured on large scale in a low-cost and eco-friendly process. The pigments obtained can be analyzed for their application in industries including cloth or leather dyeing, cosmetics, colourant for foods, and pharmaceutical industries etc. To provide better binding capacity to the pigments suitable binders can be employed. Affinity of extracted pigments with other types of binders will also be examined to verify the efficiency of colouring capacity of the pigment to employ as an effective colouring agent.

Keywords: Natural colours, pigments, *Aspergillus niger*, *Cladosporium sphaerospermum*, SDA

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INTRODUCTION

The pigments are the most important secondary metabolites formed by microbes. Natural colours are gaining their demand on a daily basis because some synthetic colours have harmful effects. While during the prehistoric times, human beings have already utilized natural colours, the industrial revolution's wake has witnessed excessive utilisation of synthetic colours [4]. Even though it appeared quite elegant in the first place, subsequently due to the varying reports on the safety of dyes and leathers while dyeing and also due to the use of unauthorized colours without any restriction, there is an immediate requirement to find the sources of natural pigments as safe dyes for textiles. Natural colours are now the demand, and thus there exists a scope of natural colour sources from biosources. Both natural colourants and artificial pigments have been widely applied in many areas of daily life, including food, feed, textile, paper, printing ink, cosmetics and pharmaceuticals [12].

Synthetic dyes have numerous drawbacks, for example, negative degradation, more persistence, cancer-causing and allergic properties etc. and therefore the need for natural, organic and biodegradable pigments increased in today's age. Natural pigments are naturally occurring pigments that are synthesized primarily by plants, animals and microorganisms. Still, the production process of pigments from plants might not be ideal due to several issues, including seasonality, depletion of sensitive plant species due to their widespread usage, high cost of production and stability as well as solubility issues [9]. Fungal pigments have been found to be an effective and readily available alternative source of natural pigmentation. Fungi possess enormous advantages over plants like, season independent pigment synthesis, synthesize pigment with varying shades of colour, yield more stable and soluble pigments, easy processing along with possessing anticancer and antimicrobial activity [13]. These pigments themselves are not responsible for growth of fungus but are responsible for keeping the fungi safe from several negative environmental conditions. These all pigments contain a single prominent feature that is 'Bioremediation' which helps in soil detoxification. Also possesses antimicrobial activity, with potential application in pharmacy and as natural food preservatives. The overall aim of this research is to investigate fungal pigments as a source for coloring colorants [2]. The objective of current research is to identify and isolate pigment producing fungi from the 'Petroleum Contaminated Soil'. The petrol pump soil possesses features, such as extremely acidic pH, microbial growth is high, petrol has the ability to activate most enzymes in soil. *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium* and *Alternaria* are some examples of pigment producing fungi in this area. These fungi possess the ability to produce pigments commonly in response to stress from the extreme conditions.

MATERIAL METHODS

Chemicals

Chemicals such, sabouraud dextrose agar, methanol, acetone, lactophenol cotton blue were procured from HiMedia Laboratories, Mumbai, India. All chemicals were of analytical grade.

Collection of samples

Soil from petroleum area of 50-100gm was collected from the nearby area of Dr. D. Y. Patil Arts, commerce and science college, Pimpri, Maharashtra Pune, India. The samples were kept in sterile labelled container or bags. In a sterile environment condition, transfer of 10 gm of soil to a sterile flask containing 90 ml sterile water. Shake the mixture vigorously for 10 min to disperse fungal spores into the water [7].

Culture

Sabouraud Dextrose Agar (SDA) medium was used as fungal growing medium. One gm of soil sample was dissolved in 9ml of sterile distilled water in a test tube was subjected to serial dilution. Mix the blend thoroughly to form homogenized suspensions. Serial dilution was prepared by transferring 1ml of suspension into 9ml of sterile water. Seed 0.1ml from each dilution on SDA plates. Spread the suspension uniformly with a sterile spreader. Incubate the plates at 30°C for 5-6 days to permit the growth of fungus. Screening for pigment production by inspect the plates for appearance of fungal colonies. Colonies that show various pigmentation, i.e., red, yellow or green. Subculturing on a sterile inoculating loop or needle, transfer a small segment of pigmented fungal colony on to a fresh SDA plate. Incubate until growth is adequate (about 25 days) [10].

Characterization of Fungus

Prepare fungal slides for microscopic examination by placing a small amount of fungal culture on a slide, staining with lactophenol cotton blue and examine under microscope. Record morphological features for identification under microscope at 40X and 100X [11].

Pigment extraction

Harvest the fungal mycelium from the agar plates, wash the mycelium with sterile water to remove residual agar. Place the washed mycelium in a sterile container with an organic solvent (eg., methanol, acetone) for pigment extraction. Allow extraction to occur for 1-2 hrs with occasional shaking. Filter the extract by using whattman's filter paper to remove fungal debris. Analyse the pigment using a spectrophotometer to determine concentration and characteristics at 390–410 nm [3].

Statistical Analysis

The collected data were stored in Microsoft Excel and analysed using statistical software (STATA version 14). All the, samples were analysed in triplicate and the results were expressed as mean $\pm$ SD.

RESULTS AND DISCUSSION

Screening after 7 days incubation period on SDA media at room temperature, the fungal isolates derived from soil. Colonies are powdery or velvety likely *Aspergillus*, or cottony and fluffy. Dark brown, greenish, orange, and white colonies, which signify varied fungal growth figure 1a [6]. The colonies are observed to be in competition, exhibiting overlapping growth and pigment diffusion into the agar. Following subculturing incubation for 25 days, homogenous growth colony is seen to have spread out evenly over the plate, a good sign of adaptation of a fast-growing fungus strain Figure 1b. Powdery or Velvety the appearance on the surface indicates a fungus that sporulates, and probably belongs to such genera as *Aspergillus*, brownish-grey the colour may be due to spore pigmentation or production of metabolites, depending on the species of fungus[1] (Figure 2a, b).

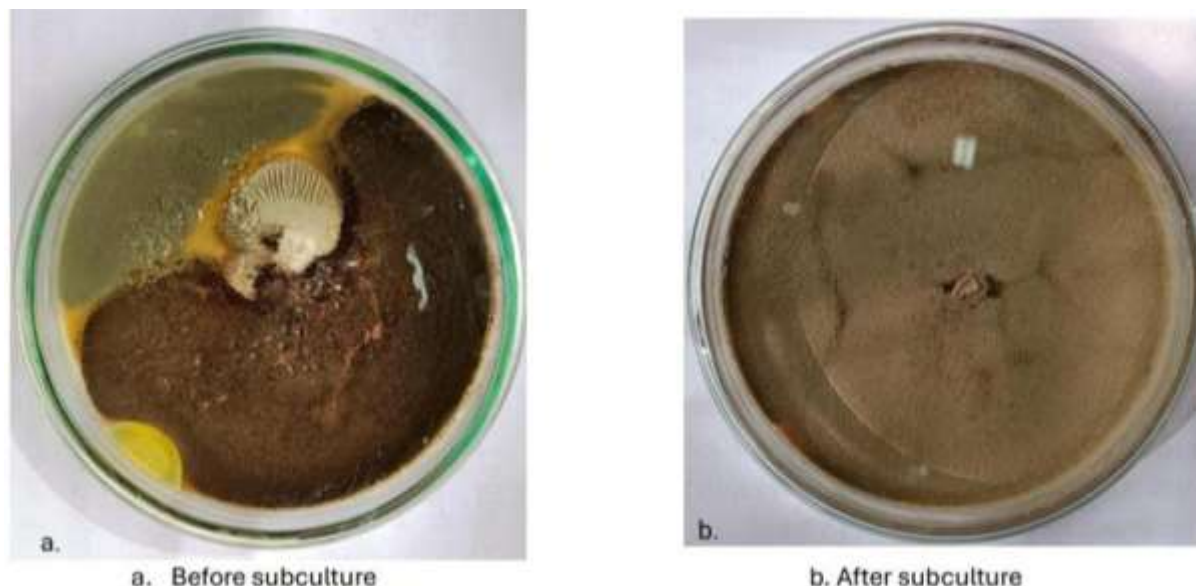


Figure 1: Culture plates of *Aspergillus flavus* on Sabouraud Dextrose Agar after primary culture (a) and subculture (b)

Minimum contamination in contrast to the earlier plate containing numerous colonies, this plate seems to have only a single dominant strain of fungus, implying that it has possibly been subculture for purity. Purified fungal pigments using fungal strains, the mycelium was collected and filtered with whattmans filter paper. Yellow Filtrate the liquid present in the beaker is colored yellow, possibly indicating extracellular pigments or metabolites secreted by the fungus (Figure 5). When measured

through spectrophotometer, absorbance was between $\sim 390\text{--}410\text{ nm}$. Mycelial biomass, the solid retained in the filter paper is dense and fibrous in nature, typical of fungal mycelia. Subsequently, colour pigment evaporated during direct sunlight and used on fabric. Fungal identification dense mycelial network (Figure 3) [5]. The picture depicts a complicated network of fungal hyphae, which seem to be interwoven. Ultrastructure in the microscope is thread-like, indicating that it is made up of fungal hyphae (Figure 4). The hyphae coalesce to create a network of mycelium characteristic of filamentous fungi such as *Aspergillus*, *Penicillium*, or *Fusarium*. Yellow and blue pigments indicate the production of pigment by the fungus resulting from intracellular or extracellular metabolites. Spores or Conidia as dark spots could represent spore formation, indicating the reproductive stage of the fungus [8].

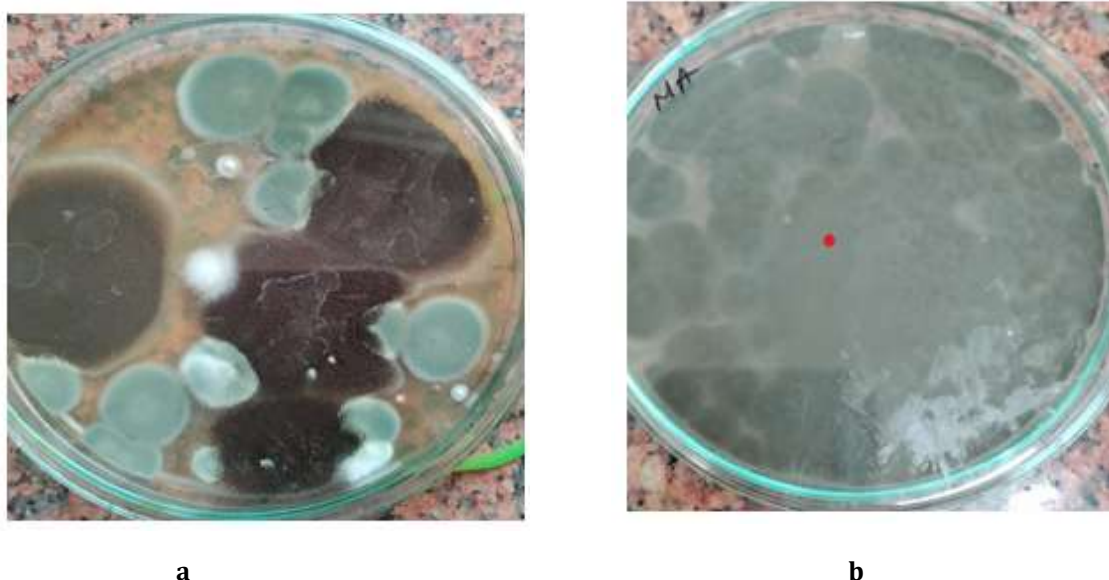


Figure 2: Culture plates of *Cladosporium sphaerospermum* on Sabouraud Dextrose Agar after primary culture (a) and subculture (b)



Figure 3: Lactophenol cotton blue staining depicting fungal spores and mycelium *Cladosporium sphaerospermum*

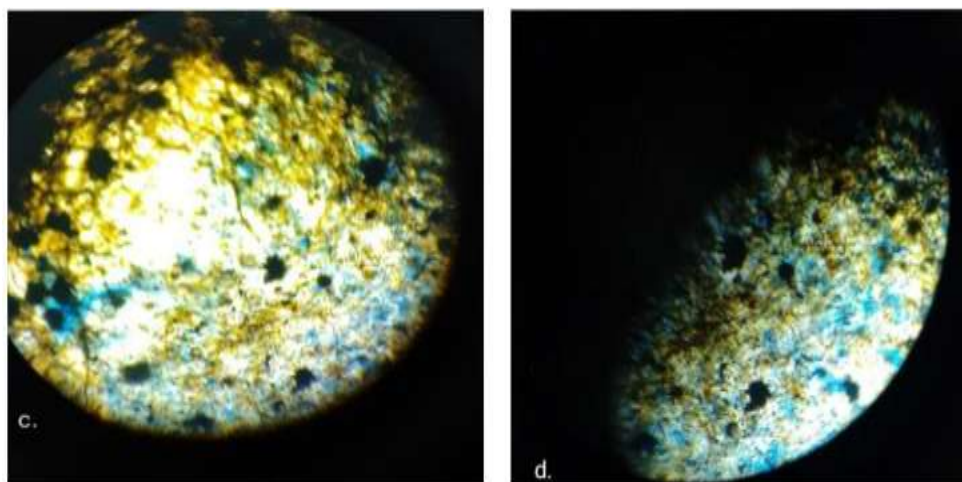
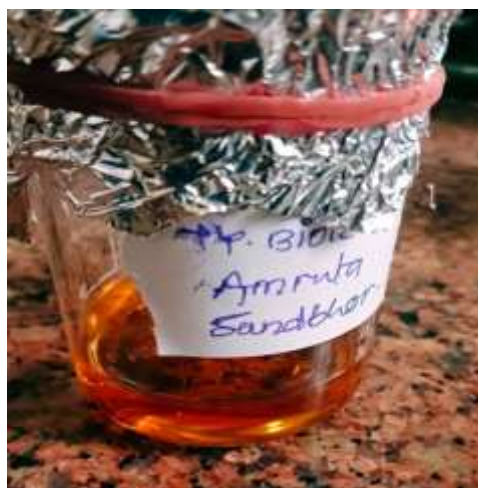


Figure 4: Lactophenol cotton blue staining depicting fungal spores and mycelium of *Aspergillus niger*



a



b

Figure 5: Fungal dye form *Cladosporium sphaerospermum* using wattman filter paper (a) pigment (b)

CONCLUSION

The screened fungal strains yield pigments that can be applied to dye textile fabrics/fibres. The extracted dye was found to be harmless for human skin. It can be inferred from current study that fungi can be a promising source of cultivating dyes that can be applied sufficiently on textile fabrics. These natural dyes can be commercially manufactured on large scale in a low-cost and eco-friendly process. The pigments obtained can be analyzed for their application in industries including cloth or leather dyeing, cosmetics, colourant for foods, and pharmaceutical industries etc. To provide better binding capacity to the pigments suitable binders can be employed. Affinity of extracted pigments with other types of binders will also be examined to verify the efficiency of colouring capacity of the pigment to employ as an effective colouring agent.

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