EXTRACTION OF FUNGAL PIGMENT MELANIN FROM *Aspergillus niger* AND ANALYSIS OF ITS ANTIMICROBIAL ACTIVITY

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Abstract

Colourful pigments are produced by microorganisms as secondary metabolites for a range of purposes in daily life. The fungal mold *Aspergillus niger* is very well known to produce the black coloured Melanin pigment. The Melanin pigment produced by *Aspergillus niger* has been studied for its potential applications in the Pharmacological science. The present research was aimed to extract the Melanin pigment from *Aspergillus niger* and evaluate its Antimicrobial potential. The *Aspergillus niger* was isolated by Open plate method and, identified by Lactophenol cotton blue (LPCB) staining method and Platting in Sabouraud's dextrose agar. The Melanin pigment was extracted and confirmed by XRD analysis. Antibacterial activity of Melanin pigment was tested by Agar well diffusion method. Bacterial pathogens were swabbed on a Mueller Hinton Agar and 10 µl of Melanin pigment extract mixed with 5 % DMSO was placed in the 6 mm Agar well and incubated at 37 ºC for 24 hrs. Melanin showed antibacterial activity against all the test bacterial pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Serratia marcescens*). Maximum activity was recorded against *Escherichia coli* (32 mm in dm). The Anticandidal activity of the Melanin was studied against *Candida albicans* and the inhibitory zone with 29 mm in dm was observed. Further research in this area may lead to the development of new and innovative uses for this pigment.

Keywords: Fungal pigments, *Aspergillus niger,* Melanin, Antimicrobial activity and Anticandidal activity.

1. Introduction

Pigments are components that can be obtained naturally or synthetically. The natural pigments are acquired from plants and microorganisms by the way the synthetic pigments are composed of various chemicals which cause numerous hazardous effects to both the environment and human health (Aghajanyan, 2011). These pigments are coloured substances which can be soluble and insoluble. The colours seen in nature come from a variety of sources, including plants, fruits, vegetables, roots, and microorganisms like

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bacteria and fungi. The term "Biocolors" refers to colours since they are made from biological sources. The practice of colouring processed foods dates back a very long time (Caro, 2017). A pigment is a material that modifies the colour of transmitted or reflected light by wavelength-selective absorption. Plants and microorganisms create pigments as secondary metabolites for a variety of uses in their daily lives. Previously, using synthetic pigments to colour food, clothing, fruit juices, and paintings was tolerated on a global scale. However, due to the dangers that these colours represent to the environment and to human health, it is now necessary to look for alternative sources of pigments that are safe to use (Joshi, 2003). Despite the availability of a wide range of pigments from fruit and veggies, microbial pigments are gaining popularity due to a number of factors, including their natural makeup and safety for use, production being independent of seasons and geographic conditions, manageable and predictable yield, and many others (Aghajanyan, 2005).

Fungi are capable of producing a wide range of colours. Many characteristics of these pigments vary, including colour and physical and chemical traits such molecule size, structure, hydrophobicity, reactivity, and biological functions. While multiple hues may be produced by the same fungus, fungi from evolutionarily distinct lineages may create pigments that are physically similar (Valenzuela Gloria, 2021). Several of these pigments, such as vitamin precursors, antibiotics, immunomodulators, and colourants have beneficial effects on human welfare. These pigments can, however, also increase the virulence and pathogenicity of human fungal diseases when they are present. The bulk of fungal pigments structure-function correlations, as well as the genes and metabolic pathways involved in their synthesis, are still unknown, despite the fact that considerable advancements in our understanding of fungal pigments have been accomplished (Mapari, 2010). Natural biopolymers called Melanin are known to support a variety of biological functions and shield organisms from harmful environmental factors. due to its use in organic semiconductors, bioelectronics, and pharmaceutical. Throughout the previous decade years, melanin has received more attention for its role in transport, photoprotection, and environmental bioremediation. Although these sectors have made significant progress, there are currently few practical uses for melanin, perhaps as a result of the scarce and expensive supply of natural melanin. However, recent biotechnology developments have made it possible to produce microbial Melanin on a somewhat big scale, which could replace the current commercial melanin (Ray and Eakin, 1975).

The melanin pigment produced by *Aspergillus niger* has been studied for its potential applications in Biotechnology and Medicine. It has been found to have antioxidant, antimicrobial, and immunomodulatory properties, which make it potentially useful for a variety of applications. Melanin pigment from *Aspergillus niger* has been utilized as a natural colouring agent in food and cosmetic goods. In order to remove heavy metals and other contaminants from wastewater, it has also been employed as a biosorbent. *Aspergillus niger* melanin pigment has been studied for its potential as

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an antioxidant and anti-inflammatory agent. It has also been investigated for its potential as a radioprotective agent, as it has been shown to reduce the damage caused by ionizing radiation in cells. *Aspergillus niger* melanin pigment is a promising natural product with a wide range of potential applications in biotechnology and medicine. Further research in this area may lead to the development of new and innovative uses for this pigment. The present study was aimed to extract Melanin pigment from *Aspergillus niger* and analysis of its Antibacterial and Anticandidal activity.

2. Materials and Methods

2.1. Isolation of Melanin pigment producing *Aspergillus niger*

The Melanin pigment producing *Aspergillus niger* was isolated in various locations of Sacred Heart College (Autonomous), Tirupattur, Tamil Nadu, India by Open plate method.

2.3. Identification of Melanin pigment producing *Aspergillus niger*

The *Aspergillus niger* isolated from various locations of Sacred Heart College will be identified by

a) Microscopic examination (Lactophenol cotton blue staining)
b) Plating on Sabouraud's dextrose agar

2.4. Optimization of Culture conditions for *Aspergillus niger* cultivation

The growth conditions of the *Aspergillus niger* was optimized according to the determination of significant parameters influencing the process particularly the pH (pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9), Temperature (20 ºC, 27 ºC, 30 ºC, 37 ºC and 40 ºC) and Incubation time (3 days, 6 days, 9 days, 12 days and 15 days).

2.5. Extraction of Melanin pigment from *Aspergillus niger*

Extraction of Melanin pigment was done according to the procedure of Zerrad *et al*. (2014). A total of 6 grams of dry dark-pigmented *Aspergillus niger* mycelia was dissolved with 10 ml of 1 N KOH and Autoclaved for 20 mins at 121 °C, then cooled and filtered with filter paper. The filtrate was then precipitated with 4 N HCl. Extraction was continued by centrifugation for 20 minutes at a speed of 5000 g. The pellets were taken and then dried in an oven at 70 °C. The dried extract was washed using Chloroform, Methanol and Ethyl acetate (1:1:1) to remove other secondary metabolites possibly toxic towards microorganisms, then dried. Melanin that has been washed and dried was dissolved in DMSO for the assay of Antimicrobial and Anticandidal activity.

2.6. Confirmation of Melanin pigment

The *Aspergillus niger* isolate was inoculated into 50 ml of 0.1 % Tyrosine substrate solution with few drops of Chloroform in 100 ml Erlenmeyer flask and incubated at 37 ˚C for 48 hrs. The deep red color shows the positive result. Further confirmation was done with the help of XRD analysis.

2.7. Determination of Antibacterial activity of Melanin pigment

Antibacterial activity of Melanin pigment was tested by Agar well diffusion method. Bacterial pathogens like

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Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa and *Serratia marcescens* were swabbed on a Mueller Hinton Agar and 10 µl of Melanin pigment extract was mixed with 5 % DMSO and poured in the 6 mm Agar well and incubated at 37 ºC for 24 hrs. After incubation, the zone of inhibition was measured and expressed as mm in dm. The Agar well with 5 % DMSO alone act as a Negative control.

2.8. Determination of Anticandidal activity of Melanin pigment

Anticandidal activity of Melanin pigment was tested by Agar well diffusion method. The fungal pathogen *Candida albicans* was swabbed on a Mueller Hinton Agar and Melanin pigment extract mixed with 5 % DMSO was placed in the 6 mm Agar well and incubated at Room temperature for 24 hrs. After incubation, the zone of inhibition was measured and expressed as mm in dm. The Agar well with 5 % DMSO alone act as a Negative control.

3. Results and Discussion

A filamentous fungus called *Aspergillus niger* is extensively utilized in industry to produceenzymes, organic acids, and other metabolites. It is also known to generate the pigment melanin, which has drawn growing interest because of its potential uses in biotechnology and medicine. The toxicity of pigments can vary depending on the specific pigment and the amount and route of exposure. Some pigments can be toxic if ingested or inhaled in large amounts, while others may be relatively safe even in high concentrations. In the case of fungal pigments, some studies have suggested that certain pigments may have toxic effects on humans and other organisms. For example, some studies have found that the melanin pigment produced by *Aspergillus niger* can induce oxidative stress and DNA damage in human lung cells, and may be a contributing factor in the development of lung diseases. Additionally, some fungal pigments have been found to be carcinogenic, mutagenic, or teratogenic in animal studies. For example, the pigment sterigmatocystin produced by *Aspergillus* species has been shown to be carcinogenic in animal studies and may pose a risk to human health if ingested in high amounts. It is important to keep in mind that other factors, such the presence of other chemicals or contaminants, can also impact how harmful pigments are. Therefore, it is important to conduct thorough safety assessments of pigmentsbefore they are used in products or exposed to the environment.

Additionally, it has been demonstrated to have potential uses in biotechnology andmedicine, including as a natural colouring agent for food and cosmetics, a biosorbent for heavy metals and other pollutants, and a therapeutic agent for a variety of illnesses, including cancer, neurodegenerative disorders, and infectious diseases. *Aspergillus niger* melanin pigment is a complicated and interesting chemical with numerous potential uses in biotechnology and medicine. Whilst further investigation is required to completely comprehend its biosynthesis, characteristics, and uses, it is obviousthat this pigment has a lot of potential for advancement in the future. Fungi are capable of producing a wide range of colours. Many characteristics of these pigments vary, including colour and

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physical and chemical traits such molecule size, structure, hydrophobicity, reactivity, and biological functions. While multiple hues may be produced by the same fungus, fungi from evolutionarily separate lineages may create pigments that are physically similar. Several of these pigments, such as vitamin precursors, antibiotics, immunomodulators, and colourants, have beneficial effects on human welfare. These pigments can, however, also increase the virulence and pathogenicity of human fungal diseases when they are present. The bulk of fungal pigments' structure-function correlations, as well as the genes and metabolic pathways involved in their synthesis, are still unknown, despite the fact that considerable advancements in our understanding of fungal pigments have been accomplished. Melanin is a naturally occurring biopolymer that is known to support a variety of biological functions and shield organisms from harmful environmental factors. Due to its uses in organic semiconductors, bioelectronics, drug delivery, photoprotection, and environmental bioremediation, melanin has attracted greater attention over the past ten years. Despite substantial advancements in these fields, there are still limited real-world applications for melanin, possibly as a result of its rarity and expensive cost in natural sources. However, new biotechnology developments have made it possible to produce microbial melanin on a reasonably large scale, which could replace the current commercial melanin.

3.1. Isolation of Melanin pigment producing *Aspergillus niger*

The Melanin pigment producing *Aspergillus niger* was isolated in various locations of Sacred Heart College (Autonomous), Tirupattur, Tamil Nadu, India by Open plate method.

3.1.1. Identification of Melanin pigment-producing *Aspergillus niger*

3.1.1.1. Microscopic examination

Conidiophore are smooth walled, hyaline or pigmented in nature. Vesicles are sub spherical in shape and conidial heads are radiate. Conidiogenous cells biseriate in arrangement. Medulla twice as long as the phialides. Conidia are brown in colour, ornamented with warts and ridges. Hyphae were septate in nature.

3.1.1.2. Colony morphology on SDA plate

Colonies are black in colour and consisting of a dense felt of conidiophores all over the plate.

3.2. Optimization of Culture conditions for Melanin producing *Aspergillus niger*

The growth of the *Aspergillus niger* was optimized under different pH, Temperature and Culture conditions for studying the best growth conditions for the Black pigment Melanin production.

3.2.1. Effect of pH on the growth of Melanin producing *Aspergillus niger*

The effect of pH on the growth of Melanin producing *Aspergillus niger* was studied at different pH *viz*., pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9, and the results were given in the Table – 1. The growth of *Aspergillus niger* was very good and luxuriant at pH 6 and pH 7, and moderate at pH 4 and pH 5. No fungal growth was observed at pH 8 and pH 9.

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3.2.2. Effect of Temperature on the growth of Melanin producing *Aspergillus niger*

The effect of Temperature on the growth of Melanin producing *Aspergillus niger* was determined at 20 °C, 27 °C, 30 °C, 37 °C and 40 °C, and the results were furnished in the Table – 2. The growth of *Aspergillus niger* was very good and luxuriant at 27 °C and 30 °C, and moderate at 20 °C and 37 °C. No *Aspergillus niger* growth was observed at 40 °C.

3.2.3. Effect of Incubation period on the growth of Melanin producing *Aspergillus niger*

The effect of Incubation period on the growth of Melanin producing *Aspergillus niger* was determined at 3rd Day, 6th Day, 9th Day, 12th Day and 15th Day, and the findings were presented in the Table – 3. It was observed that the growth of *Aspergillus niger* was very good and luxuriant at 6th Day, 9th Day, 12th Day and 15th Day, and moderate at 3rd Day.

Table – 1: Effect of pH on the growth of Melanin producing *Aspergillus niger*

pH	*Aspergillus niger* Growth
4	+
5	+
6	++
7	++
8	-
9	-

(++ Good and luxurious growth; + Moderate growth and - No growth)

Table - 2: Effect of Temperature on the growth of Melanin producing *Aspergillus niger*

Temperature (°C)	*Aspergillus niger* Growth
20	+
27	++
30	++
37	+
40	-

(++ Good and luxurious growth; + Moderate growth and - No growth)

Table - 3: Effect of Incubation time on the growth of Melanin producing *Aspergillus niger*

Incubation Time (Days)	*Aspergillus niger* Growth
3	+
6	++
9	++
12	++
15	++

(++ Good and luxurious growth; and + Moderate growth)

3.3. Confirmation of Melanin pigment

A 100 ml Erlenmeyer flask containing 50 ml of 0.1 % Tyrosine substrate solution and a few drops of chloroform was used to inoculate the *Aspergillus niger*, which was then isolated and cultured at 37 °C for 48 hours. Further confirmation was done with the help of XRD analysis (Figure – 1).

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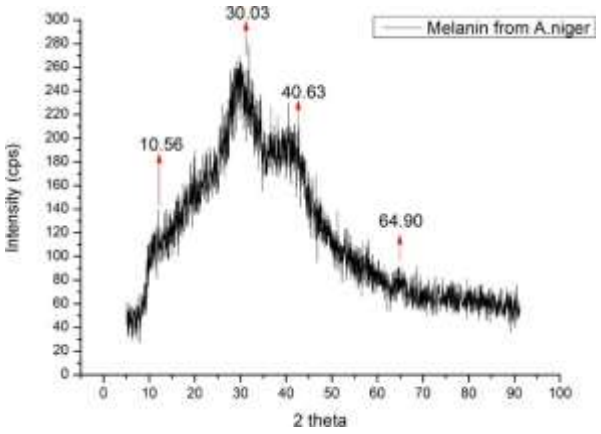

Figure – 1: Confirmation of Melanin pigment by XRD

3.4. Determination of Antibacterial activity of Melanin pigment

The Antibacterial activity of the Melanin was determined against selected human pathogens and the results were given in Table – 4. Agar well diffusion method was employed for the determination of Antibacterial activity at two different concentrations, 100 µl and 200 µl. Comparatively, antibacterial activity was excellent at 200 µl concentration when compared to 100 µl concentration. Melanin showed antibacterial activity against all the test bacterial pathogens. Maximum activity was recorded against *Escherichia coli* (32 mm in dm) followed by *Salmonella typhi* (23 mm in dm), *Streptococcus agalactiae* (21 mm in dm), *Staphylococcus aureus* (20 mm in dm) and *Pseudomonas aeruginosa* (18 mm in dm). Least zone was observed against *Klebsiella pneumoniae* (16 mm in dm). No zone of inhibition was noticed in the Negative control 5 % DMSO.

3.5. Determination of Anticandidal activity of Melanin pigment

The Anticandidal activity of the Melanin was studied against *Candida albicans* and the findings were furnished in Table – 5. Agar well diffusion method was employed for the determination of Anticandidal activity at two different concentrations, 100 µl and 200 µl. Comparatively, anticandidal activity was excellent at 200 µl concentration when compared to 100 µl concentration. Melanin showed the inhibitory zone with 29 mm in dm. No zone of inhibition was noticed in the Negative control 5 % DMSO.

Table – 4: Antibacterial Activity of Melanin pigment against Bacterial pathogens

S.No	Bacteria	Concentration of Melanin and Zone of Inhibition (mm in dm)	
		100 µl	200 µl
1	*Staphylococcus aureus*	13	20
2	*Streptococcus agalactiae*	19	21
3	*Escherichia coli*	26	32
4	*Salmonella typhi*	18	23
5	*Klebsiella pneumoniae*	14	16
6	*Pseudomonas aeruginosa*	17	18

Table – 5: Anticandidal activity of Melanin pigment against *Candida albicans*

S.No	Fungi	Concentration of Melanin and Zone of Inhibition (mm in dm)	
		100 µl	200 µl
1	*Candida albicans*	25	29

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4. Conclusion

In conclusion, a filamentous fungi *Aspergillus niger* has the capacity to synthesize the Melanin, a dark brown pigment. Tyrosine metabolism, oxidative polymerization, and enzymatic oxidation are only a few of the intricate enzymatic processes that go into the synthesis of the Melanin pigment. In Biotechnology and Medicine, Melanin pigment synthesized by fungi has number of applications, including Tissue engineering, Radioprotection, and UV protection. Due to its high productivity and simplicity of growing, *Aspergillus niger* is an organism that hold the potential for the manufacture of Melanin. Microbially synthesized pigments have several advantages over chemically synthesized pigments, making them a promising and sustainable alternative in various industries. We also conclude that the Melanin pigment have an excellent Antibacterial and Anticandidal activity.

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