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Identification of fungal melanin production and the role of anti-melanin agents

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Abstract: Accumulation of melanin pigment in some specific areas of human skin leads to various skin disorders. This study includes melanin production and extraction from non-dermatophyte, filamentous fungi (*Aspergillus niger*). The effect of carbon, nitrogen, pH and temperature on melanin production was also observed. Qualitative and Quantitative analysis was performed to confirm melanin pigment from *A. niger* by using solubility test, precipitation test and reaction with oxidizing and reducing agent. Fungus gave maximum melanin production with dextrose, peptone, pH 5.6 at 37ºC. To avoid hyper pigmentation tyrosinase is the primary target to inhibit melanin production. Study has used A. Niger as model organism to study the melanin formation under various melanin inhibitors. Ascorbic acid showed maximum inhibition at 50% while it was 25% for Kojic acid. Curcumin inhibited the tyrosinase activity at 25% while maximum inhibition observed was 30% for Aloesin and 20% for flavonoid.

Keywords: Filamentous fungi, melanin pigment, maximum inhibition, oxidizing and reducing agents, tyrosinase activity.

Accumulation of Melanin pigment in some specific areas of human skin leads to various skin disorders. This study includes melanin production and extraction from non-dermatophyte, filamentous fungi (*Aspergillus niger*). The effect of carbon, Nitrogen, pH and temperature on melanin production was also observed. Qualitative and Quantitative analysis was performed to confirm melanin pigment from *A. Niger* by using solubility test, precipitation test and reaction with oxidizing and reducing agent. Fungus gave maximum melanin production with dextrose, peptone, pH 5.6 and Temperature of 37ºC. To avoid hyper pigmentation tyrosinase is the primary target to inhibit melanin production including culturing method and micro titer plate assay. Ascorbic acid showed maximum inhibition at 50% while it was 25% for Kojic acid. Curcumin inhibited the tyrosinase activity at 25% while maximum inhibition which was observed in the research was 30% for Aloesin and 20% for flavonoid.

INTRODUCTION

Melanin is a secondary metabolite that is composed of complex heterogeneous polymers. Melanin present in fungus is considered to be biodegradable natural pigment, sustainable and contain variety of functional properties and various biological activities. Due to its specific properties it can play the role of anti-radiation, antioxidant, photoreception and adsorption. In addition to this, apparent pigmentation of the hair, skin, and eyes depends mainly on the presence of melanin in those particular tissues (Hearing, 2011). Melanin, a dark pigment that is produced by about 10% of skin cells in the dermis of skin (Hashemi & Emami, 2015). It is the most vital determinant for skin color. It is ranked as one of the great natural pigments because these are synthesized by all biological kingdoms, including a wide variety of fungi, helminthes and bacteria that is the main cause of disease in humans (Joshua, 2015). Melanins are polymerized from indolic and phenolic compounds that are negatively charged hydrophobic pigments of high molecular weight (White, 1958). Hyper pigmentation in some specific areas of the skin can lead to aesthetic issues (Lee *et al*., 2015).

The extraction and inhibition of melanin can be done using different methods. The method of extraction includes acid-alkali method (K, 2011) that is based adjustment of pH and by using enzymes (Pal, 2014). Sabouraud Dextrose Agar medium has considered being the best medium for melanin production and RPMI-1640 (Roswell Park Memorial Institute Medium) is considered as medium that works best for MTP assay. Enzyme Tyrosinase is the direct target for inhibition of melanin production (Masum *et al*., 2019). The mechanism of melanin inhibition involves Micro titer (MTP) Assay and Petri plate method.

There are various melanin sources; many common fruits and vegetables, like apples, bananas, garlic, potatoes and persimmons can also produce melanin. It can also be obtained from plants, including *Mucuna monosperma callus*. However, these methods have the disadvantage low maneuverability, high production cost and environmental pollution risk. For this reason, the bio production of melanin using microorganisms like bacteria and fungi as alternative sources for melanin production has attracted great attention. This is because they grow relatively fast rand can be applied for the scale-up process for mass production (Choi, 2021). Various fungi producing melanin have been identified so far (Tran-Ly *et al*., 2020). The great amount of knowledge about melanin

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in Aspergilli has been studied *Aspergillus Fumigatus* and, more recently, *A. bridgeri*, *Aspergillus nidulans* and *A. niger* (Goncalves *et al*., 2012). For the past decades, inhibitors of tyrosinase have been a great concern mainly due to the vital role of the enzyme in mammalian melanogenesis and fungi or fruit enzymatic browning. Melanogenesis by definition is the complete process leading to the formation of dark macromolecular pigments, i.e., melanin (Saghaie, 2013). Melanin and pathways of melanin synthesis are indeed potential targets for antimicrobial drug discovery. Hence, a thorough understanding of melanin structure will help in the identification of new approaches to target the enigmatic polymer (Joshua, 2015).

This study focused on filamentous fungus, *A. niger* for purification and extraction of melanin. Analysis of the melanin was performed using many qualitative and quantitative tests. In addition, impact of various agents as tyrosinases inhibitors was also investigated.

MATERIALS AND METHODS

Growth conditions and spores collection

The study was undertaken in single fungal isolate obtained from Civil Hospital Bahawalpur which was previously identified as haploid filamentous fungus belonging to phylum Ascomycota known as *Aspergillus niger*. Spores were inoculated on Sabouraud Dextrose Agar medium (SDA) plates. Antibiotic chloramphenicol (0.5g/L) was added in SDA medium to avoid any kind of microbial contamination. Plates were incubated at 37˚C for 7-10 days. After the completion of incubation time spores were shredded from the plate by using 0.85% saline and transferred to 1.5ml Appendorf tubes. Then it was centrifuged and pallet was saved in 40% glycerol for longer period.

Sub culturing and melanin extraction

A. niger spores were transferred to conical flasks having Sabouraud Dextrose Broth (SDB) medium (step 2 of fig. 2). Flasks were kept in shaking incubator at 37˚C for 10 days until pigmentation started. After the incubation period, SDB medium was filtered by using sterile filter paper (step 3). Biomass was dried at room temperature and converted to fine powder. Melanin was extracted by using acid-alkali method as described by (Pal *et al*., 2014) with certain modifications (as shown in fig. 2). Mycelium biomass was crushed with 2M NaOH until pH 10.5 was obtained (step 4). Then it was incubated at room temperature for 48 hours. After that mixture was centrifuged at 3500g for 15 min (step 5). Supernatant was obtained and was mixed with 2M HCl, pH 2.5 was attained. It was given the overnight incubation and centrifuged again at 3500g for 15 min. Precipitate was purified by Acid hydrolysis using 6M HCl by attaining 200^0C temperature for 2 hours (step 6). Carbohydrates and proteins were removed by acid hydrolysis. The precipitate was then treated with organic solvents (chloroform, Ethyl acetate, Ethanol 2ml from each) as shown in fig. 2 step no. 7. Resulting precipitate was dried at room temperature, again centrifuged at 3500g for 15 min (step 8). The supernatant was acidified with 6M HCl and incubated overnight (step 9). The precipitate was obtained by centrifugation and washed with distilled water. This precipitate was purified melanin (step 10) and used for Qualitative and Quantitative analysis.

Optimization of culture conditions

Optimization for carbon, nitrogen, pH and temperature was done(Pombeiro-Sponchiado *et al*., 2017). Glucose, Fructose, Dextrose, Lactose, Sucrose, Maltose and soluble starch were used as carbon sources while peptone, yeast extract; sodium nitrate and potassium nitrate were used as Nitrogen sources. A category of different temperature (37˚C, 42˚C, 47˚C and 52˚C) and pH (3.6, 5.6, 6.6, 7.6, 8.6 and 10.6) was also accustomed to see the distinction in melanin production.

Inhibition of fungal melanin

Melanin was inhibited by using different inhibitors i.e., Kojic acid, Ascorbic acid, Turmeric, Peanuts and Aloe Vera. Inhibition was performed by two methods i.e., 1) by using Petri plates (Freitas *et al*., 2019; joshua D. Nosanchuk1, 2015). 2) By using Microtiter plate (MTP) Assay (Masuda *et al*., 2005).

Inhibition through petri plates

Kojic acid was used as first melanin inhibitor. Stock solution of Kojic acid was prepared as 32mg/ml of DMSO. Different concentrations of Kojic acid stock solutions as 5µl, 10µl and 15µl were added in 15ml of Petri plate SDA medium. In the same way Ascorbic acid stock solution was prepared as 20mg/ml of ddH_2O and three different concentrations of 5µl, 10µl, 15µl were added in petri plates. Third inhibitor used was turmeric powder. Its stock solution was prepared as 20mg/ml of ddH_2O. Multiple concentrations of 30µl, 60µl, 70µl and 80µl were added in each Petri plate. Then after inoculation plates were incubated at 37ºC for 7-10 days.

Inhibition through microtiter plate assay

Second inhibitory mechanism which applied for melanin inhibition was MTP Assay. Inhibitors as Kojic acid, Ascorbic acid, Turmeric, Peanuts, Aloe Vera were used. Their percentage solutions were prepared in RPMI-1640 medium. Concentrations of 5%, 10%, 15%, 20% and 25% of Kojic acid and Turmeric (table 1) were used while concentrations of 10%, 20%, 30%, 40% and 50% were used for Ascorbic acid (table 1). Peanuts concentrations which we prepared were 2%, 4%, 6%, 8%, 10%, 12%, 14%, 16%, 18% and 20% (table 1). Aloe Vera concentration used are 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% (table 1). 200µl of each inhibitor and 100µl of

inoculum were added in each well of 96-well plate. Control was added too by adding 200µl of just RPMI medium and 100 µl of inoculum. Plate was incubated at 37°C for 72 hours. After incubation plates were washed with 0.85% saline or 1X PBS and reading was taken in ELISA reader at 480nm.

STATISTICAL ANALYSIS

All data analysis and statistical procedures were performed using Microsoft Excel version 2018. The results were presented as mean ± standard deviation of mean values.

RESULTS

Culture morphology

A. Niger spores were grown on SDA medium. *A. Niger* gave white cottony appearance at start which turned green at the end of its incubation period (7-10 days) as shown in fig. 1(a).

Microscopy

Growth characteristics and microscopic characteristics were observed to confirm the *A niger*. Macroscopic observations proved *A. niger* shows white cottony appearance at start and gradually changes to green color that is a characteristic feature of filamentous fungi (fig. 1b).

Qualitative and quantitative analysis

Melanin pigment was extracted by acid-alkali method as shown in (fig. 2). Pigment was confirmed as melanin by various physical and chemical tests i.e., color, solubility test, Precipitation test, reaction with oxidizing and reducing agents. Pigment was blackish brown in color which was primary confirmation for melanin. Pigment was easily soluble in bases e. g. NaOH, KOH and Borate buffer while completely insoluble in water and all organic solvents (Ethyl acetate, Alcohol, Acetone, Benzene, Chloroform). Pigment gave precipitates quickly when it mixed with by 1% $FeCl_3$ and HCl. Pigment was confirmed completely when it reacted with oxidizing and reducing agents decolorizing its brown color as mentioned in table 2.

Optimization of culture conditions for melanin production

Different culture conditions were furnished for melanin production i. e carbon sources, Nitrogen sources, pH and Temperature. Different quantity of biomass production and extracted melanin was detected in each growth condition. Melanin production was maximum for dextrose, Peptone, at pH 5and Temperature of 37˚C that was 3.21mg, 4.2mg, 4.84mg and 6.43mg respectively as mentioned in table 3.

Inhibition assay

Melanin inhibition was performed by using two methods named as inhibition through culturing and inhibition through microtiter plate Assay.

Table 1: Percentage solutions of different inhibitors prepared in RPMI-1640 medium.

Percentage solutions of Kojic acid	Kojic Acid/RPMI
5%	25mg/1ml
10%	50mg/1ml
15%	75mg/1ml
20%	100mg/1ml
25%	125mg/1ml
Percentage Solution of Ascorbic acid	Ascorbic acid/ RPMI
10%	100mg/ml
20%	200mg/ml
30%	300mg/ml
40%	400mg/ml
50%	500mg/ml
Turmeric powder percentage solutions	Turmeric/ RPMI
5%	50mg/ml
10%	100mg/ml
15%	150mg/ml
20%	200mg/ml
25%	250mg/ml
Percentage solutions of peanuts powder	Peanuts/RPMI
2%	40mg/ml
4%	80mg/ml
6%	120mg/ml
8%	160mg/ml
10%	200mg/ml
12%	240mg/ml
14%	280mg/ml
16%	320mg/ml
18%	360mg/ml
20%	400mg/ml
Percentage Solutions of Aloe Vera	Aloe Vera/RPMI
10%	100µl/ml
20%	200µl/ml
30%	300µl/ml
40%	400µl/ml
50%	500µl/ml
60%	600µl/ml
70%	700µl/ml
80%	800µl/ml

Inhibition through culturing

Stock solutions of inhibitor were prepared and included in each petri plate before inoculation. Plates were observed day by day. Melanin inhibition started when complete discoloration of fungus was obtained and fungus din not convert its white cottony growth to green color. Presence of white mycelial growth after increased incubation time was a signature mark of the melanin inhibition. Kojic acid was found as showing best inhibitory mechanism after 72 hours of incubation at the concentration of 10µl. Hence ascorbic acid showed melanin inhibition at the concentration of 10µl after the 48 hours of incubation while turmeric showed no melanin inhibition at multiple concentrations by this method (fig. 3).

Table 2: List of physical and chemical tests to confirm extracted pigment as Melanin

S. No.	Test	Result
1	Color observation	Blackish brown
2	Solubility test	
	NaOH	Soluble
	KOH	Soluble
	Borate buffer	Soluble
	Distilled water	Insoluble
	Chloroform	Insoluble
	Ethyl Acetate	Insoluble
	Alcohol	Insoluble
	Acetone	Insoluble
	Benzene	Insoluble
3	Precipitation Test	
	1% $FeCl_3$	Brown Precipitate
	HCl	Readily Precipitate
4	Reaction with oxidizing and reducing agent	
	H2 NaOCl h	Decolorized
	H_2O_2	Decolorized
	KMnO4	Decolorized
	Bromine water	Decolorized

Table 3: Optimization of different sources for maximum melanin production. Highlighted rows are the optimized conditions for maximum melanin production. Melanin production by different carbon sources proved dextrose as best carbon source, peptone as nitrogen source, pH 5.6 and temperature of 37˚C.

S. No.	Carbon Source	Biomass production (g)	Melanin extracted (mg)
1	Glucose	8.05	2.17
2	Dextrose	10.52	3.21
3	Fructose	9.84	3.07
4	Lactose	3.45	2.19
5	Sucrose	4.87	1.78
6	Starch	3.65	1.06
7	Maltose	3.73	1.95
	Nitrogen Source		
1	Peptone	8.46	4.2
2	Yeast extract	3.86	3.43
3	Peptone + yeast extract	3.86	2.13
4	Sodium nitrate	2.54	0
5	Potassium nitrate	1.36	0
	pH		
1	3.6	0	0
2	5.6	12.09	4.84
3	6.6	8.34	2.57
4	7.6	4.3	1.07
5	8.6	0	0
6	10.6	0	0
	Temperature		
1	25^0C	0	0
2	37^0C	13.34	6.43
3	42^0C	8.32	4.68
4	47^0C	8.47	0
5	52^0C	5.76	0

Inhibition through microtiter plate Assay (MTP)

Second method which exploited to check melanin inhibition was MTP Assay. Kojic acid showed minimum absorbance at the concentration of 25% while ascorbic acid showed maximum inhibition at 50%. Turmeric showed maximum inhibition at 25%, peanuts at 20%, while Aloe Vera at 30% as shown in fig. 4.

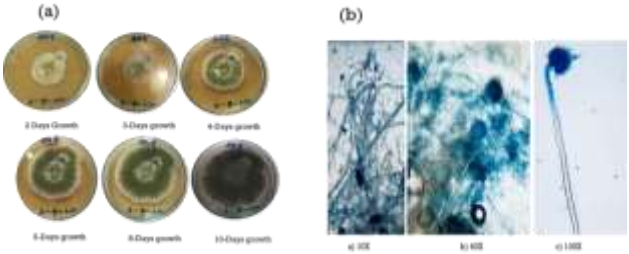

Fig. 1: (a) *Aspergillus Niger* growth with increased incubation time. (b) Microscopic view of *A. niger* at different magnification of 10X, 40X and 100X

Fig. 2: Step by step mechanism of Melanin extraction from fungus by using acid-alkali method.

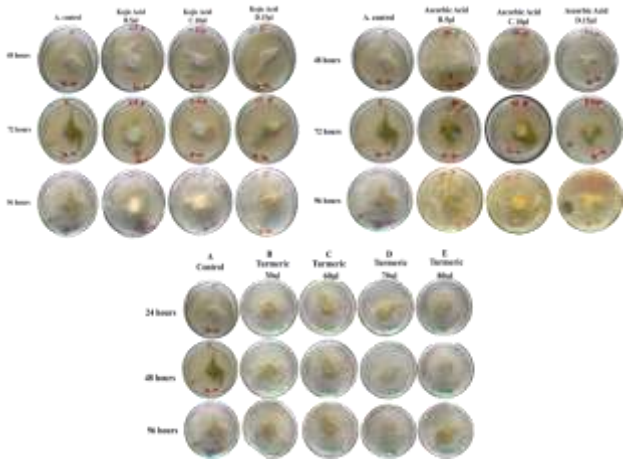

Fig. 3: Melanin inhibition by using petri plates. (a) inhibition by kojic acid at different concentrations. Inhibition observed after 72 hours. (b) inhibition by Ascorbic acid observed after 48 hours. (c) while no melanin ihibition was seen at different concentration of Turmeric.

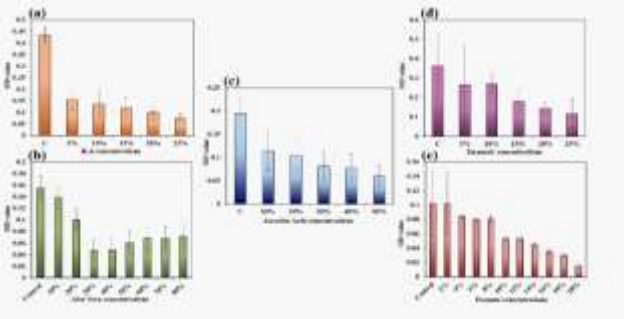

Fig. 4: OD value for different inhibitors at 480nm. (a) kojic acid showing maximum inhibition at the concentration of 25%. (b) Ascorbic acid showing maximum inhibition at 50%. (C) Turmeric showing maximum inhibition activity at 25%. (d) peanuts showing maximum inhibition activity at 20%. (e) Aloe Vera as Tyrosinase inhibitor at the concentration of 30%.

DISCUSSION

The major objective of the research was identification of the suitable fungal model for synthesis of melanin. The current study has investigated melanin content from fungi *A. niger*. Because some carbon and nitrogen sources are much more easily accumulated and promote better output of the desired products, optimizing medium constituents is an excellent strategy for increasing pigment production. SDA was investigated as the best medium for production of biomass and melanin pigment formation (Pombeiro-Sponchiado *et al*., 2017). In the study, best carbon source is found to be dextrose and best nitrogen source peptone was the most optimized sources for melanin production(Tamie, 2016). 37^0C temperature and 5.6 pH were demonstrated as the optimized conditions for the production of melanin. (Pombeiro-Sponchiado *et al*. (2017).

The increase in melanin synthesis and its accumulation occurs in different skin diseases, such as melasma, Periorbital hyperpigmentation, Acanthosis nigricans Lentigines, Cervical Poikiloderma, neurodegeneration associated with Parkinson's disease and even skin cancer risk(A. S. ElObeid *et al*., 2017). Melanogenesis is considered to be a complicated process including most of chemical and enzymatic reactions, the enzymes such as tyrosinase and tyrosinase-related proteins (TYRP1 and TYRP2) have a crucial and primary role in melanin production (Zolghadri *et al*., 2019).

Another target of undertaken research was to find the best anti melanin agents. Much time been spent to find potentially unhazardous tyrosinase suppressors with maximum effect. A variety of naturally present as well as artificially synthesized inhibitors were reported(Lee *et al*., 2015). However, little of them can be applied on skin and can be used in cosmetics. Major cause to avoid such inhibitors is that these artificially synthesized suppressors have multiple side effects and less safe for direct usage on skin. That's why there is utmost need to produce or develop such melanin inhibitors which have lowest skin disorders and maximum ability to suppress pigmentation (Chang, 2009). Many pigment inhibitors have been used in the current research including Kojic acid, Ascorbic acid, Turmeric, Peanuts and Aloe Vera. The maximum and effective inhibition of pigment was demonstrated when complete or partial discoloration of the fungus was observed. Kojic acid and Ascorbic acid was claimed as the strong inhibitors showing melanin inhibition when has been added to petri plate. Kojic acid has found to contain minimum stability at room temperature and maximum inhibition producing toxicity. Above mentioned drug shows side effects like skin irritation, skin burns, mutagenic impact on mammalian cells, poor efficacy and lower production stability (Seyedeh Mahdieh Hashemi, 2015). Aloesin, a chromone derivative that is a derivative of aloe vera and extracted from it. Its treatment is found to cause inhibition of hyperpigmentation(S. Choi, 2002). Based on our present results we demonstrated that Aloesin has a therapeutic effect on melanin formation suppression through its inhibitory impact on the tyrosinase activity.

Curcumin also down-regulates the expression of MITF (Microphthalmia associated transcription factor), TRP-1 (Tyrosinase related protein 1) and TRP-2 (Tyrosine related protein 2), which suggests that the curcumin- induced reduction of melanin content is due to the decreased MITF and melanogenic enzymes. It mainly suppresses melanogenesis in melanosomes and normal human melanocytes tissues. Curcumin-produced phosphorylation of Akt/GSK3β, ERK and p38 may lead to the anti melanogenic impact of the compound (Tu *et al*., 2012).

The peanut contains saponin, flavonoid and tannin which are phenolic compounds. When peanut shell or peanut is mixed with organic compounds like Ethyl acetate it gives flavonoid in the extract solution (Fithria *et al*., 2019). This phenolic compound has direct effect on melanin inhibition which is due to hydroxal group of phenolic compound forming hydrogen bond at the enzyme's active site which ultimately results in the lower tyrosinase activity at either first step or second step of melanin synthesis pathway (Alam *et al*., 2011) The production of dopachrome which is a substrate for melanin synthesis, is decided by activity level completed by tyrosinase. Brown color explains the reaction is processing at which level. The brown color of the product is the major determinant to check melanin inhibition. When skin color is darker it means more dopachrome is being synthesized in the melanocytes. But if skin color goes lighter it means dopachrome formation is low, Tyrosine is inhibited and not converting L-DOPA to dopachrome and then melanin the appearance of the brown color produced by the reaction between L-DOPA and the tyrosinase enzyme.

The brown color intensity will determine the dopachrome compound that is formed, meaning that the higher the intensity of the brown color, the more dopachrome compounds will be produced. If tyrosinase is inhibited, the formation of dopachrome will also be inhibited. The higher the inhibition by flavonoid, the lower the intensity of the brown color will be (Fithria *et al*., 2019; Soares *et al*., 2014).

CONCLUSION

Hyperpigmentation due to over production of melanin, in some specific areas of skin produce esthetic issues so inhibition of this pigment is necessary. Present study was designed to demonstrate the optimized production of melanin form filamentous fungi and its inhibition methods. Melanin extracted by Acid-Alkali method and its qualitative and quantitative analysis proved its production by *A. niger*. Melanin production was increased at pH 5.6 and 37°C. Results revealed that fungi have more tendency to produce melanin when dextrose and peptone were used as carbon and nitrogen sources respectively. Kojic acid and ascorbic acid inhibit melanin production at the concentration of 25% and 50% respectively. Turmeric showed maximum inhibitory mechanism at 25%. Maximum inhibition was seen by Aloesin and flavonoid which was 30% and 20% respectively. Study suggests that structural analysis of flavonoid and Aloesin may contribute to a better understanding of the mechanisms that may assist in the development of new techniques to treat melanin pigmentation.

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