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Original article

Effect of Different Incubation Temperatures, Times, and Colored Lights on Fungal Biomass and Black Pigment (Melanin) Production in *Exophiala crusticola*

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Abstract

Background: Adverse effects of synthetic pigments used in pharmaceutical and food industries and etc, have created a tendency toward the application of natural pigments. Environmental conditions are important factors in the growth and physiological function of different organisms. The aim of this study was to evaluate the growth rate of fungal biomass and production rate of black pigment (melanin) in fungus *Exophiala crusticola* under different incubation time, temperature, and light conditions to obtain an optimal condition for their production.
Materials and Methods: After obtaining an optimal incubation temperature, cultured fungus in potato dextrose agar and broth media was exposed to blue, yellow, white, red, green, and darkness light conditions with 14-35 days of incubation times. The average amount of produced dry weight of fungal biomass and pigment were measured, and the results were statistically analyzed with SPSS software ver.22.
Results: Suitable incubation temperature for fungal growth was 22°C.The maximum average amount of fungal biomass (0.17 g)and pigment production (OD = 0.94) were after 35 days of incubation ($p< .05$) and under yellow light (0.135 g) (OD= 0.98), respectively ($p< .05$).
Conclusion: By optimization the incubation time, temperature, and light for the growth and production of pigment in fungus *E. crusticola*, it is possible to produce a large amount of fungus and its related pigment in order to be utilized in a variety of industrial and pharmaceutical use, and etc. Also, due to the fungus rapid growth in response to the yellow light, it is possible to use this feature in isolation and early diagnosis of this fungus in suspected pathogenesis cases.

Key words: Fungal, Light, Biomass, Pigment, *Exophiala*

1. Background

Natural and chemicals pigments are applicable in many fields such as food, textile, cosmetic, pharmaceutical industries, and etc. (1-5). In recent years, the problems associated with synthetic origin pigments and their adverse effects on humans and environment have created a tendency toward the application of natural pigments. The main production sources of natural pigments are plants and microorganisms. But the use of plant pigments is problematic. For example, these pigments are unstable and sensitive to light, heat, and PH changes; they are also insoluble in water (6). But pigments derived from microorganisms have several advantages; they are more usable than the plant pigments due to their convenient, fast, and cheap production; independency to environmental conditions; and their color variations. Hence, there is a growing interest in industries for the bacterial pigments production. Pigments such as melanin are produced by microorganisms (4, 5, 7). Melanin has several functions in fungi, including resistant to ionizing radiation (8-10), resistance to UV radiation, resistance to lubricating enzymes and temperature changes, connecting to drug toxins (6, 11-14), and neutralize oxidizing agents (15-16). Nowadays, melanin is used in pharmaceutical industry for different purposes (6, 17-20), in radiotherapy (21), agriculture (22), cosmetic (23-25), and etc. *Dematiaceous* fungi and *Exophiala* species are among fungi species having melanin, and *Exophiala crusticola* as a novel emerging yeast-like fungus has attracted the attentions of many scholars (26-27). Because of the fungi important roles in nutrient cycles associated with plants, food and pharmaceutical industries for humans, understanding the role of environmental signals affecting their production can be useful in increasing the use of their beneficial effects and reducing their production cost (28). Light could have diverse effects on the microorganisms. Most of the studies conducted on light effects were concerned with mutagenic and lethal effects of UV radiation on microorganisms, and less attention has been paid to the effects of visible light. Recently, it has been reported that wavelengths above 400 nm have specific physiological and metabolic effects on the microorganisms (29).

2. Objectives

The aim of this study was to evaluate the growth rate of fungal biomass and the production rate of black pigment (melanin) in fungus *E. crusticola* under different incubation time, temperature, and colored light conditions to achieve an optimal condition for their production and growth.

3. Materials and Methods

3.1. Subjects

This laboratory trial study was carried out in medical mycology laboratory in Tehran University of Medical Sciences, Tehran, Iran. Standard strains of fungus *E. crusticola* (ATCC MYA3639) were cultured on the plates of Potato Dextrose Agar (PDA) (Merck, Germany) culture medium, prepared based on the instructions, in order to obtain the optimal incubation temperature required for the fungus growth. Plates were incubated in temperature conditions of 22, 28, 30, 33, and 37°C.

After 7 days, the growth rate or lack of growth was evaluated, and the optimum growth temperature for subsequent works was obtained.

3.2. The effect of different incubation times and colored lights on the production rate of fungal biomass

For this purpose, one milliliter of homogeneous fungal suspension grown up in PDA culture medium was added to each one of the tubes containing equal volumes of Potato Dextrose Broth (PDB) (Merck, Germany) medium with colored filters of yellow, red, blue, green, white (without colored filter), and darkness (covered with aluminum foil) (Figure 1). Cultures were incubated for 14, 20, 24, 28, 32, and 35 days in optimal incubation temperature while exposing to the moonlight and sunlight, then the cultures were passed through the Whatman filter paper, and their fungal biomass was dried in105°C temperature for 12-15 hours (3), then the dry weight of fungal biomass along with the control filter paper in each incubation time and color was measured by means of a sensitive digital scale (AND Scale EJ-303, 0.001 g, Japan). Then the average dry weight of the fungal biomass in all colors was calculated for each incubation time, as well as the average dry weight of fungal biomass in all incubation times was calculated for each color. From the beginning, tubes of each color were chosen as binary, and at the end, their average results were calculated.

Figure 1. Covering the PDB tubes media with different colored filters.

3.3. The effect of different incubation times and colored lights on the production rate of black pigment (melanin)

For this purpose, after culturing the fungus in PDA medium plates with colored filters of yellow, red, blue, green, white and darkness (Figure 2), they were incubated for 14, 20, 24, 28, 32, and 35 days at optimum incubation temperature while exposing to the moonlight and sunlight, then the amount of pigment produced in each incubation time and color was measured. For this purpose, 0.1 g of fungal biomass grown up on PDA medium was placed in 3 mL of distilled water and frozen at -20°C for 24 hours. After leaving the freezer and boiling in 121°C and the pressure of 15 atmospheres for half an hour, it was autoclaved for 15 minutes. Then lysis buffer with compounds of 1.0 $mg.mL^{-1}$ Proteinase K (Roche Laboratories, Japan) in reaction buffer (10.0 mMTris, 1.0 mM CaCl2, and 0.5% SDS, pH 7.8) was added to it and incubated in 50°Cwater bath for 1 hour, then 1 M NaOH was added in vitro tube and kept for 1 hour and centrifuged at 5000 rpm for 5 min, the supernatant was removed (30-32), and its optical density against the distilled water was recorded at a wavelength of 230 nm (30, 32) with a spectrophotometer (Unico UV / Vis 2100, USA). At the end, the average rate of pigment production in all colors was calculated for each incubation time, as well as the average rate of pigment production in all incubation times was calculated for each color. From the beginning, pipes associated with each color were selected as binary, and at the end, their average results were calculated.

Figure 2. Covering the PDA plates media with different colored filters.

3.4. Statistical analysis

Normality of data was assessed using the One-Sample Kolmogorov-Smirnov Test. After collecting and classifying the data, they were analyzed using One-way ANOVA or Repeated Measures ANOVA and Regression. The value of $P \leq .05$ was considered statistically significant. Statistical analyses were performed using SPSS software ver. 22.0 (SPSS Inc., Chicago, IL).

4. Results

Based on the obtained results, the fungus grew only at 22°C on PDA medium, and optimum growth temperature was reported to be 22°C. With increasing the incubation time, the production rate of fungal biomass and pigment increased in each colored light. Regression analysis showed significant correlation between incubation time and production rate of both fungal biomass and pigment (Figures 3, 4) (in both figures: $p < .05$, $R^2 = 0.9$). The highest average rate of fungal biomass (0.17 g) and pigment (OD = 0.94) production were after35 days of incubation time in sum of the colored lights (Figures 3, 4). Increase in production rate was different across the different colored lights.

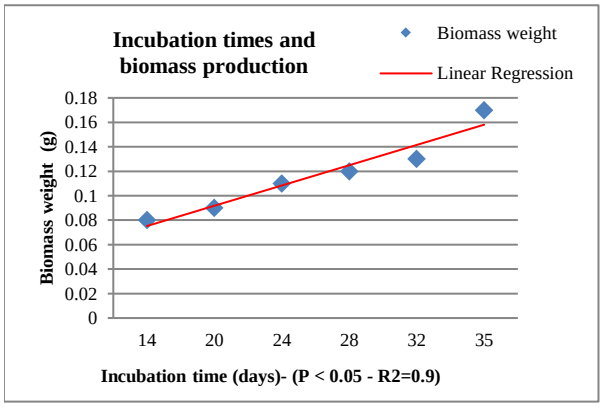

Figure 3. Effect of incubation times on fungal biomass production in *Exophiala crusticola*.

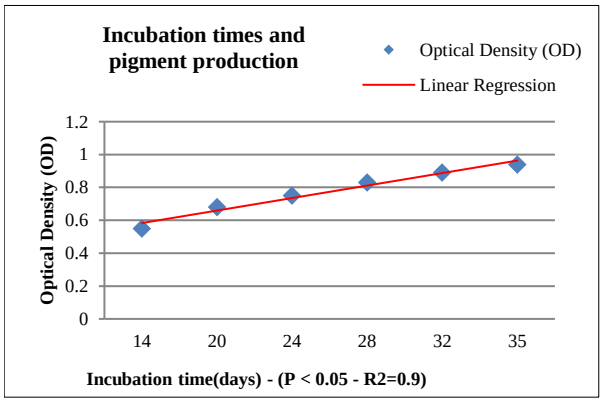

Figure 4. Effect of incubation times on fungal pigment production in *Exophiala crusticola*.

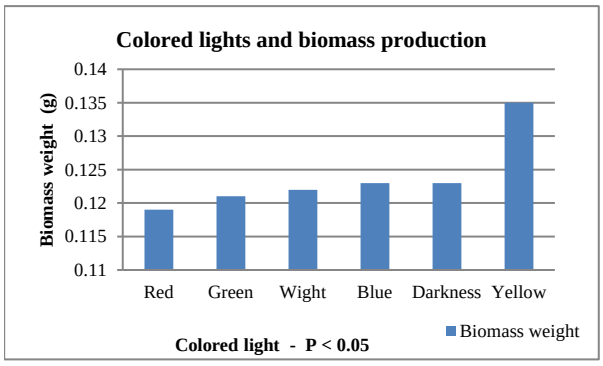

Figure 5. Effect of different colored lights on fungal biomass production in *Exophiala crusticola*.

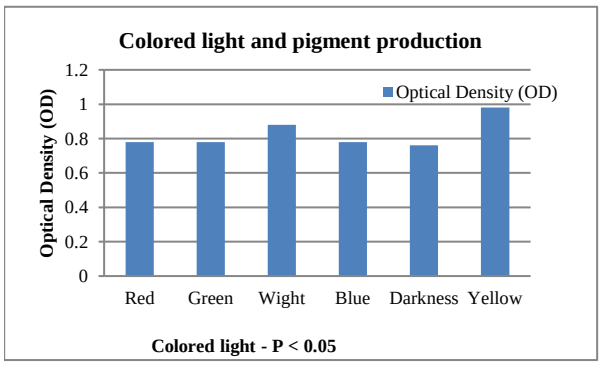

Figure 6. Effect of different colored lights on fungal pigment production in *Exophiala crusticola*.

One-way ANOVA analysis showed significant difference between colored light groups ($p< .05$), but in multiple comparisons of paired colored light (Post Hoc Tests), significant difference was observed only between some of them. The maximum average rate of fungal biomass production after 35 days of incubation (the total weight of fungal biomass in all incubation days associated with each colored light) was in response to the yellow light (0.135 g), followed by darkness, blue, white, green, and red-light conditions, respectively (Figure 5). There was a significant difference between the results of yellow light and other colored light conditions ($p< .05$), but in pair comparisons of other colored lights together, no significant differences were observed (in all: $p> .05$).

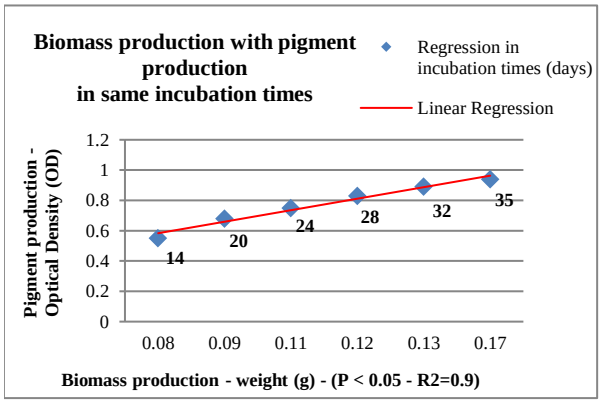

Figure 7. Regression analysis of fungal biomass and fungal pigment production in the same incubation times in *Exophiala crusticola*.

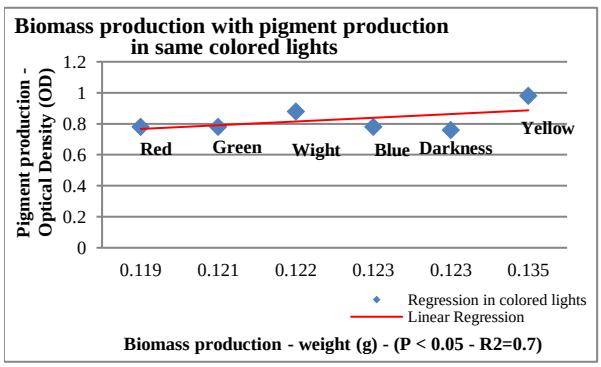

Figure 8. Regression analysis of fungal biomass production and fungal pigment production in the same colored lights in *Exophiala crusticola*.

The maximum average rate of pigment production after 35 days of incubation (the total optical density of all days associated with each colored light) was also in response to the yellow light (OD = 0.98), followed by white, green, red, darkness, and blue light conditions, respectively (Figure 6). There was a significant difference between the results of yellow light and other colored light conditions ($p< .05$). There was also a significant difference between the results of white light (without colored filter) and other colored light conditions ($p< .05$), but in pair comparisons of other colored lights together, no significant differences were observed (in all: $p> .05$). Regression analysis showed that in the same incubation times, there was a significant correlation between fungal biomass production and pigment production ($p< .05$, $R^2 = 0.9$)(Figure 7). Also, in the same colored light conditions, there was a moderate significant correlation between fungal biomass production and pigment production ($p< .05$, $R^2 = 0.7$)(Figure 8).

5. Discussion

For a long time, human societies have been interested in pigments. Passing the time and familiarity with microbes and sciences such as biotechnology made it possible to use microorganisms producing dye factors in various industries such as textiles, pharmaceutical, food, cosmetics industries, and etc (1-5), among which melanin pigments due to their interesting properties have attracted the attentions of many scholars. These pigments are produced in the body of many organisms such as humans, animals, plants, and microorganisms. In humans, due to pigments involvement in diseases such as Parkinson, Alzheimer, melanoma, and so on,

they are of great interest, and because of having various properties in the organisms' body such as fungi, they are considered as controversial (20). At present, in addition to those applications mentioned in the introduction of this study, the melanin pigments have other applications, for example, in protecting the environment (8, 33), paint industry (34), detoxification (6), making most of the appliances and devices that are in use today such as anti-UV filters, LCDs, and eye protectors (sunglasses and welding glasses) (25, 35-36). Therefore, it can be useful to study and research about them.

Fungi play important roles in many aspects of human life. As food sources in nutrient cycles associated with the plants and also from the medicinal viewpoint, they are of great importance for humans (28). For this reason, understanding the role of environmental signals affecting their production rate is very important in increasing the use of their beneficial effects and reducing their production costs. *Exophiala* species are commonly known as the black yeasts, and regardless of their pathogenic role in human diseases such as onychomycosis, subcutaneous lesions, chromoblastomycosis, eumycotic mycetoma, keratitis, sinusitis, allergic fungal Bronchopulmonary, infections emissions, and brain abscess (27, 36-37), they are considered as a group of fungi having melanin (36). *E. crusticola* as a known species is an opportunistic fungus. By taking into account its possible role in human disease (27, 36-37), *E. crusticola* species was used in this study for investigating the production rate of fungus biomass and black pigment (melanin).

Light is considered as a main source of energy required for the life on Earth and as an environmental signal for all living things. Light has several impacts on fungi; for example, it can regulate and guide the fungal growth, cause sexual and asexual reproduction, and cause formation of pigment. All these happenings are important in transmission and survival of fungi (28). Several studies have been conducted on the impact of light on fungi. *Idnurm* et al. (2005) studied molecular changes caused by lightening effects in fungus *Neurosporacrassa* and showed that White collar gene was responsible for light sensitivity, and the blue light was responsible for regulating carotenoid pigment production (28). In another study conducted by Deacon (1997), it was determined that the presence or absence of light affected proliferation or differentiation events in fungi *Trichoderma spp.* and *N. crassa*, and produced regional growth caused by asexual sporulation. It was also determined that this kind of growth was sometimes caused by the blue light (450nm), in which receptor containing flavin played an important role, and that Fruit body was produced in many of the Basidiomycetes in response to the light (38). In another study conducted by Osman and Valadon (1979), it was reported that spores of fungus *Verticillium agaricinum* were increased in response to the blue light (320-450nm), but their growth was delayed (39) while in the present study, it was revealed that most of the growth of the fungus *E. crusticola* was in response to the yellow light, and the maximum delay in its growth was in response to the red light (Figure 5).The low biomass production of *E. crusticola* under the red light in this study was in accordance to other studies conducted by *Babitha* et al. (2008) and Soumya et al. (2014) on *Monascus purpureus* and C*haetomium cupreum*, respectively (40-41); however, maximum fungus biomass production in mentioned studies was under white light, which was not in agreement with current study result about *E. crusticola*. In another study conducted by *Velmuruganet* al. (3) on five filamentous fungi *(Monascus purpureus, Isaria farinosa, Emericella nidulans, Fusarium verticillioides and Penicillium purpurogenum)*, the maximum fungus biomass production was in darkness condition, and minimums production was observed under the yellow light (3). These differences between the results of the present study and mentioned studies can be due to the cellular, physiological, and molecular characteristics of each fungus (e.g. existence or lack of responsive photoreceptors) in response to different lights.

Miyake et al. (2005) showed that red and blue lights affected the growth of fungus *Monascus* and caused in the production of secondary metabolites such as λ-aminobutyric acid, red pigment, Monacolin K, and Citrinin (42). Also, in another study conducted by Haggblom et al. (1979), it was found that in fungus *Alternaria alternate*, the blue light inhibited the production of mycotoxins alternariol and atlernariol monomethyl ether to the extent of 69-77%, but the red color had no effect on their production (43). In the current study, it was revealed that the yellow light caused in maximum production of black pigment (melanin) in fungus *E. crusticola* while the blue light and darkness reduced pigment production (Figure 6). These results are or are not in agreement with others studies. Therefore, in Soumya et al. (2014) study (41) on *C. cupreum*, the maximum fungus pigment production was under green and darkness light conditions while the minimum production was under white and yellow light. Of course, according to the OD value of 530 nm, *C. cupreum* pigment should be red pigment which is different from *E. crusticola* pigment. This difference seems to be justifiable as for the production of each colored pigments, special wavelengths of light are needed. In Babitha et al.'s study (2008) (40) conducted on *M. purpureus* to produce red and yellow pigments, the maximum production was under darkness condition, and minimum production was under the white light. In their study, blue and green lights had decreasing effects on pigment production, which is in accordance to current study. Another study conducted by Velmurugan et al. (3) on five filamentous fungi showed that the maximum average rate of pigment production in total of five fungi was under darkness condition, and minimum production was under yellow light. However, each fungus needs a selective wavelength of light for producing a special pigment. These observations indicate that every fungus is capable to sense and differentiate between light ranges and select and response to a special light for producing pigments (3).

Thus, considering the important roles of pigments in human life and in various industries, it is necessary to take into account the effect of different temperatures, incubation times, and colored lights on the growth and metabolite production of different fungi. These affecting factors, causing more activation or inactivation or less activation of certain genes, produce various results in each fungus. Incubation times give time to fungi for more growth, and consequently, more pigment production. In the fungal kingdom, light can regulate the growth rate and direction, sexual or asexual reproduction, and pigment formation. All of these are important features for the survival and dissemination of each fungal species. About colored light effect on biomass and pigment production, it could be explained by proposing the presence of photoreceptors responsible for selecting certain lights in each fungus (3, 40). In *E. crusticola*, it is suggested that incubation in yellow colored light was most effective in inducing the biomass and pigment production. The significant variation in pigment and biomass production in yellow light could be explained by the hypothesis of the existence of

photoreceptors that respond to yellow light. Despite the importance of light effect in fungal development and metabolism, there is a long way to explain the involved mechanisms and its influence on pigment production in *E. crusticola*. Thus, due to the fungi important roles in human pathogenesis, and the importance of their isolation and rapid diagnosis by using the environmental signals affecting their biological properties(the effect of incubation time and temperature and light on the growth and isolation in culture medium), it is suggested that further studies be carried out on the biological and molecular effects of lights on each fungus separately at different temperature and incubation time conditions in order to obtain useful and valid results in intended fields. Of course, the hue and amount of pigment produced by the fungi varies based on the type of strain, medium composition, and growth condition such as incubation times, temperature and type of lights (41). We evaluated the effects of three factors in our study. To evaluate the effect of several nutrition factors in medium composition, separate studies should be conducted to obtain and present comprehensive results.

6. Conclusion

As fungi play important roles in many aspects of the human life, for example, as food sources in nutrient cycles associated with the plants and pharmaceutical uses, understanding the role of environmental signals affecting their production can be useful in increasing the use of their beneficial effects and reducing their production costs. In the case of fungus *E.crusticola*, by optimizing temperature (22 ° C), incubation time (35 days), and special colored light (yellow light) for fungal growth and pigment production, it is possible to make use of various industrial and pharmaceutical benefits of the aforementioned fungus by mass production and extracting its related pigment. Also, by taking into account the fungus rapid growth in response to the yellow light, it is possible to make use of this feature in isolation and early detection of this fungus (*E.crusticola*) in suspected pathogenesis cases.

Conflicts of interest

There was no conflict of interest in the present study.

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Authors' Contributions

All of the authors contributed to this study.

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