

Original Research Article

Light influences Pigment, Biomass and Morphology in *Chaetomium cupreum* - SS02 - A Photoresponse Study

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A B S T R A C T

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Increasing consumer awareness on toxic synthetic dyes has invoked interest in study and production of natural colors. Fungi are known to be potential source for natural colors due to their easy culturing and downstream production. Pigments produced by these organisms are influenced by many environmental factors; one of them being light. The present work focuses on the photoresponse in *Chaetomium cupreum* to different wavelengths of light. The organism was isolated from soil and selected for the studies owing for its ability to produce extracellular pigment. The present work aims to study the influence of different wavelengths of visible spectrum on pigment and biomass production in *Chaetomium cupreum*. The study observes that green light incubation induced maximum pigmentation whereas yellow and white light incubation recorded low intensity and reduced pigment yield. In contrast, white and blue wavelength exhibited increased biomass production and red wavelength showed least biomass yield. There was a significant difference in the radial growth of the mycelia on solid media although the morphology showed less variation. This infers that photoreceptors are active as in other fungi and play a major role in pigment and biomass production. These findings are important and would likely assist in providing clue to the further research in *Chaetomium cupreum*.

Introduction

Light has a crucial influence on microorganisms due to its capability of inducing morphological and behavioral changes (Casas-Flores *et al.*, 2006). It induces varied response in nearly all forms of life including filamentous fungi. Fungal Photobiology shows the existence of greater convolution in responses for

visible spectrum of light (Herrera-Estrella *et al.*, 2007). Several types of photoreceptors (Molecules that receive and transduce the photon energy to promote a cell response) have been described in fungi (Corrochano 2007; Herrera-Estrella *et al.*, 2007). Many physiological processes such as growth,

the direction of growth, asexual and sexual reproduction, and pigment formation, all of which are very crucial aspects of survival and dissemination of fungi are regulated by light (Idnurm *et al.*, 2005). It is best studied and understood in *Neurospora crassa*, where in blue light is the type of light most associated with fungal photomorphogenesis, metabolic pathways (Liu *et al.*, 2003) and regulates circadian rhythms and other processes such as the synthesis of photo-protective pigments and spore formation (Miyake *et al.*, 2005).

A number of natural colorants though present, only a few are available in adequate quantity and are of industrial importance, as they are straightly extracted from different parts of the plants (Lauro 1991). Microbial pigments are interesting as potential alternative, owing to their nature, medicinal properties, nutritive value, expected yield, easy handling, safety, production being independent of season and geographical conditions [Latha *et al.*, 2010]. Increased consumer awareness of the safer natural colors has led producers to move on to the natural and non-synthetic colorants in textile industry (Nagia *et al.*, 2007), foods and cosmetics (Chiba *et al.*, 2006). It is therefore beneficial to produce colors from natural sources such as microbes. Some of the pigments successfully produced are from *Monascus* (Wong *et al.*, 1983; Yoshimura *et al.*, 1975) and *Serratia* (Trias *et al.*, 1988), however many microorganisms are yet to be explored for pigments.

C. cupreum is a widely distributed and abundantly found soil fungus that exhibits antagonism against numerous phytopathogens (Mao *et al.*, 2010). A commercial product Ketomium® has been

developed from this species, and is widely used as broad spectrum bio- fungicide for the disease control in various plants (Soytong *et al.* 2001). It is also known to biodegrade catechin, a well-known recalcitrant compound and a related enzyme catechin oxygenase (Arunachalam *et al.*, 2007), Azaphilones, a novel bicyclic anthraquinone has been successfully isolated from it. The red oosporein, from *C. cupreum* is known to have antifungal effects against *Rhizoctonia solani*, *Botrytis cinerea*, *Pytium ultimum* and many pathogenic fungi. It has also shown antitumor activity against HL-60 and A549 and acute toxicity against *Artemia salina* (Kanokmedhakul *et al.*, 2007). Its antifungal activity is being exploited for natural medicines (Kanokmedhakul *et al.*, 2006). Four compounds, rotiorinols A, C, stereoisomer (-) -rotiorin and rubrorotiorin isolated from *C. cupreum* is known to exhibit antifungal activity against *Candida albicans* (Vengurlekar *et al.*, 2012), the halogenated compound rubrorotiorin being the most active (Saleem *et al.*, 2010). Some species of *C. cupreum* are very good cellulase, laccase (Ankudimova *et al.*, 1999; Mimura *et al.*, 1999), and chitinase producers (Inglis *et al.*, 2002) and are implicated in biotechnological industry. Earlier the focus was on production of pigments from *Monascus* spp in various media. Its light-dependent growth and food industry applications were ornately studied by Pandey *et al.*, Carvalho *et al.*, and Babitha *et al.*, (1994, 2003; 2008). However, very little has been known about the influence of different colors of light on the growth and pigment production of *Chaetomium cupreum*. To overcome this lacuna, in this study, we have made an effort to examine the effect of different colors of light for the production of pigments and biomass by growing the fungus in submerged fermentation.

Materials and Methods

Isolation and Identification

Chaetomium cupreum was isolated from a litter sample collected from the GKVK campus, Bangalore. Isolation was carried out by the serial dilution method (Aneja 2003) on Potato Dextrose Agar (PDA) medium. The colony was subjected to morphological and microscopic observations. The morphological identity was confirmed by NFCCI, Agharkar Research Institute, Pune, India. To confirm the species, sequence analysis of the ITS region using universal primers (Forward primer, ITS 1 - TCCGTAGGTGAACCTGCGG and Reverse primer, ITS 4 - TCCTCCGCTTATTGATATGC) was performed. Nucleotide Blast to the obtained sequence was performed in NCBI (www.ncbi.nlm.nih.gov/) using blastn suite. The sequence was deposited in NCBI Genbank.

Extracellular Pigment Extraction and Quantification

For the extraction of the pigment, the culture was grown in Potato Dextrose Broth (PDB) at 30°C. The flasks were incubated for 7 days for maximum pigment production. The pigmented culture broth was filtered and was directly quantified (Dhale *et al.*, 2009), at 530nm in a UV-Visible spectrophotometer (Schimadzu UV – 1700, pharماسpec) to determine the absorption maxima (λ_{max}). The units were expressed as units of absorbance ml⁻¹ of the broth (Lee *et al.*, 2001). The uninoculated Potato Dextrose Broth was used as blank. The pigment was extracted to ethyl acetate, according to the protocol of Cho *et al.*, [Cho *et al.*, 2002] from the fermented broth. The extract was vacuum evaporated to dryness

to get the final yield.

Color stability at varying pH

The color sensitivity of the pigment in aqueous state was determined by changing the pH of the extract to 1, 3, 5, 7, 9, 11, 12 and 14 individually and quantifying the extract as discussed above. Absorbance maximum was noted to confirm the change in color of the pigment at varying pH.

Effect of Visible Spectrum of Light

The organism was cultured in 100ml of Potato Dextrose Broth (PDB) to study the effect of different wavelengths of light on biomass and pigment production. For the studies on cultural morphology, the organism was cultured on PDA in petriplates. Five millimeter mycelial discs bored out from the periphery of six days old culture were used for inoculation. The flasks and plates were wrapped in color glass papers of red, blue, green and yellow colors. A set of flasks and plates were covered with black art paper to completely cut off the light and hence incubated in darkness. Another set of flasks and plates were exposed completely to light source to record the effect of white light. The Principle behind the use of colored glass papers was that a colored glass paper allows only its particular color of light to pass through it whereas it filters out the other colors of the spectrum. All the flasks (Fig. 1a) and plates (Fig. 1b) were placed at equidistant (15cm) from the illuminated light source (Philips CFL 50 watts). After seven days of incubation, the pigment was quantified and extracted. The extract was concentrated in rotary vacuum evaporator and the concentrated extract was dried in a pre-weighed beaker to estimate dry solid. The final yield was recorded as mg l⁻¹.

Pigment Hue/ color

The final crude pigment was visually compared for color and texture variability.

Biomass estimation

Mycelium was separated from the broth by centrifugation and filtration after seven days of incubation. The separated mycelial mass was washed thrice with distilled water and dried overnight at 105°C in hot air oven. The dry weight was recorded as g l⁻¹. The radial growth (colony diameter) of the organism cultured on PDA plates was measured after incubation.

Cultural Morphology

The morphology of the culture was compared with respect to the hyphae, pigmentation and texture of the colonies after 7 days of incubation in PDA plates.

Statistical Analysis

All the results were analyzed by means of Multivariate ANOVA using SPSS for windows (SPSS Inc.) 11.5 version. Post Hoc analysis was performed using Scheffe test with significance $p < 0.05$.

Results and Discussion

Isolation and Identification

The isolated fungus was identified as *Chaetomium cupreum* based on morphological (Fig. 2a & 2b) and microscopic characteristics (Fig. 2c & 2d). Diffusion of deep red pigment into the media was observed around the colony on PDA plate, which suggested that it is water soluble.

The identification was further confirmed

by National Fungal culture collection of India and Fungal Identification Service (NFCCI & FIS), Agharkar Research Institute, Pune. The Culture was deposited in the National Fungal Culture Collection of India (NFCCI), with accession Number NFCCI – 3117. BLAST search performed for the sequence of ITS analysis, showed 99% homology with other strains of *Chaetomium cupreum* available in Gen bank. The sequence was deposited in NCBI Genbank with accession Number KF668034.

Quantification

The pigment showed absorption maxima (λ_{max}) at 530 nm indicating that it is a red pigment. The absorbance was expressed as Units of Absorbance ml⁻¹ (Fig. 3).

Color stability at varying pH

The pigment color at varying pH varied significantly. As the pH of the aqueous solution shifted to alkaline, the maximum absorption wavelength increased, associated with a variation in color. It turned yellow at acidic pH and deep orange at alkaline pH, though it remained deep red from pH 6 to 9 (Fig. 4).

Effect of Visible Spectrum of Light on pigment and biomass production

The results in this study indicated that pigment and biomass production varied significantly depending on the different wavelengths of light. Here, the culture under submerged condition was exposed to different colors and thus different wavelengths of light (Blue 492–455 nm, Green 577–492 nm, Yellow 597–577 nm, Red 780–622 nm, White and Darkness). Maximum pigment production was observed in green light incubation (Fig. 5),

followed by blue light and dark incubation. Though yellow and white light incubation led to least pigment production, they did not completely inhibit the synthesis of pigment. Increased pigment production was observed in total dark incubation as compared to the flasks completely exposed to white light.

The visual comparison of the extracted pigment exhibited varied hue (shades of color) to different wavelengths of light (Fig. 6). The white light incubation yielded a deep red colored pigment whereas yellow light incubation yielded a light red to orange colored pigment. The texture of the pigment also varied largely, yellow light incubated pigment was completely amorphous and the pigment from white light incubation was semi crystalline. Overall, as the shade of the pigment grew darker, the texture turned towards crystalline and the lighter shaded pigments exhibited amorphous nature.

On the contrary to the above observation, white and blue light, as well as dark incubation favored biomass production whereas red light incubation reduced the biomass yield (Fig. 7). The growth was also measured on solid media in terms of colony diameter. White light incubated colony exhibited highest radial growth and red light incubated colony showed the least colony diameter on the solid media. However, green and white light incubation did not have much effect on biomass production. This observation suggests that white light induced the biomass production in this organism, unlike in pigment synthesis where it reduced the pigment yield.

Cultural morphology

The effects of different wavelength of the visible spectrum on the cultures were

analyzed by comparing the visible phenotypic characters observed on the PDA plates after 7 days of incubation (Fig. 8). The colonies grown under blue and white showed profuse growth of mycelia, denser and pigmented towards the center. The colonies grown under red, yellow and green light showed denser mycelial growth at the center and scanty growth towards the outer circle, whereas the colonies under dark incubation showed very dense mycelia growth at the center and completely hyaline and scanty growth at the periphery. This observation showed that the incubation in white light and darkness increased the biomass production and also exhibited variation in cultural morphology.

Regulation of fungal growth and behavior is a prominent example of the effect of light on fungi (Corrochano *et al.*, 2006). In several model fungal species the effect of light has been studied. While the morphological effects and spectral analyses of light have been well characterized in basidiomycetes (*Coprinus*) and zygomycetes (*Phycomyces*), it is best understood based on the functions of the white collar genes (WC-1 and WC-2) in light sensing. (Kues 2000; Cerda –Olmedo 2001; Liu *et al.*, 2003). Despite the importance of the light in fungal development and metabolism, there is a lot left unexplored to explain the mechanisms and its influence on pigment production in *C. cupreum*.

The color of the pigment was found to be sensitive to pH. Similar variation in the color of red pigment isolated from *Paecilomyces sinclairii* and *Isaria farinosa* was also observed by Cho *et al.*, (2002) and Velmurugan *et al.*, (2010) respectively. This shows that this pigment

Figure.1 (a) Experimental set up to study the effect of light on pigment production and growth in *Chaetomium cupreum* in submerged fermentation (b) On Solid Media.



Figure.2(a) Morphology and Pigmentation of *Chaetomium cupreum* on PDA plate. (b) Reverse side of the plate. (c) Microscopic view of the ascocarp and (d) ascospores of *Chaetomium cupreum*.

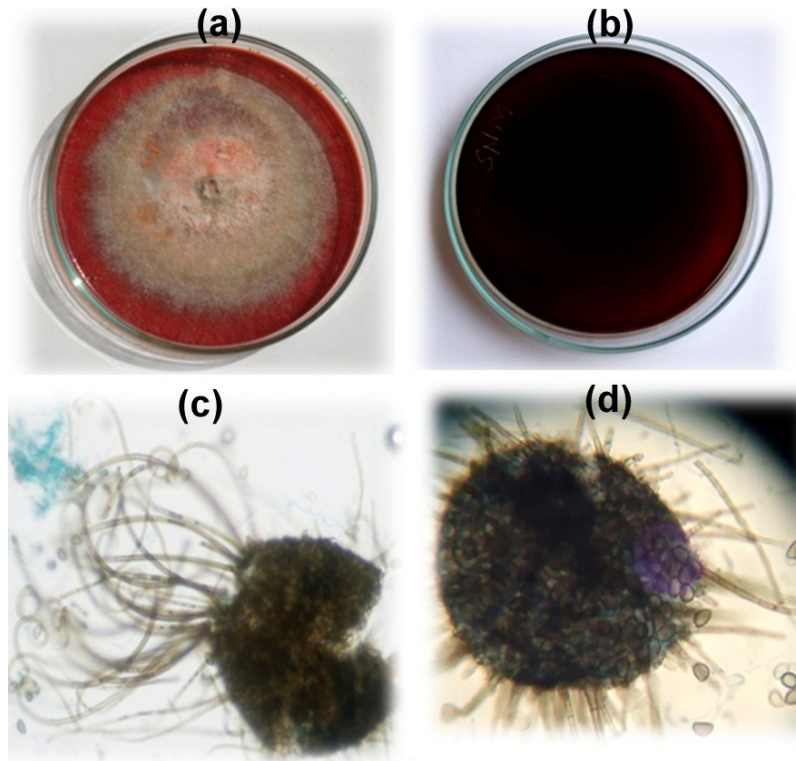


Figure.3 Spectrophotometrical Quantification of the pigment of *Chaetomium cupreum*

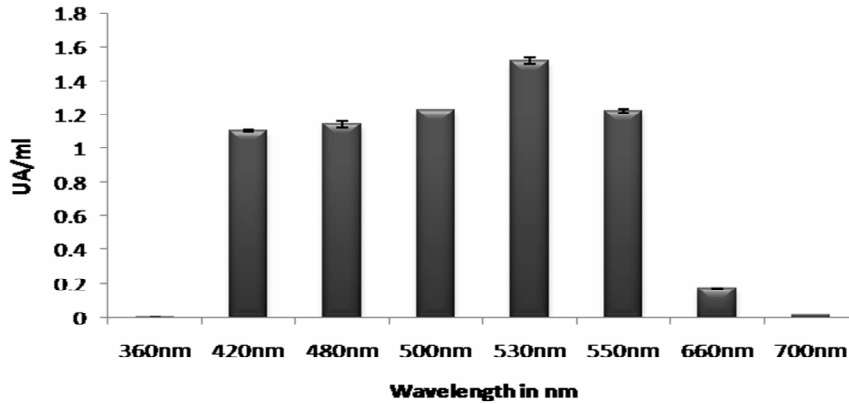


Figure.4 Sensitivity of the pigment of *Chaetomium cupreum* to varying pH

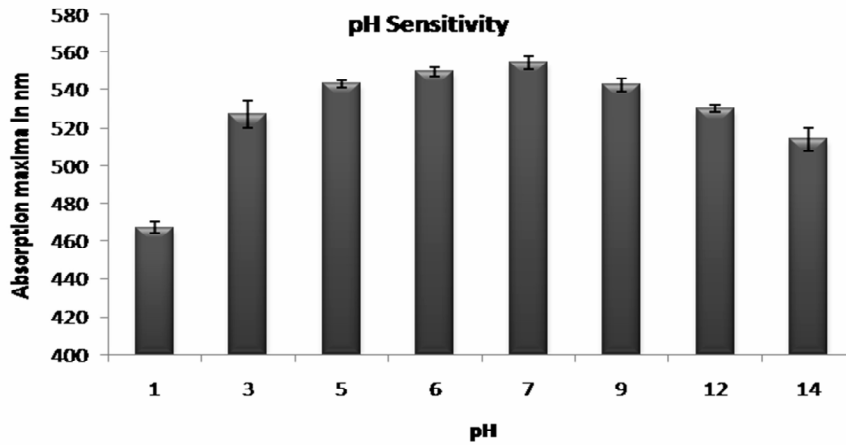


Figure.5 Effect of different wavelengths of Light and darkness on pigment production of *Chaetomium cupreum*: Scheffe post hoc test: Means sharing different superscripts are significantly different (P<0.05)

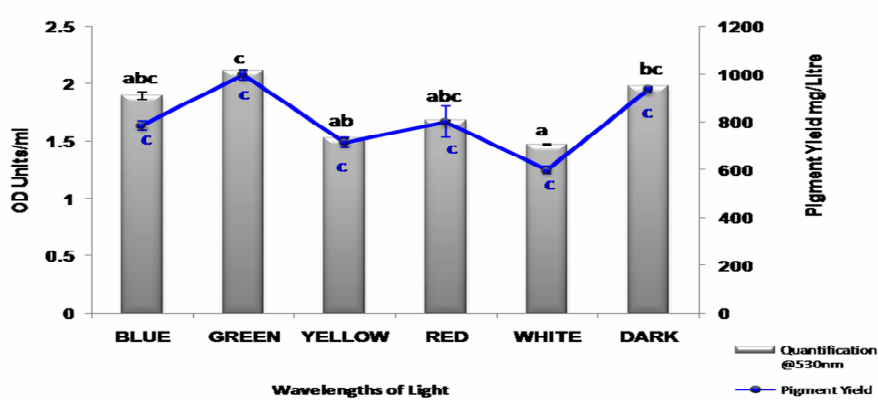


Figure.6 Effect of different wavelengths of Light and darkness on pigment hue of *Chaetomium cupreum*

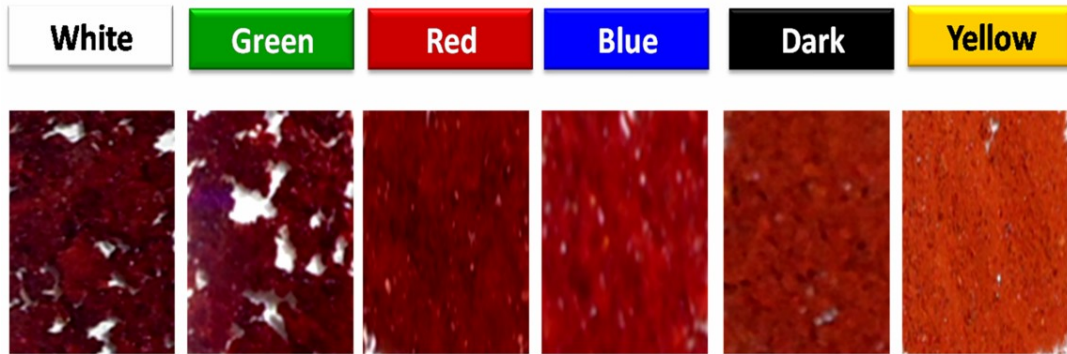


Figure.7 Effect of different wavelengths of Light and darkness on growth and biomass production of *Chaetomium cupreum*: Scheffe post hoc test: Means sharing different superscripts are significantly different (P<0.05).

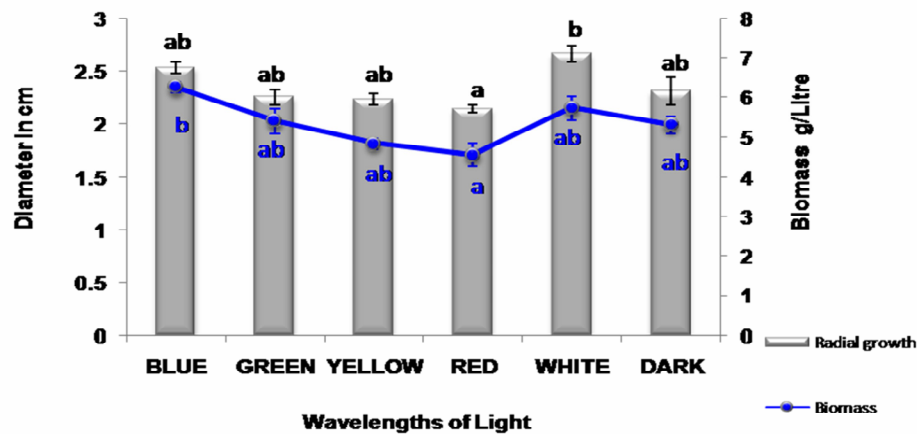


Figure.8 Effect of different different wavelengths of Light and darkness on morphology of *Chaetomium cupreum*



is sensitive to pH. This property of the pigment can be exploited for various industrial demands.

The results of effect of visible spectrum of Light on pigment and biomass production, agrees with that of (Babitha *et al.*, 2008) and is significant as it is in contradiction to the postulated photo-protective role of pigments according to (Yong *et al.*, 1991; Salih *et al.*, 2000; Seagle *et al.*, 2005). In *N. crassa*, blue light induces the carotenoid pigment production.

The significant variation of pigment and biomass production in white light and darkness could be explained by the hypothesis of the existence of photoreceptors responsive to darkness and the presence of light in this fungus (Velmurugan *et al.*, 2010). Understanding the role of environmental stimuli in fungal development is very important to increase the benefits and reduce the costs that fungi present (Idnurm *et al.*, 2005). The pigment hue produced by the fungi varies by strain, medium composition, and growth Condition and is greatly affected by the medium composition [Jung *et al.*, 2003]. It is evidenced in *Trichoderma atroviride*, that the blue-light perception system establishes a cross-talk with that involved in red light perception, which is reflected at the level of mycelial growth. Although the blue region of the spectrum provides the dominant signal, the green and red photoresponses have often been overlooked (Herrera-Estrella *et al.*, 2007). The wavelengths of light from UV to far-red can induce responses in the members of the fungal kingdom. However, of late only one photoreceptor class (blue light sensors) had been identified in the fungi. Photoresponses mediated by the photoreceptors that absorb blue light constitute the majority of photoresponses

(from the growth responses to phototropism) studied in the fungi (Kumagai 1988; Lauter 1996). Other classes of photoreceptors are also being identified recently. Blumenstein *et al.*, (2005) have reported a red light sensing via phytochrome in the model fungus *Aspergillus nidulans*, Hoff *et al.*, (2010) have reported two components of a velvet-like complex that control hyphal morphogenesis in *Penicillium chrysogenum*. Hurley *et al.*, (2012) have worked on the light inducible photoreceptor system for tunable protein expression in *N. crassa*. Retinal-reconstituted Nop-1 is a green-absorbing pigment while *Neurospora* responds to blue light; Saranak and Foster (1997) concluded that in the chytrid *Allomyces reticulatus*, a rhodopsin is responsible for zoospore phototaxis. WCC complex, Opsin and velvet photoreceptors have been identified in *Chaetomium globosum* (Rodriguez-Romero *et al.*, 2012). Very recently a Poly Ketide Synthetase (PKS) gene – pks-1 has been identified to be involved in chaetoglobosin biosynthesis, pigmentation and sporulation in *Chaetomium globosum* (Hu *et al.*, 2012), but the photoresponse studies in *C. cupreum* have not yet been reported. The phytochromes were thought to be found in photosynthetic organisms only, but recently it has been discovered even in the fungi and heterotrophic bacteria, where little is known about their functions.

The experiments on the effect of different wavelengths of visible spectrum in this study revealed that the incubation in green light was most effective followed by darkness and blue light, in inducing the pigment production. The hue and texture of the pigment also varied greatly with white light incubation producing the darkest shade of the pigment. The colonies

grown under the direct white light exhibited reduced pigment production; thus, postulating the existence of photoreceptors in this fungus, responsive to darkness and light. This photoresponse on pigment production, when compared with the other reported photoresponses in fungi, is quite different. The operation of the phytochrome type of system in this fungus is shown by the physiological and morphological responses. Varying pigment concentrations suggested the loss of pigments which is unexplainable as a change in pigment location. One possible explanation for degradation in pigments might be because of an enzymatic pathway, which may be induced by nutrient exhaustion. A common phenomenon observed in fungi is that the secondary metabolites are degraded by enzymes (Johns *et al.*, 1982). The pigment of *Chaetomium cupreum*, in the present study is extracellular and hence significant as it is water soluble and possesses simpler and cheaper downstream processing. Probably the induction and reduction of pigment synthesis could be due to the varied light intensities (intensity of the green light usually lower than white light). Therefore, to gain comprehensive insight into the regulation of pigment biosynthesis induced by light, many parameters need to be explored and we expect that the present study provides a strong approach towards attaining this goal. The study also assures the possibility of establishing a relation between the photoreceptors, light regulated genes and the photoresponse in *Chaetomium cupreum* and may probably unravel the possible functions interplaying between the different light control systems. Furthermore, these studies may also help in significant commercial applications pertaining to the use of pigments.

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