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Light Requirement for the Carotenoids Production by *Mucor hiemalis*

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Abstract

Objective
Fungi produce many different carotenoids and some are attractive in medical and industrial sources. In this work the ability of *Mucor hiemalis* (PTCC 5292) to produce carotenoids in media with different nitrogenous and carbon sources and incubation with white, yellow, blue and red lights (15W, 220V, E27) against dark-grown were studied.
Materials and Method
The microorganism cultivation in SDA medium, with or without aeration (120 rpm) at 25 °C. Mycelia were collected and dried at 50 °C. The dried mycelia were homogenized in hexane, acetone and H_2SO_4 (0.5 M) solvents. The carotenoids determined by TLC and HPLC methods.
Results
The results showed that *M. hiemalis* accumulated astaxanthin (mono-esters, di-esters and free), echinenone and canthaxanthin in the mycelia in different conditions. Blue and white lights incubation was the best for production of carotenoid pigments with 1.2 and 1.33 mg/g dried mycelia respectively, but the red light incubation not only did not have an amplifying effect on the production of carotenoid but also slightly reduced this effect. Also, the effect of intervention of lactose sugar showed more effectiveness in producing carotenoid than yeast extract and dextrose or in the presence of both of them.
Conclusion
The information reported in this study on the comparative ability of *M. hiemalis* for producing carotenoids, should be useful for assessing the biotechnological production of carotenoid pigments if it incubates with white or blue lights.

Keywords: Carotenoids, HPLC, Light, *Mucor hiemalis,* TLC

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Introduction

Carotenoids are members of the terpenoid family of compounds which are characterized by their polyunsaturated nature. The most common terpenoids in nature are carotenes *(α,* β, γ and δ), lycopene and some alcohol derivatives, such as xanthophyll, zeaxanthin, lycophyll, and cryptoxanthin (1). These compounds are the most widespread naturally occurring yellow, orange and red pigments. The abundance of carotenoids in nature is probably due to their relatively simple biosynthetic pathway, which has been demonstrated in higher plants and algae, also, in bacteria and yeasts but not in humans (2). Carotenoids are considered to provide a wide range of beneficial effects. Reducing the risk of certain cancers as well as of cardiovascular and ocular diseases are postulated (3). Various natural carotenoids such as alpha-carotene, lutein, zeaxanthin, lycopene, beta-cryptoxanthin, fucoxanthin, astaxanthin, capsanthin, crocetin and phytoene were proven to have anticarcinogenic activity (4). Sesso *et al* showed that, higher plasma lycopene concentrations are associated with a lower risk of cardiovascular disease (CVD) in women (5). Serum carotenoid levels were inversely related to coronary risk. Nonsmokers with high carotenoid levels showed a 72% reduction in coronary events. These findings suggest that carotenoids may be protective against coronary heart disease in middle-aged men with high cholesterol levels (6).

Carotenoids are used as pigments to color the skin or egg yolks in poultry, to color the flesh of fish grown under aquaculture conditions, and to color the shells of crustaceans and also, widely used as colorants in food for human consumption and also, as constituents in vitamins and dietary supplements (7). Thus, a comprehensive screening of the microbial carotenoid spectrum could help to identify novel compounds, providing beneficial effects. The production of carotenoids from biological sources has been an area of intensive investigation. Because of the inherent biosynthetic capacity of a variety of different organisms, there has been a considerable effort to develop systems to produce carotenoids commercially from biological hosts. In bacteria and yeast, carotenoids have been considered as typical secondary metabolites, playing a certain role in the survival of the fittest microorganism. Recently, staphyloxanthin was related to the survival of *Staphylococcus aureus* in infected hosts and in evading the immune system (8-9).

There are several commercial operations currently used to produce carotenoids for human as well as animal consumption. The production of β-carotene by the algae *Dunaliella* sp., astaxanthin from *Phaffia* sp. and *Haematococcus pluvialis*, canthaxanthin from *Corynebacterium* sp. and zeaxanthin by *Flavobacterium multivorum* also, was extensively studied (7).

Eubacteria such as fungi are of special interest to carotenoid researchers because they are easily grown and manipulated in the laboratory. They may also serve as producers of carotenoids of commercial value, notably astaxanthin, a pigment which is essential for the aquaculture of salmonid fish and is also used as a neutraceutical.

Light is a very important environmental signal that regulates development and metabolism in most organisms (10). The capacity of sensing and responding to light is also widespread in non-photosynthetic organisms such as bacteria and fungi. In fungi, several developmental and physiological processes have been reported to be influenced by light (11). The most prominent light response in the ascomycete *Neurospora crassa* is the light-regulated biosynthesis of the photo-protective carotenoids (12). Only traces of carotenoids can be detected in dark-grown mycelia, resulting in an almost white phenotype. Upon illumination with blue light, all carotenoid biosynthesis genes are up-regulated on a transcriptional level, leading to a fast accumulation of orange-colored carotenoids (13). Other *Neurospora* responses to light include the light entrainment of the circadian clock, the formation of spores and phototropism (14). Formation of spores in

fungi like *Mucor* sp. was determined by black color and carotenoids extraction from black colony of microorganisms will be unexpected.

So in this work the ability to produce carotenoids from *M. hiemalis* was studied. We described a Light requirement for carotenoid biosynthesis in *M. hiemalis*. The protocol requires only standard laboratory equipment and access to an analytical TLC and HPLC; for extraction and purification of pigments.

Materials and Methods

Microorganism and culture media

Mucor hiemalis (PTTC 5292) was originally obtained from Persian Type Culture Collection (PTCC), Tehran-Iran. The strain conserved by routine inoculation and incubation in Sabouraud dextrose agar (SDA) medium (Merck) at 25 °C for 72 hr, and then kept by refrigerating for 3 to 4 months.

Medium, inoculation and different light incubation for pigment comparison

From the stock cultures of *M. hemalis* an inoculation, was made over the surface of sterile SDA in petri dishes, and incubated for 3 days. After incubation, a spore suspension was prepared by addition of 5 ml of Tween 80 at 0.1%, directly over the surface of inoculated petri dishes. The spore suspensions were standardized to 1×10^6 spores/ml by addition of sterile water, counted also as "spores" intact mycelial fragments. The count was performed using a Neubauer chamber (on a microscope). The inoculum (0.5 ml of spores suspension) transferred to 500 ml fermentation flasks [containing 100 ml sabouraud dextrose broth medium (SDB) (Merck)] and solid medium (SDA) in petridishes, respectively. These flasks and petri-dishes were incubated under different light condition such as white, yellow, blue and red light (15 W, 220 V, E 27) and dark-grown at 25 °C for 10 days, then mycelia collected and dried in 50 °C oven for 48 hr and the content of pigments was determined.

Pigment extraction and TLC analysis

M. hiemalis biopigments were extracted from mycelia collected from fermented media and petri-dishes using, hexane, acetone and 0.5 M H_2SO_4, as solvent separately with the proportion of 5 ml solvents / g dry and wet fermented mass, with and without occasional agitation, for 24 hrs, then centrifuged for 20 mins at 4000 rpm. The colorful supernatant concentrated by solvent evaporation at room temperature and dry mass of pigment yield was calculated. Concentrated pigment subjected to TLC, using silica gel 60 F Merck TLC paper spotted on TLC plates and eluted with a mobile phase of petroleum ether: acetone (10:4), hexane: acetone (3:1), benzene: ethyl acetate (1:1) (15). The fractions were collected and compared with pure astaxanthin as a standard carotenoid sample.

Spectrophotometeric assay

The concentrated pigment dissolved separately in acetone and hexane. λ_{max} estimated using UV-VIS scanning spectrophotometer (UV 2101 pc, SHIMADZU) and measuring the absorbance at 200 - 500 nm. Total pigment content determined in dark-grown and illuminated with different lights.

Optimization of conditions for carotenoid extraction

The conditions for extraction were optimized with the effect of a combination of variable processes (factors), such as lactose 1%, dextrose 1%, yeast extract 1% and the combination of them (with or without shaking at 120 rpm) added to the fermentation medium and their interactions with the variable result was determined after incubation with white, yellow, blue, red lights and dark-grown. Each sample mixture at three equidistant levels, and the variable result was the carotenoid yield. In total, combinations of factors were used. The extraction and determination of carotenoid pigments were carried out as explained earlier.

Quantification of carotenoid production by HPLC

The microorganism was cultivated in a medium (1 liter) containing 60 g xylose, 3 g asparagine, 0.008 mg thiamine, 0.50 g potassium phosphate and 0.25 g magnesium sulphate (pH=6.5). Fermentations were carried out on a rotary shaker (120 rpm) at 25 °C. For carotenoids extraction, the harvested dry mycelia were homogenised in hexane/methanol (1:1, v/v)

and the hexane phase containing carotenoids obtained by adding distilled water, followed by centrifugation. The carotenoids were determined by HPLC (stainless steel C_{18}) and elution was performed using an isocratic solvent of methanol at 1 ml/min. The column was pre-equilibrated with 87.5% (v/v) methanol. At 6 and 16 mins post-injection the eluent was changed to 95% (v/v) methanol and to 100% methanol respectively. Pure astaxanthin was eluted by methanol at 1 ml/min and analysed using PMT detector (SPD-6AV) at λ_{max} 490 nm as a standard sample (16).

Statistical analysis

All determinations were carried out in triplicate. Data expressed as mean±SD, analyzed by variance analysis (P<0.05) and the means separated.

Results

Colonies of mold were very fast growing, cottony to fluffy, white, became dark grey with the development of sporangia. The solvent extracted carotenoid after concentration changed to the form of a paste with a yellow-orange color. Dried mycelia showed better result than the wet one, for extraction of carotenoid pigments. The highest number of carotenoid fractions (in TLC method) from treated mycelia with different lights, obtained when the carotenoids were extracted with hexane, followed by the solvent group (1 or 2), acetone and hexane (Table 1).

Table 1. Extraction of carotenoids in different lights incubation by 3 groups of solvents.

Solvent / Light	Group 1	Group 2	Group 3
	Rf values (mm)		
White	0.35[2], 0.5[1], 0.6[6], 0.77[3]	0.36[2], 0.52[1], 0.63[6], 0.78[3]	0.42[4], 0.34[2]
Blue	0.41[4], 0.52[1], 0.72[3]	0.49[1], 0.65[6]	0.48[1], 0.83[5]
Yellow	0.56[6], 0.72[3], 0.84[5]	0.41[4], 0.53[1], 0.76[3]	0.42[4], 0.87[5]
Red	0.61[6], 0.86[5]	0.48[1], 0.85[5]	0.47[1]

*Group (1): acetone, *Group (2): hexane, *Group (3): Sulfuric acid, *Mobile phase: acetone: hexane (1:1) / Ethanol for acid solvent
* [1]: Astaxanthin monoesters, [2]: Astaxanthin free, [3]: Astaxanthin di-esters, [4]: Canthaxanthin, [5]: Echinenone, [6]: Unknown (standards to confirm the carotenoids according by Lorenz and Todd, 1998).

The lowest carotenoid yield was obtained by using the solvent group 3 (H_2SO_4). Mycelia of *M. hiemalis,* which were treated with blue, white light and dark-grown, showed a series of positive peaks at 476, 518 and 478 nm, respectively (Figure 1a-d). Results of HPLC, indicated that the retention time of fraction (RT= 5 min) was identical with the standard astaxanthin (Figure 1-d).

Blue and white light incubations were the best for canthaxanthin and astaxanthin production respectively. These fractions had fluorescence property under UV cabinet at 366 nm. The red light incubation not only did not have an amplifying effect on the production of carotenoid but also, slightly reduced this effect. Rf values varied slightly, using the standards to confirm carotenoids (Table 2) (15). Rf value 0.33, was determined for the pure astaxanthin.

Table 2. Rf values using standards to confirm the carotenoids.

Carotenoid	Typical Rf value
β-carotene	0.99
Echinenone	0.87
Astaxanthin Di-esters	0.75
Astaxanthin Monoesters	0.50
Canthaxanthin	0.40
Astaxanthin Free	0.33
Lutein	0.25

Our results showed that the carotenoids were the predominant in dry mycelia, with incubation in red, dark, yellow, blue and white lights, in the amount of 0.3, 0.45, 0.83, 1.2 and 1.33 mg/g dry mycelia, respectively. Optimization of conditions for carotenoid extraction in white or blue light incubation showed that, when lactose was added to the fermentation medium without aeration, it gave a higher carotenoid yield than added the yeast extract or dextrose with aeration (at 120 rpm) (Table 3). However, the combination of them had a decreasing effect on the carotenoid yield.

Table 3. Optimization of carotenoids extraction in white light incubation by 3 groups of solvents.

Solvent / Media component	acetone	hexane	Sulfuric acid
	Rf values(mm)		
Fermentation medium+Lactose with aeration	0.85[5],0.71[2], 0.61[4]	0.84[3]	-
Fermentation medium+Lactose without aeration	0.83[3], 0.68[4], 0.57[4]	0.78[2], 0.61[4], 51[1]	-
Fermentation medium+Lactose+Dextrose with aeration	0.79[2]	0.83[3], 0.66[4]	-
Fermentation medium+Lactose+Dextrose without aeration	0.82[3]	0.75[2]	-
Fermentation medium+Lactose+Yeast extract with aeration	0.85[3]	0.85[3]	0.86[3]
Fermentation medium+Lactose+Yeast extract without aeration	0.7[2], 0.68[4]	0.71[2], 0.47[1]	0.47[1]
Fermentation medium+Dxtrose+Yeast extract with aeration	0.83[3], 0.78[2]	0.82[3]	0.66[4]
Fermentation medium+Dxtrose+Yeast extract without aeration	0.82[3],0.78[2]	0.79[2], 0.75[2]	0.7[2]

*Mobile phase: acetone: hexane (1:1) / Ethanol for acid solvent
*[1]: Astaxanthin Monoesters, [2]: Astaxanthin Di-esters, [3]: Echinenone, [4]: Unknown (standards to confirm the carotenoids according by Lorenz and Todd, 1998).

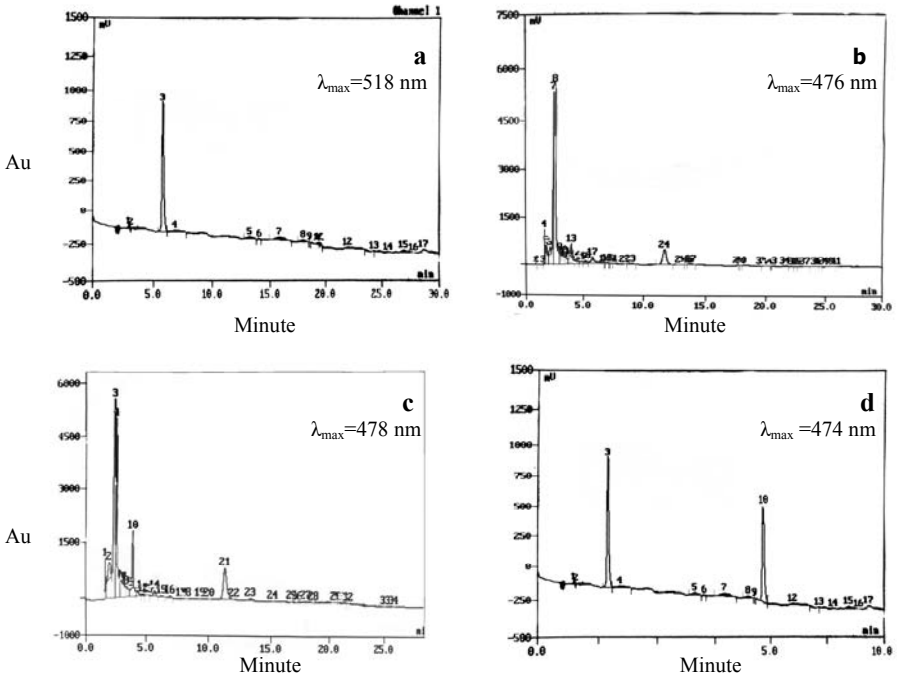

Figure 1. HPLC chromatograms of the carotenoids obtained from *Mucor hiemalis* mycelia incubated in different conditions (a) white light, (b) blue light, (c) dark and (d) pure astaxanthin as a blank sample. Detection was at 450 nm. Peak identification: 4, 8-canthaxanthin, 3, 10-astaxanthin, 1- ß-caroten, 21- lutein.

Discussion

Determination of microbial carotenoids, gains an increasing importance in biotechnology, food industries and in medicine. Hence, development of a method to determine correct amounts of carotenoids and changes in its spectrum appear to be of particular interest. To date, several methods have been developed for pigment extraction, including mechanical breakage and acid or alkaline hydrolysis (16, 17).

In this study, some of polar and non-polar solvents used for the extraction of carotenoids from *M. hiemalis* mycelia and they produced the highest yield. The extraction yield differed significantly ($P<0.05$) between solvents. Group 1 and 2 solvents gave significantly ($P<0.05$) higher yield than group 3. Sulfuric acid could produce stronger colored phase than the other two solvents, from mycelia but there was no significant band observed in TLC method. Some pigments could be damaged by acidic pH. Britton *et al* recommended the use of water miscible polar organic solvents, usually acetone, methanol or ethanol, for the extraction of carotenoids (18). Delgado-Vargus *et al* discussed the advantages and disadvantages of various organic solvents for the extraction of carotenoids and suggested that polar solvents are generally good extraction media for xanthophylls but not for carotenes (19). The results showed that dried mycelia were the best for carotenoid extraction. According to Delgado-Vargus *et al* use of non-polar solvents is not recommended for the wet sample as their penetration through the hydrophobic mass that surrounds the pigment is limited (19). De Ritter and Purcell (1981) postulated that, the complete extraction of carotenoids from plant tissues could be achieved with samples of low moisture content, by the use of slightly polar plus non-polar solvents (20).

The results indicated that astaxanthin monoesters, astaxanthin di-esters and astaxanthin free were in *M. hiemalis* mycelia, incubated with white light. However, the major pigments in incubation with blue, yellow and red lights the major pigments were astaxanthin monoesters, astaxanthin di-esters and echinenone respectively. Otherwise astaxanthin di-esters and canthaxanthin, canthaxanthin and echinenone, could also, be separated in incubation with blue and yellow lights respectively. Casas-Flores *et al* reported that blue light is a potent stimulus with the potential to induce harmful photosensitized reactions inside cells; most organisms compensate for this in ways that are not always obvious (21). Fungi are no exception: *N. crassa* responds by synthesizing screening pigments and the soil fungus *T. atroviride* sporulates. The effect of light on growth rate shown, resembles that of stressful conditions such as nutrient limitation and high temperature. It is thus possible that the blue-light regulators (BLR) proteins have a more general role in dealing with stress (21).

Dark-grown incubation had already a carotenoid content of 0.45 mg/g weight which increased 3-fold within 72 h of continuous white-light illumination and 2.6 and 1.8-fold for blue and yellow light illumination during the same period, respectively. Woitsch and Romer reported significant accumulation of these pigments occurred after a lag phase at later stages of light-dependent chloroplast differentiation (22). The carotenoid content was not significantly different between white or blue incubation, although there was a tendency toward elevated carotenoid levels in incubation with white light. In contrast, only trace amounts of carotenoid pigment could be detected in red-grown incubation. Carotenoid composition was investigated by HPLC. For screening purposes as well as assays of the effects of physiological or genetic manipulations on carotenoid production, a fast, reliable, widely applicable and inexpensive method to characterize the carotenoid profile of a test organism must be available. Such a method consists of two parts, viz. extraction which must be total and chromatographic analysis which must be unambiguous. The latter aspect is particularly important in view of the many mis-identifications of carotenoids in the past, based mainly on their UV–Vis absorption spectra and migration patterns on TLC plates. HPLC-based analyses of crude samples for carotenoid composition are

important also, in food chemistry and medicine (23). HPLC chromatograms indicated that astaxanthin, cantaxanthin, ß-caroten and lutein were the major carotenoids in the extract from mycelia, incubated with different lights. There was a significant change in the relative amounts of ß-carotene, lutein, cantaxanthin and astaxanthin, concerning the various light regimes.

This experiment conducted to prove that the static condition was more preferable to the test organism than the shaker grown condition to produce carotenoid pigment, otherwise, fungi are aerobic microorganisms. So *M. hiemalis* also, needs aeration but in fermentation medium fungi grow like a sheet on the surface. Shaking incubation could damage and fragment the mycelia. Many microorganisms reported to produce carotenoid pigments, such as strain TM (24) and *Agrobacterium auranticum* (25), which are aerobic.

Conclusion

The information reported in this study, on the comparative ability of *M. hiemalis* for producing carotenoids, should be useful for assessing the biotechnological production of carotenoid pigments.

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