Light Activation of *Xanthophyllomyces dendrorhous* Metabolism

A Major Qualifying Project

Submitted to the Faculty of Worcester Polytechnic Institute

in partial fulfillment of the requirements for the Degree in Bachelor of Science

in Chemical Engineering

Prepared by: Celeste Marsan

Submitted on: April 25, 2019

Project Advisor: Dr. Eric M. Young

This report represents work of WPI undergraduate students submitted to the faculty as evidence of a degree requirement. WPI routinely publishes these reports on its web site without editorial or peer review. For more information about the projects program at WPI, see http://www.wpi.edu/Academics/Projects.

Abstract

Antioxidants play an important role in human health, and are industrially relevant as pigments, cosmetics, and food additives. There has been an increase in natural production of these molecules from plants and fungi, such as the carotenoid producing basidiomycete *Xanthophyllomyces dendrorhous*. Previous studies show that *X. dendrorhous* carotenogenesis can be light-induced, but there is little information on the mechanism or specificity of this regulation. This project investigated the preferential light activation of *X. dendrorhous* carotenoids. We find that β-carotene production is greatest under UV radiation and that of astaxanthin is greatest in red/green light. This is relevant for optimization of industrial production and fundamental understanding antioxidant regulation and mechanisms.

Acknowledgements

I would like to acknowledge the following people for their support and assistance throughout this project and the remainder of my time at Worcester Polytechnic Institute.

Professor Eric M. Young, for the opportunity to work on this project and his guidance throughout my time at WPI and beyond

Joe Collins and Kevin Keating, for their mentorship over the past few years

WPI Biomedical Engineering student Gillian Nadeau, for her invaluable contribution to assembling the Light Plate Apparatus

Table of Contents

Background

Reactive Oxygen Intermediates and Antioxidants

Reactive oxygen intermediates (ROIs) have an almost unparalleled ability to disrupt surrounding molecules by stealing a hydrogen molecule or donating an electron or radical. These species are highly unstable and reactive within cellular systems, acting as strong electrophilic centers. The existence of ROIs, also known as reactive oxygen species (ROS), was first published in 1954 as a part of Gershman's free radical theory of oxygen toxicity.[3] ROIs occur within cells as partially reduced forms of oxygen, most commonly appearing in the form of superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals.[1,2] Especially under stress conditions, the half-lives of superoxides and hydroxyl radicals approaches 10^{-9} seconds, making them highly reactive.[3]

ROIs are a major form of intracellular damage that leads to aging, due to oxidative degradation of DNA, proteins, and lipids.[2] Their impact on human health was originally documented by Denham Harman in 1956 based on their role in the aging process.[3] In DNA, oxidation by ROIs has been associated with many cancers and degenerative diseases.[2] ROIs readily react with the purine and pyrimidine bases, as well as the sugar-phosphate backbone of DNA molecules.[3] The most studied mechanism of this is the lesion formation of oxidation of the 8'-hydroxyl group on guanines, which causes permanent damage to the molecule.[3] Peptides are also vulnerable to oxidative damage from ROIs, especially at methionine and cysteine residues.[3] Oxidation at these amino acids alters the thiol bonding of proteins, which can alter both their structure and functions. Finally, ROIs also affect cellular lipids, specifically polyunsaturated fatty acids (PUFAs) of cell membranes.[4] This process, called lipid peroxidation, begins a self-perpetuating chain reaction, where lipids are broken down into biologically-active aldehydes that spread the attack.[4] In fact, lipids are the group of macromolecules that are most likely to undergo oxidative damage within cells, and have been closely associated with tissue injury and a variety of diseases.[4,5] Lipid peroxidation is especially dangerous because it can alter membrane permeability of essential molecules, and because the aldehyde side-products can attach to and impair functioning of proteins.[5,8]

However, as Mittal and Murad found in 1977, ROIs have important secondary messenger functions within cells.[3] Plants and fungi produce ROIs as signaling molecules to control cell death, abiotic stress response, pathogen defense, and other systematic signaling.[1] Though ROIs are commonly formed under stress conditions (such as drought, salt, heat shock, pathogenic attack, UV radiation, etc.), a low level is maintained in cells due to leakage from oxidative phosphorylation in mitochondria and due to breakage of peroxisomes.[3] There is strong evidence that ROIs play a dual role within cells. While at high concentrations they are toxic, at low concentrations they play important physiological roles for cellular stress response.[3] However, due to the high toxicity of partially reduced oxygen, cells systems have evolutionarily developed mechanisms of redox homeostasis to regulate and neutralize ROIs. ROI mitigation involves several lines of defense: cellular preventative mechanisms, oxidative damage repair mechanisms, physical defenses to stressors, and finally a set of regulatory molecules.[4] These regulatory molecules have been given the umbrella-term antioxidants.

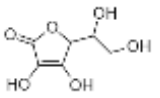
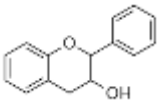
The first antioxidant, superoxide dismutase (SOD), was discovered in 1969 by McCord and Fridovich.[3] Since then, thousands of antioxidant molecules have been characterized with a variety

of different structures and functions. They occur as enzymes, aromatic rings, and long hydrocarbon rings.[4] They may be soluble in water or lipids, and may be sequestered to cellular organelles or allowed to scavenge through the cytoplasm.[1] Despite their many differences, all antioxidants have an ability to neutralize ROIs and prevent further oxidative damage to cells. The various classes of antioxidants are summarized in Table 1 below.

Of the main branches in antioxidant phylogeny is a group of enzymes that are able to reduce oxygen radicals into stable molecules.[4] This group, which includes SOD, as well as catalase and ascorbate peroxidase, are able to absorb free radicals and prevent attack on essential proteins.[1,4] Using a variety of metallic co-factors, they reduce ROIs into hydrogen peroxide and subsequently water molecules.[1,4] These enzymes often use non-enzymatic antioxidants as stabilizing reagents for redox reactions due to their ability to be readily oxidized and reduced.[1,3]

In addition to their ability to work with enzymatic antioxidants, non-enzymatic antioxidants act as ROI scavengers in cells. Upon finding the reactive molecules, they interrupt free radical chain reactions by donating a hydrogen or accepting an electron.[4] Non-enzymatic antioxidants appear in many forms, including Vitamin C (ascorbic acid), Vitamin E (α-tocopherol), polyphenols (such as flavonoids and phenolic acids), and carotenoids (carotenes and xanthophylls).[6] Vitamin C acts as a ROI scavenger and can form resonance-stabilized, unreactive ascorbyl radicals after absorbing a radical electron.[6] Vitamin E is a major membrane protectant, and acts as a redox chain-breaking electron donor or acceptor.[6] Polyphenols inactivate free radicals via hydrogen transfer and single electron transfer. Phenolic acids specifically protect against low-density lipoprotein (LDL) oxidation and DNA oxidative damage.[6] Finally, carotenoids are strong singlet oxygen quenchers and free radical scavengers due to their high number of conjugated double bonds.[6] Each group of molecules plays a specific role in a complex network, and is not interchangeable with any others.[8] A tight balance is maintained between different types of antioxidants, as well as between their oxidized and reduced states.[1] These equilibriums act as signaling pathways for homeostasis and stress response within cells.

Table 1: Summary of common antioxidants

Class of Antioxidants	ROI Protectant Mechanism	Common Forms	Structural Example
Enzymatic	Catalyze ROI reduction by radical absorption; use metallic co-factors	Superoxide dismutase, catalase, ascorbate peroxidase	Large polypeptide molecules
Vitamins	Absorb radicals to form unreactive radical intermediates	ascorbic acid (Vitamin C), α-tocopherol (Vitamin E)	
Polyphenols	Aromatic ring systems inactivate free radicals by hydrogen or electron transfer	Flavonoids, phenolic acids, polyphenolic amides	
Carotenoids	Heavy allylic chains stabilize radicals through resonance structures	Carotenes, Xanthophylls	

Antioxidants first came to the attention of public health in the 1990s, when diets of antioxidant-rich fruits and vegetables were shown to decrease the risk of cardiovascular disease, cancer, and vision loss, among many other diseases.[8] Since then, a series of nutritional studies have shown an inverse relationship between antioxidant consumption and rates of oxidative DNA damage, cell division and mutagenesis (cancer); increased recognition of LDL (cardiovascular disease); and decreased rate of cataracts, some brain pathologies (Parkinson's disease and Lou Gehrig's disease), and birth defects (from sperm mutagenesis).[2] In fact, antioxidants have been thought to provide protection against over one hundred different diseases and disorders.[4,5]

However, recent trials have shown mixed results.[8] Two of the most publicized antioxidant dietary studies on disease prevention in recent years (β-Carotene and Retinol Efficacy Trial and α-Tocopherol/β-Carotene Cancer Prevention Trial) did not show any conclusive results.[7] In fact, some trials have found that high levels of exogenous antioxidants can be toxic to cells.[8] Conversely, recent trials focused on skin cancer and age-related eye disease have shown prospects of success.[8] However most trials to this point have been limited due to short trial durations and pre-existing diseases in populations.[8]

Despite these conflicting results, antioxidants are still advertised as health additives in many cereals, protein bars, energy drinks, and other processed foods, and sold as nutraceuticals.[8] They are often added as food preservatives as well, due to their high stability and low volatility.[9] In 2018, the carotenoid industry alone was expected to be worth 1.4 billion dollars, and is projected to grow exponentially before 2025.[8,21] While they are best known for use in the food and pharmaceutical industries as nutritional supplements, they are also sold as potent pigments, cosmetics, and preservatives.[8-10] Currently, the majority of antioxidants on the market are produced synthetically. However with a market-base shifting towards natural, renewable products, and given shortcomings of past synthetic antioxidants such as butylated hydroxytoluene (BHT), there has been a push in recent years for natural methods of harvesting these secondary metabolites.[10,11]

Of the antioxidants, carotenoids are among the best at protecting against lipid peroxidation, as well as photosensitivity (cellular excitation via light-activation). The remainder of this paper will focus on carotenoids as antioxidants and fungal secondary metabolites.

Carotenoids in Nature

Carotenoids are a class of lipid-soluble, non-enzymatic antioxidants.[12] They tetraterpenoids (C_{40}) characterized by eleven sets of conjugated double bonds, which allow them to easily stabilize free radicals until they can decay into non-radical products.[7,12,14] There are over 750 different species of carotenoids, that vary in pigment and antioxidant activity due to alterations in the number of double bonds outside of the conjugated system, and addition of cyclic and oxygen functional groups.[7,14] Because of their long hydrophobic chains, they are very prevalent in cellular membranes.[4] Due to this localization and, they are the most efficient scavengers of peroxyl radicals that cause lipid peroxidation.[4] Carotenoids help to stabilize ROIs by fluctuating between reduced and oxidized states, and can interact with free radicals in three main ways: electron transfer, hydrogen abstraction, or radical addition.[5,7]

Carotenoids can be divided into two main categories: xanthophylls and carotenes. Xanthophylls have similar structures to those of carotenes, but with additional oxygen functional groups. These groups limit their ability to act as reducing agents, but do not limit their photoprotective abilities.[14] In some organisms, xanthophylls and carotenes are localized to different organelles. For example, in higher plants, xanthophylls are restricted to light-harvesting areas, where carotenes are found in protective roles against photosensitivity.[7]

Photosensitivity occurs when cellular and extracellular molecules are excited to higher energy states due to light activation. While photosensitivity of some molecules (such as those involved in photosynthesis) are necessary for life, this process can also catalyze the formation of ROIs.[13] While ultraviolet radiation (UVR, 290 – 400 nm) is best known for its ability to form ROIs, recent studies have shown that visible light (400 – 700 nm) produces these reactive species as well.[15] While publications have shown that visible light has a significant effect on photoaging and production of ROIs in tissues, there has been little investigation into the differences in ROIs produced between exposure to UVR and visible light.[16]

Nonetheless, the mechanism of ROI production by ultraviolet radiation has been well characterized.[16] Upon irradiation with UVR, oxygen surrounding and within the cellular matrix is excited to superoxide or singlet oxygen radicals. These ROIs come into contact with the lipid bilayer surrounding the cells and oxidize the PUFAs via lipid peroxidation.[13] From here, the ROI pathway continues in two ways: aldehyde byproducts from lipid peroxidation propagate the redox chain by attaching and altering proteins; and the oxidized lipid bilayer becomes more soluble to hydrogen peroxides, which diffuse into the cell and perform oxidative damage to other macromolecules.[13] Because of this, photoprotection from carotenoids, as well as other membrane-soluble antioxidants, is critical to the survival of many organisms.[5]

Carotenoid biosynthesis originates from the terpene synthesis pathway, as shown in Figure 1 below. Beginning with pyruvate-derived isoprene units, carotenoids are comprised of four terpene units (each of two isoprenes) in a linear chain.[17] The resulting base compound, phytoene, undergoes unsaturating reactions until there are eleven conjugated double bonds in molecule *all-trans*-lycopene.[14] The majority of carotenes and xanthophylls are derived from lycopene. This paper will focus on the pathway that incorporates β-carotene and astaxanthin, two of the most industrially relevant carotenoids. The core structures of lycopene, β-carotene, and astaxanthin are similar, but incorporate different functional groups on either end.[17] Where lycopene has no cyclic nature, the distal six carbons on each end of β-carotene form a tri-substituted cyclohexene ring, maintaining rotational symmetry.[17] Astaxanthin maintains the same structure as β-carotene, but with the additional substitution of a carbonyl and alcohol group on each ring.[18]

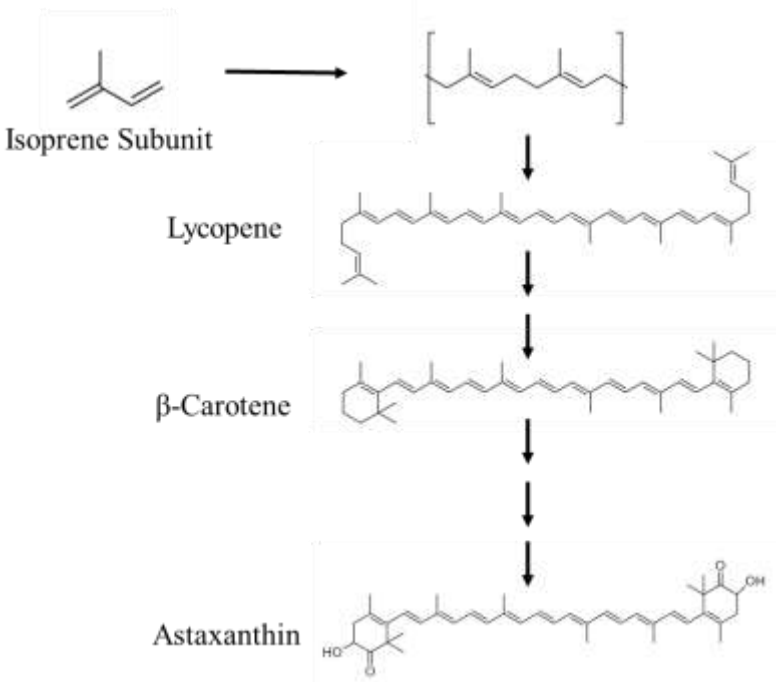


Figure 1: Carotenogenesis pathway in *Xanthophyllomyces dendrorhous* incorporating three common carotenoids: lycopene, β-carotene, and astaxanthin

The core allylic structures of β-carotene and astaxanthin are very similar, which allow them to maintain the same mechanism of antioxidation. However, the varied functional ends may affect their orientation in the membrane, and therefore their functionality.[19] Where fully hydrophobic carotenoids such as lycopene and β-carotene are oriented parallel to the membrane surface among the hydrophobic tails, the polar end groups of astaxanthin orient the molecule perpendicular to the membrane so that it spans the bilayer.[19] Though previous studies have shown that lycopene is the most potent exogenous antioxidant supplement, orthogonal orientation of astaxanthin allows it to more effectively protect lipids against oxidation from peroxyl radicals.[19,20]

Carotenoids, similar to many other complex molecules, are produced naturally by a variety of organisms, including animals, plants, fungi, and microorganisms.[21] For instance, astaxanthin biosynthesis is found in salmon, crustaceans, microalga, and fungi to name a few.[7] Currently, the majority of carotenoids are extracted from vegetables or produced synthetically.[6,21] However each of these methods has shortcomings. For the former, inconsistencies in geographical and seasonal variation do not allow for a dependable product.[21] The latter requires hazardous byproducts, and the synthetic analogs may lack molecular structural components that provide major health benefits.[21]

Instead, industries have been turning to production via fermentation of microorganisms, namely microbes and fungi.[21] While some are using genetic engineering techniques to introduce complex carotenoid pathways into model organisms, others are working to optimize production in non-model hosts that already make molecules such as β-carotene and astaxanthin.[21,22] This work focuses on *Xanthophyllomyces dendrorhous*, a high-carotenoid producing yeast with a native astaxanthin biosynthetic pathway.

Light Activation of Fungal Metabolism

X. dendrorhous (telomorphic state of *Phaffia rhodozyma*) is a heterobasidiomycetous yeast native to several temperate regions around the world including South and North America, and Eastern Asia.[23,24] Discovered in the forests of Japan and Alaska in the 1970s, it is characterized by a bright red-orange pigment, given by astaxanthin, its major antioxidant product.[18,25] Interestingly, though the *X. dendrorhous* carotenoid biosynthetic pathway has been characterized, there is not much known about its regulation. While the growth and carotenogenesis of the wildtype strain is shown to be inhibited in laboratories when grown under high-intensity light, carotenoid synthesis of high-carotenoid producing mutants is shown to be stimulated by white light and UVR.[25] Additionally, it has been proposed that the presence of different ROIs may inhibit or enhance the amount or composition of membrane carotenoids. For instance, Schroder and Johnson from the University of Wisconsin postulated that the presence of singlet oxygen radicals increased total carotenoid accumulation, whereas presence of peroxyl radicals decreased the amount of astaxanthin and increased accumulation of β-carotene.[26] However, recent studies have also shown that presence of hydrogen peroxide may induce astaxanthin production.[25] Though it may play a large role in optimization of industrial astaxanthin production, to this point regulation of carotenogenesis in *X. dendrorhous* remains largely unexplored.

Light Activation of Antioxidant Production in Xanthophyllomyces dendrorhous

While light induction of antioxidant metabolism in *X. dendrorhous* has been previously observed, there have been no publications that examine the wavelength specificity of this interaction. A light plate apparatus, based on a design from the laboratory of Dr. Jeff Tabor at Rice University, was used to shine selective wavelengths of light on cultures of *X. dendrorhous*. After set periods of time, the normalized production of total carotenoids, β-carotene, and astaxanthin were determined based on the absorbance or fluorescence of each culture.

Methodology

Assembling the Light Plate Apparatus

The light plate apparatus (LPA) was assembled following a user's manual published by Dr. Jeff Tabor's laboratory at Rice University (see Figures 2 and 3).[27] The LPA is an instrument capable of shining two individual LED lights on cell cultures in a 24-well plate. It is comprised of a 3D-printed shell surrounding a soldering board with 48 LED light sockets oriented below a 24-well plate with a clear bottom. This allows each culture to be exposed two unique LED lights without disrupting neighboring cultures.

The LPA is programmed using the platform IRIS, which was also developed and published by the Tabor Lab.[27] This program, which is downloaded onto an SD card nested in the soldering board, can manipulate the LED inputs and intensities. This gives the LPA endless programmable possibilities. For the sake of this report, one LED light was shined continuously on each cell culture for a set time interval before readings were taken. The LPA was fitted onto a cell culture shaker to ensure that cultures did not form aggregates of films on the bottom.

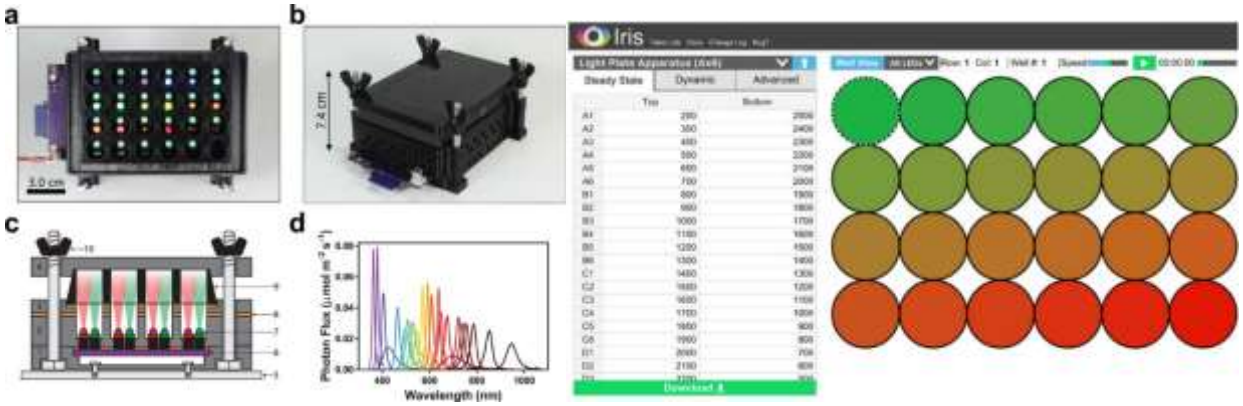


Figure 2: Model of the Light Plate Apparatus (left) and digital view of the LPA programming platform IRIS as proposed by Dr. Jeff Tabor's laboratory at Rice University.[27]

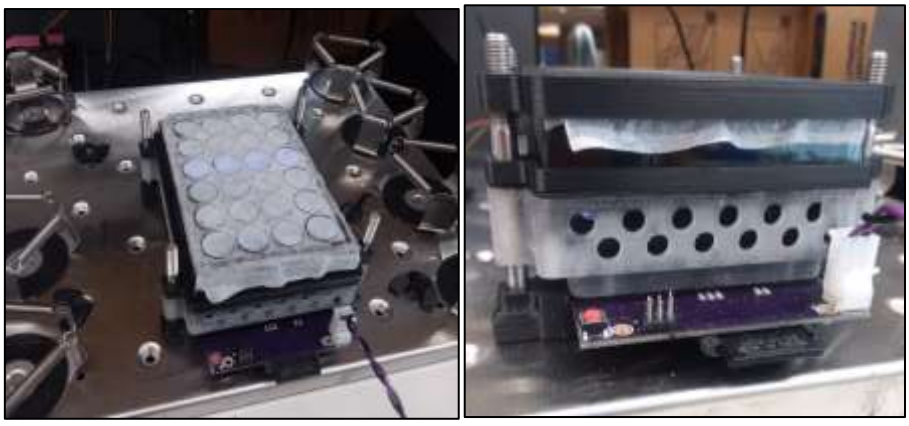


Figure 3: Top and side view of the Young Lab light plate apparatus

Culturing of Xanthophyllomyces dendrorhous

X. dendrorhous cultures were grown up in a flask in CSM/YNB media for 5-7 days at 30^{o}C before being back-diluted and distributed in a 24-well plate on the LPA. This media consists of the following: 30 mL deionized water, 10 mL filtered CSM, 5 mL filtered glucose, and 5 mL filtered YNB.

Light Induction of Xanthophyllomyces dendrorhous

X. dendrorhous cultures were grown in the LPA under six different light conditions for intervals of up to seven days, after two weeks, and after three weeks. These light conditions were: dark (no LED light), UV light (λ = 400 nm), blue light (λ = 470 nm), green light (λ = 565 nm), red light (λ = 660 nm), and white light (visible spectrum only). During the growth period, the LPA was placed on a shaker at 170 RPM.

Determination and Analysis of Metabolite Concentrations

After set time points, absorbances and fluorescences of each culture on the LPA was read on a Synergy H100 plate reader. Cell density of each culture was determined as the absorbance at 600 nm. Total carotenoid production was determined as the absorbance at 485 nm of each well. β-carotene production of each culture was determined as the fluorescence with excitation at 561 nm and emission at 605 nm. Finally, astaxanthin production was determined as the fluorescence of each well with excitation at 488 nm and emission at 570 nm. Normalized values for each secondary metabolite production were determined by dividing the absorbance or fluorescence reading by the cell density of each well.

Results and Discussion

β-carotene Biosynthesis Induction

β-carotene production levels were determined by normalizing the fluorescence at 561nm/605 nm with the cellular density of each well. Production levels were tracked up to a week, after two weeks, and after three weeks. Figure 4 compares the fluorescent emission of each light condition after one week (left bar) and after three weeks (right bar).

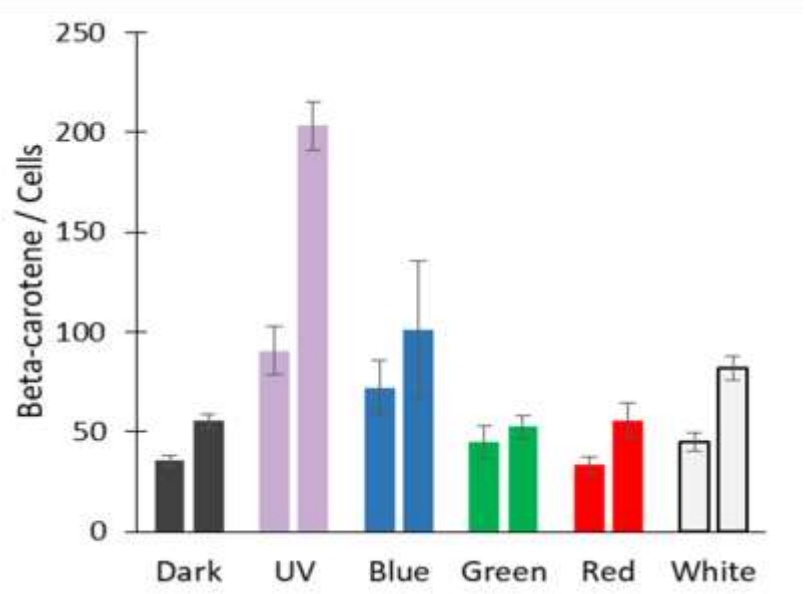


Figure 4: β-carotene biosynthetic production under six light conditions after one week (left bar) and after three weeks (right bar). Values were determined as the quotient of the fluorescence measured of β-carotene divided by the cell density in each well. The means of four replicates are plotted with error bars representing one standard deviation from the mean.

While production of β-carotene after one week was similar across all conditions, after three weeks cell cultures grown under UV radiation produced nearly two-fold more than any other light condition. This supports the conclusion that production of β-carotene in *X. dendrorhous* is induced in the presence of UV radiation. This claim is supported by the remainder of data collected throughout experimentation. A production curve of β-carotene biosynthesis is shown below in Figure 5.

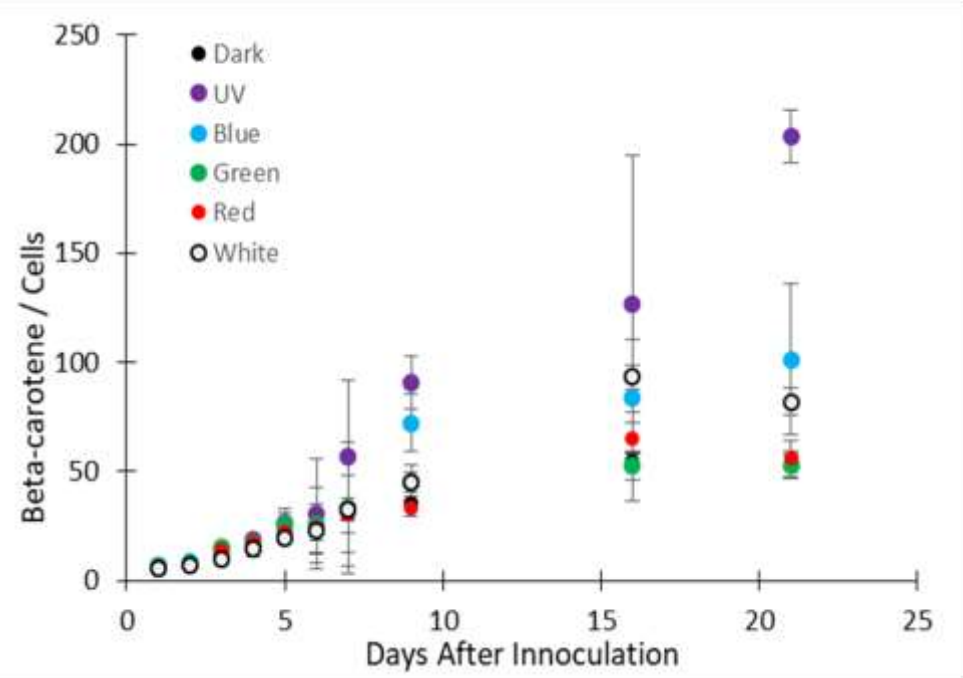


Figure 5: β-carotene biosynthetic production curve over all testing periods for each of the six light conditions. Values were determined as the quotient of the fluorescence measured of β-carotene divided by the cell density in each well. The means of four replicates are plotted with error bars representing one standard deviation from the mean.

This production curve shows the progression of normalized β-carotene biosynthesis among *X. dendrorhous* cultures over the entire time interval tested. This data also shows that increased production levels of β-carotene under UV radiation began between seven and nine days after testing began.

Astaxanthin Biosynthesis Induction

Astaxanthin production levels were determined by normalizing the fluorescence at 488nm/570nm with the cellular density of each well. Production levels were tracked up to a week, after two weeks, and after three weeks. Figure 6 compares the fluorescent emission of each light condition after one week (left bar) and after three weeks (right bar).

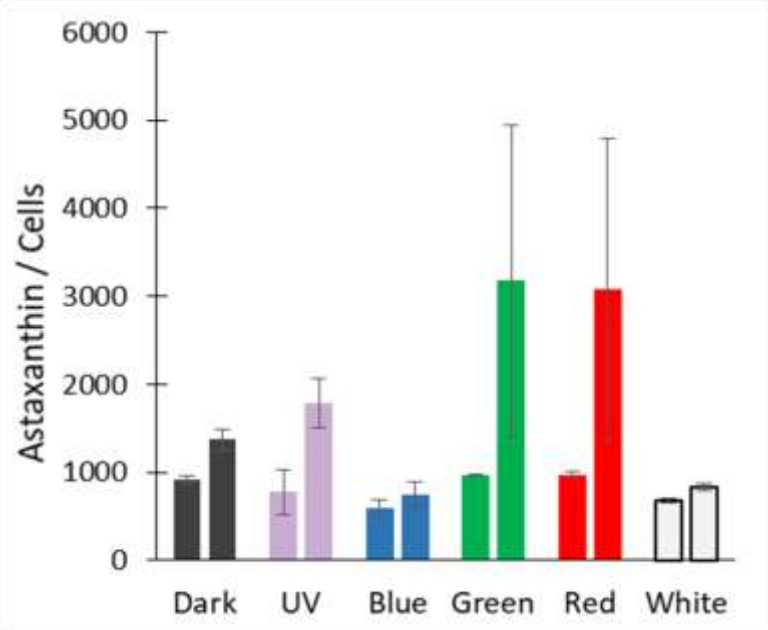


Figure 6: Astaxanthin biosynthetic production under six light conditions after one week (left bar) and after three weeks (right bar). Values were determined as the quotient of the fluorescence measured of astaxanthin divided by the cell density in each well. The means of four replicates are plotted with error bars representing one standard deviation from the mean.

Similar to β-carotene, production of astaxanthin after one week was similar across all conditions. However, after three weeks cell cultures grown under green and red light (565, 660 nm respectively) produced nearly two-fold more than any other light condition. This supports the conclusion that production of astaxanthin in *X. dendrorhous* is induced in the presence of green and red light. This claim is supported by the remainder of data collected throughout experimentation. A production curve of astaxanthin biosynthesis is shown below in Figure 7. It is critical to note that while this result was seen, high variation between samples does not produce a statistically significant finding. Additional trials must be performed at longer time intervals to support this conclusion.

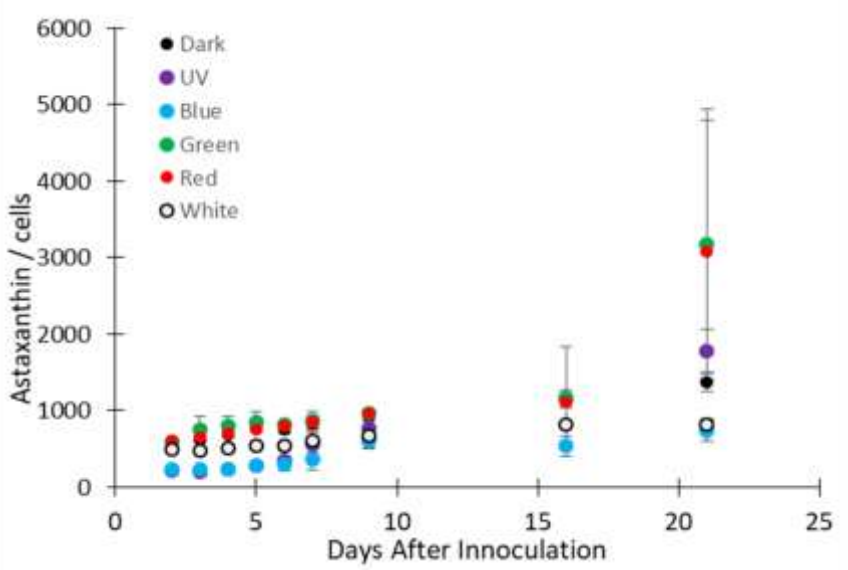


Figure 7: Astaxanthin biosynthetic production curve over all testing periods for each of the six light conditions. Values were determined as the quotient of the fluorescence measured of astaxanthin divided by the cell density in each well. The means of four replicates are plotted with error bars representing one standard deviation from the mean.

This production curve shows the progression of normalized astaxanthin biosynthesis among *X. dendrorhous* cultures over the entire time interval tested. This data also shows that increased production levels of astaxanthin under green and red light conditions began close to two weeks after testing began.

Preferential Light Induction of Xanthophyllomyces dendrorhous Carotenogenesis

It is clear that light activation of *X. dendrorhous* carotenogenesis acts at multiple levels. While astaxanthin production is highest under green and red light conditions, its precursor β-carotene is produced best under UV radiation. This shows that *X. dendrorhous* light induction occurs preferentially under different wavelengths of light. However, the cause and mechanism of this disparity is not apparent.

Environmentally, there are few situations in which sunlight separates into its component wavelengths; therefore evolutionarily, there should be no reason for light activation of metabolism to have preferential wavelengths. It is far more likely that this induction is caused by reactive byproducts (such as ROIs) caused by UV and visible light radiation. In the case, further investigation into the different ROIs produced under each condition is necessary. Additional knowledge about differential production of ROI species in UV radiation and visible light could not only help elucidate the regulation mechanism of carotenogenesis, but could also provide insight into the specific antioxidant mechanisms of β-carotene and astaxanthin.

Conclusions and Future Work

While no statistically significant conclusions can be drawn from this work, it provides a solid groundwork for additional studies into the mechanisms of preferential light activation of carotenogenesis in *X. dendrorhous*. Though this research does propose trends for light induction of β-carotene and astaxanthin, additional data, especially at later time points, must be found in order to support these conclusions. Additional research that provides evidence that biosynthesis of β-carotene and astaxanthin in *X. dendrorhous* are induced by UV radiation and green or red light respectively would be important both industrially and experimentally.

Industrially, any information about regulation or activation of carotenoid biosynthesis can be used to optimize fermentation conditions and improve yields. This is especially true if a pure sample of either β-carotene or astaxanthin is preferred. For instance, a fermentation reaction for the production of astaxanthin may want to follow subsequent intervals of darkness (to promote cell division within the reactor), UV radiation (to promote β-carotene biosynthesis, an astaxanthin precursor), and finally green or red light (to promote astaxanthin biosynthesis). Determination of an optimal wavelength for the production of astaxanthin between green and red light (550-650 nm) would also be critical information in this regard.

Further, evidence of light activation of *X. dendrorhous* carotenoids is important experimentally for the wide spread study of antioxidant mechanisms and regulation. For instance, a comparison of transcriptomics data from *X. dendrorhous* cultures growing in green and UV light may be useful to see if there is a difference in transcription of the β-carotene and astaxanthin genetic pathways. This will give important information about regulation of carotenoids at the transcriptional level. Any similar information concerning the mechanisms of antioxidant regulation and homeostasis may prove critical to human health concerns.

References

1. Mittler, R (2002). Oxidative stress, antioxidants, and stress tolerance. *Trends in Plant Science*. 7(9): 405-10.
2. Ames, B. N. et al. (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Procedures of the National Academy of Sciences, USA*. 90: 7915-22.
3. Valko, M et al. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*. 39(1): 44-84.
4. Nimse, S. B. (2015) Free radicals, natural antioxidants, and their reaction mechanisms. *Royal Society of Chemistry*. 5: 27986-8006.
5. Pisoschi, A. M. et al. (2015). The role of antioxidants in the chemistry of oxidative stress: a review. *European Journal of Medicinal Chemistry*. 97: 55-74
6. Oroian, M. et al. (2015). Antioxidants: Characterization, natural sources, extraction and analysis. *Food Research International*. 74: 10-36
7. Young, A. J. et al. (2018) Carotenoids – Antioxidant Properties. *Antioxidants*. 7(2): 28.
8. Harvard T. H. Chan School of Public Health. (2019). Antioxidants: Beyond the Hype. *The Nutrition Source*. < https://www.hsph.harvard.edu/nutritionsource/antioxidants/>
9. Wilson, D. W. et al. (2017). The role of food antioxidants, benefits of functional foods, and influence of feeding habits on the health of the older person: an overview. *Antioxidants*. 6(4): 81
10. Galasso, C. et al. (2017). Carotenoids from marine organisms: biological functions and industrial applications. *Antioxidants*. 6: 96.
11. Lanigan, R. S. et al. (2002). Final report on the safety assessment of BHT. *International Journal of Toxicology*. 21: 19-94.
12. Miller, N. J. (1996) Antioxidant activities of carotenes and xanthophylls. *Federation of European Biochemical Societies*. 384:240-2.
13. Theile, J. et al. (2001). Oxidants and antioxidants in cutaneous biology. *Current Problems in Dermatology*. 29.
14. Nisar, N. et al. (2015) Carotenoid metabolism in plants. *Cell: Molecular Plant*. 8(1): 68-82.
15. Liebel, F. et al. (2012) Irradiation of skin with visible light induces reactive oxygen species and matrix-degrading enzymes. *Journal of Investigative Dermatology*. 132(7): 1901-7.
16. Duteil, L. et al. (2014) Differences in visible light-induced pigmentation according to wavelengths: a clinical and histological study in comparison with UVB exposure. *Melanoma Research*. 27(5).
17. Fraser, P. D. et al. (2004). The biosynthesis and nutritional uses of carotenoids. *Progress in Lipid Research*. 43(3): 228-65.
18. Visser, H. et al. (2003) Metabolic engineering of the astaxanthin-biosynthetic pathway of *Xanthophyllomyces dendrorhous*. *FEMS Yeast Research*. 4(3): 221-31.
19. Jomova, K. et al. (2013). Health protective effects of carotenoids and their interactions with other biological antioxidants. *European Journal of Medicinal Chemistry*. 70: 102-10.
20. Rao, A. V. et al. (2007). Carotenoids and human health. *Pharmocological Research*. 55(3): 207-16.

21. Mata-Gomez, L. C. et al. (2014). Biotechnological production of carotenoids by yeasts: an overview. *Microbial Cell Factories*. 13:12.
22. Marienhagen, J. et al. (2013). Metabolic engineering of microorganisms for the synthesis of plant natural products. *Journal of Biotechnology*. 163(2): 166-78.
23. Tognetti, C. et al (2013). Favored isolation and rapid identification of the astaxanthin-producing yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) from environmental samples. *Journal of Basic Microbiology*. 53:766-72.
24. Baeze, M. et al. (2009) Isolation, characterization and long term preservation of mutant strains of *Xanthophyllomyces dendrorhous*. Journal of Basic Microbiology. 49: 135-41.
25. Rodriguez-Saiz, M. et al. (2010). *Xanthophyllomyces dendrorhous* for the industrial production of astaxanthin. *Applied Microbiology and Biotechnology*. 88(3): 645-58.
26. Schroeder, W. A. et al. (1995). Singlet Oxygen and Peroxyl Radicals Regulate Carotenoid Biosynthesis in *Phaffia rhodozyma*. *Journal of Biological Chemistry*. 270(31): 18374-9.
27. K. P. Gerhardt et al. (2006**)** An open-hardware platform for optogenetics and photobiology. *Scientific Reports*. 6.

Appendices

Appendix A: Additional β-Carotene Production Data

	Days after Inoculation	1	2	3	4	5	6	7	9	16	21
Dark	**Mean βcar/cell**	7.15	9.08	12.26	16.06	20.48	24.06	30.62	35.63	54.52	55.36
	Standard Deviation	0.84	1.33	1.52	0.41	2.11	11.06	17.85	2.73	17.82	3.82
UV	**Mean βcar/cell**	5.79	7.99	13.39	19.02	26.18	30.81	56.85	90.83	126.84	203.33
	Standard Deviation	2.42	0.99	2.41	2.13	6.75	25.21	34.62	12.24	67.66	12.08
Blue	**Mean βcar/cell**	6.83	8.43	11.71	14.95	22.12	23.66	32.04	72.35	83.87	101.25
	Standard Deviation	2.89	0.79	0.80	3.76	5.15	11.26	25.30	13.35	26.31	34.55
Green	**Mean βcar/cell**	6.25	7.80	15.33	17.76	25.50	25.43	33.21	45.05	52.43	52.49
	Standard Deviation	2.42	0.08	1.09	1.57	5.45	17.44	30.23	8.25	6.31	5.65
Red	**Mean βcar/cell**	6.67	7.74	13.98	17.14	22.15	24.02	30.87	33.35	65.23	56.01
	Standard Deviation	0.63	0.76	1.34	1.94	3.85	2.26	3.75	4.13	11.84	8.18
White	**Mean βcar/cell**	5.92	7.23	10.07	14.98	19.51	23.02	32.92	45.25	93.17	82.04
	Standard Deviation	0.83	1.22	0.85	1.72	0.89	4.78	4.75	4.62	5.51	6.24

Appendix B: Additional Astaxanthin Production Data

	Days after Inoculation	2	3	4	5	6	7	9	16	21
Dark	**Mean Ast/cell**	577.7	618.9	682.2	774.1	745.7	828.1	912.9	1150.8	1369.8
	Standard Deviation	5.4	16.8	11.0	13.3	46.3	108.4	37.9	84.3	114.4
UV	**Mean Ast/cell**	212.6	206.1	236.9	288.9	339.8	552.8	774.2	1163.1	1783.4
	Standard Deviation	8.4	8.9	14.2	36.1	131.1	214.1	254.9	668.8	274.4
Blue	**Mean Ast/cell**	238.8	228.5	236.9	283.7	300.8	369.4	599.2	534.8	744.7
	Standard Deviation	18.3	17.7	28.0	17.5	61.1	140.0	93.4	128.6	145.7
Green	**Mean Ast/cell**	592.7	756.6	805.7	854.2	818.9	865.8	970.8	1155.0	3175.0
	Standard Deviation	18.0	167.9	115.7	130.6	31.3	124.1	7.5	109.5	1767.6
Red	**Mean Ast/cell**	603.3	651.6	704.9	759.3	808.1	851.3	963.4	1109.2	3081.1
	Standard Deviation	13.4	11.4	27.4	30.3	18.1	21.7	43.8	72.6	1707.4
White	**Mean Ast/cell**	487.9	475.0	503.5	536.1	549.9	614.7	674.6	823.0	825.0
	Standard Deviation	21.7	29.4	22.3	23.1	42.4	161.6	28.1	14.1	42.9