PIGMENT ANALYSIS OF *RHODOBACTER SPHAEROIDES* IN PHOTOBIOMODULATION

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ABSTRACT

Photobiomodulation (PBM), commonly known as red-light therapy, involves the exposure of light onto tissues for increased cellular activity. Examples of PBM include light-induced stimulation of damaged tissue, reduced inflammation, and increased metabolic activity (De Freitas, F.L, Hamblin, R.M. 2017). Past research proposes that light stimulation of mitochondria yields increased ATP production when supplemented with photosynthetic pigments and light. Other studies have suggested the uptake of photosynthetic pigments into mammalian tissues as a mechanism for increased light harvesting. While PBM therapy is still under investigation, similarities between energy harvesting in photobiomodulation and photosynthesis are proposed explanations for increased metabolic activity. Photosynthetic bacteria and eukaryotic mitochondria potentially have similar energy-harvesting mechanisms (Alberts, B. et. al. 2002.), thus supplementing a eukaryotic organism, such as yeast, with bacterial pigments could provide insights to improved cell repair in humans. This project focuses on isolating bacterial pigments under various wavelengths and intensities of light to enhance yeast growth. Bacterial pigments were grown under white light and subsequently implemented into a culture of yeast. The yeast were grown in front of a supercontinuum light source to observe light absorption as a function of wavelength. The overarching hypothesis of this work is that supplementation of yeast with bacterial pigments could expedite yeast growth rate through stimulation of the electron transport chain and increased ATP production. The results could offer a greater understanding of PBM therapy for possible noninvasive medical applications, improvements in healing, and supplementation of photobiomodulation with bacterial pigments as a promising avenue of medical research.

ACKNOWLEDGEMENTS

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INTRODUCTION

Photobiomodulation is a light-exposure treatment that utilizes laser beam radiation for induced healing in humans and other mammals (Hamblin, R.M., 2018). Biological tissues are exposed to a specific (infrared or near-infrared) wavelength of light for increased cellular activity and possible stimulation of an electron transport chain. Chromophores, such as cytochrome c oxidase, are regions in the electron transport chain that absorb light and allow for excitation of electrons to further generate ATP production. Past studies suggest that light stimulation expedites ATP production similar to exercise-induced ATP production. This suggests that light can act as an "exercise mimetic" to have similar effects on ATP production without doing any physical activity. Recent applications of photobiomodulation therapy include enhanced tissue repair and cancer therapies, pain alleviation, and relieved oxidative stress (Luodan, Y. et. al. 2021; Hamblin, R.M., 2016). Penetration of light into biological tissues has also been shown to increase production of ATP in mammalian mitochondria (Luodan, Y. et. al. 2021). While the cause of increased healing is still under investigation, we suggest biochemical similarities between photobiomodulation and photosynthesis. Similarities in energy harvesting between eukaryotic mitochondria and photosynthetic bacteria via an electron transport chain involve shuttling of electrons and generation of a proton gradient.

Studies have found improvements in mitochondrial ATP activity and lifespan of eukaryotic organisms when cultured with chlorophyll pigments and subsequent exposure to light (Zhang, D. et. al. 2016). Chlorophyll pigments are found in photosynthetic organisms to shuttle energy from sunlight to a reaction center for ATP production. Possible similarities between

eukaryotic mitochondria and photosynthetic chloroplasts could allow for enhancements of photobiomodulation when supplemented with pigments. Based on past studies which show the uptake of chlorophyll pigments into mammalian tissues, it is possible that supplementing photosynthetic pigments to yeast samples could also yield pigment absorption into yeast mitochondria (Xu, C. et. al. 2014). Yeast was used in this study because of its replicability and use as a model organism for mitochondrial activity in biological research (Malina, C. Larsson, C. Nielsen, J. 2018). Photosynthetic bacteria, *Rhodobacter sphaeroides*, was studied for pigment isolation and analysis because of its high data yield and years of research. Additionally, *R. sphaeroides* show optimal absorbance patterns at longer wavelengths of light, which penetrate eukaryotic tissues even deeper than red light (Hamblin, R.M., 2018; Brandis, S.A. et. al. 2006) . Deeper penetration in mammalian tissues could have a greater impact on mitochondrial activity within those deep tissues. Thus, it is predicted that deeper penetration will stimulate cell growth upon addition of bacterial pigments. Addition of photosynthetic pigments and subsequent exposure to light could promote energy harvesting to the mitochondrial electron transport chain to ultimately increase ATP output. Identifying optimal bacterial pigments to enhance photobiomodulation could offer novel ways of improving ATP production in eukaryotic organisms.This could advance biomedical research as a non-invasive method for improved healing and cell repair.

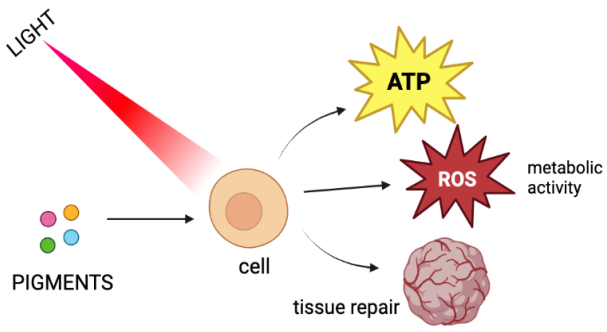

Figure 1. Possible effects of coupling pigment supplementation and light exposure to eukaryotic cells. Similar energy harvesting mechanisms between chloroplast and mitochondria may upregulate ATP output in eukaryotes after addition of photosynthetic pigments and light (Alberts, B. et. al. 2002.).

METHODS

Isolation and Analysis of Bacterial Pigments

In this study we used a gram-negative, photosynthetic bacterium, *Rhodobacter sphaeroides*, to extract carotenoid and bacteriochlorophyll pigments after growth under a tungsten lamp. *Rhodobacter spaheroides* were cultured in a sistrom medium inside of an anaerobic chamber to prevent exposure to oxygen. Exposure to oxygen would have intrusive effects on bacterial growth and pigment concentration, thus it is imperative to keep the bacteria inside the anaerobic chamber until its exponential growth phase. The bacteria were grown under a tungsten lamp to analyze pigment composition under white light. After growth, isolation of bacterial pigments was achieved via centrifugation and thin layer chromatography. 150 μL of the bacteria were centrifuged for 10 minutes at 5000 relative centripetal force (RCF). After the first round of centrifugation, the supernatant was discarded and magnesium sulfate and sand were added to the pellet (6v:4v:2v) and ground inside of the centrifuge vial. 5 mL of acetone was added to the vial and the bacteria were centrifuged again for 10 minutes at 5000 RCF. The supernatant was collected and dried under gaseous nitrogen to obtain the crude pigment.

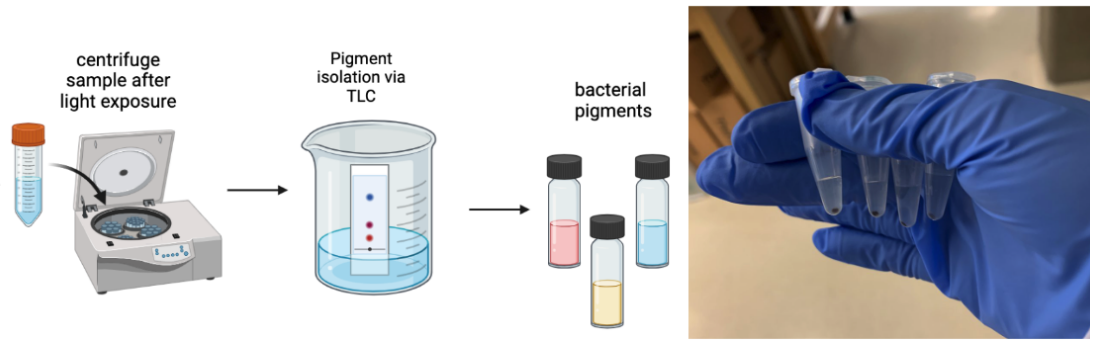

Figure 2. After bacterial inoculation, 150 µL samples were collected and centrifuged for further isolation via thin-layer chromatography

Thin-layer chromatography (TLC) is an isolation technique that relies on polarity differences to separate components within a sample. A glass plate lined with a polar silica gel formed the stationary phase, while a hexane-acetone (7v:3v) eluant served as the mobile phase. Components of the sample that are polar remain towards the bottom of the plate due to forming polar bonds with the silica. Nonpolar pigments move farther along the plate due to weaker forces between nonpolar and polar molecules. Because carotenoid pigments are more nonpolar in chemical structure, it was proposed that the pigments that moved farther along the plate were carotenoids and pigments that remained closer to the starting position were bacteriochlorophyll pigments. After isolation of the pigments, the retention factor (Rf value) of each pigment was determined to measure a quantitative analysis of pigment polarity. Retention factor is a value between 0 and 1 which reflects the polarity of the specific compound in a sample based on how far the compound travels given the maximum path length it can possibly travel. If the Rf value is higher (closer to 1) then the molecule represents a more nonpolar chemical structure, and vice versa. We used

TLC as our isolation technique because from the data we could better characterize how each pigment reacts to the same light using spectral analysis (TLC reflectance).

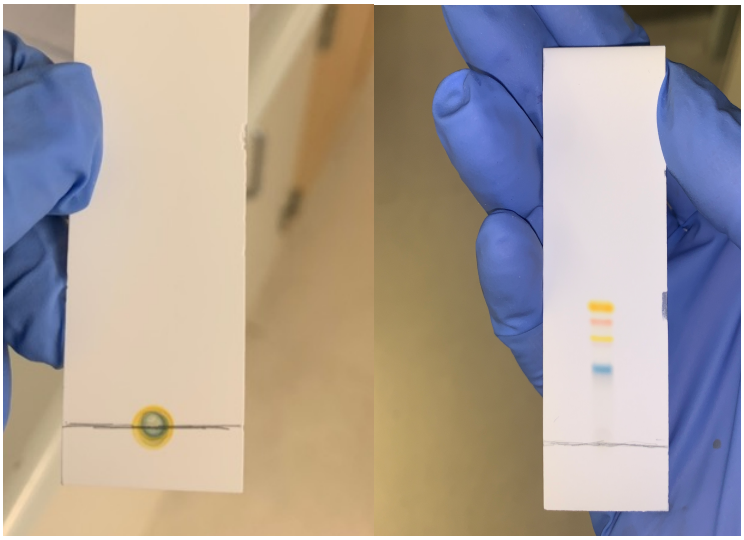

Figure 3. TLC plate of *R. sphaeroides* pigments. A glass pipette was used to spot a sample of the crude bacterial pigment (after drying supernatant using N2 gas). The photo on the right shows the separation of each pigment in the sample using TLC after suspending the TLC plate into the eluent for about 3 minutes.

For further analysis of each pigment band, liquid chromatography-mass spectrometry was used. The TLC plate allowed for physical visibility of each pigment band, but ultra-performance liquid chromatography and mass spectrometry are purification techniques coupled together to help identify the chemical composition of each pigment metabolite. After separating the pigments on the TLC plate, 3 different bands were scraped off and each displaced into a separate 1.5 mL centrifuge vial. 150 µL of acetonitrile was added to dissolve the silica gel in each vial, vortexed and centrifuged to separate the silica gel pellet from the supernatant (pigment). 100 µL of the

colored supernatant was collected into a new vial for each sample. Ultra-performance liquid chromatography (UPLC) was first performed to separate the individual components of the pigments. Similar to TLC, UPLC involves separation via polarity but instead involves inserting a small sample volume into a tube with tiny resin beads to offer higher sensitivity for pigment metabolites within a sample. Pigment metabolites are a broken-down, derived form of the complex pigment. After running each sample through the UPLC device, the pigment metabolites were ionized using mass spectroscopy to determine the mass-charge-ratio of each isolated metabolite. The parent and daughter ions were identified and compared to a database for potential candidate molecules. Understanding the polarity, spectral properties, and chemical composition of each pigment and metabolite after growing *R. sphaeroides* under various wavelengths and intensities of light can help to supplement the yeast sample with specific pigments under specific wavelengths of light for optimal growth.

Yeast Growth

Yeast samples were grown on an agarose gel and surrounded by a water bath to maintain temperature and humidity. The water bath was lined with glass and neoprene on a flat plane to which we referred to as our “yeast farm”.

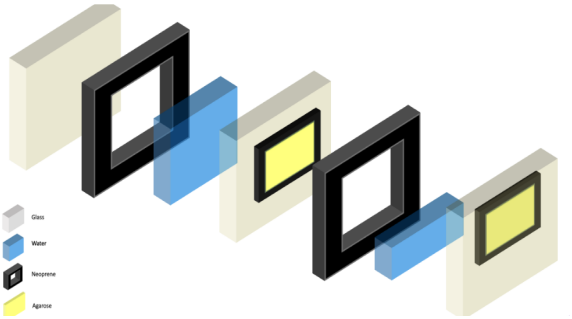

Figure 4. Model of yeast farm grown on agarose gel. The yeast farm was placed in front of a supercontinuum light source to measure growth as a function of wavelength. Future directions for this project involve isolation of the bacteria pigments after growth under different wavelengths and intensities of light to supplement different pigments to yeast to compare growth rates under various wavelengths and intensities of light.

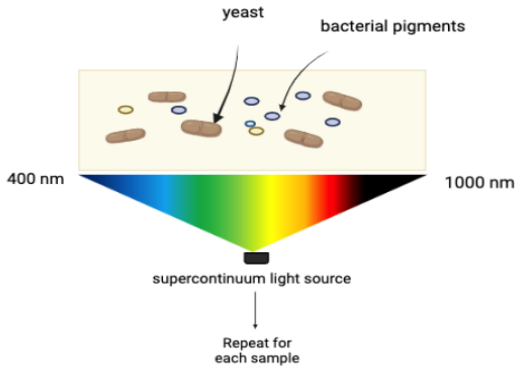

Figure 5. Model for yeast growth cultured with various bacterial pigments and exposed to a supercontinuum light source. Future samples include *R. sphaeroides* growth under various wavelengths and intensities of light

Future treatments will investigate potential differences in mitochondrial activity by culturing yeast cells in a yeast extract pentose and dextrose (YPD) media and measuring cell growth of varying samples of bacterial pigments and exposure to light. Research suggests that R. sphaeroides bacteria show optimal growth patterns at around 850 nm (Saer, G.R. Blankenship, E.R. 2017). Understanding how yeast growth is affected by supplementation with bacterial pigments under different wavelengths of light, i.e. outside of the 850 nm range, may yield

enhancements in PBM. Each yeast sample will be inoculated in YPD media, cultured on a glass plate, cultured with bacterial pigments, and placed in front of a rainbow generated by a supercontinuum source. The supercontinuum source allows for a visual measurement of cell growth as a function of wavelength. With the yeast farm experimental setup, future experimentation includes calculating yeast growth rate upon addition of bacterial pigments and under different wavelengths and intensities of light. If yeast growth increases with the bacterial pigments, it is predicted that mitochondrial activity has improved (Xu, C. et. al. 2014). Yeast growth will be measured using Beer Lambert's Law, which suggests that concentration is linearly correlated with absorbance. A fitting function model will then be used to illustrate yeast growth. If there is a higher absorbance, it is assumed that more yeast cells are present at that point.

$$A = \varepsilon c l$$

Equation 1. Beer Lambert's Law

A indicates the absorbance of a molecule. *ε* signifies molar absorptivity. *c* reflects concentration. *l* reflects path length.

Calculation of yeast growth rate each yeast sample and comparison with various controls (no pigments added, no light exposure) and experimental groups (adding R. sphaeroides pigments under varying wavelengths, etc) will allow for better understanding of pigment supplementation to enhance PBM. We predict that if yeast mitochondria show traces of light-dependent photosynthetic activity, then adding bacterial pigment(s) to a yeast culture while radiated with a specific wavelength and intensity of light will increase growth rate.

RESULTS

Isolation and Analysis of Bacterial Pigments

After obtaining isolation of pigment bands on the TLC plate, each band was analyzed for its spectral reflectance. We built a setup that would allow for detection of TLC plate reflectance as a function of wavelength. Were able to use this information to couple it to the band's retention factor. Multi-dimensional analysis of each band would give us more information about how the pigments and their metabolites could interact with light when supplemented to the yeast culture.

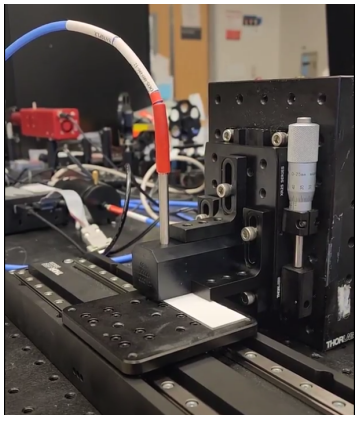

Figure 6a. Apparatus that detected TLC reflectance as the TLC plate ran along the flat plane. As each band was exposed to the light source, the detector recorded reflectance of light at different wavelengths according to the band's retention factor. The results are shown in Figure 6b.

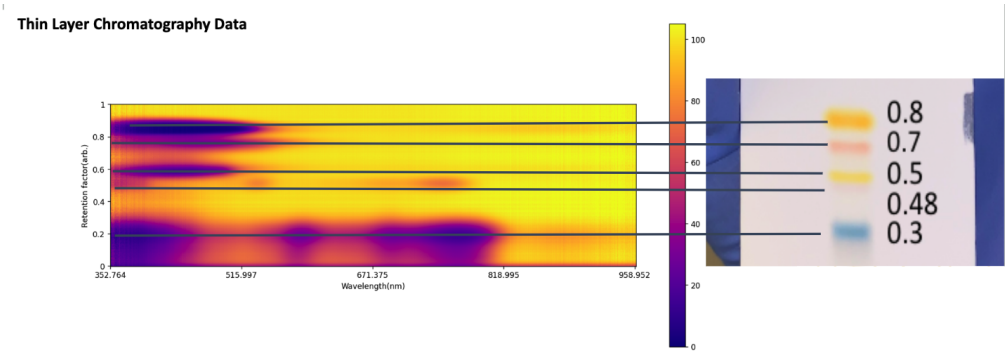

Figure 6b. 3D heat map of TLC plate showing reflectance (left y-axis) as a function of retention factor (right y-axis) and wavelength (x-axis)

The 3D heat map displays data from each of the five bands on the TLC plate. The wavelengths with greatest absorbance (least reflectance) are shown by the purple regions and the wavelengths with least absorbance (greatest reflectance) are shown by the yellow regions. The reflectance patterns correspond to each of the bands aligned on the TLC plate and their retention factors reflect relative polarity of the specific band. The bottom-most band had a retention factor of about 0.3 and had the greatest absorption at the 352-490 nm range, 600 nm, and around 700-800 nm. Because of its blue pigment, relative non-polarity compared to the other bands, and its absorbance in the 800 nm region which was not seen in any of the other bands, we predicted that the bottom-most band contained bacteriochlorophyll or bacteriochlorophyll metabolites. We also saw an absorbance peak in the 900 nm region that was not previously seen in *R. sphaeroides* without exposure to light. This could suggest that 900 nm, infrared light, could stimulate cellular activity in the bacteria that would allow it to better harvest the light for increased absorption. The top 4 bands (see Figure 6b) primarily displayed absorbance peaks in the 352-500 nm region. Due to their relative polarity, with the top band showing an rf value of 0.8, a yellow/ pink colored pigment, and absorbance patterns in the 400 nm region, we predicted that the other bands had carotenoid or carotenoid metabolite candidate molecules (Ashenafi, E.L, et. al. 2023).

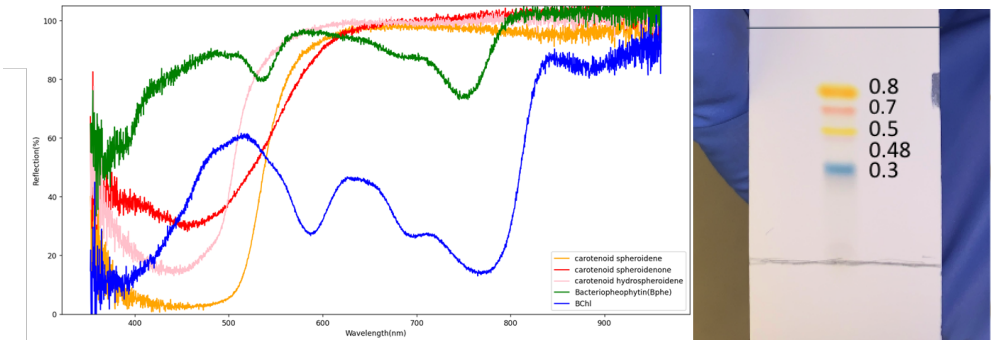

Figure 6c. TLC reflection plot as a function of wavelength. Each line on the plot represents a band on the TLC plate with potential candidate molecules predicted from previous studies isolating *R. sphaeroides* pigments (see legend on bottom right corner; Zhihua, L. et al. 2014).The bottom-most pigment shows an additional absorbance peak at around 900 nm which was not typically seen in *R. sphaeroides* (see Figure 7). Increased absorption could suggest increased light-stimulated activity in the 900 nm region when supplementing yeast samples with the blue pigment from the TLC plate. Each pigment band aligns to one of the lines on the plot in the order shown in the legend.

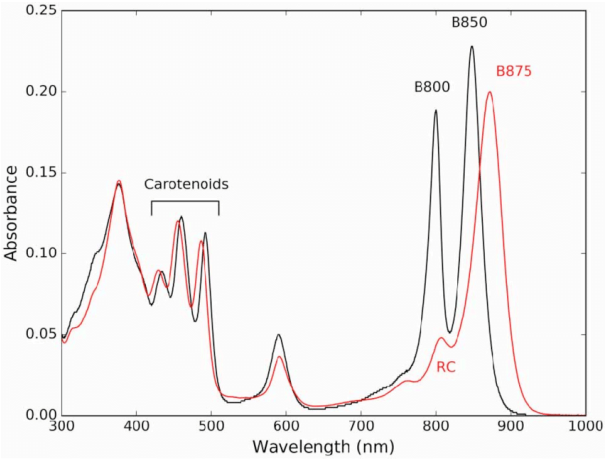

Figure 7. Absorption spectra of *R. sphaeroides*. Each peak shows optimal wavelengths for absorption of a distinct bacterial pigment. The black line indicates the bacteriochlorophyll pigments (B800, B850) and the carotenoids, while the red line indicates the reaction center pigment (Saer, G.R. Blankenship, E.R. 2017).

As shown in Figure 6c, the blue line (blue pigment) aligns similarly to the absorbance spectra in Figure 7, with the exception of an additional absorbance peak in the 900 nm region. The TLC reflection plot shows that each pigment band on the TLC plate has its own spectral capacities and absorbs light at different wavelengths. The top band on the TLC plate, corresponding to the orange line, primarily absorbs light within the 400-500 nm range which

aligns to the carotenoid pigments in Figure 7. Observing the spectral properties of each pigment band gave a better understanding to how these pigments interact with specific wavelengths of light. It was predicted that if a specific pigment absorbs light at a particular wavelength, and subsequently supplemented to a yeast culture, yeast growth rate may increase due to light stimulation of the electron transport chain and increased ATP production. Similar to photosynthetic organisms, supplementation of pigments could help absorb light energy for optimal energy harvesting of the yeast's electron transport chain to ultimately improve ATP generation. Understanding how bacterial pigments affect yeast growth may have implications for enhanced PBM therapy in eukaryotes.

In addition to spectral analysis, chemical composition of the pigments and pigment metabolites was assessed via LC-MS. The samples were compared to a *Progenesis* database to identify potential candidate molecules in each band. Mass-to-charge ratios for each molecule (parent and daughter ions) were assessed for and compared to the control group (silica gel). See Figure 8 for the pigment bands that were analyzed and used as treatments.

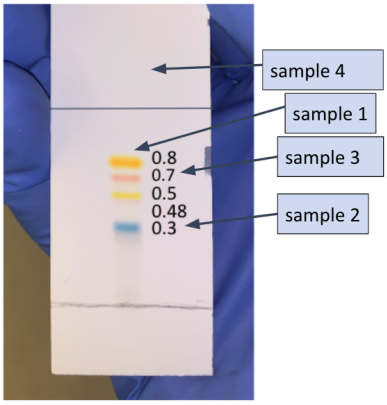

Figure 8. Samples taken from TLC plate for LC-MS analysis. Sample 1 represents the top-most band (predicted carotenoid pigment), sample 2 represents the blue band (predicted to be bacteriochlorophyll), sample 3 represents the pink-colored band, and sample 4 serves as the control group (silica gel).

Candidate molecules for each sample were identified. It is important to note that while TLC is useful for separating pigments based on polarity, each band could have had remnants of other molecules or contaminants from other pigment bands. Thus isolation via TLC is not as precise and further separation via ultra-performance liquid chromatography was used to distinguish chemical composition of each band. Sample 1 contained lycopene and spherodene-like molecules. Both candidate molecules were carotenoid pigments, which did align with our spectral analysis and Rf value. Sample 2 contained remnants of biliverdin and bacteriochlorophyll A. Sample 3 contained ubidecarenone, also known as coenzyme Q, a significant carrier molecule in the electron transport chain (Orozco, D. et. al. 2007). Distinguishing candidate molecules in each pigment band allowed us to better understand how these pigment metabolites could interact with various wavelengths of light to improve PBM in yeast growth. Future directions for this project include comparing candidate molecules after bacterial growth under various wavelengths of light to observe changes in absorbance peaks and chemical composition. The bacteria used in the study were grown under a white tungsten lamp.

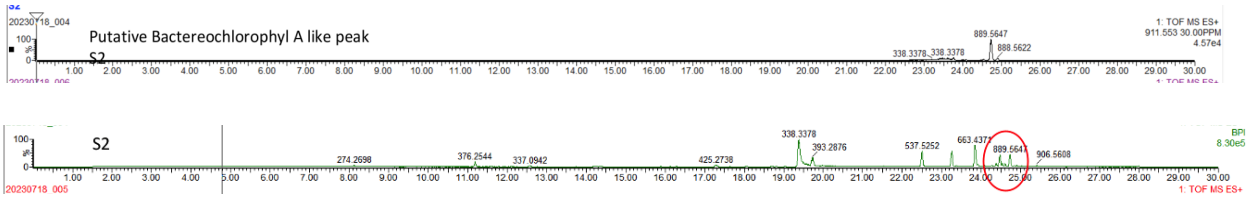

Figure 9. The chromatograms above show mass-to-charge ratio and relative abundance (y-axis) as a function of retention time (x-axis) for one of the candidate molecules in sample 2 (blue band). The top chromatogram is taken from the *Progenesis* database to compare parent and daughter ions in sample 2 for identification of chemical composition. The bottom chromatogram depicts our sample. As shown in the two chromatograms, there is a peak for a parent molecule at around 889.5647 amu which aligns with a bacteriochlorophyll metabolite. This process was completed for each sample and possible molecule matches were identified.

Yeast Growth

Yeast were cultured on an agarose gel using a yeast farm model (see Methods section). Before supplementation with pigments, our goal is to observe PBM in yeast by measuring growth in front of a supercontinuum light source. Video footage was taken to observe yeast growth over time and we saw a depression of intensity that moves radially at a rate different from other regions near 767 nm. This suggests that light stimulation of yeast without supplementation of bacterial pigments can alter growth rate. Future treatments include supplementing the yeast with bacterial pigments that optimally absorb a particular wavelength of light and measuring growth as a function of wavelength.

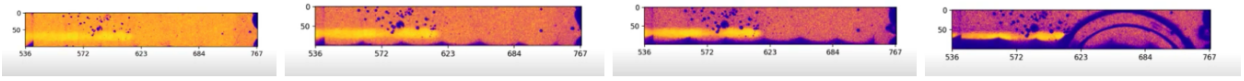

Figure 10. Screenshots taken from video of yeast sample in front of a supercontinuum light source. The x-axis represents wavelength and the y-axis represents absorbance of light, or yeast growth (see Equation 1). The purple regions indicate absorbance of light (yeast growth). As shown in the images, light exposure stimulates radial yeast growth at around 767 nm at a faster rate than other wavelengths. This could suggest that at around 767 nm there is expedited yeast growth, and thus supplementing the yeast with a pigment that optimally absorbs light in the 767 nm range could enhance PBM in yeast.

The results show yeast growth affected by light. Future research suggests that when adding isolated bacterial pigments to the yeast farm and subsequently exposing the yeast to light

that is absorbed by the pigments, yeast growth rate could increase as a result of improved light harvesting. For example, supplementing the yeast farm with pigment from the blue band (see Figure 6b) could stimulate increased yeast growth rate if exposed to a 767 nm laser beam. The blue pigment was specifically identified to have biliverdin and bacteriochlorophyll candidate molecules (see Figure 9), which both have absorbance peaks in the 700-850 nm range.

CONCLUSIONS

Photobiomodulation therapy has opened doors to light-induced healing in humans and mitigation of damaged tissues (Dompe, C. et. al. 2020). Supplementing light therapy with bacterial pigments under exposure to varying light conditions could offer novel methods of improving ATP production in eukaryotic organisms. In humans, light and pigment applications may be used to enhance medical treatments including cancer therapies, stem cell proliferation, and alleviate conditions related to oxidative stress, such as hypoxic ischemia (Luodan, Y. et. al. 2021).

Some challenges we ran into with this project include finding the best way to isolate the bacterial pigments. Although TLC helped visualize separation between pigment bands, it was difficult to analyze the chemical composition of each band by scraping off the pigment bands because little sample volume was obtained. Repeated measures were taken to undergo isolation through UPLC. Separation of the bands on the TLC plate took multiple trials due to difficulties finding the right eluent and ratios of hexane and acetone to separate the sample. *R. sphaeroides* was also grown in an anaerobic chamber to mitigate any exposure to oxygen which would alter pigment composition and metabolism. If left in the centrifuge vial for too long, separation was not clear and the bacteria would have to be inoculated again which took several days. We conducted TLC analysis once the bacteria were grown to saturation, so to stay consistent with our results it was important to remove the bacteria once it reached exponential growth. Yeast growth is also challenging to observe due to easy contamination and maintenance of humidity and temperature. The yeast farm was modified to prevent the agarose gel from being exposed to air and evaporating. This caused air bubbles to form inside of the yeast farm and fermentation

because yeast can grow both aerobically and anaerobically. Exposure to air made it difficult to measure yeast growth.

Future directions for this project include inoculating the bacteria under various light conditions to observe changes in pigment bands on the TLC plate. Changes could include color, rf value, spectral properties, and chemical composition. Similar isolation and analysis with the TLC plate and LC-MS would be done to each experimental treatment to investigate whether growth under various wavelengths of light would alter pigment composition. The pigments would then be supplemented to the yeast farm with a laser beam that would radiate a light onto the yeast sample. The laser would shine a wavelength of light that is best absorbed by the pigments to measure differences in yeast growth rate. Understanding how yeast growth is affected by pigment uptake and PBM could have promising applications for human medical research. Another area of research that this project can focus on is mitochondrial activity. In the future, yeast mitochondria could be analyzed upon exposure to light and supplementation of bacterial pigments to measure ATP production and pigment uptake. Isolation of yeast mitochondria would clarify whether PBM has direct impacts on ATP production. Long-term applications for this project could be used to promote stimulation of cellular activity with the uptake of bacterial pigments and exposure to various wavelengths of light.

This research project provided greater clarity of PBM by isolating and analyzing *R. sphaeroides* pigments to understand how eukaryotic cell growth could be affected by implementing pigments to harvest light energy, a data-dense measurement that has yet to be done in the field of PBM research. The results could offer insights to future non-invasive treatments in

humans for improved metabolic activity, cell repair, energy production, and mitochondrial function.

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