Antioxidant Production by Xanthophyllomyces dendrorhous Under Different Stress Conditions

A Major Qualifying Project

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Abstract

Antioxidants are significant contributors to the aquaculture, medical, food and cosmetic industries. Previously these molecules have been produced synthetically, but in recent years there has been a push for their natural development through plants and fungi like *Xanthophyllomyces dendrorhous*, a basidiomycete yeast. The red yeast *X. dendrorhous* naturally makes large amounts of the antioxidant astaxanthin - a carotenoid. Previous work has proven *X. dendrorhous*' capability to produce carotenoids, but the most optimum growing conditions have yet to be determined. This study investigated the stressor induced metabolite production by two different *X. dendrorhous* strains when exposed to hydrogen peroxide, ultraviolet light, and various temperatures. Ultimately, it was determined that stress response is strain dependent, with the over producer *X. dendrorhous* strain always producing more astaxanthin. It is also possible that other secondary metabolites are being produced by *X. dendrorhous*, especially at room temperature (~23ºC). The determination of optimum growing conditions that facilitate the maximum production of metabolites by *X. dendrorhous* is essential for a wide range of industries whose consumers are more responsive to the natural production of antioxidants because bio-based products are more sustainable and environmentally friendly.

Acknowledgements

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Background

1.1 Reactive Oxygen Species and Antioxidants

Trillions of cells in the human body face formidable threats that range from insufficient nutrition from foods, viral attacks, and chemicals called free radicals (*Antioxidants*, n.d.). At extremely high levels, they are capable of damaging cells and their genetic material. The generation of free radicals by our bodies is an inevitable byproduct of turning food into energy. The effects of exercising, smoking, air pollution, and radiation from sunlight can substantially increase free radical levels (*Antioxidants*, n.d.). The structure of free radicals can vary in many different shapes, sizes, and chemical configurations. However, they all share an attraction for stealing electrons from any nearby substances (*Antioxidants*, n.d.). As a result, the structure and function of the free radical who lost an electron can alter drastically. The stealing of electrons can cause changes in DNA sequence and even morph the shape of a cell membrane (*Antioxidants*, n.d.). Excessive amounts of these reactive oxygen species (ROS) in the body can inhibit various chronic diseases. As a response, the human body produces regulatory molecules to quench the negative effects of these free radicals.

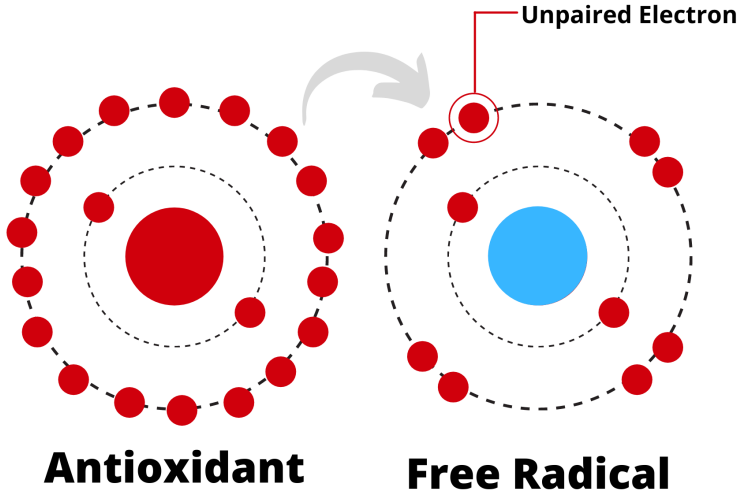

***Figure 1**: Antioxidant reducing free radical through electron donation*

These regulatory molecules are known as antioxidants. Today, there are thousands of different types of antioxidants that have been identified with various structures and functions. Antioxidants tend to be characterized by three main characteristics; size, solubility, and enzymatic activity (Satish Balasaheb & Dilipkumar, 2015). When organized based on size, they are split into two groups; small molecule antioxidants and large molecule antioxidants. Small molecule antioxidants (vitamin C, vitamin E, and carotenoids) neutralize the reactive oxygen species in a process called radical scavenging and carry them away (Satish Balasaheb & Dilipkumar, 2015).

On the contrary, large molecule antioxidants are enzymes and sacrificial proteins that absorb the ROS in order to prevent them from attacking essential proteins (Satish Balasaheb & Dilipkumar, 2015).When characterized by solubility, antioxidants are split into two categories: soluble in water or lipids. Water soluble antioxidants (vitamin C) are present in cellular fluids like the cytosol or cytoplasmic matrix, while lipid soluble antioxidants (vitamin E, carotenoids, lipoic acid) are predominantly located in cell membranes(Satish Balasaheb & Dilipkumar, 2015).

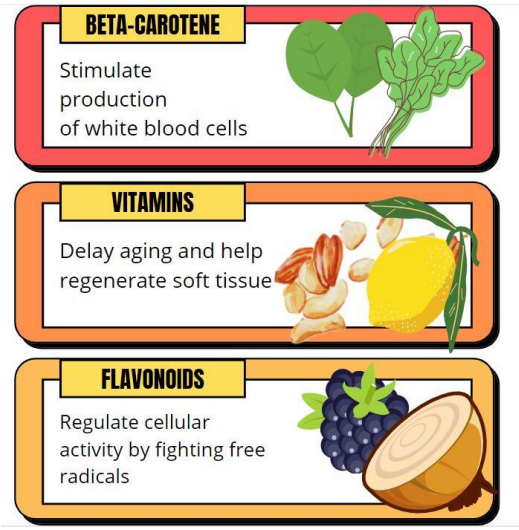

Figure 2: *Diagram of common antioxidants with corresponding functions and common foods they are found in*

When characterized by enzymatic activity antioxidants are split into non-enzymatic and enzymatic antioxidants. Non-enzymatic antioxidants (vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione) work by interrupting free radical chain reactions (Satish Balasaheb & Dilipkumar, 2015). During the free radical chain reactions repeating reactions take place that lead to the production of multiple ROS. The antioxidants are able to intercept and terminate these reactions using mechanisms specific to the antioxidant. Enzymatic antioxidants work by breaking down and removing free radicals (Satish Balasaheb & Dilipkumar, 2015). An example of this process is breaking down dangerous oxidative products to hydrogen peroxide and then water, in a multi step process with cofactors such as manganese, iron, and copper (Satish Balasaheb & Dilipkumar, 2015).

1.2 Primary and Secondary Metabolites

In animals as well as humans, it is known that antioxidant supplementation can help increase metabolism. Metabolism is characterized as a multitude of essential chemical reactions that an organism uses to convert food to energy, use energy for growth, and eliminate waste. They are intermediates and products of metabolism that are typically characterized by small molecules with various functions (*Primary and Secondary Metabolites*, 2020). Generally, metabolites are split into two broad categories; primary metabolites and secondary metabolites. Primary

metabolites, like ethanol, lactic acid, and citric acid, are considered essential to microorganisms and help support the overall development of a cell (C, 2017). In contrast, secondary metabolites are not directly involved in growth, development, or reproduction, but typically have an important ecological function, including defence mechanisms such as acting as antioxidants (*Primary and Secondary Metabolites*, 2020). Secondary metabolites are but not limited to pigments, alkaloids, essential oils, toxins, and polymeric substances such as rubber and cellulose (*Antioxidants*, n.d.).

1.2.1 Carotenoids

Carotenoids are part of a class of secondary metabolites acting as antioxidants, known as terpenoids. They are plant pigments responsible for bright red, yellow, and orange hues in many fruits and vegetables and due to this pigmentation, carotenoids are widely used in food industries (Szalay, 2015). Pharmaceuticals widely use carotenoids due to their cancer fighting properties, anti-inflammatory properties, and cardiovascular disease prevention (Szalay, 2015). There are more than 600 recorded types of carotenoids, with the most well known being β-Carotene and astaxanthin (Szalay, 2015).

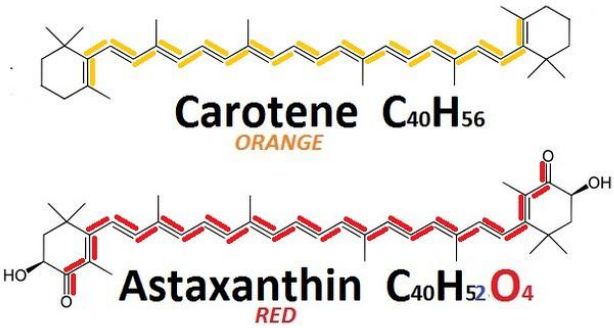

Figure 3: *Chemical structure and formula of carotene versus astaxanthin [17]*

β-Carotene is the most prominent member of the group of carotenoids abundant in fungi, plants, and fruits, like carrots and sweet potatoes. This non-enzymatic hydrocarbon is an antioxidant characterized by its orange and red color and the most important precursor to Vitamin A (Grune et al., 2010). Based on β-Carotene's structure and experimental data demonstrating it as an efficient singlet oxygen quencher, it has been characterized as an antioxidant. However, there has been conflicting research about the true antioxidant properties β-Carotene might have and whether or not it displays prooxidant properties (Grune et al., 2010). Prooxidants are defined as chemicals that induce oxidative stress, through the formation of reactive oxygen species or by inhibiting the antioxidant system (Sotler et al., 2019). Despite the contrasting evidence, β-Carotene is still marketed as a powerful antioxidant and in 2019 its estimated market size was $498 million (*Beta Carotene Market Size 2020: Industry Overview by Size, Share, Future*

Growth, Development, Revenue, Top Key Players Analysis and Growth Factors up to 2024, 2020).

Astaxanthin, another prominent carotenoid, differs from carotenes due to its structure. Unlike β-Carotene, astaxanthin is not a hydrocarbon because it contains oxygen (Szalay, 2015). Additionally, astaxanthin is more yellow than β-Carotene and is not a precursor to Vitamin A (Szalay, 2015). It is most commonly found in marine organisms such as microalgae, trout, krill, shrimp, and crustaceans and it is most abundantly sourced from the green microalgae, Haematococcus pluvialis (Natural Medicine Journal, 2012). When salmon farming increased in the 1980's, it created a large market for astaxanthin, the leading pigment in salmon (E. A. Johnson & Gil-Hwan, 2008). Thus, making the fish more desirable for consumer purchase. Along with the natural pigment astaxanthin provides to the aquaculture industry, its antioxidant potential has become extremely valuable commercially across other industries such as the food, pharmaceutical, and cosmetic industries. In 2019 astaxanthin's global market size was estimated to be approximately 1 billion (*Astaxanthin Market Size, Share & Trends Analysis Report By Source, By Product (Dried Algae Meal, Oil, Softgel), By Application (Nutraceutical, Cosmetics, Aquaculture and Animal Feed), And Segment Forecasts, 2020 - 2027*, 2020).

1.2.2 Additional Secondary Metabolites that Act as Antioxidants

Apart from carotenoids, there are other secondary metabolites that act as antioxidants, such as phenols and flavonoids. Phenolic compounds are the most abundant antioxidants in the human diet and are characterized structurally by containing hydroxyl groups on aromatic rings (Vuolo et al., n.d.) . They are found ubiquitously in plants and play important roles as internal physiological regulators, chemical messengers, and protectors against pathogen infection and UV radiation. Additionally, phenolic compounds have become a subject to a tremendous amount of research due to their anti inflammatory properties and potential involvement in lowering the risk of chronic diseases, such as cardiovascular diseases, and certain types of cancer (Zhang et al., 2020).

Flavonoids are a type of phenols that are characterized structurally by containing multiple phenol groups and a heterocyclic ring. Research on flavonoids orginated in 1936 by Albert Szent Gyorgi who studied citrus fruits and worked to identify vitamin P (a complex compound of flavonoids) and investigated its use in strengthening capillaries (*Albert Szent-Gyorgi*, n.d.). Today flavonoids are thought to have antioxidant, antibacterial, antiviral, antifungal, anti inflammatory, and anticancer properties. As an antioxidant, flavonoids are able to quench ROS by reducing the singlet oxygen's, hindering enzymes involved in ROS generation, chelating transition metal ions that trigger ROS production, and helping with the recycling of other antioxidants. Within plants, flavonoids help protect against abiotic (salt, drought, UV radiation, and heat) and biotic stressors. Additionally, they help regulate subcellular activities, provide pigments to flowers, and help

develop the Rhizobium symbiotic relation between plants and microbes (Khalid et al., 2019). In 2015, the flavonoid market size was estimated to be approximately $410 million (*Flavonoids Market Size, Share & Trends Analysis Report By Product (Anthocyanins, Flavones, Anthoxanthins), By Application, By Region, And Segment Forecasts, 2018 - 2025*, 2016).

1.3 Xanthophyllomyces dendrorhous

Xanthophyllomyces dendrorhous is a basidiomycetous yeast originally found in tree exudates in the northern hemisphere at high altitudes and latitudes (Libkind et al., n.d.). Originally discovered in Alaska and Japan in the 1970's *X. dendrorhous* is characterized by its bright red-orange pigment provided by astaxanthin, its main antioxidant product (Visser et al., 2003) (Rodriguez-Saiz et al., 2010). *X. dendrorhous* is the only known yeast to produce astaxanthin, which is naturally created in order to provide protection against reactive oxygen species (E. Johnson & Echavarri-Erasun, 2011). The identification of this native carotenoid biosynthetic pathway has made *X. dendrorhous* a highly sought after organism due to astaxanthin's importance in the aquaculture, food, pharmaceutical, and cosmetic industries.

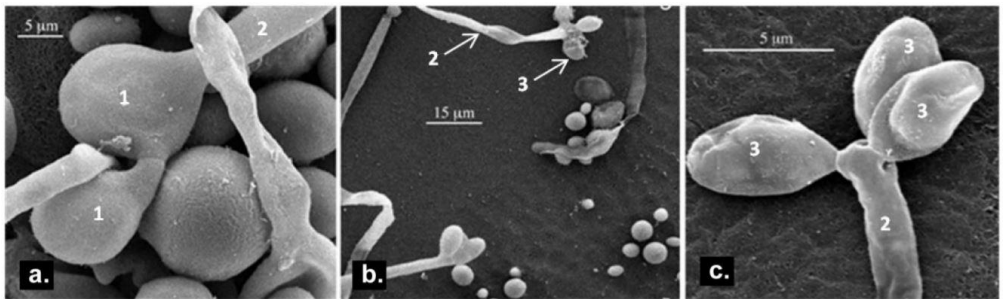

Figure 4: *Sexual state of Xanthophyllomyces dendrorhous. (a) Scanning electron microscopy photo indicating two conjugate cells (b) aerial basidium with apical basidiospores (c) detail of basidium apex and basidiospores Source: American Society of Microbiology*

Since wild strains of *X. dendrorhous* produce low quantities of secondary metabolites, a significant amount of research has been conducted in order to develop strategies for enhancing yeast metabolite production. Through mutagenization and the creation of new culture mediums, new strains of *X. dendrorhous* are able to produce 20 to 50 times more antioxidants (E. Johnson & Echavarri-Erasun, 2011) (Stachowiak, 2012). While a considerable amount of research has been focused on the enhancement of *X. dendrorhous'* biosynthetic pathways, the potential production of other secondary metabolites as well as *X. dendrorhous'* behavior under different stressors is relatively unexplored.

1.4 Identifying Metabolites in Yeast

Due to globalization and an evolving world population with an increasing resistance to existing drugs, there will always be a need for the development of new pharmaceutical products. Today, a wide variety of modern medicine is derived from natural products and only 27% are completely synthetic in origin. Since World War 2 with the discovery of the 'wonder drug' penicillin, fungi have been described as a rich source of biologically active natural compounds like secondary metabolites (Hoeksma et al., 2019). One past literature survey found that of the 1,500 compounds that have been isolated from fungi between 1993 and 2001, more than half displayed antibacterial, antifungal, or antitumor activity (Keller, 2019). A more recent study covering fungal natural products that were discovered between 2009 and 2013 further confirmed this phenomenon (Keller, 2019). However, given the large biodiversity within the fungal kingdom, only a small fraction of fungi have ever been tested for the production of biologically active compounds.

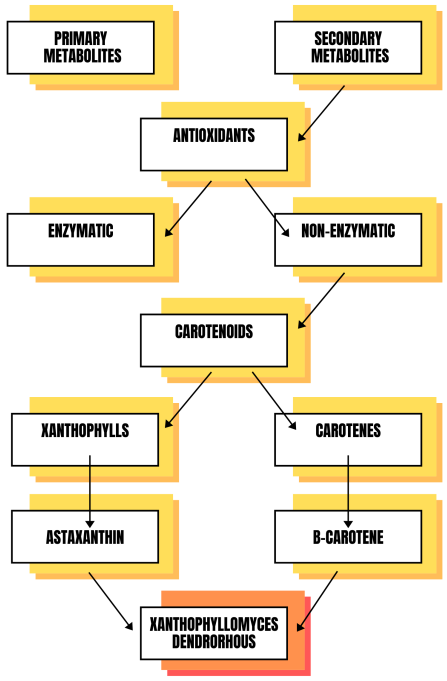

Figure 5: *Chart summary of relationship between secondary and primary metabolites*

1.5 Stressor Induced Metabolite Production

1.5.1 Light Activation of Fungal Metabolism

Previous studies have shown that *X. dendrorhous* carotenogenesis could be induced by ultraviolet light (Marsan, 2019.). In recent years, the wild type strain has been grown in laboratories under

UV to induce the production of secondary metabolites (Rodriguez-Saiz et al., 2010). While there have been previous observations, limited information on the wavelength specificity of this interaction, more trials need to be performed for higher accuracy. UV radiation is harmful for organisms because it has the ability to break bonds and form free radicals in the organism. The metabolites produced block the effects of these free radicals. Although the *X.dendrorhous* carotenoid biosynthetic pathway has been identified, not much is known about how it is regulated. When grown under high intensity light, the production of the carotenoid synthesis of the wild type strain showed to be simulated by white light and UVR 25 (Rodriguez-Saiz et al., 2010).

1.5.2 Thermal Activation of Antioxidant Production

Researchers have wanted to increase astaxanthin production for commercial use by chemical mutagenesis or genetic engineering for decades (Shiwen et al., n.d.). In a 2018 study, results concluded that *X. dendrorhous* could not grow past 25°C. To achieve a continuous industrial production of astaxanthin in *X. dendrorhous,* moderate temperature of 25–37°C fermentation process was needed (Shiwen et al., n.d.). This 2018 study used a two-step process with a 20°C pre‑culture for 18 hours and a 30°C culture for 30 hours to achieve desired astaxanthin yields (Shiwen et al., n.d.).

Changing the culture process was a viable way to increase the overall culture temperature (Shiwen et al., n.d.). Since *X. dendrorhous* grows the most optimally at 21ºC (Collins, 2020), there have not been many investigations regarding *X. dendrorhous* growth and secondary metabolite production at low temperatures. However, since *X. dendrorhous* has been found naturally in cold climates like Alaska and Antarctica, investigations into metabolite production in cold temperatures, would be prudent (Contreras et al., 2015).

1.5.3 Hydrogen Peroxide of Secondary Metabolites

A few studies have also been performed to investigate hydrogen peroxide induced astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous*. A 2006 study analyzed astaxanthin production in *X. dendrorhous* when exposed to varying concentrations of H_2O_2 over two days (Shuai Liu & Yong Wu, 2006). Exposing *X. dendrorhous* to hydrogen peroxide is of interest because in microorganisms, secondary metabolites are known to accumulate during the exposure to biotic and abiotic stressors. The stimulated carotenoid biosynthesis by oxidative stress from reactive oxygen species has been observed in a multitude of carotenoid-producing microorganisms In the 2006 study, hydrogen peroxide (30% w/w) was added to the *X. dendrorhous* cultures to a concentration of 10 or 20 mmol/L at 0, 24, and 48 hours post inoculation. The flasks were then harvested at selected time intervals and the overall culture period for each trial was 120 hours. In order to measure the carotenoid contents, the yeast cells were separated by centrifugation, dried overnight at 105ºC, extracted with hexane-ethyl acetate (50:50 v/v) and quantified using UPLC. It was found that the hydrogen peroxide caused slightly lower cell growth, but significantly

higher astaxanthin yield compared to the control culture - especially at 10 mmol/L H_2O_2 at 24 hours of culture. It was also found that β-Carotene content decreased the most at 24 hours of exposure (48 hours post inoculation) (Shuai Liu & Yong Wu, 2006).
A second study, also from 2006, investigated the production of astaxanthin by *X. dendrorhous* when exposed to hydrogen peroxide (as well as ethanol and acetic acid). On day five of a ten day culture, when the residual glucose was nearly depleted, hydrogen peroxide in a range of 0-1.5% (v/v) was added. After 48 hours, the amount of astaxanthin production was quantified and it was found that at a concentration of 1% hydrogen peroxide the total carotenoids content increased compared to the control. Both the 0.5% and 1.5% hydrogen peroxide trial had less carotenoid content compared to the control. Interestingly, out of ethanol, acetic acid, and hydrogen peroxide; ethanol caused the largest increase in carotenoid content in the yeast (Jeong-Hwan & Chang, 2005).

This study aimed to investigate the impact of stress response on *X. dendrorhous* secondary metabolite production. Five different stressors were analyzed, temperature (4ºC, room temperature, 37ºC), hydrogen peroxide, and UV light. A range of temperatures were chosen because while *X. dendrorhous* is normally cultured at room temperature, it is found in nature at regions that experience significantly lower temperatures, so it was hypothesized that *X. dendrorhous* would be able to produce antioxidants at 4ºC (Contreras et al., 2015). While it was not expected for *X. dendrorhous* to be most efficient in metabolite production at warmer temperatures, previous work has shown that *X. dendrorhous* is able to grow at 37ºC, so it became of interest to investigate metabolite production significantly above and below normal culturing conditions.

As outlined above, previous studies have also found that exposure of *X. dendrorhous* to UV light and hydrogen peroxide resulted in an increase in astaxanthin production (Rodriguez-Saiz et al., 2010)(Marsan, 2019.)(Shuai Liu & Yong Wu, 2006). This study aimed to compare the amount of astaxanthin produced by *X. dendrorhous* while exposed to these different stressors in order to determine the most ideal growing conditions for *X. dendrorhous.*

Methodology

2.1 Exposing the Xanthophyllomyces dendrorhous to Stressors

To assess the antioxidant production of wild type and over producer strains of *X. dendrorhous*, different cultures of *X. dendrorhous* were exposed to UV light, hydrogen peroxide, and a range of temperatures (4ºC, 23ºC, and 37ºC). To ensure accuracy, each strain was run in triplicate. Overall for each stressor there were two strains and three replicates per strain, leading to a total of six trials per stressor. Throughout the experiments, growth curves of each stressor were constructed using optical density measurements taken at different timepoints, as indicated in figure 6. In order to measure the optical density, 100 µl of sample was removed from each trial and mixed with 900 µl of Milli Q water in a cuvette, creating a 1:10 dilution of the yeast culture which was then measured with a spectrophotometer at 600nm. Ultra performance liquid chromatography (UPLC) and a plate reader was later used to quantify the antioxidant production of both strains of *X. dendrorhous*. To prepare for the UPLC/plate reader, an additional 1 mL of sample was removed during the density measurements, and placed into a cryotube. In an effort to obtain more reliable data the stressor experiment was run twice. During the first experiment all five stressors were run (UV, H_2O_2, 4ºC, 23ºC, and 37ºC). During the second run of the experiment three of the stressors were run (UV, H_2O_2 and 23ºC). The initial data for the 4ºC stressor was adequate thus the cold temperature stressor was not run a second time. Originally it was planned to run the 37ºC trial a second time, however due to limited lab availability this did not occur.

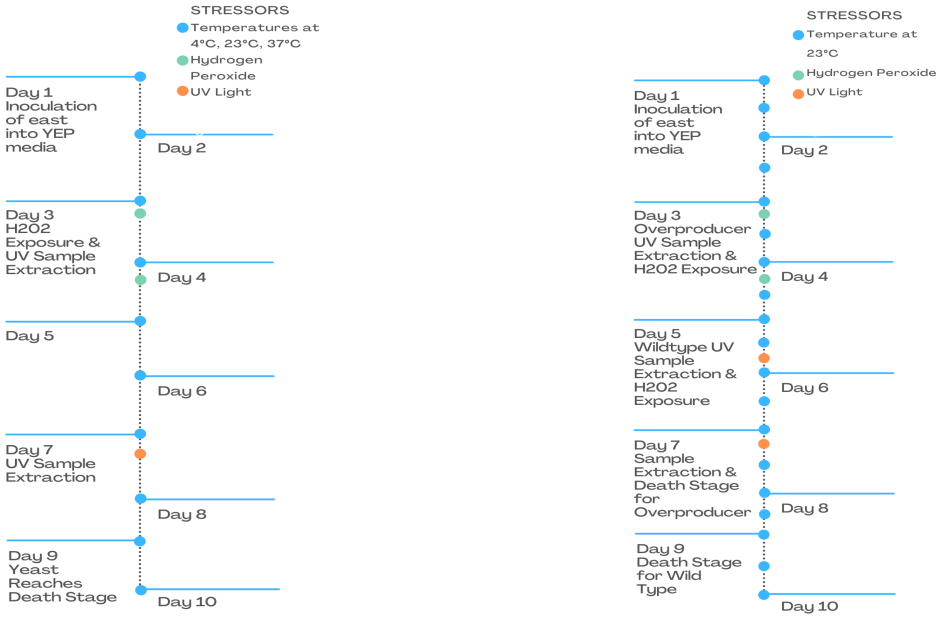

Figure 6*: Timeline for the first stressor experiment (left) versus the timeline for the second stressor experiment (right)*

2.2 Culturing of the Xanthophyllomyces dendrorhous

To prevent cross contamination every trial of each stressor was grown in its own suspension culture. The temperature and hydrogen peroxide trials were cultured in 125 mL erlenmeyer flasks and the UV trials were cultured within a well plate in the light plate apparatus. Yeast Extract Peptone (YEP) media was created by adding 30 grams of YEP powder to 1000 mL of Milli Q water and then autoclaving the solution for fifteen minutes at 121ºC. Each trial culture was inoculated with *X. dendrorhous* at 0.1M on day one of the experiment. 25 mL of YEP media was added to each flask and 2 mL of media was added to each well. To reach the desired *X. dendrorhous* concentration 0.1M, preliminary density readings of each strain of *X. dendrorhous* were measured and then used to calculate the amount of *X. dendrorhous* needed to be added to each flask/well plate. For the wild type strain 806 µl of *X. dendrorhous* was added to each flask and 64.5 µl of *X. dendrorhous* was added to each well plate. For the over producer strain 610 µl of *X. dendrorhous* was added to each flask and 48.8 µl of *X. dendrorhous* was added to each well plate.

2.3 Temperature (4ºC, 23ºC, 37ºC) Exposure

Exposure of the *X. dendrorhous* to various temperatures was conducted using six 125 mL erlenmeyer flasks per temperature (three replicates per strain and two strains). To increase cell growth all of the flasks were placed on a stir plate. The 4ºC trial flasks were placed in a freezer, the room temperature flasks were placed on a lab bench, and the 37ºC trial flasks were placed within an incubator. In order to create the growth curves, samples from each trial were removed at regular time intervals. During the first run, samples were taken every twenty-four hours. In an effort to better depict the exponential phase of the growth curves, for the second run of the experiment samples were taken every twelve hours (this run only included the 23ºC trials). The experiment ended once it was apparent on the growth curves that the yeast had reached the death stage and were no longer growing. For the first run this occurred on day nine and for the second run this occurred on day nine for the wildtype strain and day seven for the over producer strain.

2.4 Ultraviolet Light Exposure

Exposure of the *X. dendrorhous* to UV light was conducted using a light plate apparatus constructed in a past MQP by Celeste Marsan (Marsan, 2019). The light plate apparatus was located on a stir plate at room temperature. To further verify the UV light results, control trials were added, where a section of the light plate apparatus was programmed to expose the control trials to no light. Figure 7 depicts the well plate set up of the UV light/dark trials. Samples for density measurements and the UPLC were only drawn at one point throughout the experiment due to the limited amount of volume available in the wells on the light plate apparatus. The samples were drawn during the peak growth period for the yeast (day seven for the first run and

day five for wildtype/ day three for over producer for the second run), which was determined using the growth curves constructed from the room temperature trials.

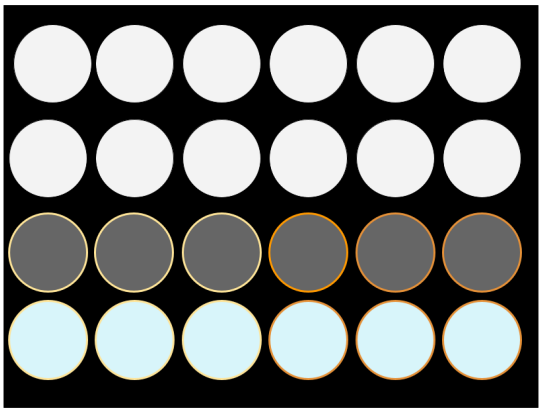

Figure 7: *Light plate apparatus set up. Light blue circles indicate UV light exposure, dark gray circles indicate no light, and white circles indicate not used well plates. Orange rings indicate wells with the overproducer strain and light yellow rings indicate wells with the wild type XD strain.*

2.5 Hydrogen Peroxide Exposure

Exposure of the *X. dendrorhous* to hydrogen peroxide was conducted using six, 125mL erlenmeyer flasks on a stir plate at room temperature. The yeast do not live long when exposed to hydrogen peroxide. Instead of exposing the yeast to hydrogen peroxide on day one, they were exposed when the color of the solution became clear on the room temperature growth curves that the yeast were in their exponential growth phase (day three for the first run and day five for wild type/day three for over producer for the second run). To create the desired hydrogen peroxide solution, 5.9 µl of hydrogen peroxide was added to each sample to create a 0.01M solution. In order to properly assess the impact of the hydrogen peroxide on the yeast, sample extraction for density measurements and the UPLC/plate reader were taken directly before exposure and twenty four hours later. In addition, during the second run of the experiment samples were recorded after twelve and twenty-four hours.

2.6 Preparing Samples for Extraction

Samples were prepared for extraction through the execution of two wash steps. Samples obtained from the first round of stressor experiments were first thawed through the suspension of a sample in 1 ml of warm water. Due to the increased volume of solution, the solution was then split into two 1 mL centrifuge tubes. Samples obtained from the second round of stressor experiments were prepared for extraction before frozen, so the thawing process was unnecessary. The first wash step was completed once the sample was at room temperature which included the sample being centrifuged for two minutes at 15,000 rpm, and excess water being removed and discarded from the centrifuge tube. Approximately 1 mL of distilled water was added into the centrifuge

tube and the vortexed to dislodge and mix the pellet back into the solution. This wash step was completed a second time. After the second wash step, the sample was centrifuged for two minutes at 15,000 rpm for a third and final time. The sample was then prepared for extraction once the excess water was removed from the centrifuge tube, leaving only a pellet behind.

2.7 Extraction of the Antioxidants

Prior to the start of the extraction process, samples obtained from the second round of stressor experiments had to be thawed since they were frozen in between the preparing samples for extraction and the extraction steps. In order to facilitate the lysis of the yeast cells , a solvent (1 mL of DMSO or dimethyl sulfoxide) was added to the samples and vortexed. The extraction process was then conducted with sodium chloride or NaCl (100 µl of 20% w/l) and acetone (1 mL). The solutions were vortexed and centrifuged for 5 minutes at 3250 rpm. The centrifuge separated the extracted antioxidants (organic phase) with the rest of the cell material, which formed a pellet. The organic phase material was removed and stored in a 2 mL centrifuge tube for later use with the UPLC and plate reader. In order to extract any remaining antioxidants, DMSO (1 mL), NaCl (100 µl), and acetone (1 mL) were added back into the original centrifuge tubes which were vortexed in order to fully mix the solvents and dislodge the pellet. The solution was centrifuged again for 5 minutes at 3250 rpm and then the organic phase was removed and stored in the same 2 mL centrifuge tube that had the previous extracted antioxidants added to it.

2.8 Creating Standard Curve for Plate Reader

In order to analyze the samples, a standard concentration curve had to be created for astaxanthin and b-carotene. The concentration curved included five different points at 50% dilutions ranging from 5 µg/mL to 0.313 µg/mL. A blank was also created. In order to discern the difference between astaxanthin and β-carotene, three different 5 µg/mL standards were created, all in 1.5 mL centrifuge tubes. One 5 µg/mL that was pure astaxanthin, one 5 µg/mL that was pure β-carotene, and one 5 µg/mL that consisted of both astaxanthin and β-carotene. The pure astaxanthin and β-carotene samples were prepared by adding 0.5 µl of the astaxanthin or β-carotene stock solutions into 999 µl of DMSO The 5 µg/mL standard of both astaxanthin and β-carotene was prepared by adding 0.5 µl of both astaxanthin and β-carotene into 999 µl of DMSO. The rest of the standard curve solutions were then made using the 5 µg/mL standard that had both astaxanthin and β-carotene through serial dilutions. To prepare for the serial dilutions, four other 1.5 mL centrifuge tubes were obtained and filled with 0.5 mL of solvent (DMSO). The serial dilution was then conducted by removing 0.5 mL of solution from the 5 µg/mL tube and adding it into the next tube (creating a concentration of 2.5 µg/mL). After mixing thoroughly with a pipette, 0.5 mL of the 2.5 µg/mL solution was removed and added into the next tube (creating a concentration of 1.25 µg/mL). This process continued for all five centrifuge tubes,

creating concentrations of 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.313 µg/mL. A blank (0 µg/mL) was then created by added 0.5 mL of DMSO into a sixth 1.5 mL centrifuge tube.

2.9 Running Samples on Plate Reader

Samples were run on the plate reader in a clear plastic 96-well plate. To provide further accuracy on absorbance value, each sample was run in triplicate (the same sample was added into three wells). 100 µl of sample was added into each well. Additionally, 100 µl of the solutions prepared for the standard curve were added onto the well plate, also in triplicate.

2.10 Preparing Samples For Ultra Performance Liquid Chromatography (UPLC)

Similarly to the plate reader, in order to quantify the concentration of the samples with the UPLC, a standard curve had to be created. The solutions for the standard curve were prepared in the same manner as described for the plate reader in section 2.8. Each extracted antioxidant sample and standard curve solution were prepared for the UPLC in a similar manner. In order to remove any particulates a syringe filter was used. This syringe was assembled by using a 1 mL syringe, a Millipore Millex-GV PVDF 0.22µm filter, and a 25G1.5 PrecisionGlide needle. Either 100 µl of extracted antioxidant or 1 mL of standard curve solution was added into the syringe, by removing the plunger. The plunger was then used to push the material into 2mL glass autosampler vials. For vials that had only 100 µl of sample added to them, a small glass insert was added into the vials in order to create a deep enough volume within the vial that the UPLC needle can properly remove liquid. Once material was added to the vials, a cap was secured using a hand cap crimper. In order to clean the syringe between samples, 1 mL of methanol was run through the syringe. Additionally in order to prevent contamination, the samples were prepared from lowest concentration to highest concentration (if the concentration was known).

2.11 Running Ultra Performance Liquid Chromatography (UPLC)

Once ready to run, the samples were loaded into the 96 well plate in the UPLC. The samples were run for eight minutes each and once the process was finished they were analyzed at 500nm by calculating the area of absorbance peaks.

Results and Discussion

3.1 Growth Curve Results

The *Xanthophyllomyces Dendrorhous* yeast cells were cultured on a plate shaker. Each stressor was run in triplicate to provide us with more reliable data and give a higher likelihood that the cells would grow from the inoculated yeast cells. Growth data was collected at 4°*C* and room temperature (~23°*C)*. The room temperature stressor was run two times, the second time with data collected at more frequent time intervals in order to better depict the different stages of the growth curve. We also ran the experiment at 37°*C* but many of the yeast cultures did not grow which will be discussed further.

3.1 4°C Data

The 4°*C* temperature trails were only run once. The lag phase for the wild type was shorter as it took two days for the wild type strain and four days for the over producer strains to start their growth phase as can be seen by figure 8 below.

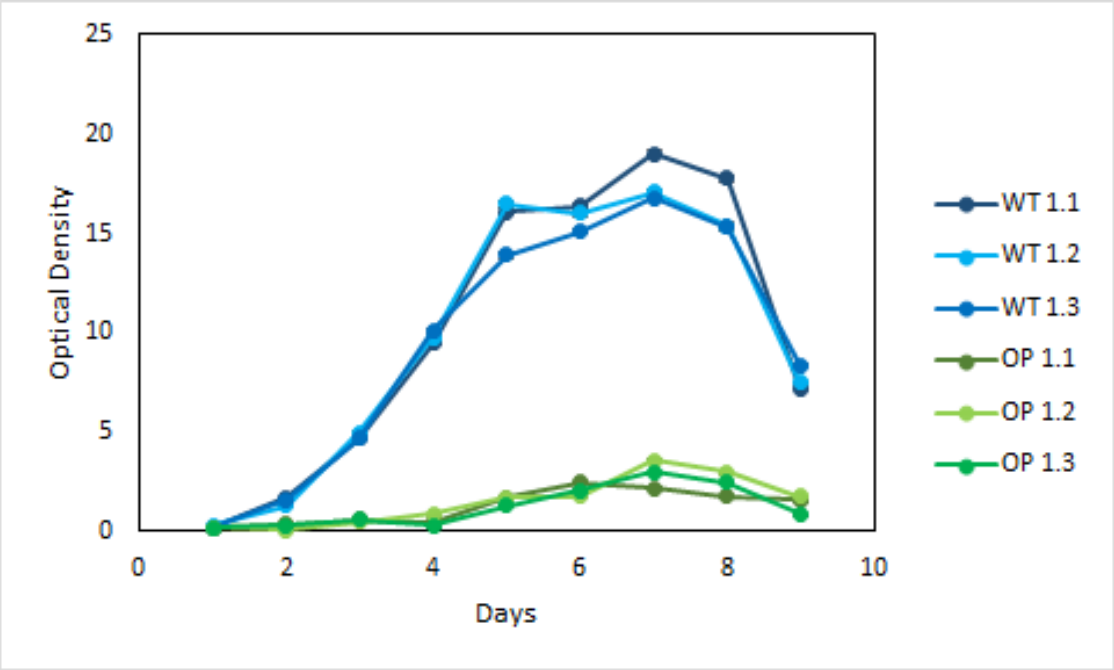

Figure 8*: Optical density readings of the wild type (blue) and overproducer (green) strains at 4°C The first number after each strain represents the run number and the second number represents the replicate number, so WT 1.3 means wild type strain, run 1, replicate 3.*

The yeast cells stayed in the exponential growth phase for about three days. Once peak cell density readings were reached the stationary phase kept cell density levels mostly constant. By the ninth day the yeast cultures had reached the death phase when they ran out of media, which signified the end of the experiment. Since the trails were run in triplicate all three flasks remained

consistent with no outlier trends and small error. The peak density varied significantly between the two strains with the wild type strain reaching an optical density reading of seventeen Au/OD600 while the overproducing strains only achieved an optical density reading of three Au/OD600.

3.2 Room Temperature Data

The room temperature experiments were run twice. During the first run for the wild type strains (WT 1.1, 1.3) there was an extremely short lag period (only a couple of hours) and the yeast very quickly started to grow up, reaching stationary phase by day two. The second run (WT 2.1 and 2.3) had a very long lag phase of approximately four days before the yeast started to grow up. The peak density readings were about the same for both wild type runs and the stationary phase was longer during the first run (~four days) than the second (~2 days). The wild type growth curve is depicted in figure 9 below.

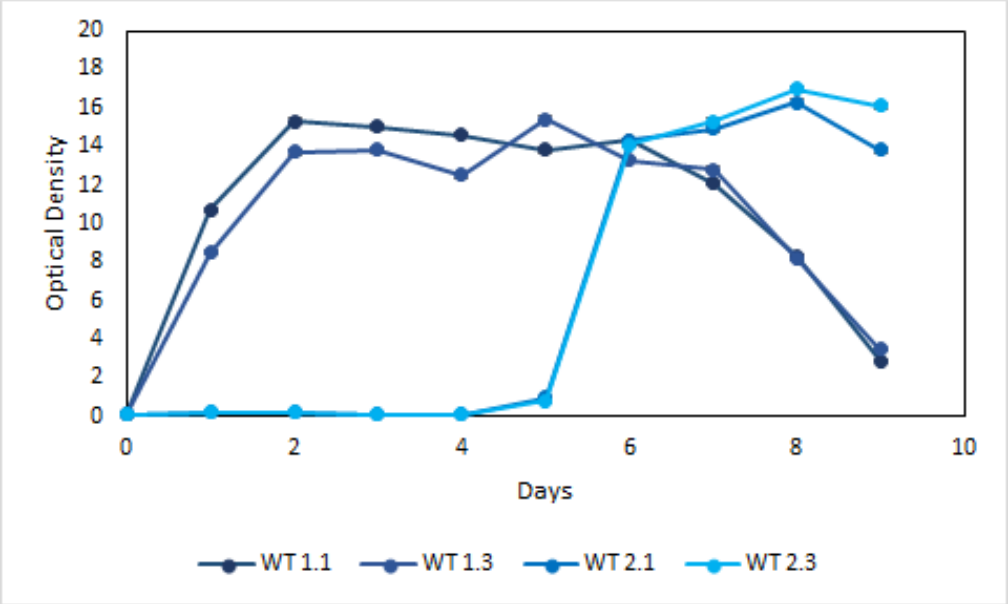

***Figure 9**: Optical density readings of the wild type strain at ~23ºC. The first number after each strain represents the run number and the second number represents the replicate number, so WT 1.3 means wild type strain, run 1, replicate 3.*

Unlike the wild type strain, the over producer strain took longer to grow up and had an exponential phase of approximately three - five days (as visible in figure 10). Between run one and run two the over producer strain was more consistent and did not have one run with a much longer lag phase. Additionally, with both runs the over producer reached it's death phase at around the same time of six days.

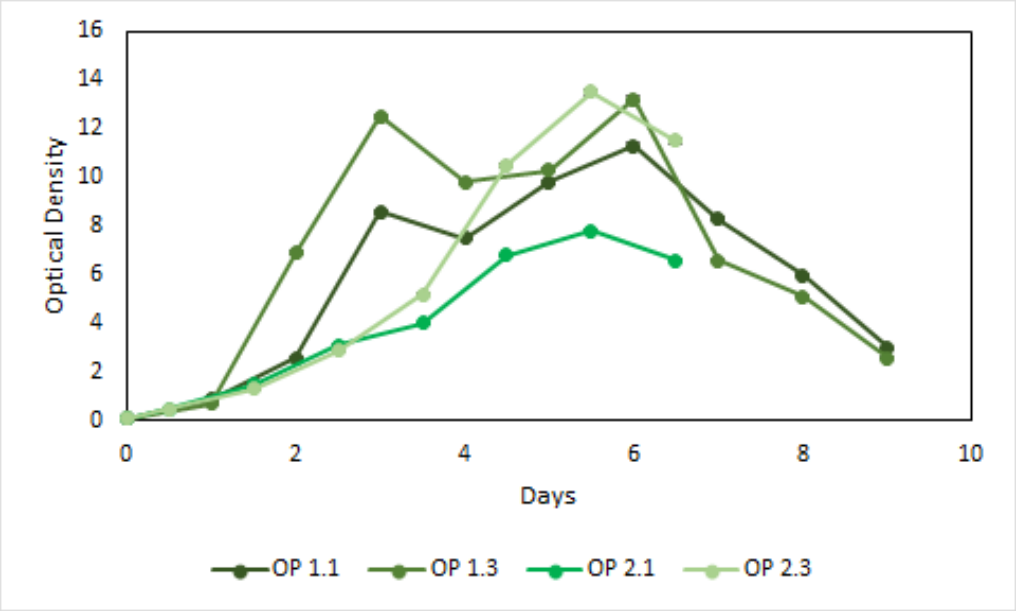

***Figure 10**: Optical density readings of the over producer strain at ~23ºC. The first number after each strain represents the run number and the second number represents the replicate number, so WT 1.3 means wild type strain, run 1, replicate 3.*

The peak optical densities of both runs for each strain were about the same with the wild type reaching peak optical densities of about fifteen Au/OD600 and the over producer reaching peak optical densities of twelve Au/OD600. The wild type strain reached similar optical densities in the room temperature and the four degree temperature trials while the over producer had much higher optical densities in the room temperature trials.

Overall the growth curves acted as expected with the wild type strains growing up very quickly and the over producer strains slower. It was not expected that every replicate would grow at the exact same rate, it is the nature of growing cultures for there to be some fluctuations in growth. When analyzing antioxidant production with the plate reader and UPLC samples were chosen based on their growth phase, not time since exposure so all replicates would be at the same point of growth when analyzed.

3.3 37°C Data

The 37°C experiments did not inhibit the expected growth trends since initial tests showed the yeast cells survived in the 37ºC environment. The second wild type trial is the only cell culture to have growth. We were only able to run the experiment once since we weren't able to get access to a stir plate during our growth phase. Since we were not able to gather significant data from this stress condition we left it out of our other results in the plate reader and UPLC analysis. If we were to run this experiment again we would test for inoculation at 37ºC before taking growth

measurements so we could know more accurately if the *X. dendrorhous* yeast can grow in high temperature atmospheres.

3.2 Extraction Protocols + Concentration Curve Determination

In total, two different extraction protocols were tested. One protocol was a DMSO lysis + acetone/NaCl extraction (Sedmak, 1990) and the other protocol was a blucanex lysis + acetone/NaCl extraction (Zuharlida, 2020). The exact procedures tested for each extraction method is described in Appendix B. In order to quantify which extraction method was most effective, extracted samples from each method were run on the plate reader and the relative levels of absorbances were analyzed. The same yeast samples were used for each extraction method, so the protocols could be comparable. It was found that samples extracted from the blucanex lysis method resulted in larger absorbances meaning more antioxidants were able to be extracted. Due to human error, the DMSO lysis protocol was used as the extraction method for the remainder of the study. In the future the team recommends the use of the blucanex lysis protocol.

Additionally, multiple solvents were tested for use when preparing the standard concentration curves. Originally, pure acetone was used as the solvent, but once samples were loaded into the plastic 96-well plates for the plate reader it became quickly apparent that the acetone was melting the well plate. In order to determine the correct solvent for the concentration curve, two different solvents; 50:50 acetone/DMSO and 100% DMSO were tested. Concentration curves for astaxanthin and β-carotene were prepared. The curves were 50% dilutions starting at 10 μg/ml to 0.313 μg/ml (and a blank). In order to quantify which solvent was the most effective, concentration curves created from each solvent were run on the plate reader and absorbance values, regression lines and R^2 values were compared.
Overall, when analyzing total carotenoids both solvents were similarly effective with large R^2 values (0.9997 for 50:50 solution and 0.9995 for pure DMSO) meaning that data was highly correlated and fits well to the regression line.

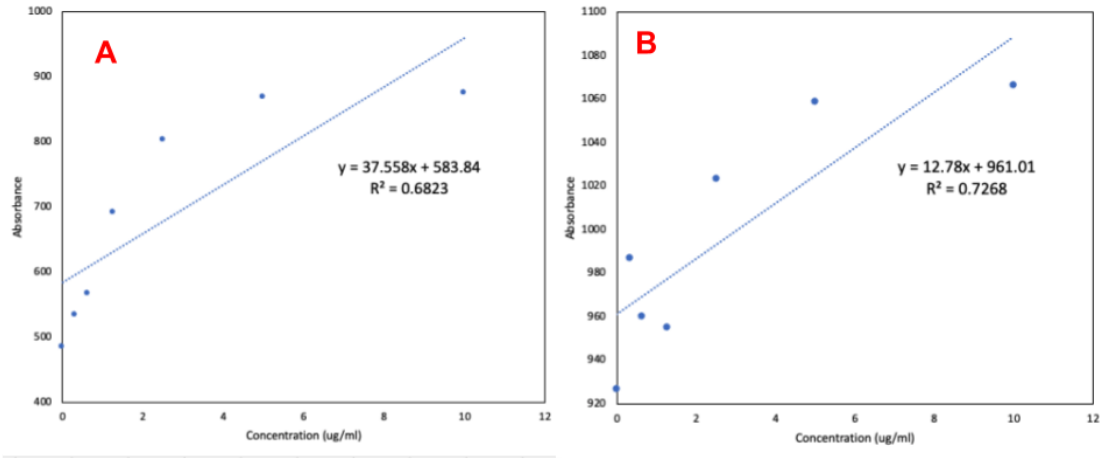

Figure 11: *Astaxanthin plate reader results for concentration curve solvent determination with linear regression lines and R^2 values visible. Figure 11A: Absorbance Vs Concentration graph for 100% DMSO as solvent. Figure 11B: Absorbance Vs Concentration graph for 50:50 DMSO/Acetone as solvent.*

As visible in figure 11 above, the R^2 values for just the astaxanthin concentration curves were more varied. Even though the R^2 value for 50:50 acetone/DMSO solution was higher (0.7268 compared to 0.6823 for the pure DMSO) it was ultimately decided that the pure DMSO would be a more effective solvent because the raw absorbance values for the pure DMSO trial had less outliers.

3.3 Plate Reader Analysis

The plate reader uses a 96 well plate to measure absorbance of many different carotenoids and cell growth. Our experiment measured the total number of carotenoids as well as astaxanthin and beta-carotene levels. We also tested standard curves of known concentration using astaxanthin, beta-carotene, and a mix of both. Using the standard curve data we were able to plot the data and find the linear trend which was used to find the replicate concentrations. The plate reader was run at two separate times once for run 1 and once for run 2 containing the UV, H_2O_2, and 4ºC temperature stress replicates. Due to variations in the standard curves the standard curve used for run 2 was used to assess the run 1 concentration data. The resulting concentration of total carotenoids can be seen in figure 12 below.

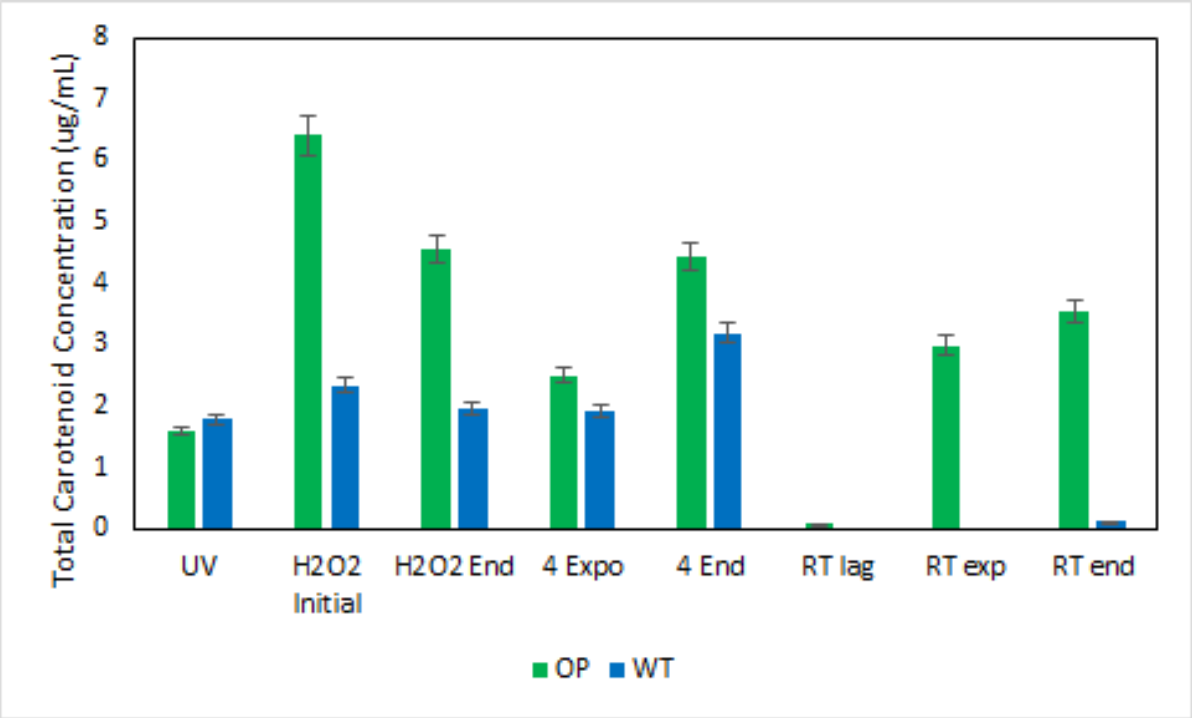

Figure 12: Total carotenoid concentration for each stress condition experimented with the overproducer strain in green and the Wild-type strain in blue

As figure 12 shows the over producer strain consistently had more carotenoid production for each stress condition except for the UV stress experiment. It was expected that the over producer strain would produce more carotenoids than the wild type strain because it was genetically engineered to do so. For the UV trial it is thought that contamination could have led to the unexpected results.. Exposure to hydrogen peroxide appears to have had a significant impact on the carotenoid concentration in the over producer strain. Based on previous studies it was expected that hydrogen peroxide exposure would decrease cell density, but ultimately increase antioxidant concentration (Shuai Liu & Yong Wu, 2006). Since the data obtained from the plate reader was not consistent with our expectations or UPLC data (further explained in section 3.4.3), further analysis is recommended.

For the 4ºC and room temperature trials, in both cases the most carotenoids were produced during the end/stationary phase, which was consistent with the growth curve data where the highest cell density was measured during the end/stationary phase. At 4ºC the wild type strain produced significantly more carotenoids than at room temperature which makes sense since X. dendrorhous is naturally found at high altitudes so it most likely has naturally evolved to be the most efficient at cold temperatures. It was expected that the over producer would have the largest measured carotenoid concentration at room temperature, because it is engineered to grow at normal lab growing conditions which is room temperature, and we believe that inaccuracies of the plate reader has led to the unexpected data.

As outlined above there were numerous unexpected/potentially inaccurate results obtained from the plate reader, because of this it was decided that our main results will be based off of the UPLC data described below in section 3.4. The UPLC is a more accurate assessment of antioxidant production because it directly measures carotenoids, while the plate reader measures any chemical in the light range of astaxanthin.

3.4 UPLC Analysis

In total 48 samples were run on the UPLC. From the second run of the stressor experiment, eighteen samples were run. For each replicate from each strain one sample from the lag phase, mid exponential phase, and stationary phase were quantified. There were three replicates per strain and two strains. From the first run of the stressor experiment, 30 samples were run. Six samples were run from the UV stressor, three from each strain, one per replicate. For the hydrogen peroxide stressor, twelve samples were run. Each replicate from each strain had a sample run from directly before the hydrogen peroxide addition and from twenty four hours later. Lastly, from the 4ºC stressor twelve samples were run.
Each replicate from each strain had a sample run from mid exponential phase and one from stationary phase. It was decided not to run any samples from the lag phase for the 4ºC stressor, because after the analysis from the room temperature samples it was found that a negligible amount of antioxidants were being produced in the lag phase. No samples were run from the 37ºC trial because only one replicate showed growth during the stressor experiment. Astaxanthin and β-carotene concentration curves were prepared, however when conducting UPLC analysis it was found that the β-carotene standard did not produce any peaks. The team noted this as an issue with the β-carotene standard. For the rest of the analysis, astaxanthin is the only antioxidant analyzed quantitatively, but there is a qualitative discussion about other potential secondary metabolites.

3.4.1 Room Temperature Trials

Eighteen samples from the room temperature stressor experiment were run on the UPLC. For each strain (wild type and over producer) three samples were run at three main points of the *X. dendrorhous* growth curve (lag phase, mid-exponential phase, and stationary phase). Using absorbance values from these samples and data from an astaxanthin standard curve, the concentration of astaxanthin was calculated for each sample. The average concentration of each strain at each point of the growth curve is depicted below in figure 13.

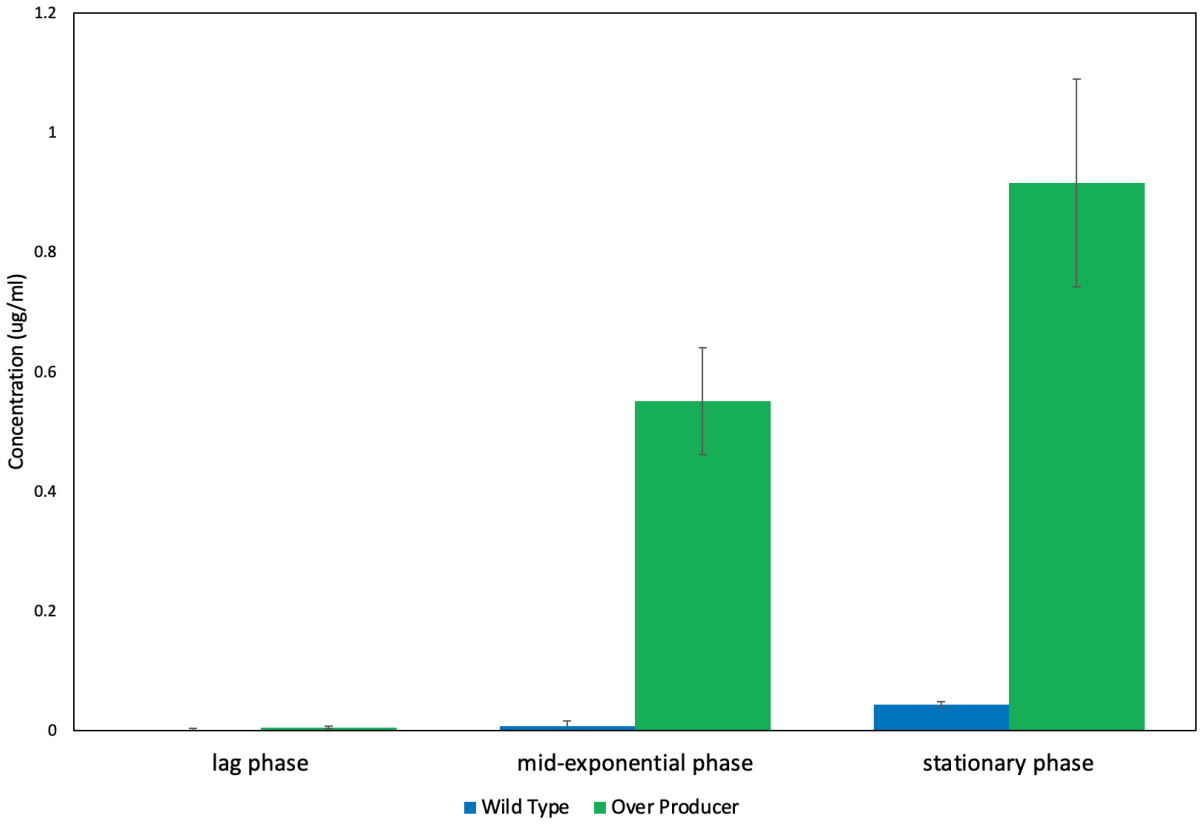

Figure 13: *Concentration (µg/ml) of Astaxanthin in the X. dendrorhous strains at room temperature. Each concentration value is the average concentration of the three replicates.*

As visible in the figure above, a very small amount of astaxanthin is produced during the lag phase. This was expected since the lag phase occurs before any real growth in the culture. By mid-exponential phase there was a large increase in astaxanthin production and by stationary phase an even larger concentration of astaxanthin was produced. This makes sense since, as seen in the growth curve data, stationary phase had the largest cell density, so there was a larger quantity of cells available to produce the antioxidant. Also as expected, the over producer stain had significantly larger astaxanthin concentrations. The over producer strain is engineered to 'over produce' antioxidants compared to the wildtype strain and at room temperature this is occurring by approximately 20 fold in stationary phase and 73 fold in mid-exponential phase.

For the wild type strain, the increase in astaxanthin concentration between stages stayed pretty consistent (concentration increased by about 500% between each stage). For the over producer strain, there was a much larger increase in astaxanthin concentration between the lag and mid-exponential stage (10,000%) than the mid-exponential to stationary phase (200%). Part of this discrepancy could be related to the time point of the sample used in analysis - it could have

been more towards the end of exponential phase instead of mid. Or the overproducing yeast cells were producing more astaxanthin early on in growth compared to the end.

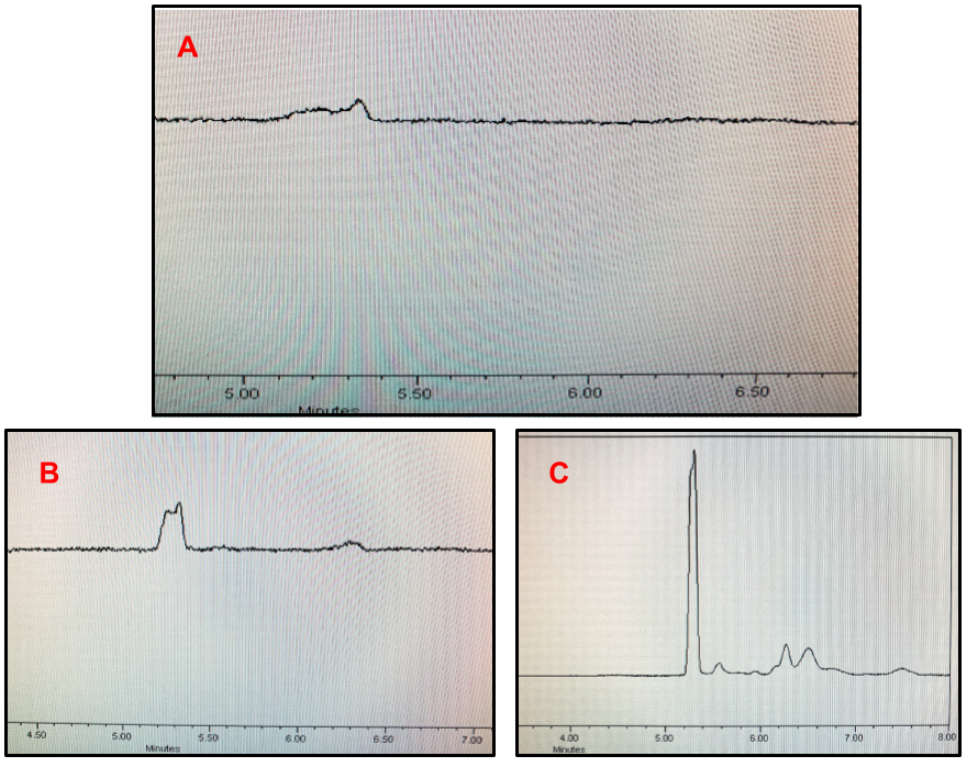

Figure 14: *Qualitative analysis of room temperature samples. Figure A is absorbance peaks from wild type mid exponential phase. Figure B is absorbance peaks from wild type stationary phase. Figure C is absorbance peaks from over producer stationary phase.*

In addition to the quantitative analysis performed to calculate the concentration of astaxanthin in each sample, a qualitative analysis was performed to discuss the potential of other secondary metabolites being produced by each strain, as depicted above in Figure 14. The three pictures from this figure are pictures taken from the UPLC during analysis of mid-exponential phase (Figure 14A), and stationary phase (Figure 14B, 14C). For both the wild type and over producer strain during lag phase and mid-exponential phase there was only one main peak, as seen in Figure 14A. This indicates that for the most part only astaxanthin is being produced from either strain. During stationary phase however, more peaks are visible. For the wild type this mostly included one other small peak (Figure 14B). This peak might be β-carotene since it is known that *X. dendrorhous* produces both astaxanthin and β-carotene. The over producer during stationary phase has seven different peaks (Figure 14C). This means that the over producer is producing other types of secondary metabolites, besides astaxanthin and β-carotene. A further investigation into the identification of these secondary metabolites is recommended.

3.4.2 4ºC Trials

Similarly to the room temperature trials, three samples from each *X. dendrorhous* strain (wild type/ over producer) were run based on different points in their growth curve. For the 4ºC analysis, samples were run from mid-exponential phase and stationary phase. UPLC was used to calculate the concentration of astaxanthin in the samples, the average for each phase indicated on Figure 15, as well as to conduct a qualitative analysis of secondary metabolite production (Figure 16).

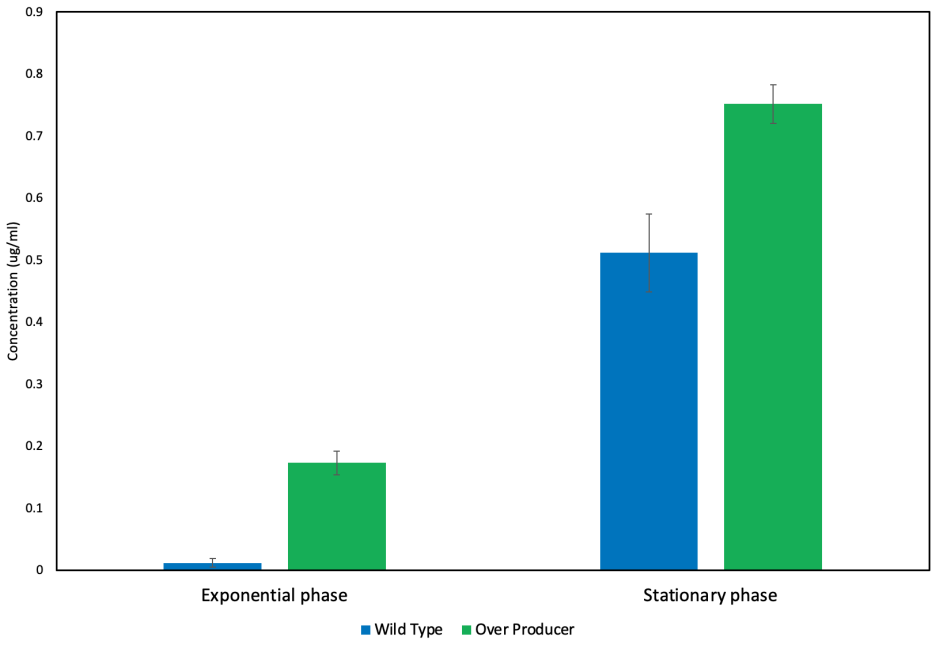

***Figure 15**: Concentration (µg/ml) of Astaxanthin in the X. dendrorhous strains at 4ºC. Each concentration value is the average concentration of the three replicates.*

As expected based on the cell density data and previous room temperature UPLC analysis, the concentration of astaxanthin was significantly higher in both strains in stationary phase. Additionally, the over producer stain had a larger astaxanthin concentration compared to the wild type strain. The difference in astaxanthin concentration between the two strains at each phase is smaller compared to the room temperature samples. This is because the average astaxanthin concentration for the over producer strain was lower in both phases at 4ºC compared to room temperature. This is probably due to the fact that the over producer yeast at 4ºC did not grow up as well and had lower cell densities compared to the room temperature trial. Even though the samples were taken at the same point of the growth curve at each temperature, since the 4ºC samples had lower cell densities in general, it makes sense that the astaxanthin concentration would also be lower.

On the other hand, the average astaxanthin concentration in the wild type strain was larger in both phases at 4ºC compared to room temperature. The cell densities for the wild type strain at

each temperature were similar, so that was probably not a significant contributing factor to the difference in astaxanthin concentration. *X. dendrorhous* has been found in Antarctica, so it is not extremely surprising that the wild type strain produces more astaxanthin at lower temperatures.

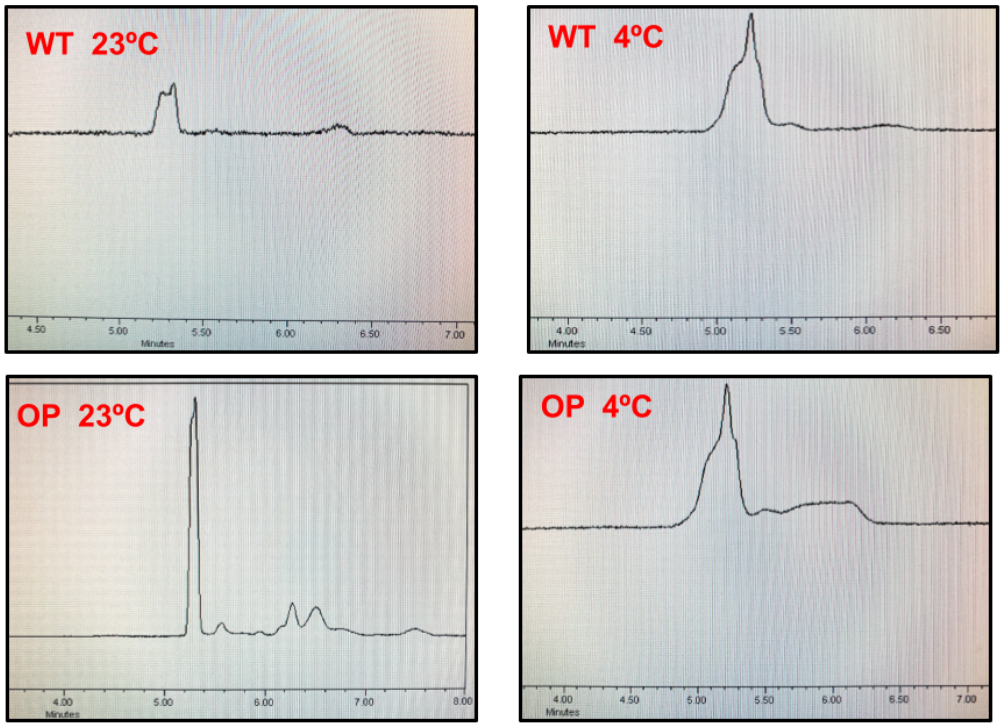

***Figure 16**: Qualitative analysis of wild type and overproducer stationary phase peaks at room temperature (23ºC) and 4ºC*

Depicted above in Figure 16, is a qualitative analysis comparing the production of secondary metabolites produced by X. dendrorhous at room temperature and 4ºC. All four of the pictures taken from the UPLC are from stationary phase. The two photos on the top are the wild type strain at room temperature (left) and at 4ºC (right). The two photos on the bottom are of the over producer strain at room temperature (left) and at 4ºC (right). For the wild type strain it is apparent that at 4ºC, not only is there a greater amount of astaxanthin (the main peak), but also there are three peaks visible, instead of the two at room temperature. This means that at 4ºC the wild type strain is producing more secondary metabolites.

For the over producer strain at room temperature there are significantly more peaks compared to 4ºC. This makes sense considering the over producer strain was designed to over produce at normal growing conditions which is room temperature. Overall, the wild type strain was able to produce more astaxanthin at 4ºC compared to room temperature, especially by the stationary phase (concentration of astaxanthin was ten times larger at 4ºC). Additionally, more secondary metabolites are produced by the wild type strain at 4C. The over producer strain produced more

astaxanthin at room temperature and at stationary phase produced significantly more secondary metabolites.

3.4.2 UV light Trials

For the UV analysis six samples were analyzed, three from each strain, all from the same point in time (day seven, stationary phase). Unfortunately, no reliable data was able to be obtained. As indicated in Table 1, all except one sample had either no peak visible on the UPLC (indicated by the dash) or had a very small peak that resulted in a negligible astaxanthin concentration. The cell density of the wild type samples were very small (<0.1) , so these results are not surprising. Most likely, the wild type samples were contaminated at some point during the experiment or since there was no cell growth during both trials of the stressor experiment, wild type *X. dendrorhous* might just not grow when exposed to UV. The over producer samples did have larger cell densities (~10) so either the samples were also contaminated and were growing something that wasn't *X. dendrorhous* or there was an issue with the extraction process and the astaxanthin was never extracted or the exposure to UV caused the over producer strain to stop producing antioxidants. Either way, it is recommended to repeat this portion of the study to further investigate *X. dendrorhous'* relationship with UV light.

Table 1*: Astaxanthin concentration values from wild type and over producer strains (run in triplicate) after exposure to UV light.*

Sample	WT 1	WT 2	WT 3	OP 1	OP 2	OP 3
Astaxanthin Concentration (μg/ml)	0.248	-	-	0.005	0.014	-

3.4.3 Hydrogen Peroxide Trials

The last stressor run on the UPLC was hydrogen peroxide. For each strain, three samples were run from directly prior to the hydrogen peroxide being added and three samples were run twenty-four hours after hydrogen peroxide exposure. The average astaxanthin concentration from each of these conditions is depicted in Figure 17.

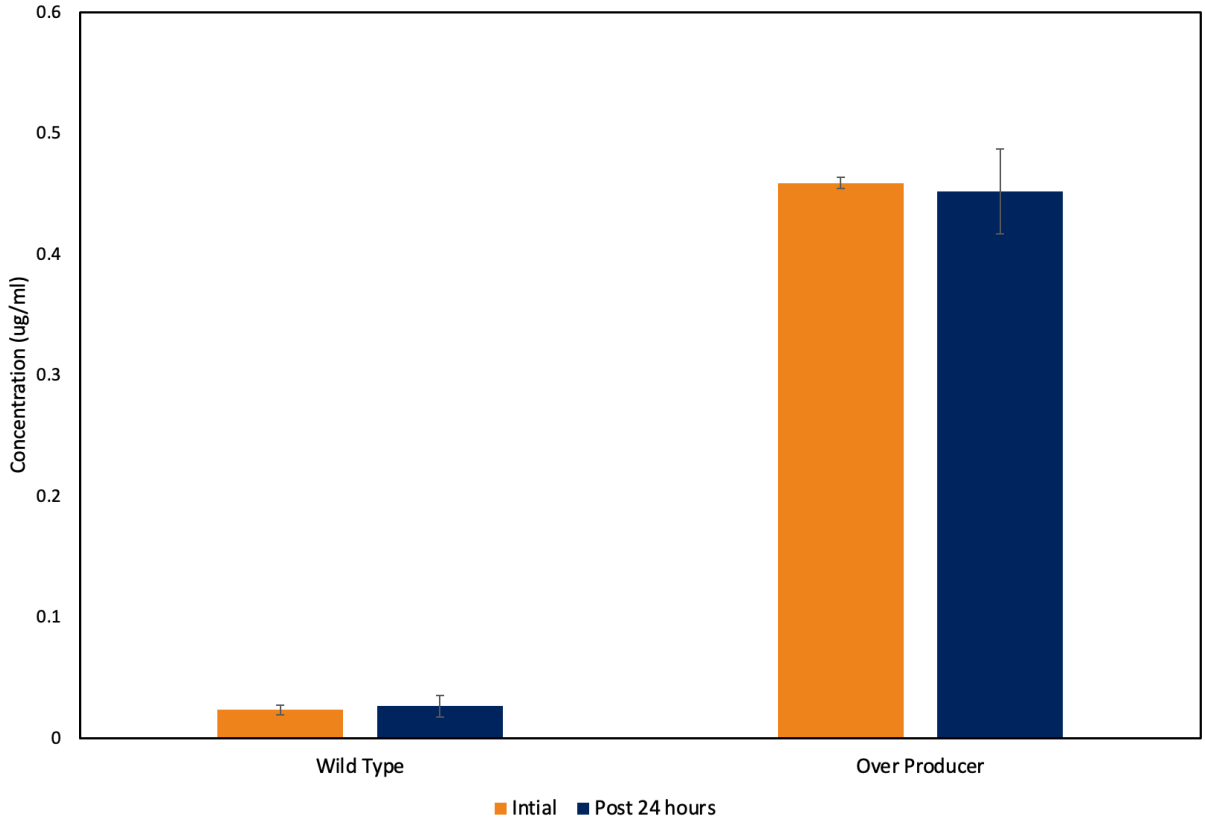

***Figure 17**: Concentration of astaxanthin in wild type and overproducer X. dendrorhous samples immediately before the addition of hydrogen peroxide (orange) and 24 hours after (navy).*

As visible in Figure 17, the addition of hydrogen peroxide did not heavily impact the production of astaxanthin in either strain of *X. dendrorhous*. Based on previous work, as mentioned in the background, it was expected that the exposure to hydrogen peroxide would cause an increase in astaxanthin concentration. When conducting the experiment hydrogen peroxide was added to a concentration of 0.01M, future studies might want to try increasing this concentration. From a qualitative standpoint the wild type strain had only one peak visible and the over producer strain had three. This is consistent with the mid-exponential phase analysis from the room temperature UPLC data, so it does not appear like additional secondary metabolites were produced once the *X. dendrorhous* was exposed to hydrogen peroxide.

Conclusions and Future Work

After exposing two *X. dendrorhous* strains (wild type and over producer) to five different stressors (4ºC, 23ºC, 37ºC, UV, H_2O_2) it was apparent that stress response is strain dependent, but the over producer strain will always produce more astaxanthin. Additionally, both strains produced the largest quantity of astaxanthin during their stationary growth phase, which was consistent with cell density measurements. Stationary phase had the largest measured cell density values, so there was more yeast available to produce astaxanthin. The wild type strain produced the most astaxanthin at 4ºC, while the over producer produced the most astaxanthin at room temperature (23ºC). Through a qualitative analysis it was also determined that it is possible that other secondary metabolites, besides astaxanthin, are also being produced. Hydrogen peroxide exposure at 0.01M had no discernible effect to either yeast strain after twenty-four hours. Both the UV light and 37ºC trials resulted in no measurable cell growth or no measurable extracted astaxanthin.

It is recommended that future studies work to further verify *X. dendrorhous*' reaction to exposure to UV light, 37ºC, and hydrogen peroxide. It is believed that *X. dendrorhous* can grow under UV light and at 37ºC, and it was contamination/extraction issues that lead to no observable growth during this study. It is recommended that future studies expose *X. dendrorhous* to hydrogen peroxide concentrations above 0.01M. Since the yeast was still able to grow after exposure to the hydrogen peroxide, it is believed that the hydrogen peroxide concentration used was too small to cause any significant impact. It was predicted, based on previous studies, that exposure to hydrogen peroxide would cause some cell death, but an overall increase in secondary metabolite production (Shuai Liu & Yong Wu, 2006).

It is also recommended that a further analysis be conducted to identify which other secondary metabolites are being produced by *X. dendrorhous*. Besides astaxanthin, *X. dendrorhous* is known to produce β-carotene, but other antioxidants have yet to be identified. Examination of *X. dendrorhous* (specifically the over producer) at room temperature during the stationary growth phase would be the most optimal since that condition had the most visible peaks on the UPLC.

Lastly, it is recommended that for future investigations a different extraction procedure to be used. The study used the DMSO lysis + acetone/NaCl extraction protocol outlined in the methodology section 2.7, in order to remove antioxidants from the yeast cells for UPLC and plate reader use. This extraction protocol was successful, however it was determined that the blucanex lysis + acetone/NaCl extraction protocol described in appendix B provides a more effective extraction.

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Appendices

Appendix A: Cell Density Raw Data

Run 1 (Fall) Data:

Cell Density at 4ºC

Wild Type Strain		
Replicate	**Days Since Exposure**	**Density**
1	1	0.2
	2	1.7
	3	4.7
	4	9.5
	5	16.1
	6	16.4
	7	19
	8	17.8
	9	7.2
2	1	0.3
	2	1.3
	3	5
	4	9.8
	5	16.5
	6	16
	7	17.1
	8	15.4
	9	7.5
3	1	0.2
	2	1.6
	3	4.7
	4	10.1
	5	13.9
	6	15.1
	7	16.8
	8	15.3
	9	8.4

Over Producer Strain		
Replicate	**Days Since Exposure**	**Density**
1	1	0.2
	2	0.4
	3	0.6
	4	0.5
	5	1.7
	6	2.5
	7	2.2
	8	1.8
	9	1.6
2	1	0.2
	2	0.1
	3	0.5
	4	0.9
	5	1.7
	6	1.8
	7	3.6
	8	3.0
	9	1.8
3	1	0.2
	2	0.3
	3	0.6
	4	0.3
	5	1.3
	6	2.1
	7	3.0
	8	2.5
	9	0.9

Cell Density at Room temperature (~23ºC)

Wild Type Strain		
Replicate	**Days Since Exposure**	**Density**
1	1	10.7
	2	15.3
	3	15
	4	14.6
	5	13.8
	6	14.3
	7	12.1
	8	8.3
	9	2.9
2	1	8.2
	2	15.9
	3	14.4
	4	13.5
	5	13.7
	6	12.2
	7	11.8
	8	7.5
	9	4.2
3	1	8.5
	2	13.7
	3	13.8
	4	12.5
	5	15.4
	6	13.3
	7	12.8
	8	8.2
	9	3.5

Over Producer Strain		
Replicate	**Days Since Exposure**	**Density**
1	1	0.9
	2	2.6
	3	8.6
	4	7.5
	5	9.8
	6	11.3
	7	8.3
	8	6.0
	9	3.0
2	1	0.6
	2	2.8
	3	11.3
	4	6.1
	5	8.3
	6	7.1
	7	9.0
	8	5.6
	9	2.9
3	1	0.7
	2	6.9
	3	12.5
	4	9.8
	5	10.3
	6	13.2
	7	6.6
	8	5.1
	9	2.6

Cell Density at 37ºC

Wild Type Strain		
Replicate	**Days Since Exposure**	**Density**
1	1	7.6
	2	8.2
	3	7.8
	4	6.7
	5	10.1
	6	7.7
	7	11.4
	8	9.3
	9	1.7
2	1	0
	2	0.1
	3	0
	4	0
	5	0
	6	0
	7	0
	8	0
	9	0
3	1	0
	2	0
	3	0
	4	0
	5	1.0
	6	0
	7	0
	8	0
	9	0

Over Producer Strain		
Replicate	**Days Since Exposure**	**Density**
1	1	0.1
	2	0.1
	3	0.1
	4	0.1
	5	0.1
	6	0.1
	7	0
	8	0.2
	9	0.1
2	1	0.1
	2	0.1
	3	0.1
	4	0.2
	5	0.1
	6	0.1
	7	0
	8	0.2
	9	0
3	1	0.1
	2	0.1
	3	0
	4	0.1
	5	0
	6	0.1
	7	0
	8	0.1
	9	0

Cell Density with Hydrogen Peroxide Exposure

Wild Type Strain		
Replicate	**Days Since Exposure**	**Density**
1	3 (initial)	16
	4	13.8
2	3 (initial)	15.7
	4	15.4
3	3 (initial)	14.8
	4	15.1

Over Producer Strain		
Replicate	**Days Since Exposure**	**Density**
1	3 (initial)	11.4
	4	6.0
2	3 (initial)	10.6
	4	5.6
3	3 (initial)	11.7
	4	5.0

Cell Density with Ultraviolet Light Exposure

Wild Type Strain		
Replicate	**Days Since Exposure**	**Density**
1	3 (initial)	0.6
2	4	0.8
3	3 (initial)	0.4

Over Producer Strain		
Replicate	**Days Since Exposure**	**Density**
1	3 (initial)	11.1
2	4	10.3
3	3 (initial)	9.7

Cell Density with No Light

Wild Type Strain		
Replicate	**Days Since Exposure**	**Density**
1	3 (initial)	1.2
2	4	0.7
3	3 (initial)	0.1

Over Producer Strain		
Replicate	**Days Since Exposure**	**Density**
1	3 (initial)	10.8
2	4	11.0
3	3 (initial)	8.3

Run 2 (Spring) Data:

Growth Curve at Room Temperature (~23ºC)

Wild Type Strain			
Replicate	Date	Days Since Exposure	Density
1	3/3	12 hr (8 pm)	0.1
	3/4	1 (8 am)	0.2
		1 (8 pm)	0.1
	3/5	2 (8 am)	0.2
		2 (8 pm)	0.1
	3/6	3 (8 am)	0.1
		3 (8 pm)	0.1
	3/7	4 (8 am)	0.1
		4 (8 pm)	1.0
	3/8	5 (8 am)	1.0
		5 (8 pm)	2.4
	3/9	6 (8 am)	14.3
		6 (8 pm)	8.2
	3/10	7 (8 am)	14.9
		7 (8 pm)	10.2
	3/11	8 (8 am)	16.3
		8 (8 pm)	17.5
	3/12	9 (8 am)	13.8
		9 (8 pm)	8.5
	3/3	12 (8 pm)	0.1
	3/4	1 (8 am)	0.2
		1 (8 pm)	0.1
	3/5	2 (8 am)	0.2

Over Producer Strain			
Replicate	Date	Days Since Exposure	Density
1	3/6	12 hr (8 pm)	0.5
		1 (8 am)	0.4
	3/7	1 (8 pm)	1.5
		2 (8 am)	2.6
	3/8	2 (8 pm)	3.1
		3 (8 am)	1.1
	3/9	3 (8 pm)	4.0
		4 (8 am)	1.5
	3/10	4 (8 pm)	6.8
		5 (8 am)	6.9
	3/11	5 (8 pm)	7.8
		6 (8 am)	4.5
	3/12	6 (8 pm)	6.6
		7 (8 am)	4.8
2	3/6	12 (8 pm)	0.5
		1 (8 am)	0.4
	3/7	1 (8 pm)	1.6
		2 (8 am)	3.7
	3/8	2 (8 pm)	3.0
		3 (8 am)	1.5
	3/9	3 (8 pm)	4.8
		4 (8 am)	2.4
	3/10	4 (8 pm)	5.7

2		2 (8 pm)	0.2
	3/6	3 (8 am)	0.4
		3 (8 pm)	0.9
	3/7	4 (8 am)	10.5
		4 (8 pm)	14.8
	3/8	5 (8 am)	13.9
		5 (8 pm)	8.6
	3/9	6 (8 am)	15.2
		6 (8 pm)	9.3
	3/10	7 (8 am)	16.8
		7 (8 pm)	15.1
	3/11	8 (8 am)	14.3
		8 (8 pm)	8.9
	3/12	9 (8 am)	13.2
		9 (8 pm)	7.7
3	3/3	12 hr (8 pm)	0.1
	3/4	1 (8 am)	0.2
		1 (8 pm)	0.1
	3/5	2 (8 am)	0.2
		2 (8 pm)	0.1
	3/6	3 (8 am)	0.1
		3 (8 pm)	0.1
	3/7	4 (8 am)	0.1
		4 (8 pm)	0.1
	3/8	5 (8 am)	0.8
		5 (8pm)	2.2
	3/9	6 (8 am)	14.1

		5 (8 am)	3.5
	3/11	5 (8 pm)	6.6
		6 (8 am)	4.0
	3/12	6 (8 pm)	6.6
		7 (8 am)	4.2
3	3/6	12 hr (8 pm)	0.5
		1 (8 am)	0.4
	3/7	1 (8 pm)	1.3
		2 (8 am)	2.6
	3/8	2 (8 pm)	2.9
		3 (8 am)	1.2
	3/9	3 (8 pm)	5.2
		4 (8 am)	4.4
	3/10	4 (8 pm)	10.5
		5 (8 am)	5.9
	3/11	5 (8pm)	13.5
		6 (8 am)	7.0
	3/12	6 (8 pm)	11.5
		7 (8 am)	11.6

		6 (8 pm)	14.3
	3/10	7 (8 am)	15.3
		7 (8 pm)	11.1
	3/11	8 (8 am)	17.0
		8 (8 pm)	13.2
	3/12	9 (8 am)	16.1
		9 (8 pm)	10.9

Cell Density with Hydrogen Peroxide Exposure

Wild Type Strain			
Replicate	Date	Days Since Exposure	Density
1	3/8	5 (8 am) (intial)	0.1
		5 (8 pm)	0.1
	3/9	6 (8 am)	0.1
2	3/8	5 (8 am) (intial)	12.4
		5 (8 pm)	7.0
	3/9	6 (8 am)	12.6
3	3/8	5 (8 am) (intial)	0.1
		5 (8 pm)	0.1
	3/9	6 (8 am)	0.1

Over Producer Strain			
Replicate	Date	Days Since Exposure	Density
1	3/9	3 (8 pm) (intial)	2.4
	3/10	4 (8 am)	7.2
		4 (8 pm)	4.0
2	3/9	3 (8 pm) (intial)	1.4
	3/10	4 (8 am)	4.6
		4 (8 pm)	2.8
3	3/9	3 (8 pm) (intial)	1.6
	3/10	4 (8 am)	3.7
		4 (8 pm)	1.9

Cell Density with UV Exposure

Wild Type Strain			
Replicate	**Date**	**Days Since Exposure**	**Density**
1	3/8	5 (8 am)	0
2	3/8	5 (8 am)	0
3	3/8	5 (8 am)	0.1

Over Producer Strain			
Replicate	**Date**	**Days Since Exposure**	**Density**
1	3/9	3 (8 pm)	0.5
2	3/9	3 (8 pm)	0.5
3	3/9	3 (8 pm)	0.2

Cell Density with No light

Wild Type Strain			
Replicate	**Date**	**Days Since Exposure**	**Density**
1	3/8	5 (8 am)	0.1
2	3/8	5 (8 am)	0.1
3	3/8	5 (8 am)	0.1

Over Producer Strain			
Replicate	**Date**	**Days Since Exposure**	**Density**
1	3/9	3 (8 pm)	0.4
2	3/9	3 (8 pm)	0.4
3	3/9	3 (8 pm)	0.3

Appendix B: Extraction Protocols

Blucanex lysis + acetone/NaCl extraction:

*This procedure was found to be the most effective. Our study used blucanex for the lysis due to enzyme availability in the lab, but the original source used glucanex. Additionally this procedure assumes that the samples were already prepared for extraction as outlined in section 2.7.

1. Add 0.75 mL of sodium citrate buffer pH 4.5 into the 2 mL centrifuge tube
2. Add 0.25 mL of blucanex 5% w/v into the 2 mL centrifuge tube
3. Vortex the sample
4. Put centrifuge tubes in flask - the entire tube, do not empty contents into flask, this is just to make it easier to put tubes on stir plate
5. Put flask into incubator (on stir plate) for 1 hour at 30ºC
6. Remove centrifuge tubes from flask
7. Centrifuge the centrifuge tubes for 2 minutes at 15,000 rpm
8. Remove and discard liquid (anything that's not part of the pellet) from every tube
9. Add approximately 1 mL of DI water into each tube
10. Centrifuge the centrifuge tubes for 2 minutes at 15,000 rom
11. Remove and discard liquid (anything that's not part of the pellet) from every tube
12. Add 1 mL of acetone and 100 μl of sodium chloride into each tube
13. Vortex samples
14. Centrifuge for 2 minutes at 15,000 rpm
15. Remove and save liquid (organic phase) from each centrifuge tube - this is the extracted antioxidants (our group saved the organic phase in new 2 mL centrifuge tubes)
16. Repeat steps 12 - 15 until pellet has lost color (for our group this was two times)

DMSO lysis + acetone/NaCl extraction:

*This procedure assumes that the samples were already prepared for extraction as outlined in section 2.7. Also, the DMSO should ideally be heated up to 55ºC.

1. Add 1 mL of DMSO to each centrifuge tube
2. Vortex samples
3. Add 100 μl of 20% w/v NaCl to each centrifuge tube
4. Transfer material to larger 15 mL centrifuge tubes
5. Add 1 mL of acetone to each centrifuge tube
6. Vortex samples
7. Centrifuge for 5 minutes at 3234 rpm (max speed)
8. Remove organic phase at put into 2 mL centrifuge tubes (this is extracted antioxidant)
9. Repeat steps 1-8 (except for switching centrifuge tubes, step 4) until pellet has lost color - for our group this was occurred after 2 rounds

Appendix C: Plate Reader Raw Data- Data Analysis

Room Temperature Data (total carotenoids - astaxanthin +β-carotene):

Sample	Average Absorbance (from plate reader)	Concentration (calculated)
WT lag phase 1	0.045	-0.164
WT lag phase 2	0.044	-0.182
WT lag phase 3	0.048	-0.114
WT exponential phase 1	0.049	-0.102
WT exponential phase 2	0.057	0.059
WT exponential phase 3	0.048	-0.115
WT stationary phase 1	0.060	0.115
WT stationary phase 2	0.061	0.133
WT stationary phase 3	0.061	0.133
OP lag phase 1	0.057	0.059
OP lag phase 2	0.060	0.102
OP lag phase 3	0.059	0.090
OP exponential phase 1	0.288	4.341
OP exponential phase 2	0.299	4.538
OP exponential phase 3	0.335	5.212
OP stationary phase 1	0.295	4.458
OP stationary phase 2	0.250	3.624
OP stationary phase 3	0.194	2.590

4ºC Data (total carotenoids - astaxanthin +β-carotene):

Sample	Average Absorbance (from plate reader)	Concentration (calculated)
WT exponential phase 1	0.152	1.807
WT exponential phase 2	0.164	2.036
WT exponential phase 3	0.155	1.869
WT stationary phase 1	0.228	3.229
WT stationary phase 2	0.221	3.086
WT stationary phase 3	0.228	3.216
OP exponential phase 1	0.183	2.388
OP exponential phase 2	0.193	2.580

OP exponential phase 3	0.190	2.512
OP stationary phase 1	0.294	4.452
OP stationary phase 2	0.275	4.087
OP stationary phase 3	0.310	4.736

Hydrogen Peroxide Data (total carotenoids - astaxanthin +β-carotene):

Sample	Average Absorbance (from plate reader)	Concentration (calculated)
WT initial 1	0.19	2.487
WT initial 2	0.20	2.672
WT initial 3	0.16	1.943
WT end 1	0.17	2.197
WT end 2	0.16	2.018
WT end 3	0.18	1.943
OP initial 1	0.36	5.600
OP initial 2	0.46	7.449
OP initial 3	0.37	5.941
OP end 1	0.31	4.656
OP end 2	0.30	4.545
OP end 3	0.31	4.681

UV light data (total carotenoids - astaxanthin +β-carotene):

Sample	Average Absorbance (from plate reader)	Concentration (calculated)
WT 1	0.15	1.702
WT 2	0.14	1.653
WT 3	0.15	1.684
OP 1	0.14	1.567
OP 2	0.15	1.690
OP 3	0.14	1.597

Appendix D: UPLC Raw Data

Room Temperature Data:

Sample Type	Sample Number	Area	Concentration	# of peaks (if above one)
WT lag phase 1	1	35814	0.889	
WT lag phase 2	2	1302	0.003	
WT lag phase 3	3	0	0	
WT exp phase 1	4	1013	0.003	
WT exp phase 2	5	7103	0.018	
WT exp phase 3	6	940	0.002	
WT stationary phase 1	7	17006	0.042	2 peaks
WT stationary phase 2	8	16760	0.042	2 peaks
WT stationary phase 3	9	19559	0.049	2 peaks
OP lag phase 1	10	1941	0.005	
OP lag phase 2	11	3043	0.008	
OP lag phase 3	12	1499	0.004	
OP exp phase 1	13	180322	0.447	3 peaks
OP exp phase 2	14	244322	0.606	5 peaks
OP exp phase 3	15	241402	0.599	6 peaks
OP stationary phase 1	16	416098	1.032	7 peaks
OP stationary phase 2	17	402465	0.998	7 peaks
OP stationary phase 3	18	288617	0.716	7 peaks

4ºC Data:

Sample Type	Sample Number	Area	Concentration	# of peaks (if above one)
WT exp phase 1	19	4265	0.019	2 peaks
WT exp phase 2	20	1141	0.005	2 peaks
WT exp phase 3	21	2388	0.011	2 peaks
WT stationary phase 1	22	112309	0.497	3 peaks
WT stationary phase 2	23	103301	0.457	3 peaks
WT stationary phase 3	24	131156	0.581	3 peaks
OP exp phase 1	25	34766	0.154	2 peaks
OP exp phase 2	26	43317	0.192	2 peaks
OP exp phase 3	27	38932	0.172	2 peaks
OP stationary phase 1	28	163945	0.726	3 peaks
OP stationary phase 2	29	167600	0.742	3 peaks
OP stationary phase 3	30	177692	0.787	3 peaks

Hydrogen Peroxide Data

Sample Type	Sample Number	Area	Concentration	# of peaks (if above one)
WT initial 1	7	4458	0.020	
WT initial 2	8	6253	0.028	
WT initial 3	9	5095	0.023	
WT end 1	10	7003	0.031	
WT end 2	11	6067	0.027	
WT end 3	12	4832	0.021	
OP initial 1	13	91227	0.404	3 peaks
OP initial 2	14	106512	0.471	3 peaks
OP initial 3	15	113234	0.501	3 peaks
OP end 1	16	124179	0.550	3 peaks
OP end 2	17	96960	0.429	3 peaks
OP end 3	18	85202	0.377	3 peaks

UV light Data

Sample Type	Sample Number	Area	Concentration	# of peaks (if above one)
WT 1	1	56021	0.248	
WT 2	2		0	No peaks
WT 3	3		0	No peaks
OP 1	4	1233	0.005	
OP 2	5	3092	0.014	
OP 3	6		0	No peaks