Effect of Magnetism on *Xanthophyllomyces dendrorhous* Yeast for Astaxanthin Production

A Major Qualifying Project

Submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

degree of Bachelor of Science

in

Chemical Engineering

by

Eric Kasischke

Date:

April 25, 2024

Report submitted to:

Dr. Eric M. Young

Worcester Polytechnic Institute

Abstract

Astaxanthin is a commercially-important antioxidant and pigment chiefly produced from petroleum. The yeast species *Xanthophyllomyces dendrorhous* is a natural producer of this chemical and has come into biotechnological use in production. There is evidence of an increase in astaxanthin production when *X. dendrorhous* is exposed to a static magnetic field, but there is a lack of optimization. This project investigated the effect of magnetism on *X. dendrorhous* astaxanthin production. We find that magnetism exposure at the 72-hour time point produces the highest astaxanthin concentration.

Acknowledgements

I would like to acknowledge the following people for their support and assistance in the completion of this project and the end of my time as a student at Worcester Polytechnic Institute.

Dr. Eric M. Young, for the resources and guidance to make this project possible.

Emma Tobin, for the hands-on guidance in the lab and mentoring throughout the project.

All members of the Young lab, for offering constructive feedback towards the development of my ideas and making the lab a welcoming place.

Contents

1 Introduction

Biotechnology, or the usage of biological systems by humans to produce potentially useful chemicals, is an ancient science. From the earliest days of civilization, humans were using biotechnology to produce food and beverage products through farming and brewing, respectively [1]. Although they were not aware of it, these ancients were influencing the genetic structure of their crops, and selecting them for the production of certain chemicals that offered more nutrition or a better flavor [1]. In modern times, this idea has expanded to include the industrial production of chemicals on a large scale. Humans have discovered ways of increasing the chemical production of species found in nature, as well as modifying the genes of organisms artificially in order to produce the chemical of interest [2]. Organisms that have been modified thus are frequently known as cellular chemical factories. Instead of requiring a complicated human-scale system for the synthesis of these commodity chemicals, microbes can be engineered to conduct the difficult reactions internally, hopefully increasing production and financial efficiency [3]. Innovation in the field today largely relies on finding an organism that naturally produces the chemical, and then investigating the genetic pathway to produce the chemical, or ways to produce the chemical economically and sustainably in the original organism or by inserting the genes into another species that is more desirable [4].

1.1 Astaxanthin Overview

Recent focus of bio-production has been on the carotenoid astaxanthin. This chemical is currently used as a natural colorant added to animal feed as a stand-in for colors that the animals would ingest in their food in the wild, as well as as a micronutrient supplement to keep the animals healthy. Astaxanthin is also being explored for its antioxidant and health benefits in humans, where it could prove to be a cheaper alternative to the drugs currently available [5]. The general family of natural chemical that includes astaxanthin is the colorful carotenoid family, which includes β-carotene that gives color to autumnal leaves and orange produce. Carotenoids are a class of tetraterpenoid molecules that are widely found as pigments in nature [6]. These are important biologically as antioxidants that help maintain homeostasis by reacting with free radical oxygen and nitrogen that cause oxidative stress within the cell. Oxidative stress has been linked to many health issues, as the free radicals can react with and change lipids, proteins, and DNA, potentially changing their functions and causing the cell to malfunction [7]. Astaxanthin is a particularly strong antioxidant that selectively helps protect the mitochondria of cells from attack by reactive oxygen species [8]. These properties, as well as other health effects, such as being an anti-inflammatory and an immune modulator, make astaxanthin an attractive choice for large-scale production [9].

The complexities of the structure make astaxanthin difficult to synthesize, although that is the major

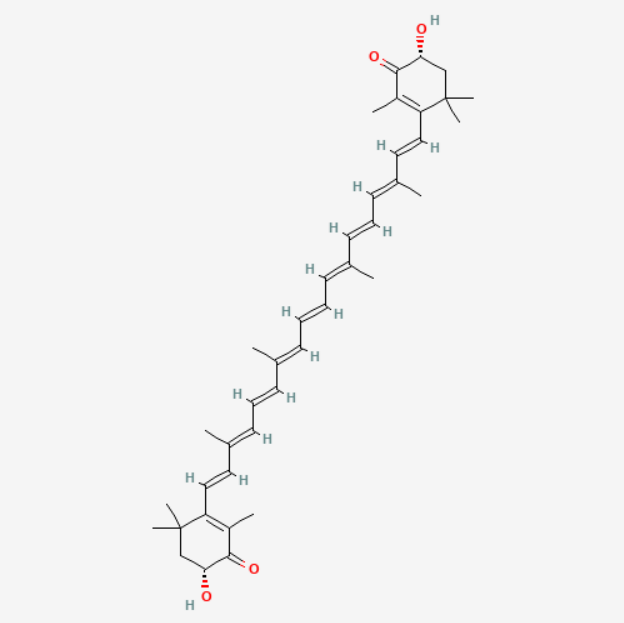

Figure 1: A 2-dimensional structural figure of the (R,R) isomer of astaxanthin from [10]

source of the chemical currently. This process involves processing petroleum derivatives, which are not sustainable and involve highly-polluting processes to obtain [8]. The structure of astaxanthin is shown in Figure 1. Any method of synthesis produces a mix of three isomers that have different biological effects, and the ratios of these three isomers varies depending on the method of production. For example, the current synthesis method from a distilled petroleum product produces more (3R, 3'S)-astaxanthin than the product from the green algae *Haematococcus pluvialis* [8]. Alternatives to the petroleum derivation involves the use of the green algae *Haematococcus pluvialis* and the red yeast *Phaffia rhodozyma (Xanthophyllomyces dendrorhous)*, which are sustainable organisms that will be available into the future. As yet, these methods have been only mildly successful because of difficulties in achieving the same yield as the synthetic method [5]. However, there have been improvements in the efficiency of the biosynthesis of astaxanthin though the study and metabolic development of *Xanthophyllomyces dendrorhous (X. dendrorhous)* [4, 11].

1.2 *Xanthophyllomyces dendrorhous* Overview

A non-conventional organism of study for bio-processing is the red yeast *Xanthophyllomyces dendrorhous*. Found living on specific species of trees in temperate regions, it is a fairly uncommon wild fungus part of the Tremellomycetes family of fungi, where it is one of the few members with bio-processing potential [12]. This yeast is differentiated in its production of astaxanthin as a crucial antioxidant to protect itself from cellular damage caused by singlet oxygen. This makes it especially useful as a source of astaxanthin, as it already contains the metabolic requirements to produce the chemical [11]. Other organisms, such as the much more

common Baker's Yeast (*Saccharomyces cerevisiae*) have been modified to produce astaxanthin, but require major genetic and metabolic engineering work [13]. Additionally, other metabolic pathways in *X. dendrorhous* offer the opportunity to produce other terpenoid molecules, such as limonene, that are used in many products and are being researched as precursors for bio-diesel and pharmaceuticals [14]. Unlike other organisms used for bio-production, *X. dendrorhous* does not require expensive culture media to produce chemicals at a similar rate. In fact, it will grow on processed fruit and vegetable waste, which has an added benefit of reducing the amount of waste going to landfills [15]. For large scale production, this means that the costs are lower compared to other organisms. For these reasons as well as others, *Xanthophyllomyces dendrorhous* is an attractive organism to use for bio-production, and is currently important to the bio-production of astaxanthin [16]. In order to fully replace the synthetic industry for astaxanthin with bio-production, the production potential of *X. dendrorhous* yeast must be increased through genetic and metabolic engineering, and culture optimization [11].

1.3 Magnetism Effects

A less-studied way of changing the metabolic expression of an organism is the use of magnetism [17]. Magnetic fields can effect the orientation of proteins or other chemicals inside cells, which can effect their function and lead to differing cell operations than no magnetic field. In fact, magnetic fields have been theorized to have an effect on human cells and complex organisms as well as single-celled microbes [18]. Magnetic fields have been seen to increase bio-production of metabolites in the algae species *Chlorella fusca* as well as the model yeast species *Saccharomyces cerevisiae* [19, 20]. The species of interest in this investigation, *Xanthophyllomyces dendrorhous*, shows an increase in the production of astaxanthin when under a static magnetic field, according to [21, 22]. Ultimately, the impact of magnetic fields on the gene expression of organisms is not well understood, and the technology is too new for there to be an industrial application. By replicating the results of an experiment where magnetism had a positive effect on the astaxanthin production of *X. dendrorhous*, the transcription changes under these conditions can be recorded and the data can be used to better inform us of the effects of magnetism on cells [17, 21]. For industrial applications to use the astaxanthin-boosting effects of magnetism, the interplay between magnetism and other culture optimizations must be investigated [17].

2 Methodology

2.1 Preculturing of *Xanthophyllomyces dendrorhous*

X. dendrorhous cultures of strain CBS 6938 were grown in a flask of yeast extract peptone dextrose (YPD) with 20 g/L glucose for 2-3 days at 21°C and 200 rpm until an optical density of at least 1 at 600nm is reached. The samples were then diluted to an optical density of 1 before being transferred to the next set of flasks. Adapted from [21].

2.2 Investigation of Literature

2.2.1 First Magnetism Exposure

The diluted preculture from section 2.1 was divided into 9 250 mL flasks by diluting further to 10% preculture with YPD media. These flasks were then incubated for 48 hours at 21° C with no magnetism and a 30mT static magnetic field to investigate if the first exposure to the magnetism stated in [21] has an effect on the final results generated at the end of the experiment. After this 48-hour period, the optical density at 600nm was measured, and the astaxanthin was extracted from a 1-mL sample following the procedure given in section 2.5.

2.2.2 Magnetism Induction

The 9 first-exposure samples were further diluted to 12 500 mL flasks, split into 4 treatments of 3 replicates each, representing 2 control treatments where there was no magnetism exposure during the first-exposure period and 2 experimental treatments where the field was applied during the first-exposure phase. Each of these 3 flasks containing 202.5 mL YPD media was inoculated with 22.5 mL of material from a different one of the first-exposure flasks for the correct treatment to continue tracking the replicate culture. These flasks were then exposed to no magnetism or a 30mT static magnetic field for the periods between either 48-72 hours or 144-168 hours. All flasks were incubated at 21° C and 200 rpm for 168 hours before the astaxanthin was extracted following the method stated in section 2.5. This is an adaptation of the method given in [21].

2.3 Magnetism Experiment Redesign

Xanthophyllomyces dendrorhous was cultured for 3 days in a 125 mL flask, as given in section 2.1 before being diluted to an optical density of 1, measured at 600 nm. The sample was then transferred to 3 250 mL flasks, representing 3 replicates, which were then diluted further to 10% preculture to YPD media. These flasks were allowed to grow for another 3 days to an optical density at 600nm of at least one, before being

diluted back down to an optical density of 1 at the same wavelength. Each of these replicates was then transferred to 4 250 mL flasks, and diluted 10% with YPD media. This results in 12 flasks, made up of 4 of each replicate. Each of these groups of 3 flasks (one of each replicate) received one of 4 treatments: no magnetism exposure at all, constant magnetism exposure for 168 hours, magnetism applied between 72 and 96 hours into the growth, and magnetism applied between 96 and 120 hours into the growth. All samples were cultured at 21° C and 200 rpm for all 168 hours. Every day, 2 1 mL samples were removed from each flask. One was used to find the optical density each day to track the growth of the cultures, and the other was put into a centrifuge tube and stored at -20° C. Later, extraction of astaxanthin, as detailed in section 2.5, was conducted on every sample to monitor the effects of the treatments on every day in the cell growth.

2.4 Further Investigation of Magnetism Results

Xanthophyllomyces dendrorhous was cultured for 3 days in a 500 mL flask until an optical density of greater than 1, measured at 600 nm. A sample of *X. dendrorhous* was transferred to the flask containing 250 mL of yeast extract-peptone-dextrose (YPD) media with 20 g/L glucose, and incubated at 21° C and 200 rpm. The culture was adjusted to an optical density of 1, measured at 600 nm. This adjusted culture was then divided into 12 250 mL flasks by combining 10 mL of the culture with 90 mL of the aforementioned YPD media with glucose. These 12 flasks represented 3 replicates each of 4 treatments: no magnetism, magnetism applied at 24 hours into the culture, magnetism applied at 48 hours into the culture, and magnetism applied at 72 hours into the culture. All magnetism applications were 30 mT and took place for 24 hours. The flasks were incubated at 21° C and 200 rpm for 5 days, with 2 samples removed everyday. One sample was 100 μ L, combined with 900 μ L YPD media with glucose in a cuvette to measure optical density and track the cell growth. The other sample was 1 mL and was frozen at -20° C for future extraction. Later, these samples were extracted, as detailed in section 2.5.

2.5 Determination and Analysis of Astaxanthin Concentration

At points throughout the experiment, stated in the individual methods sections for each experiment, the astaxanthin produced was extracted from a 1-mL sample of the cells and measured by the fluorescence values given by HPLC. First, The cell density was measured through the use of a spectrometer at a wavelength of 600nm, and the cells were diluted by a factor of 10, as needed, to ensure an accurate reading. to conduct the extraction, a well-mixed 1-mL sample of culture was pipetted into a micro-centrifuge tube and centrifuged at 10,845×g for 10 minutes. The supernatant was poured off, and then the cells were washed twice by

resuspending in distilled water, centrifuging at the same settings, and pouring off the supernatant. The cells were then resuspended in 300-μL of HPLC-grade acetone before being transferred to a pre-filled tube containing 400-μm zirconium lysing beads. The whole mixture was then carefully poured off into snap-cap Eppendorf tubes and homogenized at 12 m/s for 5 minutes using a StormPro Bullet Blender. The tubes were then centrifuged again at 10,845$\times$g for 10 minutes. The supernatant was pipetted into a nylon or polytetrafluoroethylene (PTFE) Whatman Mini-UniPrep tube with filter pore size 0.2-μm, which was then pushed to filter out any remaining lysing beads or cellular residue. The liquid was then transferred to an amber HPLC vial with a glass insert. The fluorescence areas produced by the HPLC were compared to a standard curve, seen in Figure 2, for astaxanthin in order to correctly characterize the concentration of the product in the cells.

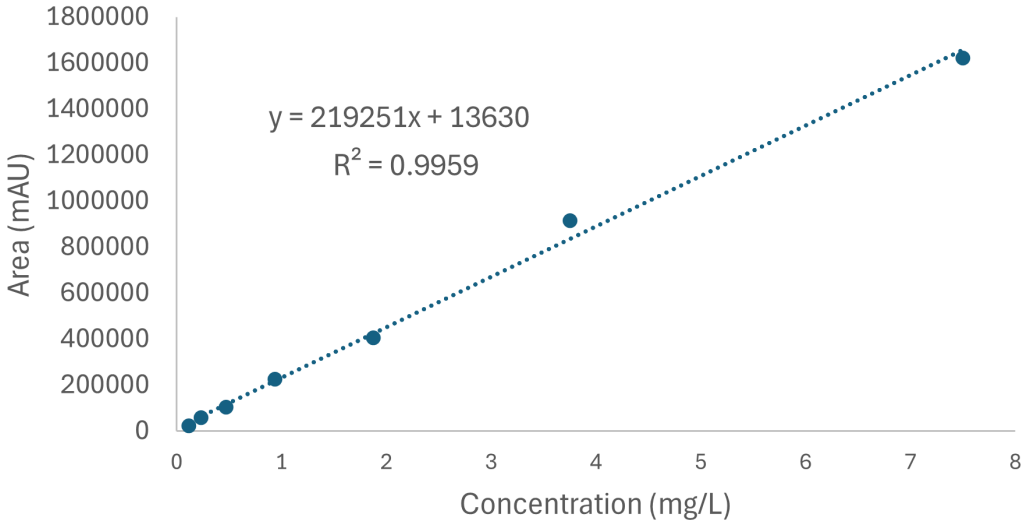

Figure 2: Standard curve for astaxanthin concentration

3 Results and Discussion

3.1 Literature Replication

Astaxanthin production was determined by using the areas given by the HPLC and converting them using a standard curve, Figure 2, to get the concentration of astaxanthin in the extracted samples. Figure 3, below, shows the average astaxanthin concentration in mg per liter for the experiment towards replicating the results found in the literature.

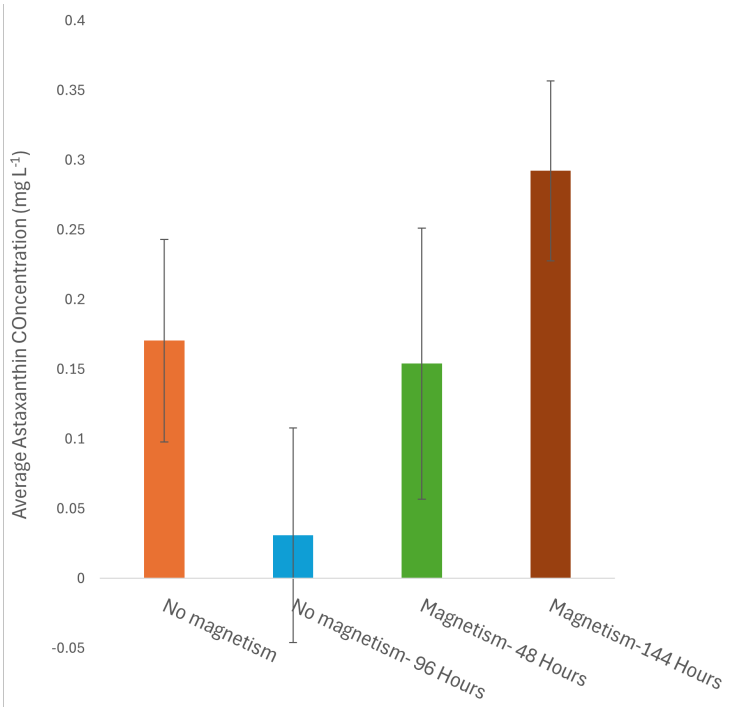

Figure 3: Astaxanthin concentration for each treatment in literature replication trial. Concentration was determined from the areas given by HPLC that were run through a standard curve.The means of three replicates are displayed, with error bars representing one deviation from the mean.

While there are some striking differences in the means of concentration measured, there was no data that was statistically significant. The experiment found in [21] was not successfully replicated. As a result, the methods were reviewed, and it was determined that the data collection in the paper may have been flawed in that the samples were all taken at the end of the incubation, giving the astaxanthin time to decay before being measured. The experiment was redesigned and then conducted to clarify the results.

3.2 Experiment Redesign

Astaxanthin production was determined in the same way as mentioned earlier, in Section 3.1. Figure 4 shows the average concentration of astaxanthin in the extract every day for the redesigned experiment.

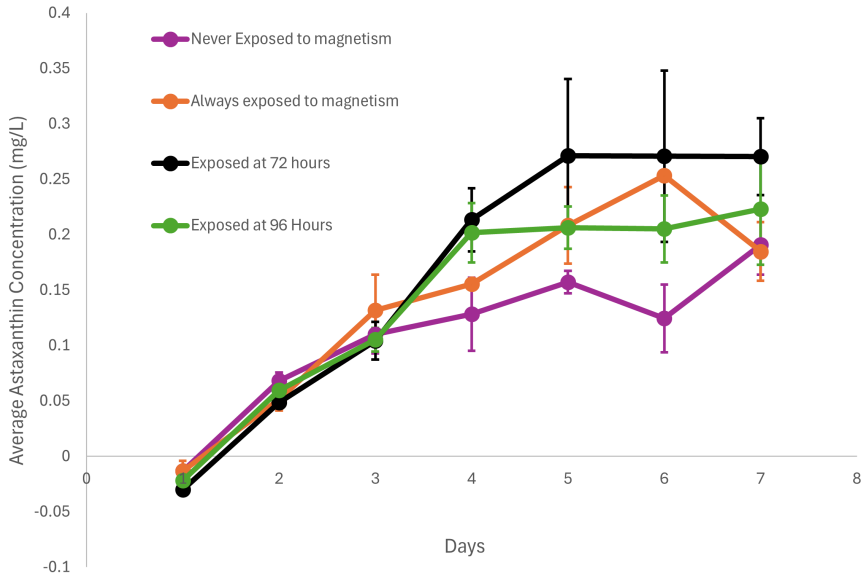

Figure 4: Astaxanthin concentration over time for the four treatments applied. Concentration was determined form the areas given by HPLC that were run trough a standard curve. Each point represents the mean of three replicates, with error bars indicating one standard deviation from the mean.

All trials start at the same level of astaxanthin production, and then begin to differentiate at day four of the culture. From day 4 of the culture onwards, the measured astaxanthin concentration in the samples that had applied magnetism increased over the control samples with no applied magnetism. While not statistically significant by the student's T-test, the difference in concentration is large, representing greater than one standard deviation of difference between the measurements. This suggests that magnetism will increase the astaxanthin production in *Xanthophyllomyces dendrorhous*. Overall, the samples exposed to a 30 mT static magnetic field showed the highest average astaxanthin concentration. This suggests that the application time of the magnets is important, and that 72 hours is closer to the ideal application time than 96 hours. All treatments showed minimal additional increase in astaxanthin concentration after day 5. This suggests that only 5 days in culture is required to reach the maximum astaxanthin production. More trials expanding the number of replicates and magnetism application times is needed to fully support these conclusions.

3.3 Further Investigation

Samples were collected to further develop the conclusions in Section 3.2, however HPLC was not conducted to enable comparison of average astaxanthin concentrations. As a result, the conclusions still remain unsupported.

It is apparent, however, that there is a link between the application of an external magnetic field and an increase in astaxanthin production by *Xanthophyllomyces dendrorhous*. The cause and mechanism of this is as-yet-unknown.

Magnetism does not frequently occur naturally. As a result, there is no clear evolutionary reason for any metabolic induction by the application of a magnetic field. It is possible that motion over the magnetic field is producing a similar stress to the organisms as experienced when they are exposed to light, due to the presence of the electromagnetic spectrum. If this is the case, more research will need to be conducted to confirm.

4 Conclusions

The data found in this work provides some evidences that magnetism can act to increase the production of astaxanthin by *Xanthophyllomyces dendrorhous*, however these conclusions are not statistically significant. Additional data to both replicate and expand the time points of application are necessary to ultimately draw conclusions about the proper time to apply magnetism to optimize production of astaxanthin in this species.

In industry, any (even minor) production improvement is likely to be used to maximize the efficiency of the process. The application of a magnetic field to a cell culture is an environmental change that is relatively cheap to implement, and, when optimized, can show increased astaxanthin bioproduction.

4.1 Future Work

Future experimentation focusing on the optimization of the applied magnetic field would be a logical next development for investigation. This would involve changing the strength of the magnetic field and changing the application time to find the conditions that lead to the highest production of astaxanthin. To assist in this, transcriptomics data can be compared between samples with and without applied magnetism to track the gene expression resulting from the magnetic field exposure. From this data, the genes that show higher expression with the magnet application can be studied and potentially modified. Finally, the integration of magnetism application with other culture optimizations such as changing light or media, or with genetic changes that cause *X. dendrorhous* to produce more astaxanthin or produce another chemical are all important interactions for further study, enabling optimization to reach a value close to the maximum production capability.

5 References

1. Bhatia, S. & Goli, D. in *Introduction to Pharmaceutical Biotechnology* (IOP Publishing, July 23, 2018). ISBN: 978-0-7503-1300-1.

2. Zhang, Y.-H. P., Sun, J. & Ma, Y. Biomanufacturing: history and perspective. *Journal of Industrial Microbiology and Biotechnology* **44,** 773–784. ISSN: 1367-5435. https://doi.org/10.1007/s10295-016-1863-2 (2023) (May 1, 2017).

3. Cho, J. S., Kim, G. B., Eun, H., Moon, C. W. & Lee, S. Y. Designing Microbial Cell Factories for the Production of Chemicals. *JACS Au* **2.** Publisher: American Chemical Society, 1781–1799. https://doi.org/10.1021/jacsau.2c00344 (2023) (Aug. 22, 2022).

4. Schmidt, I. *et al.* Biotechnological production of astaxanthin with Phaffia rhodozyma/Xanthophyllomyces dendrorhous. *Appl Microbiol Biotechnol* **89,** 555–571. ISSN: 1432-0614 (Feb. 2011).

5. Higuera-Ciapara, I., Félix-Valenzuela, L. & Goycoolea, F. M. Astaxanthin: a review of its chemistry and applications. *Crit Rev Food Sci Nutr* **46,** 185–196. ISSN: 1040-8398 (2006).

6. Maoka, T. Carotenoids as natural functional pigments. *J Nat Med* **74,** 1–16. ISSN: 1340-3443. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6949322/ (2023) (2020).

7. Lobo, V., Patil, A., Phatak, A. & Chandra, N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev* **4,** 118–126. ISSN: 0973-7847. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3249911/ (2023) (2010).

8. Snell, T. W. & Carberry, J. Astaxanthin Bioactivity Is Determined by Stereoisomer Composition and Extraction Method. *Nutrients* **14,** 1522. ISSN: 2072-6643. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9002770/ (2023) (Apr. 6, 2022).

9. Chang, M. X. & Xiong, F. Astaxanthin and its Effects in Inflammatory Responses and Inflammation-Associated Diseases: Recent Advances and Future Directions. *Molecules* **25,** 5342. ISSN: 1420-3049 (Nov. 16, 2020).

10. PubChem. *(R,R)-Astaxanthin* https://pubchem.ncbi.nlm.nih.gov/compound/12358421 (2023).

11. Torres-Haro, A., Verdín, J., Kirchmayr, M. R. & Arellano-Plaza, M. Metabolic engineering for high yield synthesis of astaxanthin in Xanthophyllomyces dendrorhous. *Microb Cell Fact* **20,** 175. ISSN: 1475-2859 (Sept. 6, 2021).

12. Palma, M. C. D. *Biogeography of Xanthophyllomyces dendrorhous, a yeast with biotechnological potential – population mapping and host associations* PhD thesis (Universidade de Nova de Lisboa, 2011).

13. Ukibe, K., Hashida, K., Yoshida, N. & Takagi, H. Metabolic Engineering of Saccharomyces cerevisiae for Astaxanthin Production and Oxidative Stress Tolerance. *Applied and Environmental Microbiology* **75.** Publisher: American Society for Microbiology, 7205–7211. https://journals.asm.org/doi/full/10.1128/aem.01249-09 (2023) (Nov. 15, 2009).

14. Yang, L. *et al.* Recent Advances in Multiple Strategies for the Synthesis of Terpenes by Engineered Yeast. *Fermentation* **8.** Number: 11 Publisher: Multidisciplinary Digital Publishing Institute, 615. ISSN: 2311-5637. https://www.mdpi.com/2311-5637/8/11/615 (2023) (Nov. 2022).

15. Gervasi, T. *et al.* Astaxanthin production by Xanthophyllomyces dendrorhous growing on a low cost substrate. *Agroforestry Systems* **94.** MAG ID: 2914418505, 1229–1234 (Aug. 1, 2020).

16. Rodríguez-Sáiz, M., De La Fuente, J. L. & Barredo, J. L. Xanthophyllomyces dendrorhous for the industrial production of astaxanthin. *Appl Microbiol Biotechnol* **88,** 645–658. ISSN: 0175-7598, 1432-0614. http://link.springer.com/10.1007/s00253-010-2814-x (2023) (Oct. 2010).

17. Albuquerque, W. W. C., Costa, R. M. P. B., de Salazar e Fernandes, T. & Porto, A. L. F. Evidences of the static magnetic field influence on cellular systems. *Progress in Biophysics & Molecular Biology* **121.** MAG ID: 2312332361, 16–28 (May 1, 2016).

18. Zhang, X., Yarema, K. & Xu, A. *Biological Effects of Static Magnetic Fields* ISBN: 978-981-10-3579-1. http://link.springer.com/10.1007/978-981-10-3579-1 (2023) (Springer Singapore, Singapore, 2017).

19. Machado, B. R., Silva, P. G. P., Garda-Buffon, J. & Santos, L. O. Magnetic fields as inducer of glutathione and peroxidase production by Saccharomyces cerevisiae. *Brazilian Journal of Microbiology.* MAG ID: 4302282045 (Oct. 6, 2022).

20. Deamici, K. M., Silva, P. G. P., Costa, J. A. V. & Santos, L. O. Low Electromagnetic Fields Applied to Chlorella fusca Cultivation to Increase Production of Microalga-Based Carbohydrates. *Bioenerg. Res.* **16,** 1548–1555. ISSN: 1939-1234, 1939-1242. https://link.springer.com/10.1007/s12155-022-10562-7 (2023) (Sept. 2023).

21. Silva, P. G. P. D., Prescendo Júnior, D., Sala, L., Burkert, J. F. D. M. & Santos, L. O. Magnetic field as a trigger of carotenoid production by Phaffia rhodozyma. *Process Biochemistry* **98,** 131–138. ISSN: 13595113. https://linkinghub.elsevier.com/retrieve/pii/S1359511320308266 (2023) (Nov. 2020).

22. Silva, P. G. P., Prescendo Júnior, D., Burkert, J. F. D. M. & Santos, L. O. Effect of magnetic field and agitation as exogenous factors for carotenoid production by Phaffia rhodozyma. *Braz. J. Chem. Eng.* ISSN: 0104-6632, 1678-4383. https://link.springer.com/10.1007/s43153-023-00396-x (2023) (Sept. 7, 2023).

Appendices

Appendix A: Additional Optical Density Data for Literature Replication

Lit Rep Diluted 10X

Flask	No magnetism-No magnetism	No magnetism- 96 hours	Magnetism- 48 Hours	Magnetism- 144 Hours
1	0.545	0.649	0.598	0.652
2	0.696	0.384	0.674	0.719
3	0.753	0.47	0.544	0.987
Average	0.664666667	0.501	0.605333333	0.786

Appendix B: Additional Optical Density Data for Re-worked Experiment

Re-Work Not diluted

Flask	Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1		1.114	1.527	1.673	1.761	1.721	1.683	1.644
2		1.175	1.413	1.507	1.636	1.614	1.558	1.524
3	No magnetism	0.953	1.388	1.513	1.618	1.582	1.551	1.493
1		1.168	1.463	1.606	2.026	1.662	1.624	1.563
2		1.143	1.501	1.568	1.734	1.692	1.643	1.596
3	Always Magnetism	0.964	1.436	1.574	1.695	1.66	1.631	1.557
1		1.076	1.413	1.495	1.591	1.604	1.575	1.52
2		0.861	1.44	1.567	1.694	1.365	1.601	1.561
3	72 Hours	1.07	1.403	1.629	1.859	1.478	1.618	1.561
1		1.13	1.434	1.568	1.664	1.459	1.592	1.54
2		0.851	1.45	1.573	1.687	1.378	1.587	1.547
3	96 Hours	1.15	1.439	1.584	1.681	1.669	1.643	1.576

Appendix C: Additional Optical Density Data for Further Experimentation

Further Diluted 10X

Flask	Treatment	Day 1	Day 2	Day 3	Day 4	Day 5
1		0.17	0.229	0.298	0.324	0.319
2		0.21	0.246	0.289	0.333	0.298
3	No magnetism	0.174	0.255	0.288	0.331	0.305
1		0.262	0.276	0.308	0.343	0.31
2		0.212	0.275	0.304	0.335	0.328
3	24 Hours	0.282	0.286	0.281	0.343	0.324
1		0.299	0.274	0.316	0.349	0.322
2		0.235	0.264	0.307	0.352	0.288
3	48 Hours	0.231	0.262	0.288	0.32	0.215
1		0.282	0.282	0.27	0.322	0.328
2		0.236	0.257	0.293	0.323	0.289
3	72 Hours	0.235	0.245	0.268	0.322	

Appendix D: Additional Raw Data for Standard Curve

Standards (mg/L)	Replicate 1	Replicate 2	Replicate 3
7.5	1725556	1655786	1485718
3.75	988595	972341	788775
1.875	456744	413957	351342
0.9375	259722	224282	193191
0.46875	135992	98799	79112
0.234375	59913	61695	53433
0.1171875	21398	25611	23475

Average	Standard Deviation
1,622,353.33	123364.8003
916570.3333	110971.9944
407347.6667	53010.92214
225731.6667	33289.18203
104634.3333	28885.49699
58347	4347.922262
23494.66667	2106.568853

Appendix E: Additional Raw Data for Literature Replication

Treatment	Non-standard	Standard	Mean	Standard Deviation
No magnetism	30154	0.075366	0.170517	0.072545771
	68732	0.251319		
	54162	0.184866		
No magnetism- 96 Hours	43272	0.135197	0.031019	0.076866688
	14906	0.00582		
	3115	-0.04796		
Magnetism- 48 Hours	44282	0.139803	0.154113	0.097200081
	74947	0.279666		
	23029	0.042869		
Magnetism-144 Hours	73656	0.273778	0.292309	0.064593801
	96735	0.37904		
	62766	0.224108		

Appendix F: Additional Raw Data for Re-Worked Experiment

Day	Replicate	Never	Always	Exposure at 72 hours	Exposure at 96 hours				
	1	9712	8293	6639	8018				
Day 1	2	10806	13247	Spilled	8807				
	3	11716	10745	7560	9869				
						T-Test P	0.9919	0.0165	0.0798
	1	30860	26070	25052	26625				
Day 2	2	27388	21771	23495	26186				
	3	27632	26868	Spilled	27262				
						T-Test P	0.135	0.0496	0.2207
	1	42457	42109	31600	39667				
Day 3	2	37590	34139	37213	36439				
	3	33252	51372	40693	33999				
						T-Test P	0.4588	0.7531	0.7537
	1	31701	46745	69089	Spilled				
Day 4	2	45250	47250	57762	63697				
	3	48348	49187	54424	52016				
						T-Test P	0.3636	0.0522	0.1517
	1	45611	63996	83158	59849				
Day 5	2	50974	48603	84454	53328				
	3	47770	65334	51633	63451				
						T-Test P	0.1646	0.1428	0.0849
	1	Spilled	68432	89459	54702				
Day 6	2	34202	69977	79902	53248				
	3	47650	Spilled	49682	67972				
						T-Test P	0.1446	0.1052	0.1649
	1	62712	52504	80555	56592				
Day 7	2	55501	48078	65284	53025				
	3	48204	61907	Spilled	77983				
						T-Test P	0.8337	0.2121	0.482

Day	Replicate	Never	Always	Exposure at 72 hours	Exposure at 96 hours		Always	72 H	96 H
	1	-0.0179	-0.0243	-0.0319	-0.0256				
Day 1	2	-0.0129	-0.0017	Spilled	-0.0220				
	3	-0.0087	-0.0132	-0.0277	-0.0172				
						T-Test P	0.9919	0.0165	0.0798
	1	0.0786	0.0567	0.0521	0.0593				
Day 2	2	0.0627	0.0371	0.0450	0.0573				
	3	0.0639	0.0604	Spilled	0.0622				
						T-Test P	0.135	0.0496	0.2207
	1	0.1315	0.1299	0.0820	0.1188				
Day 3	2	0.1093	0.0935	0.1076	0.1040				
	3	0.0895	0.1721	0.1234	0.0929				
						T-Test P	0.4588	0.7531	0.7537
	1	0.0824	0.1510	0.2529	Spilled				
Day 4	2	0.1442	0.1533	0.2013	0.2284				
	3	0.1583	0.1622	0.1861	0.1751				
						T-Test P	0.3636	0.0522	0.1517
	1	0.1459	0.2297	0.3171	0.2108				
Day 5	2	0.1703	0.1595	0.3230	0.1811				
	3	0.1557	0.2358	0.1733	0.2272				
						T-Test P	0.1646	0.1428	0.0849
	1	Spilled	0.2500	0.3459	0.1873				
Day 6	2	0.0938	0.2570	0.3023	0.1807				
	3	0.1552	Spilled	0.1644	0.2479				
						T-Test P	0.1446	0.1052	0.1649
	1	0.2239	0.1773	0.3052	0.1959				
Day 7	2	0.1910	0.1571	0.2356	0.1797				
	3	0.1577	0.2202	Spilled	0.2935				
						T-Test P	0.8337	0.2121	0.482

Means

Day	Never	Always	72	96
1	-0.01316	-0.01308	-0.02979	-0.02158
2	0.0684	0.051416	0.048545	0.059571
3	0.110085	0.131858	0.104319	0.105229
4	0.128329	0.155517	0.213431	0.201716
5	0.157301	0.20835	0.271158	0.206366
6	0.124497	0.253474	0.270851	0.205293
7	0.190842	0.18487	0.270418	0.223047

Standard Deviations

Day	Never	Always	72	96
1	0.003737	0.009225	0.0021	0.003459
2	0.007217	0.01021	0.003551	0.002015
3	0.017149	0.032118	0.017086	0.010588
4	0.03297	0.004801	0.028625	0.026638
5	0.010049	0.034624	0.069216	0.019109
6	0.030668	0.003523	0.077325	0.030216
7	0.027014	0.0263	0.034825	0.050267