Light dependency of carotenoid- and lipid production in oleaginous red yeast, *Rhodotorula toruloides*

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Ljusbehov under karotenoid- och lipidsyntes i oljeacumulerande röd jäst, *Rhodotorula toruloides*

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Sammanfattning

Den snabba befolkningstillväxten på jorden belastar planetens kapacitet att möta mänskliga behov, vilket kräver ökad industrialisering och intensiv användning av fossila bränslen. Detta har lett till klimatförändringar och miljöföroreningar. För att hantera dessa moderna utmaningar behövs innovativa lösningar, som hållbara produktionsmetoder för mat, foder och energi. En lovande strategi innefattar användning av oljeacumulerande röda jästsvampar, särskilt *Rhodotorula toruloides*, som kan producera värdefulla karotenoider och lipider. Dessa föreningar har potentiella tillämpningar inom biodiesel, livsmedels- och foderindustrin, bioplaster, biokemikalier, läkemedel och biokosmetik (Pham et al. 2021).

Denna studie undersöker ljusets påverkan på karotenoid- och lipidproduktionen i tre olika *R. toruloides* stammar: CBS 14, CBS 6016 och CBS 349, att förstå dessa faktorer kan förbättra praktiska tillämpningar. Resultaten visar skillnader i karotenoidkoncentration och lipidkoncentration mellan stammarna under ljusa och mörka förhållanden. CBS 14 producerade mer karotenoider i mörkt förhållande, medan CBS 6016 producerade mer i ljust förhållande. Vilket tyder på en stimulering av karotenoidproduktionen i CBS 6016 och en försvarsmekanism i CBS 14. CBS 349 visade ingen märkbar variation mellan förhållandena.

Lipidkoncentrationen varierade också, med högre koncentration i mörkt förhållande enligt FT-NIR-analys, men högre koncentration i ljus enligt SFE-analys, däremot är det olika prover som analyserades. Tillförlitligheten av dessa resultat ifrågasattes dock på grund av variationer mellan proverna. Studien antyder ett möjligt samband mellan lipid- och karotenoidbiosyntesvägar, vilket indikerar en samordnad reglering för att möta cellens metaboliska behov. Regleringsmekanismer som styr lipidmetabolismen kan påverka karotenoidbiosyntesen och det motsatta.

Sammanfattningsvis visade CBS 14 den högsta karotenoidproduktionen, medan CBS 349 visade den lägsta, sannolikt på grund av genetiska skillnader.

Nyckelord: Oljeackumulerande, Röd jäst, *Rhodotorula toruloides,* Karotenoid, Fett, Biosyntes, Ljusreaktionen

Abstract

The rapidly increasing population of earth is straining the planet's capacity to meet human needs, necessitating industrialization and the intensive use of fossil fuels. This has led to climate change and environmental pollution. Addressing these modern issues requires innovative solutions, such as sustainable production methods for food, feed, and energy. One promising avenue involves oleaginous red yeasts, particularly *Rhodotorula toruloides*, which can produce valuable carotenoids and lipids. These compounds have potential applications in biodiesel, food and feed industries, bioplastics, biochemicals, pharmaceuticals, and biocosmetics (Pham et al. 2021).

This study investigates the impact of light on carotenoid and lipid production in three *R. toruloides* strains: CBS 14, CBS 6016, and CBS 349, understanding these factors can enhance practical applications. The findings reveal differences in carotenoid concentration and lipid content between the strains under light and dark conditions. CBS 14 produced more carotenoids in the dark, while CBS 6016 produced more in the light, indicating a stimulation of carotenoid production in CBS 6016 and a defense mechanism in CBS 14. CBS 349 showed no noteworthy variation between conditions.

Lipid concentration also varied, with higher levels in the dark when analyzed with FT-NIR, but higher levels in the light when analyzed with SFE, although the analyzes was done with different batches. However, the reliability of these results was questioned due to variability between replicates. The study suggests a possible link between lipid and carotenoid biosynthetic pathways, indicating coordinated regulation to meet cellular metabolic needs. Regulatory mechanisms controlling lipid metabolism may influence carotenoid biosynthesis and the other way round.

In conclusion, CBS 14 exhibited the highest carotenoid production, while CBS 349 showed the lowest, likely due to genetic differences.

Keywords: Oleaginous, Red yeast, *Rhodororula toruloides*, Carotenoid, Lipids, Biosynthesis, Light dependency

Index

List of Tables

List of Figures

Abbreviations & Terminology

ALA	Alpha-linolenic acid
AMP	Adenosine monophosphate
Anthropocene	Considering human beings as the most significant entity of the universe
ATP	Adenosine Triphosphate
C/N ratio	Carbon-to-nitrogen ratio
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
Ecosystems	A biological community of interacting organisms and their physical environment.
EPA	Eicosapentaenoic acid
FADH2	Flavin Adenine Dinucleotide
FFA	Free Fatty Acid
GLA	Gamma-linolenic acid
IDA	Isocitrate dehydrogenase
MVA pathway	Mevalonate pathway
NADH	Nicotinamide Adenine Dinucleotide
OTC	Over-the-counter
Precursor	A substance from which another is formed, especially by the metabolic reaction
Quadruple Squeeze model	The degrees of freedom for sustainable human exploitation of planet Earth are severely restrained.
ROAD	Xanthophyll- and polyunsaturated lipid-deficient
ROS	Reactive Oxygen Species
SE	Sterol Ester
TAG	Triacylglycerol
TCA cycles	Citric Acid cycle

Introduction

The growing population, along with unsustainable production and consumption patterns of humanity, has had and will continue to have significant consequences for the well-being of the Earth. Current population of approximately 7.7 billion people on the planet, is expected to increase by about 2 billion by the year 2050. This results in a great demand not only on the Earth itself but also on humanity in terms of how to provide for the entire global population today and in year 2050 (SVT Nyheter 2019).

Since reports indicate that there are serious food crises in various regions of the world, there is already a great deal of concern regarding this topic. This is consistent with Anthropocene human activity, such as excessive use of the planet's ecosystems and rapid industrialization. Both of which have led to considerable climate change because of the large amounts of greenhouse gases they have emitted into the atmosphere. These greenhouse gas emissions originate from several industries and are influenced by a few different variables, transportation and food production being the expanding industrial sector. Growing populations and widespread use of fossil fuels create a significant issue, as an increasing number of people need to share the planetary resources. In addition to being a limited resource, fossil fuel emissions have serious repercussions. It is therefore important to quickly find methods to reduce emissions (Santos et.al 2022).

In this quest for innovation to transform the resource production processes, attention has been directed towards a wide range of lipid-rich microorganisms, including bacteria, microalgae, and yeasts. These are highly beneficial organisms that produce long-chain lipids with low land or food requirements. These lipids provide a flexible basis for the synthesis of various useful compounds, especially biofuels (Nagavara Nagaraj 2023).

Researchers are intensively exploring the potential of lipid-accumulating microorganisms, which are attracted by their remarkable lipid production capacity, often exceeding 20 % of dry biomass (Nagavara Nagaraj 2023). Among these organisms, *Rhodotorula toruloides*, an oleaginous yeast, proves to be a promising candidate. It shows the ability to accumulate lipids in a wide range from 20 to 80 % of its dry biomass, especially under conditions of limited nitrogen and excess carbon (Dulermo et al. 2015a; Calvey et.al 2016). Furthermore, *R. toruloides* shows an additional ability to synthesize carotenoids, secondary metabolites belonging to a class of natural pigments produced by both plants and microorganisms. Carotenoids are valued for their inherent antioxidant properties and are of great importance in various industries such as pharmaceutical, chemical, food, and animal feed production (Nagavara Nagaraj 2023).

The green economy and green growth have become important worldwide policy imperatives in recent years. According to the UN Environment programme, the green economy is characterized by its capacity to reduce ecological scarcities and environmental dangers while concurrently promoting social justice and human well-being (UN Environment Programme 2024). Fundamentally, the green economy encourages energy saving for greater efficiency and stresses the switch from fossil fuels to renewable energy sources. The bioeconomy, circular economy, and clean technology sectors are the collective terms for these changes, which include low carbon use, resource optimization, social inclusion, and innovation promotion (Pham et al. 2021).

Central to the concept of the green economy is the utilization of renewable resources to supplant non-renewable ones, driving advancements in bioproduction technologies. Consequently, effective ecosystem management on a landscape scale and the cultivation of utility organisms are paramount. The bioeconomy, characterized by sustainable production practices utilizing renewable resources, encompasses a wide array of products, ranging from bioenergy, such as biofuels and biogas, to biomaterials like bioplastics and biochemicals including pharmaceuticals and biocosmetics (Pham et al. 2021). Within this framework, lipids have emerged as adaptable resources with significant industrial applications, serving as biofuel for energy production, raw material for bioplastics, and essential components for pharmaceuticals such as DHA. Similarly, carotenoids, renowned for their diverse utility across industries, have garnered considerable attention (Pham et al. 2021).

Aim

Several reports have examined the influence of light on microorganisms, according to Pham et al. (2020), their analysis of the phenotype and gene expression of *R. toruloides* revealed that exposure to light resulted in darker pigmentation and increased carotenoid production. Although no noteworthy difference in lipid production was observed, slight changes in fatty acid composition were recorded.

This experiment is aimed at studying how light can affect the production of carotenoids and lipids in three different strains of *R. toruloides*, CBS 14, CBS 6016, and CBS 349, with CBS 6016 being a hybrid strain. Predominant inheritance and expression of genetic traits are observed from one strain, CBS 14, rather than the other, CBS 349 (Nagavara Nagaraj 2023). This research will help to develop information on the factors influencing the synthesis of carotenoids and lipids that could be used in practice and recognize their potential as vital contributors to the sustainable industrial landscape of the green economy. Where the question of issue is “How does light affect carotenoid- and lipid production in strains CBS 14, CBS 6016 and CBS 349 of *Rhodotorula toruloides*?” and “Does light maximize or minimize the carotenoid and lipid production in *Rhodotorula toruloides?”*.

Background

Oleaginous Yeast

Rhodotorula toruloides is a species of oleaginous yeast belonging to the red yeast category. It is characterized by its typical reddish-pink coloration caused by the carotenoid pigments produced. The yeast species has been detected in soil, water, and air environments, as well as on plants and fruits (Atrayee Chattopadhyay et.al 2021). Since the species is widely saprophytic, it may grow in a wide range of conditions, due to different origins of the strains, from the rotting pine wood in Sweden (*R. toruloides* CBS 14) to the soil in Japan (*R. toruloides* CBS 349) (Nagavara Nagaraj 2023).

Recently, *R. toruloides* has been in the limelight due to its possible applications in the field of biotechnology and industrial processes. Being capable of utilizing a wide range of carbon sources, such as xylose and glycerol, makes it an attractive candidate for bioremediation and biofuels. In addition, this yeast species can accumulate high amounts of lipids, making it more promising in the production of biodiesel. Since the lipids produced by *R. toruloides* have a similar fatty acid composition to vegetable oil, they may also be used to produce edible oil.

Therefore, microbial lipid production by *R. toruloides* provides an attractive source of alternate oil for the biodiesel and food industries (Atrayee Chattopadhyay et.al 2021).

In addition, *R. toruloides* has been studied to produce bioactive compounds like carotenoids, used in the food, pharmaceutical, and cosmetics industries. Being a robust-growing organism under various conditions and manipulable through genetic means, it makes it an attractive organism for biotechnological applications. In addition, they are among the bioactive phytochemicals that help reduce the risk of degenerative diseases like cancer, cardiovascular disease, macular degeneration, and cataracts. Their ability to produce carotenoids by using oleaginous yeasts makes *R. toruloides* an important organism (Atrayee Chattopadhyay et.al 2021).

Carotenoids

Classification and Nomenclature

Carotenoids are a group of naturally occurring yellow, orange, and red organic pigments synthesized by algae, fungi, yeast, and certain bacteria. Their most important biological functions include their role in photosynthesis by capturing light energy and serving as antioxidants. These compounds are typically classified and named based on specific nomenclature criteria (Meléndez-Martinez et.al 2019).

Carotenoids usually acquire informal names based on their original sources or places where they are more abundant. For example, *β-carotene* gets its name from the scientific name of the carrot (Daucus carota). Along with these customary names, a semi-systematic nomenclature is available that gives information about the structure of the carotenoid. Carotenoids are provided names with Greek letters followed by "-carotene," such as *β-carotene*, *α-carotene*, and *γ-carotene. Xanthophylls* have similar names with "xantho-" meaning oxygenation, followed by the Greek letter system and "-xanthin," as is the case with *lutein*, *zeaxanthin*, and *astaxanthin* (Meléndez-Martinez et.al 2019).

In the case of tetraterpenoids, that is, regular C40 isoprenoids, most of the carotenoids will contain a considerable number of modifications, including cyclization, with various degrees of saturation or unsaturation, and the adding of other functional groups. Carotenes, the most important representatives in this case, consist usually of only carbon and hydrogen atoms, so they are hydrocarbons; well-known examples are *α-carotene*, *β-carotene*, and *lycopene* (Meléndez-Martinez et.al 2019).

Xanthophylls are oxygen-containing carotenoids that include *lutein* and *zeaxanthin*. Their structure, in particular, conjugation length, is closely related to their color, ranging from pale yellow to deep red. The yellowish color that most often characterizes *xanthophylls* was the classification name provided for them (Meléndez-Martinez et.al 2019).

Along with the broad classifications of carotenoids as cyclic or acyclic, namely, carotenes and xanthophylls, further subgroups can be identified based on their structural characteristics. Microorganisms' carotenoids, for example, possess one of two additional isoprenoid units, resulting in molecules containing 45 or 50 carbon atoms. Decaprenoxanthin is a typical example within this category (Meléndez-Martinez et.al 2019).

There are also two subgroups of carotenoids with fewer than 40 carbon atoms, including peridinin, which lack one to three carbon atoms in the central hydrocarbon backbone. On the other hand, apocarotenoids lack fragments at one or both ends of the molecules, including crocetin, the saffron carotenoid, with 20 carbon atoms. On the contrary, secocarotenoids have a broken bond between adjacent carbons (excluding carbons 1 and 6 in rings), as in semi-carotene. On the other hand, retrocarotenoids have a shift in the system of conjugated double bonds, as in rhodoxanthin. Apart from naturally existing carotenoids, over 150 chemically synthesized variants containing heteroatoms have been synthesized (Meléndez-Martinez et.al 2019).

Biological Properties and Application

Carotenoids have a variety of functions in photosynthetic organisms, such as microalgae and plants. Carotenoids essentially serve as auxiliary pigments in the light-harvesting process during the light phase of photosynthesis. They can also shield the photosynthetic apparatus from excessive light by scavenging reactive oxygen species (ROS), including singlet oxygen and other radicals (Vílchez et.al 2011).

The primary biological roles of carotenoids in humans are associated with their antioxidant characteristics, which are a direct consequence of their molecular makeup. Comprehending the ROS-induced mechanisms of oxidative stress and devising suitable techniques to counteract it have emerged as crucial objectives in medical research in recent times. Numerous investigations have been carried out to show how oxidative stress contributes to the onset of degenerative illnesses including Parkinson's and Alzheimer's. Concurrently, a diet high in carotenoids has been shown to lower the risk of degenerative disorders (Vílchez et.al 2011).

The health benefits of carotenoids have been established by several scientific research, and their use is quickly expanding for this reason. Carotenoids have also been suggested as a value-added ingredient that might help make microalgae-based biofuel production profitable (Pham et al. 2021).

Of all the existing natural carotenoids, five can be considered the most economically important which are *beta-carotene*, *astaxanthin*, *canthaxanthin*, *lycopene* and *lutein*. However, this report is focusing on the carotenoids accumulated from *R. toruloides* (Table 1). The most important applications of carotenoids are currently food supplements, fortification, food colors, feed, pharmaceuticals, and cosmetics (Vílchez et.al 2011).

Table 1: Main accumulated carotenoids from R. toruloides and their availability.

Carotenoid	Availability
β-carotene	Synthetic and naturally extracted forms
Torularhodin	Only currently available in nature form
γ-carotene	Synthetic and naturally extracted forms
Torulene	Only currently available in nature form

β-carotene, the most well-known carotenoid, due to its presence in carrots, is also known to be a precursor to vitamin A. There is also evidence that several other carotenoids play a similar

role. Research has shown that carotenoids provide health benefits because they have antioxidant properties. Especially in older people, carotenoids are believed to reduce the risk of degenerative diseases and cancer (Pham et al. 2021).

The health industry uses carotenoids in over the counter (OTC) supplements and fortified foods. This is one of the fastest-growing segments of the industry but is still relatively small compared to the color segment. Carotenoids are also frequently used in the pharmaceutical and cosmetics industry for their coloring properties, although their use by pharmaceutical and cosmetics companies is growing rapidly due to their nutraceutical properties. An example of a new product from this segment is a 'beauty pill' containing the carotenoid *lycopene*. This product belongs to a new market segment known as 'cosmeceuticals', which aims to combine cosmetics and nutraceutical food ingredients to create products to improve skin and hair (Vílchez et.al 2011)

The industry is dominated by chemically synthesized natural equivalent carotenoids, but as consumers seek more natural goods, organically extracted carotenoids are gaining popularity. Organisms that naturally produce carotenoids include fungi, algae, and tomatoes. The most popular types include beads in oil form, emulsions in oil form, and powder that dissolves in water. The most typical concentration is 10%, although there are other ranges as well: 0.2 to 100%. It is also possible to find mixes or mixed carotenoids, which consist of two or more distinct carotenoids. Similar to the individual carotenoids, mixes are offered in a range of formats, such as powder that dissolves in water, oil suspension. Blends can have concentrations ranging from 1 to 30%, with 10% being the most popular (Vílchez et.al 2011).

Natural foods like egg yolks, chicken flesh, and fish frequently use carotenoids to enhance their color. Merely a handful of the over 400 identified carotenoids, such as *lutein*, *β-carotene*, *lycopene*, and *asthaxanthin*, have found commercial application. A primary benefit of using microalgae as a carotenoid carrier in the food business is the presence of numerous additional antioxidant chemicals in the microalgal biomass, which can occasionally operate in concert with carotenoids to improve human health (Vílchez et.al 2011).

Additionally, certain minerals whose presence is inherent to the algal biomass are provided in the formula if carotenoids are disposed of within the microalgal matrix (carotenoid-enriched dry biomass). These minerals are good for human health because they increase anabolic activity. Additionally, preservatives containing carotenoids have been found in sun protection and cosmetics (Vílchez et.al 2011).

Microalgae's increasing commercial value and wide range of applications in the food market can be attributed to their carotenoid concentration. This entails the usage of functional foods such as *Arthrospira*, *Chlorella, Dunaliella, Spirulina*, and *Aphanizomenon*, which are available in the market as pills, tablets, and capsules. Additionally, the nutritional composition of pasta, snacks, candies, drinks, and bubble gum has been combined with these microalgae. (Vílchez et.al 2011).

Microbial Carotenoids

There are various reasons why microbial carotenoids have garnered a lot of attention recently. In addition to their biological functions, microbial carotenoids have significant commercial value. They are used as natural colorants in the food and cosmetics industries and have potential pharmaceutical applications due to their antioxidant and anti-inflammatory properties.

Microbial production of carotenoids offers a sustainable alternative to synthetic carotenoids by utilizing biotechnological processes to increase yields and reduce environmental impact (Vílchez et.al 2011).

Even though over 600 carotenoids have been discovered from diverse sources, only approximately 40 are consumed by humans, and even fewer are employed commercially for cosmetics, medications, feed, and food coloring. Total chemical synthesis is utilized to create most carotenoids used in the food, feed, nutritional supplement, cosmetic, pharmaceutical, and other industrial sectors (Vílchez et.al 2011; Pham et al. 2021).

Overall, microbial carotenoid research and application represent a vibrant interface between microbiology, biotechnology, and industrial chemistry that promises scientific insights and practical benefits (Pham et al. 2021).

Lipids

Classification and Nomenclature

Lipids are a group of components that are insoluble in water but is soluble in organic solvents. Triacylglycerol (TAG), which consists of three fatty acid molecules esterified with a glycerol backbone, is the most abundant lipid component. Fatty acid molecules are made up of carbon (C) atoms bonded together in a chain, with a holding carboxyl group (-COOH) at one end of the chain. The length of the chain and the quantity and location of carbon double bonds affect the characteristics of fatty acids. Fatty acids are involved in the synthesis of numerous complex lipid compounds (Oisés Teles dos Santos et.al 2014).

Lipids are found in various compounds, including terpenes, phospholipids, fatty acids, sterols, and sphingolipids. Given the remarkable diversity of lipids in terms of structure and function, it is not surprising that there are significant differences in existing classification schemes. According to Fahy et al. (2011), the Lipid Library and Cyberlipids categorize lipids into "simple" and "complex" groups. Simple lipids, such as acylglycerols, hydrolyze to produce at most two distinct entities, like fatty acids and glycerol. In contrast, complex lipids, such as glycerophospholipids, hydrolyze to yield three or more products.

Lipid nomenclature is divided into two primary categories: common or trivial names and systematic names. The first contains acronyms that are a useful means of characterizing the acyl/alkyl chains seen in glycerolipids, sphingolipids, and glycerophospholipidsn (Fahy et.al 2011).

Biological Properties and Application

Oils and fats, often known as lipids, have been used in various fields, including food, bioenergy, bioplastics, and medicine as medications or dietary supplements. Which are some of the fields where scientist would like to maximize the use of lipid at a low cost.

Edible oil is a type of lipid that is obtained through physical extraction from seeds and tissues of plants and animals, for instance, coconut, palm, oilseed rape, soybean, sunflower, olive, and so forth. Baking, frying, and other food procedures all utilize edible oil. Moreover, edible oil makes up the recommended daily supplement component. They are the primary source of dietary fats, vital for growth, nourishment, and healthy nervous system function. Furthermore,

edible oil comes in a variety of forms and qualities. According to Shah, Aragon, and Calderon (2021), the bio-lubricant oil market is projected to increase from $2 billion in 2020 to $2.4 billion (20%) by 2025.

By chemically converting animal fats and vegetable oils through the transesterification process, biodiesel is created as a renewable energy source. As of late, photosynthetic aquatic plants known as microalgae have been shown to produce these oils, which can be used to produce biodiesel- a renewable substitute for fossil fuels. By 2020, the United States' consumption of biodiesel, which was approximately 260 million gallons in 2010, is expected to reach 1.8 billion gallons (U.S. Energy Information Administration, 2021).

Polymers made from vegetable oils and starch crops- a petroleum substitute- are known as bioplastics. Additionally, petrochemicals, microbes, renewable raw resources, or mixtures of all are used to make biodegradable plastics. Although the majority of the oils used in this technique come from fossils, crop oils or bio-oils (originally from microorganisms) are gradually taking their place (Swanson et.al 2012).

Lipids that contain important elements like omega-3 and omega-9 fatty acids are known as functional oils. Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and alpha-linolenic acid (ALA) are the three primary forms of omega-3 fatty acids. Whereas algae and marine fish include DHA and EPA meanwhile, ALA is mostly found in plant oils like flaxseed, soybean, and canola oils. During the human body's metabolic activities, DHA is produced in small amounts from other fatty acids. It is therefore necessary to consume them through diet or supplements (Braarud et al. 2018; Ferrucci and Fabbri 2018).

Heart disease and other chronic diseases are less likely to occur when DHA and EPA are present. According to reports (Allaire et al. 2016; Yamagata 2017), DHA also supports healthy eyes and cognitive functions. Increased consumption of omega-3 fats, such as DHA, has also been associated with a decreased risk of many malignancies, such as pancreatic, colon, breast, and prostate cancers (Zárate et al. 2017; Ferrucci and Fabbri 2018). Similarly, DHA is necessary for the brain and ocular development of newborns with specific needs (Braarud et al. 2018.; Swanson et.al 2012).

These applications highlight some of the reasons this study aim to maximize lipid production and utilization in oleaginous red yeasts.

Microbial Lipids

Oleaginous microorganisms, which include a wide range of taxa like algae, filamentous fungi, yeasts, and bacteria, create microbial lipids, also referred to as single-cell oils (Ewing and Msangi 2009). *Yarrowia lipolytica*, *Rhodotorula toruloides*, *Rhodotorula graminis*, *Rhodotorula glacialis*, *Chlorella protothecoides*, and *Trichosporon coremiiforme* are examples of oleaginous yeasts that can collect a significant amount of lipids within their cells (Dulermo et al. 2015a; Calvey et al. 2016).

Certain oleaginous yeasts' lipids are rich sources of polyunsaturated fatty acids that are added to a variety of foods and nutraceuticals. On the other hand, distinct oleaginous yeast is used in the industrial production of omega-3 and omega-6 fatty acids. Examples include *eicosapentaenoic acid* (EPA) (20:5, n−3) by *Mortierella alpina* ST1358 and *Yarrowia lipolytica*; *docosahexaenoic acid* (DHA, 22:6, n−3) by *Crypthecodinium cohnii* and

Schizochytrium; and *γ-linolenic acid* (GLA, C18:3, n−6) produced by *Mucor circinelloides*; *dihomo-gamma-linolenic acid* (DGLA) (20:3, n−6) (Bellou et al. 2016).

Triacyl-glycerol (TAG), which is deposited in intracellular organelles as "lipid droplets" that are generated from the endoplasmic reticulum, occupies the majority of the lipid accumulated in the cells of oleaginous yeasts (Garay et.al 2014). TAGs are naturally occurring neutral lipids produced by the biosynthesis of fatty acids. TAGs are utilized in a variety of industries, including feedstocks for chemical synthesis, feed, and food. TAG derivatives, including alkanes and long-chain fatty acid methyl esters, have garnered interest recently as potential biofuels (Zhu et al. 2012).

Filamentous fungi and yeast have been observed to exhibit significant oil content among the oil-producing microorganisms. Using a culture tank, it is feasible to scale up the production of lipids, regardless of the surrounding ecosystems and climates, as fungi grow more quickly than plants and microalgae (Bonturi et al. 2015). Furthermore, a wider variety of sugars and other carbon sources are used by fungi for growth and lipid accumulation, including alcohols, polysaccharides (starch and pectin), monosaccharides (glucose and xylose), amino sugars (N-acetylglucosamine), disaccharides (lactose, galactose, mannose, cellobiose, and sucrose), and organic acids (Galafassi et al. 2012).

General Biosynthesis Pathways

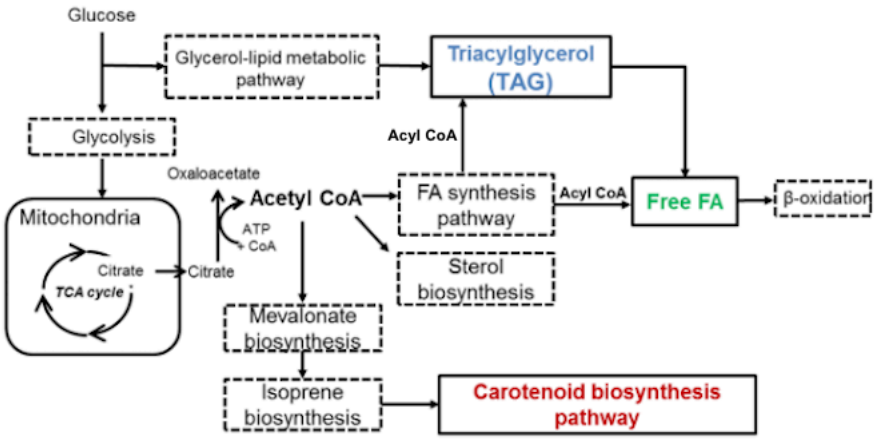


Figure 1: A schematic diagram of the metabolic pathways related to lipid production and carotenoid production (Pham et al. 2021) Modified.

Figure 1 displays a schematic diagram of the metabolic pathways connected to the synthesis and accumulation of lipids that are shared by different species (Pham et al. 2021).

Through the citric acid cycle, glucose that is taken up by cells is transformed from glycolysis to ATP, NADH, FADH2, etc. The metabolic pathway that splits off from this energy generation pathway also accumulates lipids. β-oxidation is the process of degrading lipids, where they serve as raw materials for energy conversion. These molecules enter not only the biosynthesis of TAGs, FFA, or SE from acetyl-CoA but also the biosynthesis of carotenoid pigments via the routes of isoprene and mevalonate (Pham et al. 2021).

Carotenoids Biosynthesis

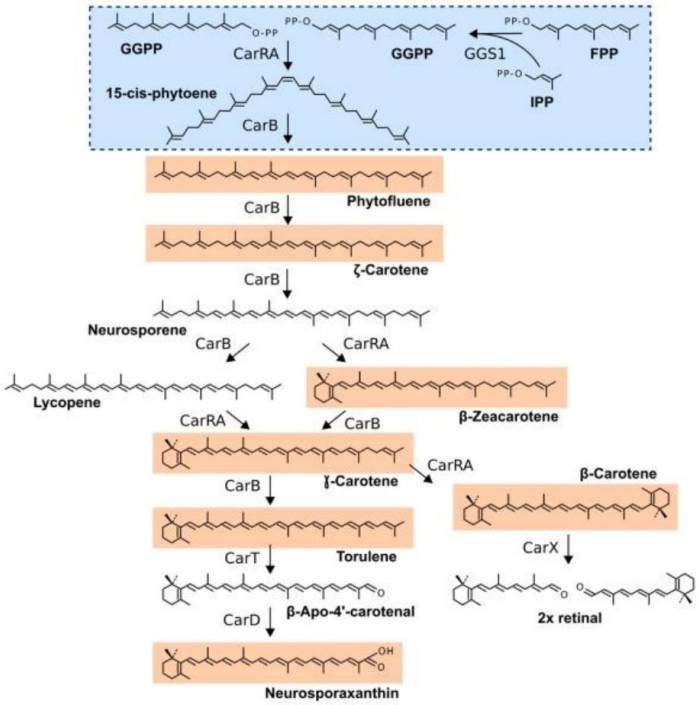


Figure 2: The carotenoid biosynthesis pathway and genes: CarRA., CarB., CarT., CarD in Saccharomyces cerevisiae (Pham et al. 2021)

Following the MVA pathway and isoprene biosynthesis, the C20 molecule GGPP produces phytoene, the first C40 carotene component produced by phytoene synthase (CAR2/CarB) in the carotenoid biosynthesis pathway (Figure 2). Phytoene desaturase (CAR1/CarRA) transforms phytoene, which is produced by the isoprenoid biosynthesis pathway, into lycopene. Following that, lycopene cyclase (CAR2/CarB) converts *lycopene* to *γ-and β-carotene*. Additionally, *γ-carotene* is used in the biosynthesis of neurosporaxanthin and *astaxanthin* (Avalos et al. 2017). Since *lycopene* is an all-trans molecule, the phytoene's first or second double bond isomerizes at the same point during the desaturation process (Mata-Gómez et al. 2014). Additionally serving as a precursor to cyclic carotenoids, lycopene passes through some metabolic processes that result in the formation of *astaxanthin*, *β-carotene, γ-carotene, torulene, and torularhodin.*

Factors Affecting Carotenoid and Lipid Production

Microorganisms that produce lipids and carotenoid compounds have several advantages over other techniques, such as the ability to produce at high rates and a reduced sensitivity to seasonal and regional fluctuations. Additionally, using inexpensive natural substrates, such as carbohydrates, they can accumulate carotenoids and lipids (Lin et al. 2017). However, a variety of parameters, such as the selection of the carbon and nitrogen sources, light, temperature, pH, aeration, metal ions, and salts, affect the synthesis process of lipids and carotenoid pigments (Mata-Gómez et al. 2014).

The most researched factor influencing the growth, or the generation of lipids and carotenoid pigments is the carbon supply. *Rhodotorula toruloides*, an oleaginous yeast, can accumulate

lipids to over 75% of its dry biomass when cultivated under conditions with limited nitrogen and surplus carbon (Nagavara Nagaraj, 2023). This basidiomycetous yeast grows effectively on cost-efficient carbon sources such as lignocellulose hydrolysates and can convert the carbon present in lignocellulosic materials into lipids and carotenoids. The yeast's ability to accumulate lipids depends on carbon availability and environmental triggers, typically the limitation of nitrogen and phosphorus. For the yeast cells to store lipids, there must be an excess of carbon sources accompanied by a limited availability of specific nutrients such as nitrogen, phosphorus, or sulfur. Additionally, lipid production in oleaginous yeasts requires adequate levels of key cellular metabolites such as citric acid and malic acid, along with available energy in the form of adenosine triphosphate (ATP), which is essential for fatty acid synthesis (Nagavara Nagaraj, 2023). Given that fast growth and pigment production are supported by glucose, sucrose, and xylose, the impact of these carbon sources on growth and pigment production was investigated in a variety of strains (Alcantara and Sanchez 1999).

Another important consideration while researching carotenoid or lipid formation was the C/N ratio. The depletion lowers the concentration of intracellular AMP under nitrogen deprivation circumstances. Isocitrate dehydrogenase (IDH), which uses isocitrate (a derivative of citric acid) as a substrate, experiences a drop-in activity when AMP is present because IDH is a complicated substrate of IDH in the citric acid cycle. This leads to the release of a significant amount of isocitric acid, which is then transformed into acetyl CoA, the primary substrate to produce fatty acids (Tang et al. 2009). As a result, it was said that a high C/N ratio supported increased lipid synthesis. Additionally, it was noted that in cultures with a high carbon-to-nitrogen ratio, R. *toruloides* accumulated both carotenoids and lipids (Tang et al. 2009).

Temperature is another parameter, that influences developing cells and metabolite production (Vijayalakshmi et al. 2001). The xanthophyll- and polyunsaturated lipid-deficient (ROAD) of *Synechocystis spp*., has also been employed as a model to investigate how low temperatures affect glycerolipid saturation levels. According to the article, the maximum biomass was 17.9 g/L at a temperature group that was comparatively higher—30 h—while the maximum biomass of 15.8 g/L was at a substantially lower temperature group—45 h. In the meantime, the yeast produced more carotenoid at 30°C than at 24°C (Zhang, Zhang et.al 2014).

Growth, including the synthesis of lipids and carotenoids by some strains of the yeast *R. toruloides* (Dias et al. 2015) has been shown to require an optimal pH.

Numerous publications contained information on carbon and nitrogen sources as well as temperature, pH, aeration, metal ions and salt. However, only a few studies have mentioned the light factor and its impact on microorganisms' production of carotenoids or lipids, and none have addressed both carotenoid and lipid production with light. This indicates that the light factor is the missing piece, in fully understanding how to maximize carotenoid and lipid production. In addition, because carotenogenic is a photoprotective mechanism, the production of carotenoid pigments in microorganisms serves as a defensive mechanism for cells against light and other environmental stresses (Mata-Gómez et al. 2014). Unsaturated fatty acids are one type of antioxidant that guards against cell damage. It has been suggested, therefore, that light contributes to the formation of lipids and carotenoid molecules, with more emphasis being placed on the yeasts that contains carotenoid oil (Mata-Gómez et al. 2014).

Material and Methods

The experiment took place at the Department of Molecular Science, SLU Uppsala Ultuna, Sweden, during the spring semester of 2024. The design of the experiment was divided into two parts and comprised of total ten steps (Figure 3). The first part of the experiment the three strains of *R. toruloides*, CBS 14, CBS 6016 and CBS 349 were cultivated in both dark and light condition. For each strain and condition, the experiment was done in triplicates. The second part of the experiment was performed the same way as part 1 but with a total of eight shaker flask for each strain and condition. The cells were harvested second and fourth day, for the study profile with UHPLC, the 10^{th} step (Figure 3).

Sterile YPD agar medium (glucose 20g/L, peptone 20g/L, yeast extract 10g/L and agar 15g/L) was prepared and autoclaved. The medium was subsequently poured into 18 Petri dishes, allowed to solidify, and labeled according to strain. Yeast cells of CBS 14, CBS 6016 and CBS 349 from separate stock culture were streaked onto the plates in triplicates and incubated at 25°C for 48 hours.

For the second step, 400 ml of prepared sterile YPD medium (glucose 20g/L, peptone 20g/L and yeast extract 10g/L) was poured into sterile shake flasks. For each strain, a loop full of isolated yeast cells was transferred into the flask, thoroughly mixed, and duplication were done. The flasks were incubated at 25°C for 72 hours at 150 rpm.

The third step included harvesting the cells by centrifugation in centrifuge bottles (4000 g, 10 min), transferring them into Falcon tubes, washing twice with sterile saline solution (NaCl, 9 g/L), and resuspending them in saline for inoculation.

Subsequently, the fourth step involved calculating OD 1 by measuring the absorbance with a spectrophotometer, and multiply with the dilution factor and then use the equation $C_1V_1=C_2V_2$, see the calculations in appendix (Tables A.1.1 and A.1.2 and calculations A.2 in the appendix).

In the fifth step, 90 ml of sterile glucose solution (glucose 70 g/L) was measured and transferred into sterile shake flasks. Followed by the addition of 10 ml of YNB (17 g/L) with MES buffer containing dissolved nutrients (ammonium sulphate 20 g/L, yeast extract 7.5 g/L and MES 0.5 M. pH adjusted to 6.0 and then filter sterilized using 0.2 µm sterile). The glucose with YNB media was inoculated with pre-cultured yeast cells (with initial OD 1, Tables A.1.1 and A.1.2 in the appendix) and incubated at 25°C for 5 days at 150 rpm.

Following incubation, the yeast cells were harvested at 4000g for 10 min, which was done in total three times, and freeze-dried for further analysis in the sixth step.

The shaker was covered with aluminum foil during the fifth step for both the dark and light samples. Additionally for the light samples, the walls and roof of the shaker were exposing a 90 µmol/m2/s white light during the incubation.

To prepare for carotenoid extraction from yeast cells, the seventh step started by dissolving approximately 100 mg of freeze-dried cells in 15 mL of milliQ water in a Falcon tube. Vortexing until pellets were fully dissolved. Then, disintegrating the cells using a French press at 40,000 KPI, running 15 mL of the sample through the press. Rinsing the Falcon tube with another 15 mL of DI water and running it through the French press. Finally, rinsing the French press with an additional 15 mL of milliQ water.

After homogenizing the cells, acetone extraction was performed immediately. Transferring an equivalent of 5 ml of homogenized cells to a glass tube, adding 2 mL of acetone. Vortexing for 5-10 seconds, then centrifuging at 2000 rpm for 10 minutes. Removing approximately 90% (keeping track of how much) of the acetone phase to a Teflon tube and adding 1 mL of acetone to the leftover sample (pellets) in the glass tube before centrifuging again.

This process was repeated until the supernatant was clear. Acetone extract was vortexed in the Teflon tube with an equal volume of cyclohexane (1:1), and centrifuging. Removing the acetone phase to a new glass tube and adding 1 mL of acetone to the samples in the Teflon tubes. Repeating this centrifugation and removal process until the supernatant was clear.

Next, the acetone phase was evaporated under nitrogen, after evaporating and ensuring samples remain unsaponified, they were dissolved in 1 mL of hexane and added to a cuvette and rinsed with another 1 ml of hexane and added to the cuvette.

Then in the eighth step, the sample absorbance was measured against hexane at 450 nm using a UV-1800 spectrophotometer. Utilizing a 6-point beta-carotene standard curve ranging from 0.25 to 2.0 μg/ml. Calculating the total carotenoid content expressed as milligrams of β-carotene equivalent (β-EQ) per 100 grams of dried yeast weight based on the standard curve (Figure 6 and Tables 5-7 in results).

In the ninth step, the total lipid quantification was determined by FT-NIR, where the same sample was measured three times, and the average was taken (Table 8-10 in results).

Lastly, in the tenth step, the study profile was conducted (which is referred to as part 2). All the steps up to the sixth step (Figure 3) were repeated; however, one strain at a time for both dark and light conditions. Also, eight shaker flasks were used for the dark conditions and eight for the light conditions, with four flasks for each condition harvested on the second day and the other four on the fourth day. Thereafter the carotenoid and lipid were extracted by SFE. Then UHPLC was used to determine the carotene identification for the two different times during incubation, the second and fourth day for both dark and light conditions (Tables 11-13).

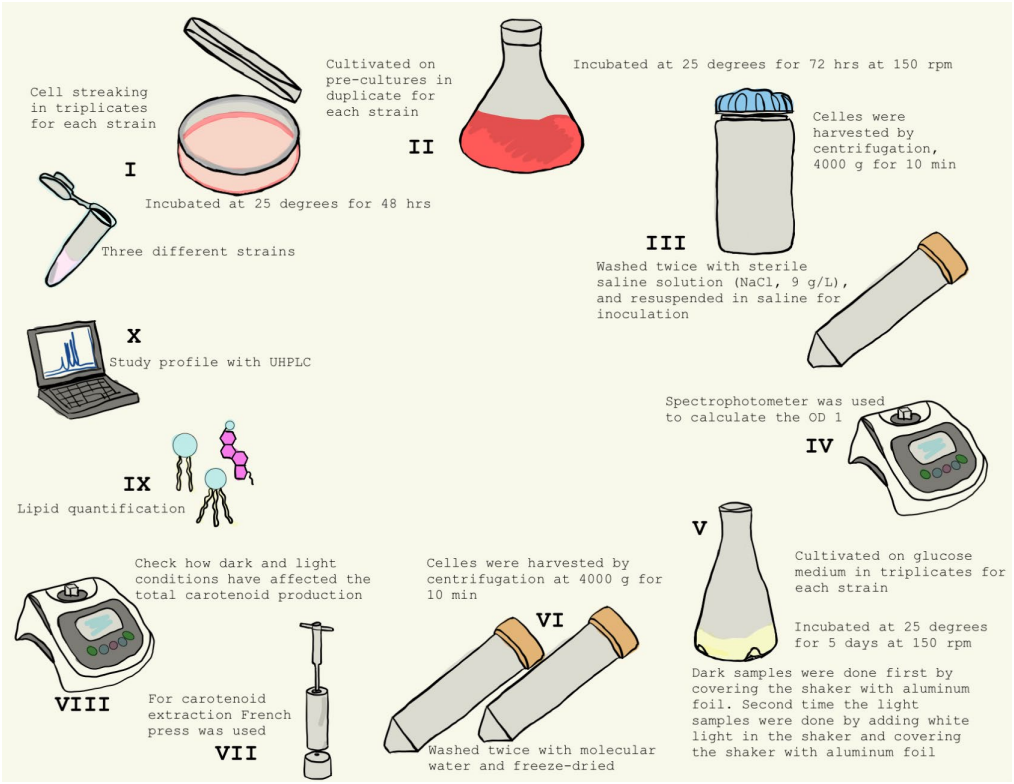


Figure 3: A schematic demonstration of the ten steps (in Roman numbers) conducted for the experiment, illustrated by Dana El Soudi

Results

Table 2 shows the color development of CBS 14 during incubation in the shaker for dark and light conditions, during step 5. (See the different shades in the appendix, A.3.).

Table 2: Color development of CBS 14 during 5 days of incubation for both dark and light conditions

CBS 14	Day 1	Day 2	Day 3	Day 4	Day 5
Dark	No color development	Coral pink color	Coral pink color	Pastel red color	Coral red color
Light	Coral pink color	Coral pink color	Pastel red color	Pastel red color	Pastel red color

Table 3 shows the color development of CBS 6016 during incubation in the shaker for dark and light conditions, during step 5. (See the different shades in the appendix, A.3.).

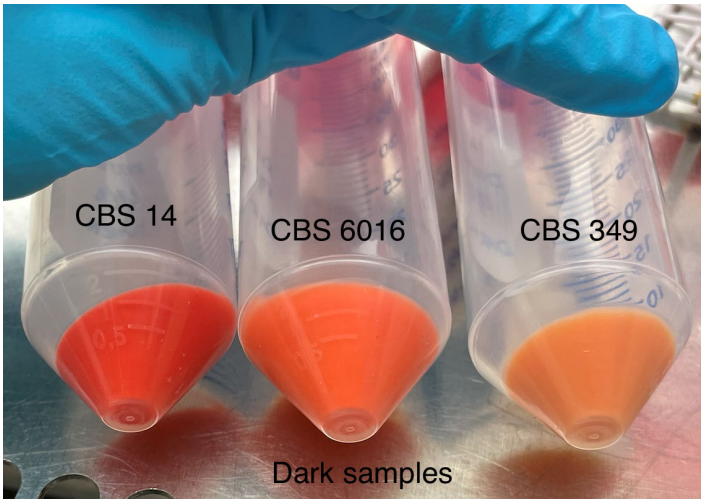
Table 3: Color development of CBS 6016 during 5 days of incubation for both dark and light conditions

CBS 6016	Day 1	Day 2	Day 3	Day 4	Day 5
Dark	No color development	Melon color	Melon color	Coral orange color	Atomic tangerine orange color
Light	Melon color	Coral orange color	Atomic tangerine orange color	Orange	Dark Orange

Table 4 shows the color development of CBS 349 during incubation in the shaker for dark and light conditions, during step 5. (See the different shades in the appendix, A.3.).

Table 4: Color development of CBS 349 during 5 days of incubation for both dark and light conditions

CBS 349	Day 1	Day 2	Day 3	Day 4	Day 5
Dark	No color development	Cream yellow color	Cream yellow color	Lemon chiffon color	Mellow yellow color
Light	Cream yellow color	Lemon yellow color	Mellow yellow color	Mellow yellow color	Light orange color



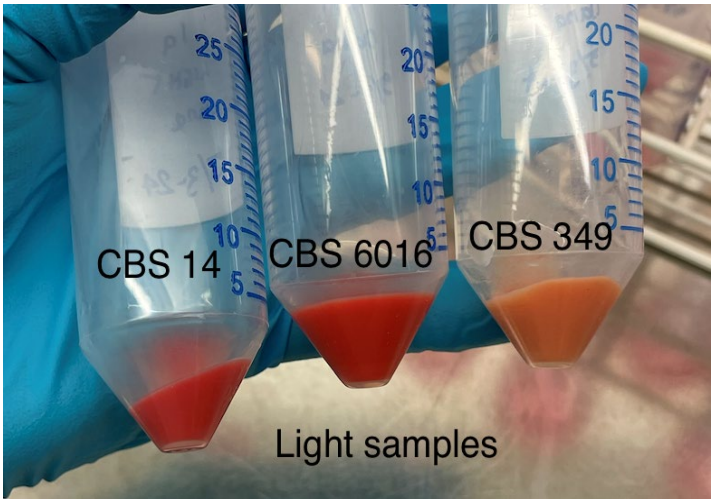


Figure 4 and Figure 5: Images of the harvested cells after dark respective light conditions

Figures 4 and 5 show the harvested cells after incubation for 5 days in the shaker, under respective dark and light conditions. As depicted in the images, CBS 14 appears slightly lighter after exposure to light, while CBS 6016 exhibits a noticeably darker color under the same conditions. However, there is not a significant difference observed for CBS 349, although the light condition does result in a slightly darker shade.

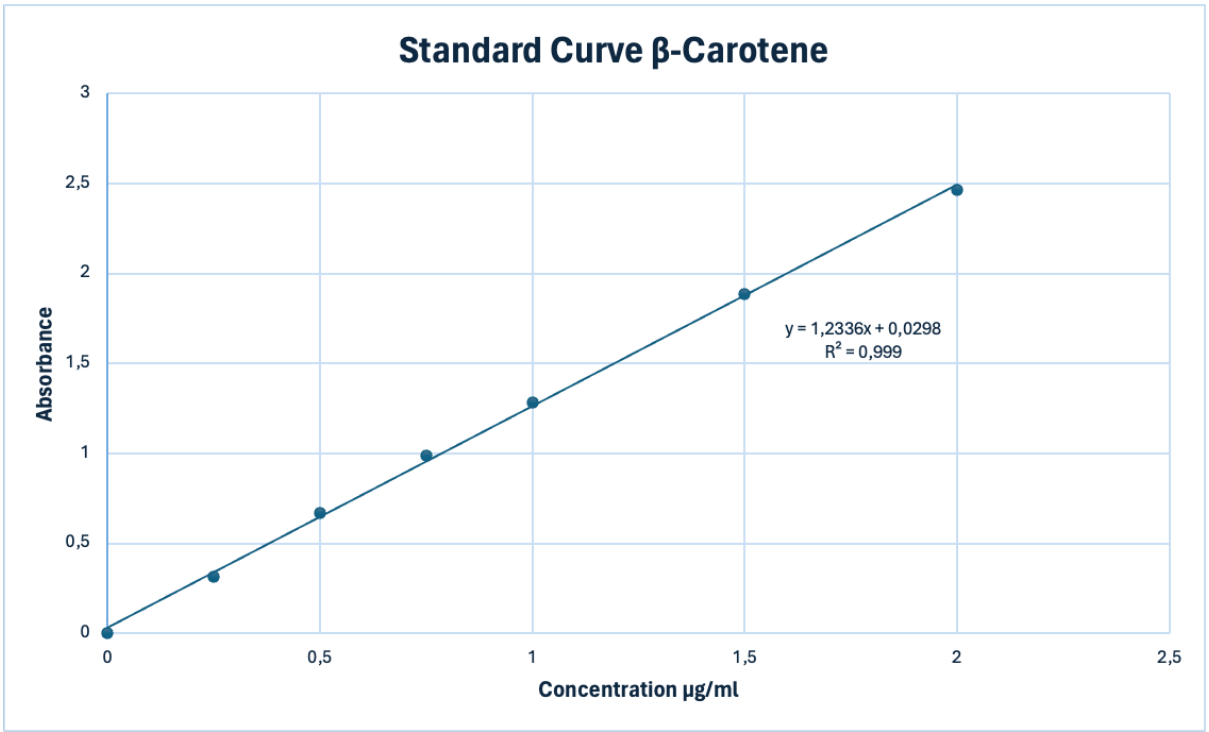


Figure 6: Diagram of the standard curve of β-Carotene done in excel

The Standard Curve of β-Carotene, depicted in Figure 6, was generated using six different concentrations: 0.25 µg/ml, 0.5 µg/ml, 0.75 µg/ml, 1 µg/ml, 1.5 µg/ml, and 2 µg/ml. By plotting

these concentrations on the x-axis against the absorbance on the y-axis, an equation was derived: $y = 1.2336x + 0.0298$, from excel.

Table 5 shows the obtained absorbance against hexane at 450 nm and the calculated concentration, derived using the equation obtained from the standard curve of β-Carotene (Figure 6): $y = 1.2336x - 0.0298$, where y represents the absorbance value and x denotes the concentration expressed in µg/ml and then converted to mg/100 g to better represent the "right" concentration due to a standard curve of β-Carotene, for CBS 14. (See the step-by-step calculation in the appendix, A.5 and A.6).

Table 5: Obtained absorbance and calculated concentration of CBS 14 in dark and light conditions

CBS 14	Dark absorbance (450 nm)	Dark concentration (mg/ 100 g)	Light absorbance (450 nm)	Light concentration (mg/ 100 g)
1.	0.265	3.284	0.141	1.60
2.	0.230	2.801	0.115	1.242
3.	0.211	2.630	0.182	2.145
Mean (SD):	-	**2.905 ± 0.339**	-	**1.66 ± 0.453**

Table 6 shows the obtained absorbance against hexane at 450 nm and the calculated concentration, derived using the equation obtained from the standard curve of β-Carotene (Figure 6): $y = 1.2336x + 0.0298$, where y represents the absorbance value and x denotes the concentration expressed in µg/ml and then converted to mg/100 g to better represent the "right" concentration due to a standard curve of β-Carotene, for CBS 6016. (See the step-by-step calculation in the appendix, A.5 and A.6).

Table 6: Obtained absorbance and calculated concentration of CBS 6016 in dark and light conditions

CBS 6016	Dark absorbance (450 nm)	Dark concentration (mg/ 100 g)	Light absorbance (450 nm)	Light concentration (mg/ 100 g)
1.	0.069	0.569	0.119	1.261
2.	0.050	0.280	0.164	1.920
3.	0.079	0.701	0.147	1.706
Mean (SD):	-	**1.55 ± 0.176**	-	**1.629 ± 0.336**

Table 7 shows the obtained absorbance against hexane at 450 nm and the calculated concentration, derived using the equation obtained from the standard curve of β-Carotene (Figure 6): $y = 1.2336x + 0.0298$, where y represents the absorbance value and x denotes the concentration expressed in µg/ml and then converted to mg/100 g to better represent the "right" concentration due to a standard curve of β-Carotene, for CBS 349. (See the step-by-step calculation in the appendix, A.5 and A.6).

Table 7: Obtained absorbance and calculated concentration of CBS 349 in dark and light conditions

CBS 349	Dark absorbance (450 nm)	Dark concentration (mg/ 100 g)	Light absorbance (450 nm)	Light concentration (mg/ 100 g)
1.	0.033	0.046	0.037	0.104
2.	0.034	0.060	0.030	0.003
3.	0.037	0.103	0.033	0.046
Mean (SD):	**-**	**0.0696 ± 0.017**	**-**	**0.051 ± 0.051**

Table 8 shows the lipid quantification for both dark and light conditions in precent (%) of cell dry weight, which was obtained in step 9 with two different methods. FT-NIR was used during the first part and the total lipid content for the cells harvested day 5, with triplicates. Meanwhile SFE was used during part 2, when the cells were harvested day 2 and day 4.

Table 8: The total lipid content (% of cell dry weight) in CBS 14

CBS 14	Dark lipid (%) with FT-NIR	Light lipid (%) with FT-NIR	CBS 14	Dark lipid (%) with SFE	Light lipid (%) with SFE
1.	≈ 31.86	≈ 13.11	Day 2	2.1	2.9
2.	≈ 33.60	≈ 11.99	Day 4	3.1	3.3
3.	≈ 30.83	≈ 11.56	-	-	-
Mean:	≈ **32.10**	≈ **12.22**	-	-	-

Table 9 shows the lipid quantification for both dark and light conditions in precent, which was obtained in step 9 with two different methods. FT-NIR was used during the first part and the total lipid content for the cells harvested day 5, with triplicates. Meanwhile SFE was used during part 2, when the cells were harvested day 2 and day 4.

Table 9: The total lipid content (% of cell dry weight) in CBS 6016

CBS 6016	Dark lipid (%) with FT-NIR	Light lipid (%) with FT-NIR	CBS 6016	Dark lipid (%) with SFE	Light lipid (%) with SFE
1.	≈ 35.27	≈ 27.82	Day 2	3.7	3.8
2.	≈ 39.88	≈ 46.66	Day 4	8.9	10.9
3.	≈ 42.68	≈ 24.83	-	-	-
Mean:	≈ **39.28**	≈ **33.10**	-	-	-

Table 10 shows the lipid quantification for both dark and light conditions in precent, which was obtained in step 9 with two different methods. FT-NIR was used during the first part and the total lipid content for the cells harvested day 5, with triplicates. Meanwhile SFE was used during part 2, when the cells were harvested day 2 and day 4.

Table 10: The total lipid content (% of cell dry weight) in CBS 349

CBS 349	Dark lipid (%) with FT-NIR	Light lipid (%) with FT-NIR	CBS 349	Dark lipid (%) with SFE	Light lipid (%) with SFE
1.	≈ 22.94	≈ 16.86	Day 2	2.3	3.4
2.	≈ 23.82	≈ 19.05	Day 4	5.4	7.5
3.	≈ 23.42	≈ 18.66	-	-	-
Mean:	≈ **23.39**	≈ **18.19**	-	-	-

Table 11 displays the study profile acquired for CBS 14 and the total carotenoid production on days 2 and 4 under both dark and light conditions. During the dark condition on days 2 and 4, β-carotene, torularhodin, γ-carotene, and torulene are expressed. Conversely, under the light condition on both days 2 and 4, only β-carotene and γ-carotene are evident. The peaks for torularhodin and torulene were too small to yield a positive concentration.

Table 11: Study profile of carotenoid and the total carotenoid production of CBS 14

CBS 14	Day 2 Dark concentration (µg/g)	Day 2 Light concentration (µg/g)	Day 4 Dark concentration (µg/g)	Day 4 Light concentration (µg/g)
β-carotene	0.106	0.040	0.089	0.040
Torularhodin	7.474	-	3.542	-
γ-carotene	1.062	0.364	0.730	0.157

Torulene	13.32	-	56.34	-
Total	**21.96**	**0.404**	**60.70**	**0.197**

Table 12 displays the study profile acquired for CBS 6016 and the total carotenoid production on days 2 and 4 under both dark and light conditions. During the light condition on days 2 and 4, β-carotene, torularhodin, γ-carotene, and torulene are expressed. Conversely, under the dark condition on both days 2 and 4, only β-carotene, γ-carotene and torulene are evident. The peaks for torularhodin were too small to yield a positive concentration.

Table 12: Study profile of carotenoid and the total carotenoid production of CBS 6016

CBS 6016	Day 2 Dark concentration (µg/g)	Day 2 Light concentration (µg/g)	Day 4 Dark concentration (µg/g)	Day 4 Light concentration (µg/g)
β-carotene	0.048	0.059	0.060	0.075
Torularhodin	-	1.328	-	2.310
γ-carotene	0.476	0.560	0.603	0.346
Torulene	6.837	2.592	9.802	0.998
Total	**7.361**	**4.540**	**10.465**	**3.728**

Table 13 displays the study profile acquired for CBS 349 and the total carotenoid production on days 2 and 4 under both dark and light conditions. During both dark and light condition on days 2 and 4, only β-carotene is expressed. The peaks for torularhodin and torulene were too small to yield a positive concentration. And no peak was found for γ-carotene when analyzing the UHPLC.

Table 13: Study profile of carotenoid and the total carotenoid production of CBS 349

CBS 349	Day 2 Dark concentration (µg/g)	Day 2 Light concentration (µg/g)	Day 4 Dark concentration (µg/g)	Day 4 Light concentration (µg/g)
β-carotene	0.013	0.015	0.029	0.013
Torularhodin	-	-	-	-
γ-carotene	No Peak	No Peak	No Peak	No Peak
Torulene	-	-	-	-
Total	**0.013**	**0.015**	**0.029**	**0.013**

Discussion

According to the findings of this study, CBS 14 exhibited a higher carotenoid concentration in samples cultivated under dark conditions, whereas CBS 6016 showed a higher concentration in samples cultivated in a light environment. Interestingly, there was no noteworthy difference in carotenoid concentration for CBS 349 between the two cultivation conditions. This discovery is particularly noteworthy because previous studies indicated that CBS 6016, the hybrid strain, primarily inherits its genetic traits from CBS 14 (Nagavara Nagaraj 2023), which should in theory exhibit the similar behavior as CBS 14.

Observing the cultivation, over the course of five days, it was evident that the color change differed between the two conditions in which the cells were cultivated. In the light condition, the color change occurred day 1, whereas in the dark condition, the color change didn't begin until day 2 (Table 2, 3 and 4). Another observation made was that it seemed like the number of harvested cells was lower for the samples cultivated in light conditions compared to those cultivated in dark conditions by the volume in the falcon tube (Figure 4 and 5).

The reason for this discrepancy could be related to stress factors, as light stimulates carotenoid production in the oleaginous yeast *R. toruloides* by activating carotenoid biosynthesis genes, as evidenced by the results from CBS 6016. However, for CBS 14, it likely involves a defense mechanism, as carotenogenic serves as a photoprotective mechanism. The production of carotenoid pigments in microorganisms acts as a defensive mechanism against light and other environmental stresses (Mata-Gómez et al. 2014).

A third notable observation was that the shake flask located in the bottom right front corner consistently exhibited lighter pigmentation colors for all three strains under both dark and light conditions. A similar occurrence has happened for the flask located closest to the window in other studies during fermentation of this strains. This phenomenon may be attributed to the flask not receiving a stable temperature, potentially influenced by external environmental factors contributing additional stress.

Additionally, a notable contrast observed between the cultivation processes in part 1 and part 2 is that strain CBS 349, under both dark and light conditions, exhibited a deeper pigmentation color in part 2 compared to part 1. However, for strain CBS 14 and CBS 6016 during cultivation under both conditions, they exhibited a lighter color in comparison with part 1. This discrepancy may be attributed to variations in seasonal environmental conditions. Also, CBS 14 displayed a darker pigmentation color under light conditions, marking a deviation from part 1. However, CBS 6016 and CBS 349 exhibited the same difference as in part 1.

Furthermore, in part 2, both CBS 14 and CBS 6016 exhibited similar behavior during cultivation, providing evidence of CBS 6016 being a hybrid that predominantly inherited genetic traits from CBS 14 (Nagavara Nagaraj 2023). As well, confirming that in part 1, the higher pigmentation color observed in CBS 14 under dark conditions compared to cultivation under light conditions is indeed a defensive mechanism against light.

The study also revealed variation in lipid content among replicates across all three strains with FT-NIR. Interestingly, all three strains exhibited higher lipid content when cultivated in dark conditions. However, due to the considerable variability in lipid content among replicates, the reliability of the results is questionable. Despite literature suggesting that lipid content for oleaginous red yeast typically falls within the range of 20-80% (w/w) (Dulermo et al. 2015a;

Calvey et al. 2016), many results, particularly for samples cultivated in light conditions, showed less than 20% lipid content. Also, the lipid amount obtained through SFE is very low, this could potentially be attributed to the insufficient quantity of samples utilized during the analysis. Furthermore, when analyzed with SFE, the samples cultivated in light exhibited slightly higher lipid content compared to those cultivated in the dark.

The optimal temperature for growth and lipid biosynthesis of oleaginous yeasts is generally reported to be 30°C (Mohammed et al. 2018); however, certain strains exhibit higher lipid synthesis efficiency at lower temperatures (20°C) (Zhang et al. 2014), possibly due to increased expression of genes coding to produce certain desaturases. Nevertheless, the experiment was carried out at a temperature of 25°C.

Regarding the ideal temperature needed for lipid production, a significant difference was found. This study showed a significant variation in lipid content following growing at 25°C, however additional literature investigations revealed that cultures incubated at a temperature of roughly 30°C resulted in the largest biomass concentration of lipids (Mohammed et al. 2018). The findings demonstrate that it is important to ascertain each yeast strain's ideal culture temperature, which in turn depends on the culture medium being employed.

The varied results that were achieved may have also been influenced by the chemical composition of the experimental medium. If a different medium is utilized, it is likely that lipid production might proceed more effectively at higher temperatures. The degree of escalation of lipid and carotenoid biosynthesis in the other conditions studied may also have been strongly influenced by the type of medium used; however, to confirm this theory, experiments should be conducted using a minimum of several different types of basal media for comparison.

Another reason for the variation in lipid content could be attributed to the fact that, at the time of cell harvesting, the medium still contained a higher amount of glucose than anticipated. Nevertheless, the cells were still harvested because if the glucose levels were to decrease overnight, the cells might resort to consuming lipids as energy source, which also would affect the total lipid content.

There might be a connection between lipids and carotenoid biosynthesis pathways, while lipid metabolism and carotenoid biosynthesis are distinct pathways, they exhibit points of convergence and interdependence, suggesting coordination and regulation to meet the metabolic needs of the cell.

For example, some intermediates in the pathways leading to lipid synthesis can also serve as precursors for carotenoid biosynthesis. For instance, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are common precursors for both lipid and carotenoid synthesis. Also, some enzymes involved in lipid metabolism can sometimes influence the production of carotenoids and vice versa (Pham et al. 2021).

Regulatory mechanisms that control lipid metabolism can also impact carotenoid biosynthesis and vice versa. Transcription factors and signaling pathways involved in lipid metabolism may regulate the expression of genes encoding enzymes involved in carotenoid biosynthesis. Carotenoids are often found in association with lipid membranes in cells. They can play roles in protecting cells from oxidative damage and in maintaining membrane integrity. Thus, the availability and composition of lipids in the membrane can influence the synthesis, stability, and function of carotenoids (Pham et al. 2021).

The study profile was as expected, the total carotenoid content of the strains comprises a mixture of *β-carotene, torularhodin, γ-carotene, and torulene*. However, the intriguing aspect lies in the quantity of each of these carotenoids in the strains on days 2 and 4.

In the dark samples of CBS 14, both on days 2 and 4, all four anticipated carotenoids were detected. Nevertheless, *β-carotene, torularhodin*, and *γ-carotene* exhibited slightly higher levels on day 2, whereas *torulene* showed a significantly higher concentration on day 4, resulting in an overall higher concentration on day 4. In contrast, in the light samples on both days 2 and 4, the concentration of *torularhodin* and *torulene* were below the limit of quantification.

CBS 6016 exhibits the opposite pattern compared to CBS 14, as in this case, the light samples on both days 2 and 4 contain all four anticipated carotenoids, whereas the concentration of *torularhodin* in the dark samples were below the limit of quantification. Nonetheless, despite the absence of *torularhodin* in the dark samples, they display a higher total carotenoid concentration.

In the case of CBS 349, the behavior is unpredictable, as only *β-carotene* was detected. The concentrations for *torularhodin* and *torulene* were below the limit of quantification (LOQ), while the peak for *γ-carotene* was not even found in the UHPLC graph. However, the concentration of *β-carotene* remained similar across both conditions and days.

Despite the tests being conducted on a new batch, all three strains exhibited expected behavior consistent with the results from part 1. Throughout both part 1 and part 2, CBS 14 consistently showed a higher total carotenoid concentration in the dark samples. CBS 349, on the other hand, did not exhibit any differences between dark and light conditions in either part 1 or part 2 of the experiment. However, CBS 6016 displayed slight variations, in part 1, the light samples showed the highest total carotenoid concentration. Whereas in part 2, although some carotenoids were not included in the total carotenoid concentration for dark samples, they still exhibited a higher total carotenoid concentration.

Further Studies

The procedure of this study could be improved and achieve better results, if several changes could be considered. Firstly, instead of conducting the study in two separate parts, it would have been more advantageous to integrate them into one cohesive process. This would involve starting with a larger number of replicates to ensure a higher amount of harvested cells. To initially ensure an ample supply for subsequent stages, particularly for the SFE carotenoid extraction.

Moreover, using the same pre-culture for both dark and light cultivation conditions would have been preferable. This approach would involve employing the same strain under both conditions simultaneously, rather than initially culturing under dark conditions and afterword cultivating under light condition.

Another aspect to consider is the variation in measurement techniques due to human error. During preparation of the different mediums such as YPD medium, glucose medium, and MES buffer may introduce inconsistencies in measurement for each time.

Another parameter worth exploring is the effect of temperature on the strains. Conducting experiments at multiple temperatures, such as 20, 25, 30, and 35 degrees Celsius, would provide a more comprehensive understanding of the impact of temperature on the study and the microorganism defense mechanism response.

Additionally, it would be intriguing to observe how the seasons affect the three different strains, CBS 14, CBS 6016 and CBS 349 because it seemed like they did kind of behave differently during part 1 (February- winter) and part 2 (April- spring). Therefore, another parameter could involve conducting the same process during fall, winter, spring, and summer to determine if these strains exhibit differing behaviors during the four seasons.

Furthermore, it might have been beneficial to harvest cells over all five days. This would enable the observation of any correlation between carotenoid and lipid production over time, providing a more comprehensive analysis and correlation of the light factor affect between carotenoid and lipid production. Thus, by addressing these considerations, the study could potentially yield more robust and insightful results.

Conclusion

Understanding the factors influencing carotenoid and lipid production in yeasts has remarkable implications for biotechnological applications, including biofuel production, pharmaceuticals, food, and feed. Optimizing growth conditions and genetic manipulation strategies based on strain-specific responses could enhance the efficiency of microbial production systems. The question this study aims to address is: "How does light affect carotenoid- and lipid production in strains CBS 14, CBS 6016 and CBS 349 of *Rhodotorula toruloides*?" Additionally, it seeks to determine whether light maximizes or minimizes carotenoid and lipid production in *Rhodotorula toruloides*.

This study reveals variations in both carotenoid and lipid concentrations among all three strains, with no discernible behavioral patterns observed among them. Consequently, predicting how the defense mechanisms will respond becomes challenging. However, based on the findings of this study, CBS 14 demonstrates a higher concentration of carotenoids when cultivated in darkness, whereas CBS 6016 exhibits elevated carotenoid levels under light conditions. In contrast, CBS 349 shows no noteworthy difference in carotenoid concentration between the two cultivation conditions. Due to the considerable variability in lipid concentration among replicates, the reliability of the results is questionable. Meanwhile, the carotenoid study showed that CBS 14 exhibited a higher carotenoid concentration in the dark samples, as did CBS 6016, even though some of the carotenoids were not expressed. In contrast, CBS 349 showed no noteworthy difference between the dark and light conditions, nor any significant difference between days 2 and 4.

The conclusion is that strain CBS 14 produces the highest level of carotenoids, while CBS 349 produces the lowest amount due to genetic differences. Additionally, light affects carotenoid production by in theory triggering the defense mechanism and depending on strain it maximizes or minimize the carotenoid production.

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Popular Science Summary

The population of the Earth is growing at an overwhelming pace, and it is already exceeding the ability of the planet to cover the needs of all inhabitants. Moreover, the growth of the population resulted in the necessity of industrialization, and the intensive use of fossil fuels led to severe climate change and environmental pollution. These modern problems require modern solutions, and one of the answers to them could be the sustainable way of producing food, feed, energy, and other essential materials.

This innovation could be achieved with the help of oleaginous red yeasts, particularly *Rhodotorula toruloides*, which can produce valuable compounds; lipids and carotenoids. These microbial resources can play key roles in the creation of a green economy since they can be used as alternatives source of oil for the biodiesel and food- and feed industries, and to biomaterials like bioplastics and biochemicals including pharmaceuticals and biocosmetics (Pham et al. 2021).

The experiment is aimed at studying how light can affect the production of lipids and carotenoids in three different strains of *R. toruloides*, CBS 14, CBS 6016, and CBS 349. This research will help to develop information on the factors influencing the synthesis of lipids and carotenoids that could be used in practice.

The results show variations in carotenoid concentration and lipid content between the three different yeast strains cultivated under different conditions: light and dark. CBS 14 showed a higher carotenoid concentration under dark condition, while CBS 6016 showed a higher concentration under light condition. CBS 349 showed no significant difference between the conditions. The response of CBS 6016 to light indicates a stimulation of carotenoid production, while the response of CBS 14 indicates a defense mechanism.

The study profiles for CBS 14 and CBS 349 exhibit the same behavior as during the first part of the experiment. However, for CBS 6016, a higher total carotenoid concentration was observed in the dark samples, despite the omission of some carotenoids.

Lipid content varied between strains and conditions, with higher levels observed in dark condition when analyzed with FT-NIR, and higher level observed in light condition when analyzed with SFE, but the reliability of the results was questioned due to the variability between the replicates.

The study highlights possible links between lipid and carotenoid biosynthetic pathways, suggesting coordination and regulation to meet cellular metabolic needs. Regulatory mechanisms that control lipid metabolism may affect carotenoid biosynthesis and vice versa.

The conclusion is that strain CBS 14 produces the highest level of carotenoids, while CBS 349 produces the lowest amount due to genetic differences.

Appendix

A.1. Tables of OD 1

Table A.1.1 shows the calculated OD 1 value, the volume of the pre-culture samples used in step five for the dark samples. (See the step-by-step calculation in the appendix).

Table 14, A.1.1: Initial OD 1 for Dark conditions samples during part 1

Dark samples	CBS 14	CBS 6016	CBS 349
Absorbance	0.21	0.41	0.35
OD	210	410	700
V (ml)	0.476	0.243	0.143
V (ul)	**476**	**243**	**143**

Table A.1.2 shows the calculated OD 1 value, the volume of the pre-culture samples used in step five for the light samples. (See the step-by-step calculation in the appendix).

Table 15, A.1.2: Initial OD 1 for Light conditions samples during part 1

Light samples	CBS 14	CBS 6016	CBS 349
Absorbance	0.41	0.41	0.31
OD	1 230	820	620
V (ml)	0.081	0.121	0.161
V (ul)	**81**	**121**	**161**

Table A.1.3 shows the calculated OD 1 value, the volume of the pre-culture samples used in step five during part 2 for the dark and light samples. (See the step-by-step calculation in the appendix).

Table 16, A.1.3: Initial OD 1 for both Dark and Light conditions samples during part 2

Dark and Light samples	CBS 14	CBS 6016	CBS 349
Absorbance	0.25	0.22	0.22
OD	250	220	220
V (ml)	0.4	0.4545	0.4545

V (ul)	**400**	**455**	**455**

A.2. Calculation of OD 1

Equation used: Absorbance x (Dilution Factor) = OD

$C_1V_1=C_2V_2$ → Where C_1= OD, V_1= Want, C_2= 1 and V_2= 100 ml → $V_1=\frac{(1\ x\ 100)}{OD}$

A.2.1Dark samples (Table 1):

A.2.1.a. CBS 14:

Absorbance: 0.21

Dilution Factor: 1000

0.21 x 1000 = 210

$V_1=\frac{(1\ x\ 100)}{210}$ = 0.476 ml = 476 µl

A.2.1.b. CBS 6016:

Absorbance: 0.41

Dilution Factor: 1000

0.41 x 1000 = 410

$V_1=\frac{(1\ x\ 100)}{410}$ = 0.243 ml = 243µl

A.2.1.c. CBS 349:

Absorbance: 0.35

Dilution Factor: 2000

0.35 x 2000 = 700

$V_1=\frac{(1\ x\ 100)}{700}$ = 0.143 ml = 143 µl

A.2.2. Light samples (Table 2):

A.2.2.a. CBS 14:

Absorbance: 0.41

Dilution Factor: 3000

0.41 x 3000 = 1 230

$V_1=\frac{(1\ x\ 100)}{1230}$ = 0.081 ml = 81 µl

A.2.2. b. CBS 6016:

Absorbance: 0.41

Dilution Factor: 2000

0.41 x 2000 = 820

$V_1=\frac{(1\ x\ 100)}{820}$ = 0.121 ml = 121 µl

A.2.2.c. CBS 349:

Absorbance: 0.31

Dilution Factor: 2000

0.31 x 2000 = 620

$V_1=\frac{(1\ x\ 100)}{620}$ = 0.161 ml = 161 µl

A.2.3. Dark and Light samples (Table 3):

A.2.3.a. CBS 14:

Absorbance: 0.25

Dilution Factor: 1000

0.25 x 1000 = 250

$V_1 = \frac{(1\ x\ 100)}{250}$ = 0.4 ml = 400 µl

A.2.3.b. CBS 6016:

Absorbance: 0.22

Dilution Factor: 1000

0.22 x 1000 = 220

$V_1 = \frac{(1\ x\ 100)}{220}$ = 0.4545 ml = 455 µl

A.2.3.c. CBS 14:

Absorbance: 0.22

Dilution Factor: 1000

0.22 x 1000 = 220

$V_1 = \frac{(1\ x\ 100)}{220}$ = 0.4545 ml = 455 µl

A.3. Color Shades

Coral pink color

Pastel red color

Coral red color

Melon color

Coral orange color

Atomic tangerine orange color

Orange

Dark orange

Cream yellow color

Lemon chiffon color

Mellow yellow color

Light orange

A.4. Sample Weight

Table A.4.1 shows the weighted samples in mg used in step 7 and conversion to gram (g) for the calculation for both in dark and light condition.

Table 17, A.4.1: Sample weight in mg used in step 7 and converted to gram for the concentration calculations

CBS 14	Dark (mg)	Dark (g)	Light (mg)	Light (g)
1.	104.7	0.1047	101.2	0.1012
2.	104.1	0.1041	100.0	0.1
3.	100.6	0.1006	103.2	0.1032

Table A.4.2 shows the weighted samples in mg used in step 7 and conversion to g for the calculation for both in dark and light condition.

Table 18, A.4.2: Sample weight in mg used in step 7, and converted to gram for the concentration calculations

CBS 6016	Dark (mg)	Dark (g)	Light (mg)	Light (g)
1.	101.3	0.1013	102.8	0.1028
2.	102.8	0.1028	102.2	0.1022
3.	102.7	0.1027	100.2	0.1002

Table A.4.3 shows the weighted samples in mg used in step 7 and conversion to g for the calculation for both in dark and light condition.

Table 19, A.4.3: Sample weight in mg used in step 7, and converted to gram for the concentration calculations

CBS 349	Dark (mg)	Dark (g)	Light (mg)	Light (g)
1.	101.7	0.1017	100.6	0.1006
2.	101.2	0.1012	100.3	0.1003
3.	102.4	0.1024	101.8	0.1018

A.5. Calculations of Carotenoid Concentration

The equation utilized (Figure 6) is y=0.0084x−0.0014 → (which yields the concentration in µg/ml). Subsequently, this value was multiplied by 2, representing the volume in ml (unit now in µg). The result was divided by the measured weight in grams and then multiplied by 9, the index factor (unit now in µg/g). Finally, the value was divided by 10, resulting in the unit being mg/100g.

A.5.1. CBS 14 Dark:

1. 0.265= 1.2336*x* + 0.0298 → x= 0.191 µg/ml
   0.191 x 2 = 0.382/ 0.1047= 3.65 x 9= 32.84/ 10= **3.284 mg/100g**
2. 0.230= 1.2336*x* + 0.0298 → x= 0.162 µg/ml
   0.162 x 2= 0.324/ 0.1041= 3.11 x 9= 28.01/ 10= **2.801 mg/100g**
3. 0.211= 1.2336*x* + 0.0298 → x= 0.147 µg/ml
   0.147 x 2= 0.294/ 0.1006= 2.922 x 9= 26.30/ 10= **2.630 mg/100g**

Mean: (3.284 + 2.801 + 2.630) / 3= 2.905 mg/100g

A.5.2. CBS 14 Light:

1. 0.141= 1.2336*x* + 0.0298 → x= 0.090 µg/ml
   0.090 x 2 = 0.18/ 0.1012= 1.778 x 9= 16/ 10= **1.60 mg/100g**
2. 0.115= 1.2336*x* + 0.0298 → x= 0.069 µg/ml
   0.069 x 2= 0.138/ 0.1= 1.38 x 9= 12.42/ 10= **1.242 mg/100g**
3. 0.182=1.2336*x* + 0.0298 → x= 0.123 µg/ml
   0.123 x 2= 0.246/ 0.1032= 2.38 x 9= 21.45/ 10= **2.145 mg/100g**

Mean: (1.60 + 1.242 + 2.145) / 3= 1.66 mg/100g

A.5.3. CBS 6016 Dark:

1. 0.069= 1.2336*x* + 0.0298 → x= 0.032µg/ml
   0.032 x 2 = 0.064/ 0.1013= 0.632 x 9= 5.686/ 10= **0.569 mg/100g**
2. 0.050= 1.2336*x* + 0.0298 → x= 0.016 µg/ml
   0.016 x 2=0.032/ 0.1028 = 0.311 x 9= 2.80/ 10= **0.280 mg/100g**
3. 0.079= 1.2336*x* + 0.0298 → x= 0.040 µg/ml
   0.040 x 2= 0.08/ 0.1027= 0.779 x 9= 7.01/ 10= **0.701 mg/100g**

Mean: (0.569 + 0.280 + 0.701) / 3= 1.55 mg/100g

A.5.4. CBS 6016 Light:

1. 0.119= 1.2336*x* + 0.0298 → x= 0.072 µg/ml

0.072 x 2 = 0.144/ 0.1028= 1.4 x 9= 12.607/ 10= **1.261 mg/100g**

2. 0.164= 1.2336*x* + 0.0298 → x= 0.109 µg/ml
   0.109 x 2= 0.218/ 0.1022 = 2.133 x 9= 19.198/ 10= **1.920 mg/100g**
3. 0.147= 1.2336*x* + 0.0298 → x= 0.095 µg/ml
   0.095 x 2= 0.19/ 0.1002= 1.896 x 9= 17.065/ 10= **1.706 mg/100g**

Mean: (1.261 + 1.920 + 1.706) / 3= 1.629 mg/100g

A.5.5. CBS 349 Dark:

1. 0.033= 1.2336*x* + 0.0298 → x= 0.00259 µg/ml
   0.00259 x 2= 0.00518/ 0.1017= 0.0509 x 9= 0.458/ 10= **0.046 mg/100g**
2. 0.034= 1.2336*x* + 0.0298 → x= 0.00340 µg/ml
   0.00340 x 2= 0.0068/ 0.1012= 0.067 x 9= 0.604/ 10= **0.060 mg/100g**
3. 0.037= 11.2336*x* + 0.0298 → x= 0.00584 µg/ml
   0.00584 x 2= 0.01168/ 0.1024= 0.114 x 9= 1.027/ 10= **0.103 mg/100g**

Mean: (0.046 + 0.060 + 0.103) / 3= 0.0696 mg/100g

A.5.6. CBS 349 Light:

1. 0.037= 1.2336*x* + 0.0298 → x= 0.00584 µg/ml
   0.00584 x 2= 0.01168/ 0.1006= 0.116 x 9= 1.044/ 10= **0.104 mg/100g**
2. 0.030= 1.2336*x* + 0.0298 → x= 0.000162 µg/ml
   0.000162 x 2= 0.000324 / 0.1003= 0.00323 x 9= 0.0290 / 10= **0.003 mg/100g**
3. 0.033= 1.2336*x* + 0.0298 → x= 0.00259 µg/ml
   0.00259 x 2= 0.00518/ 0.1018= 0.0508 x 9= 0.458/ 10= **0.046 mg/100g**

Mean: (0.104 + 0.003 + 0.046) / 3= 0.051 mg/100g

A.6. Calculation of Standard deviation:

$$SD = \sqrt{\frac{\Sigma (xi - x)^2}{n-1}}$$

A.6.1. CBS 14 DARK:

3.284 - 2.905= 0.38^2 =0.1444
2.801 - 2.905= -0.104^2 = 0.010816
2.630 - 2.905= -0.275^2 = 0.075625

(0.1444 + 0.010816 + 0.075625) = 0.230841/ (3-1) = 0.1154205 → $\sqrt{0.1154205}$ = 0.3397 ≈ **0.339**

A.6.2. CBS 14 LIGHT:

1.60 - 1.66= -0.06^2 = 0.0036
1.242 - 1.66= -0.414^2 = 0.171396
2.145 - 1.66= 0.485^2 = 0.235225

(0.0036+ 0.171396 + 0.235225) = 0.410221/ (3-1) = 0.2051105 → $\sqrt{0.2051105}$ = 0.45293 ≈ **0.453**

A.6.3. CBS 6016 DARK:

0.569 - 0.5166= 0.0624^2 =0.00274576
0.280 - 0.5166= -0.2366^2 = 0.05597956
0.701 - 0.5166= 0.1844^2 = 0.034

(0.00274576 + 0.05597956 + 0.034) = 0.0927/ (3-1) = 0.03090 → $\sqrt{0.03090}$ = 0.175807 ≈ **0.176**

A.6.4. CBS 6016 LIGHT:

1.261 - 1.629= -0.368^2 = 0.135424
1.920 - 1.629= 0.291^2 = 0.084681
1.706 - 1.629= 0.077^2 = 0.005929

(0.135424 + 0.084681 + 0.005929) = 0.226034/ (3-1) = 0.113017 → $\sqrt{0.113017}$ = 0.336180 ≈ **0.336**

A.6.5. CBS 349 DARK:

0.046 - 0.0696= -0.0236^2 =0.00055696
0.060 - 0.0696= -0.0096^2 = 9.216×10^{-5}
0.103 - 0.0696= 0.0334^2 = 0.00111556

(0.00055696 + 9.216×10^{-5} + 0.00111556) = 0.000588/ (3-1) = 0.000294 → $\sqrt{0.000294}$ = 0.01714 ≈ **0.017**

A.6.6. CBS 349 LIGHT:

0.104 - 0.051= 0.053^2 =0.002809
0.003 - 0.051= -0.048^2 = 0.002304
0.046 - 0.051= -0.005^2 = 2.5×10^{-5}

(0.002809 + 0.002304 + 2.5×10^{-5}) = 0.005138/ (3-1) = 0.002569 → $\sqrt{0.002569}$ = 0.050685 ≈ **0.051**

A.7. Study Profile of Carotenoid with UHPLC

https://docs.google.com/spreadsheets/d/1NyA1LRwKdLlnwxXiOGhqg2uKnx8QOM4n/edit?usp=sharing&ouid=109514735589596843747&rtpof=true&sd=true [Calculations of carotenoids concentration from the UHPLC]

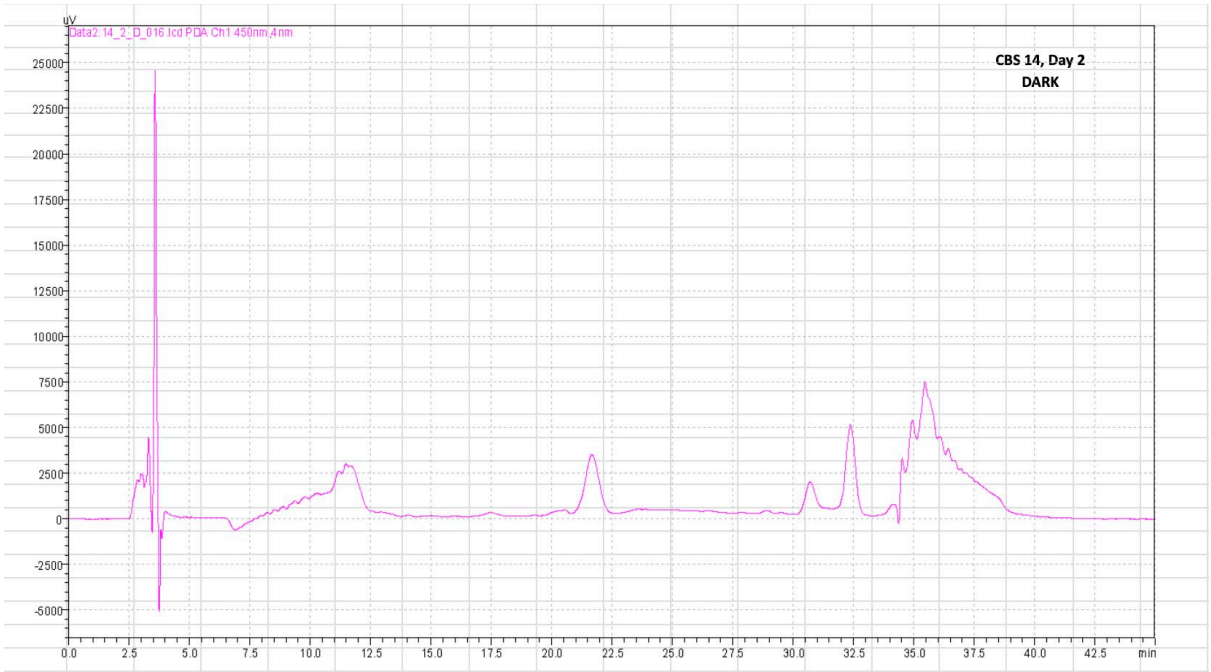

Figure 7: Chromatograph of CBS 14, Day 2, Dark

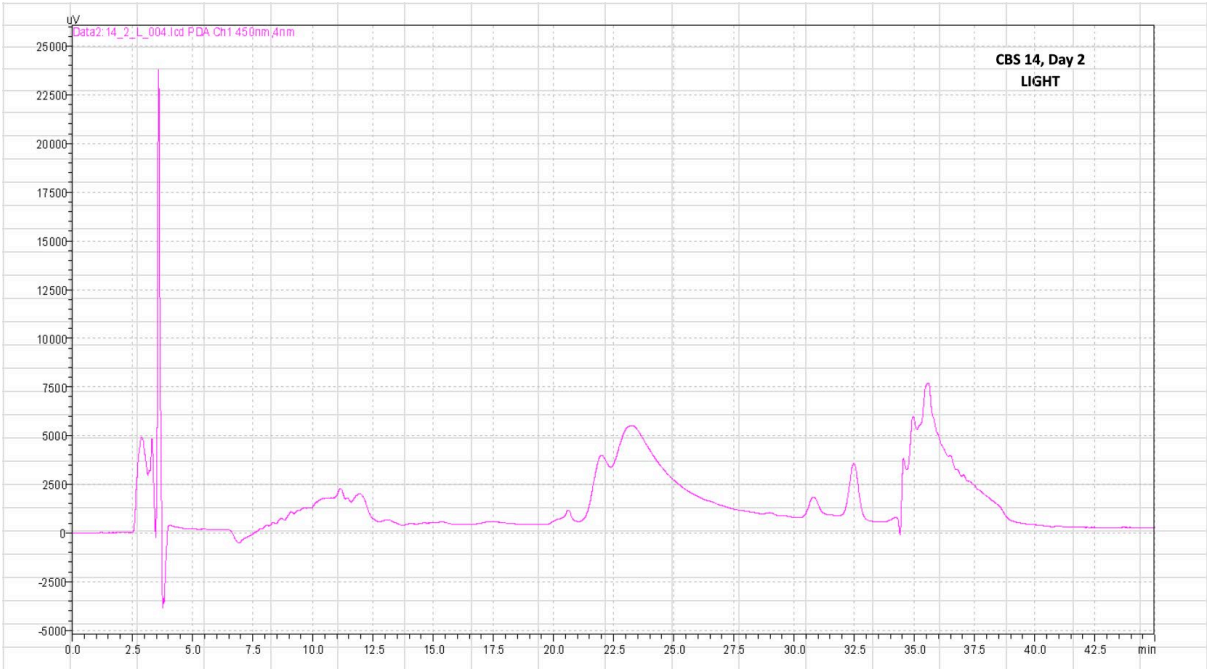

Figure 8: Chromatograph of CBS 14, Day 2, Light

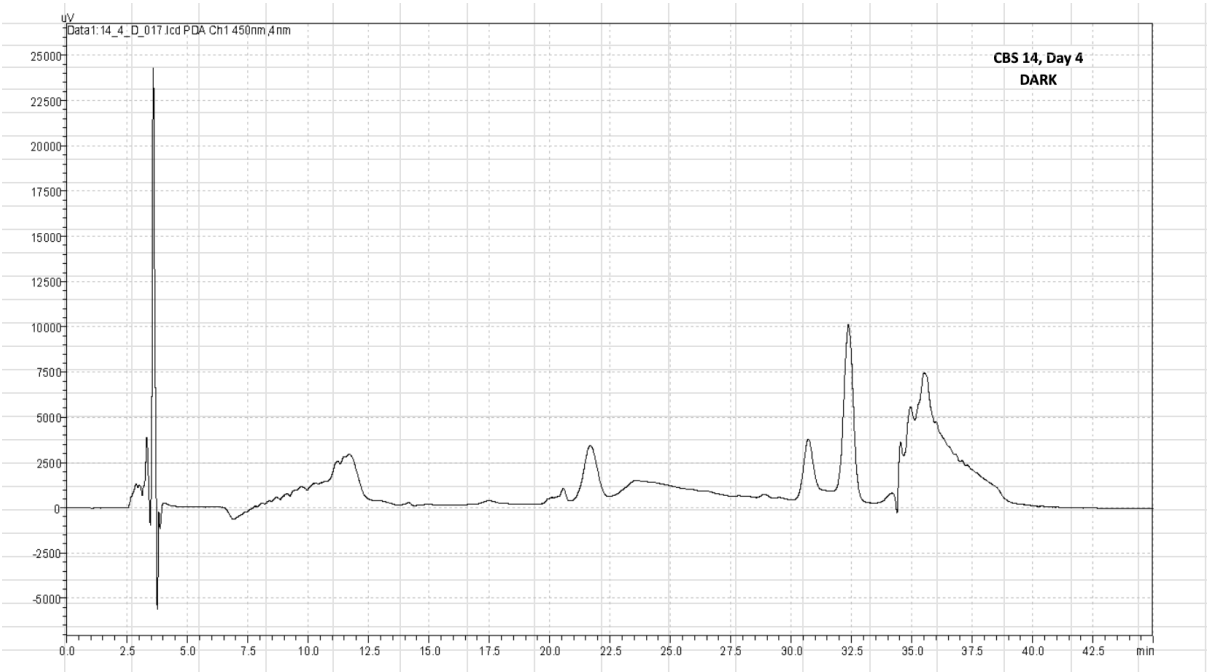

Figure 9: Chromatograph of CBS 14, Day 4, Dark

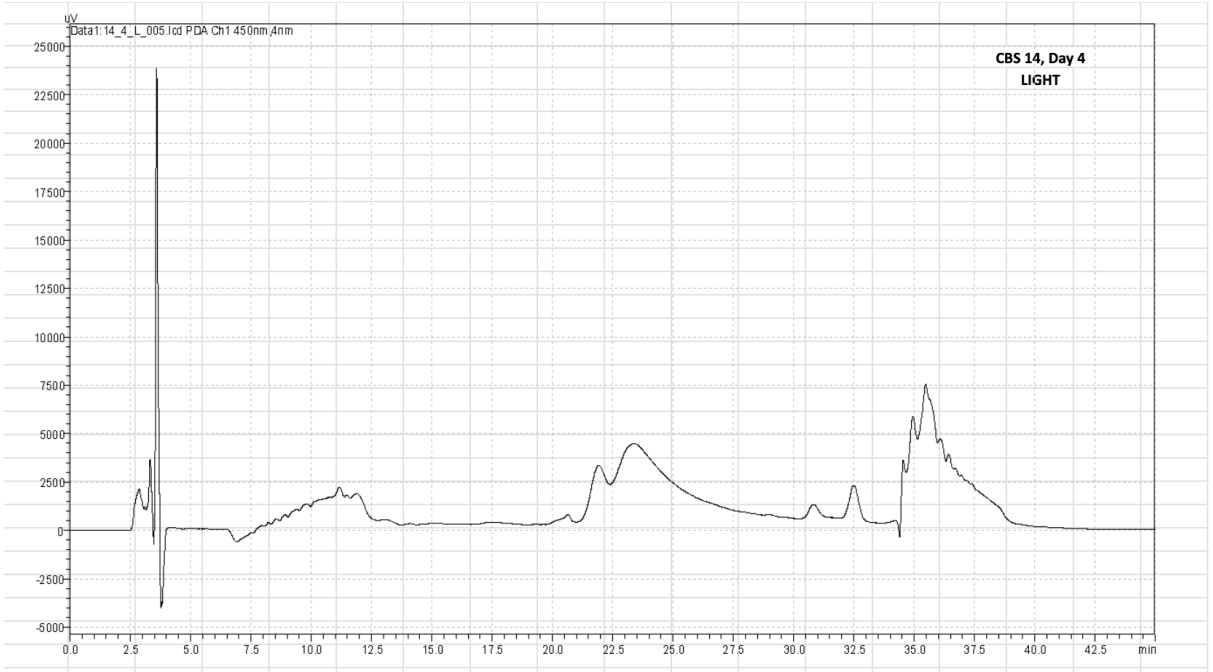

Figure 10: Chromatograph of CBS 14, Day 4, Light

Figure 11: Chromatograph of CBS 6016, Day 2, Dark

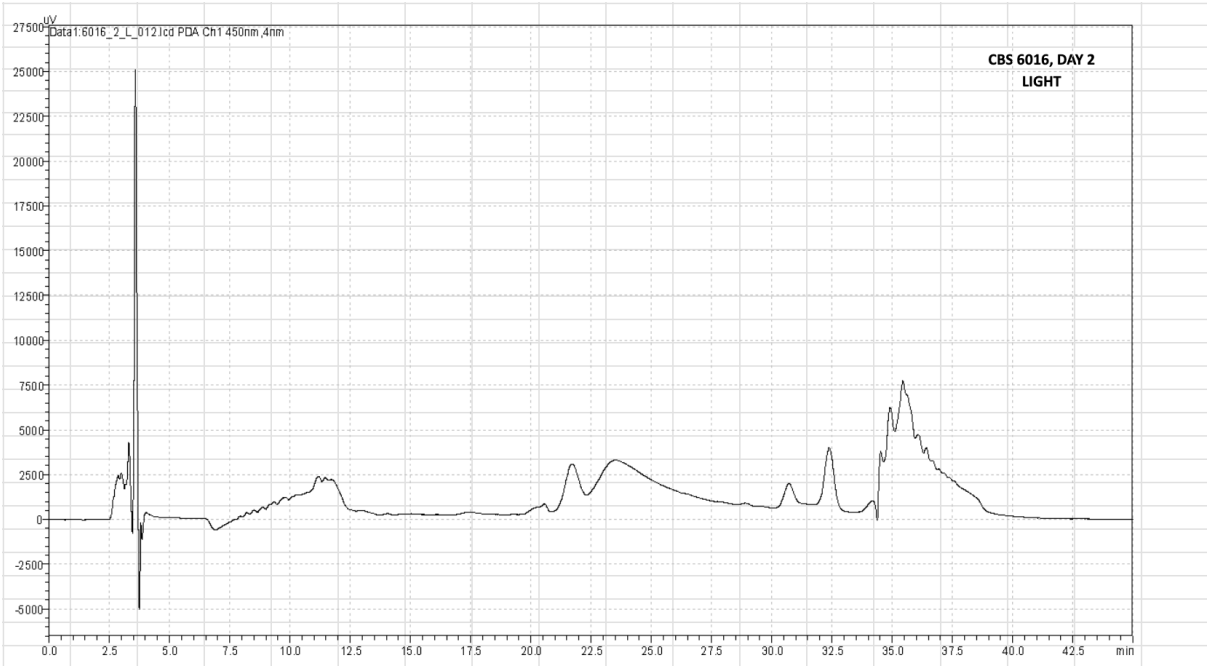

Figure 12: Chromatograph of CBS 6016, Day 2, Light

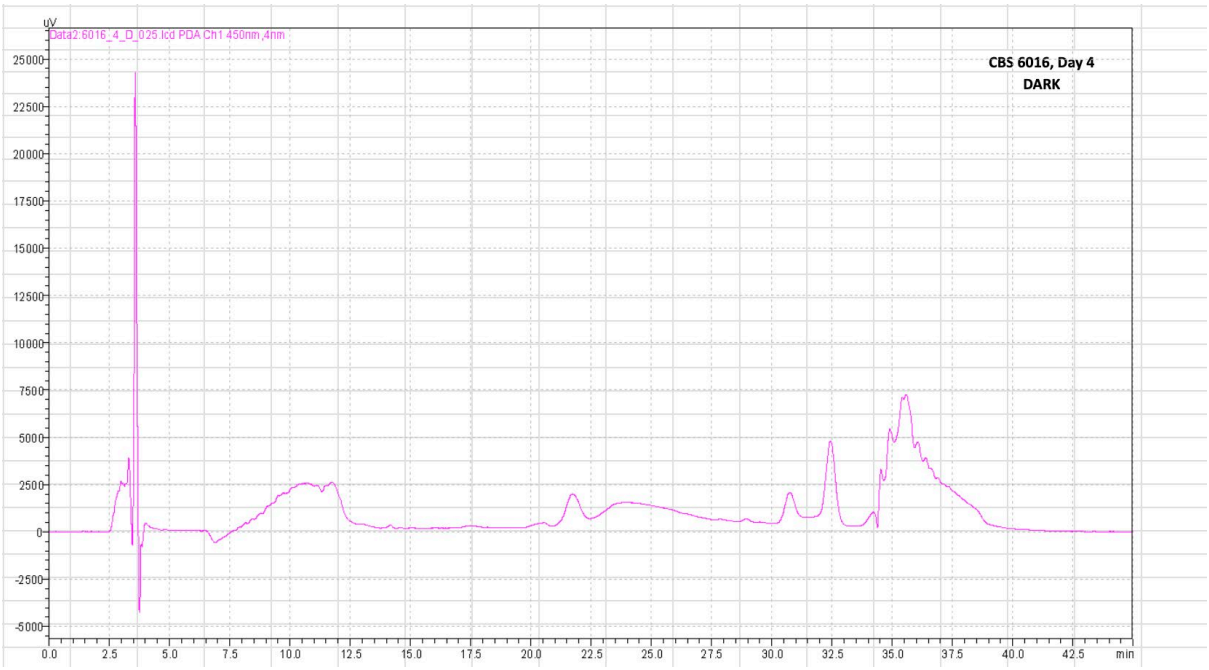

Figure 13: Chromatograph of CBS 6016, Day 4, Dark

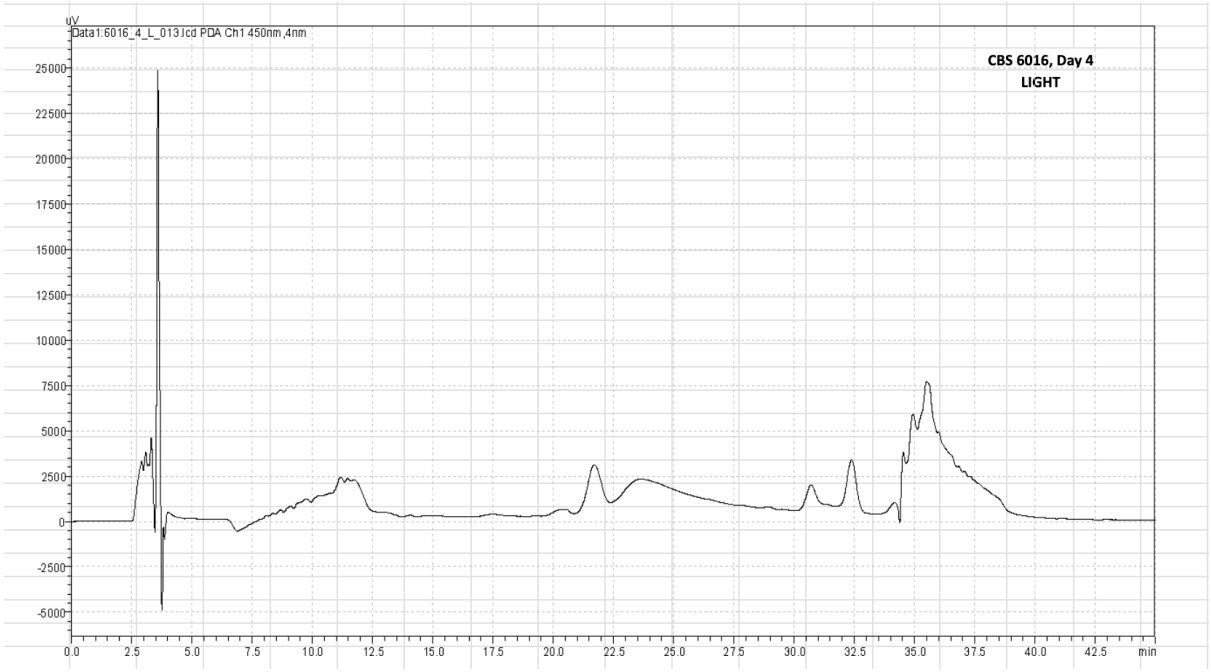

Figure 14: Chromatograph of CBS 6016, Day 4, Light

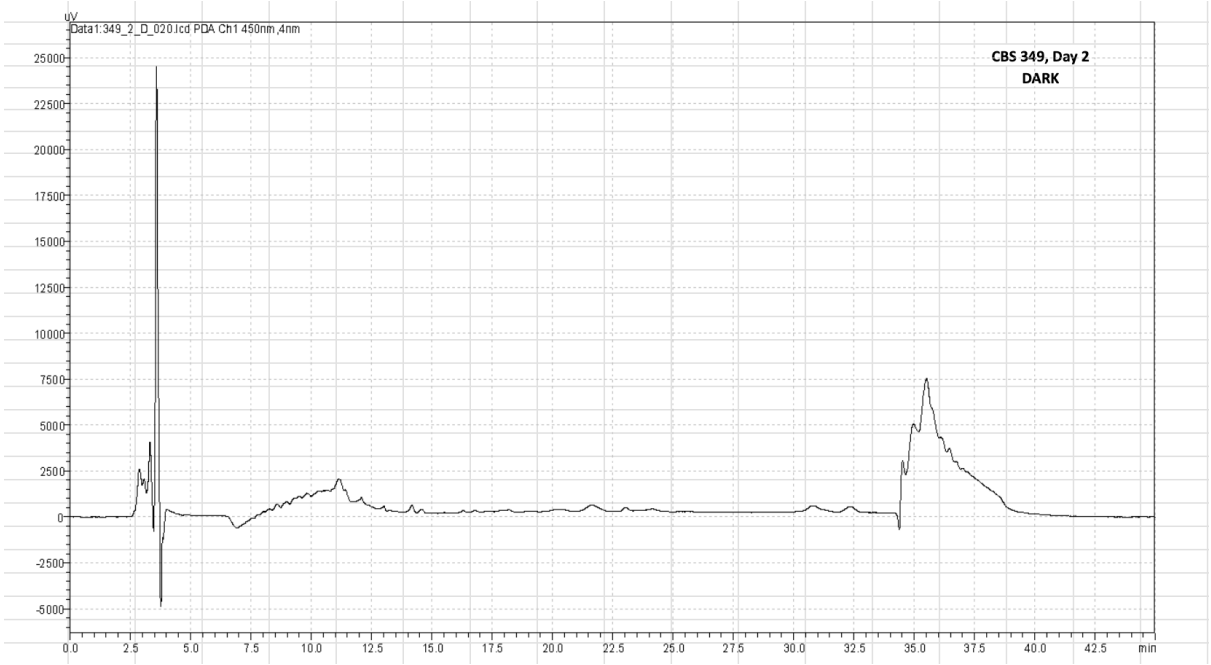

Figure 15: Chromatograph of CBS 349, Day 2, Dark

Figure 16: Chromatograph of CBS 349, Day 2, Light

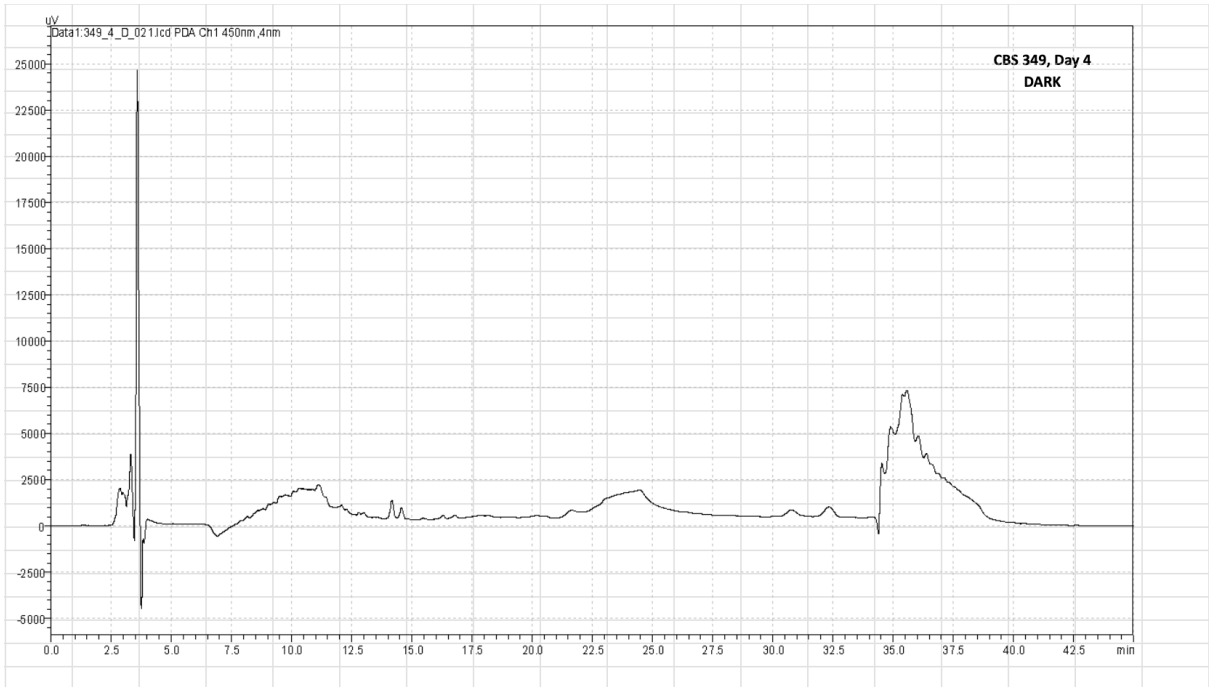

Figure 17: Chromatograph of CBS 349, Day 4, Dark

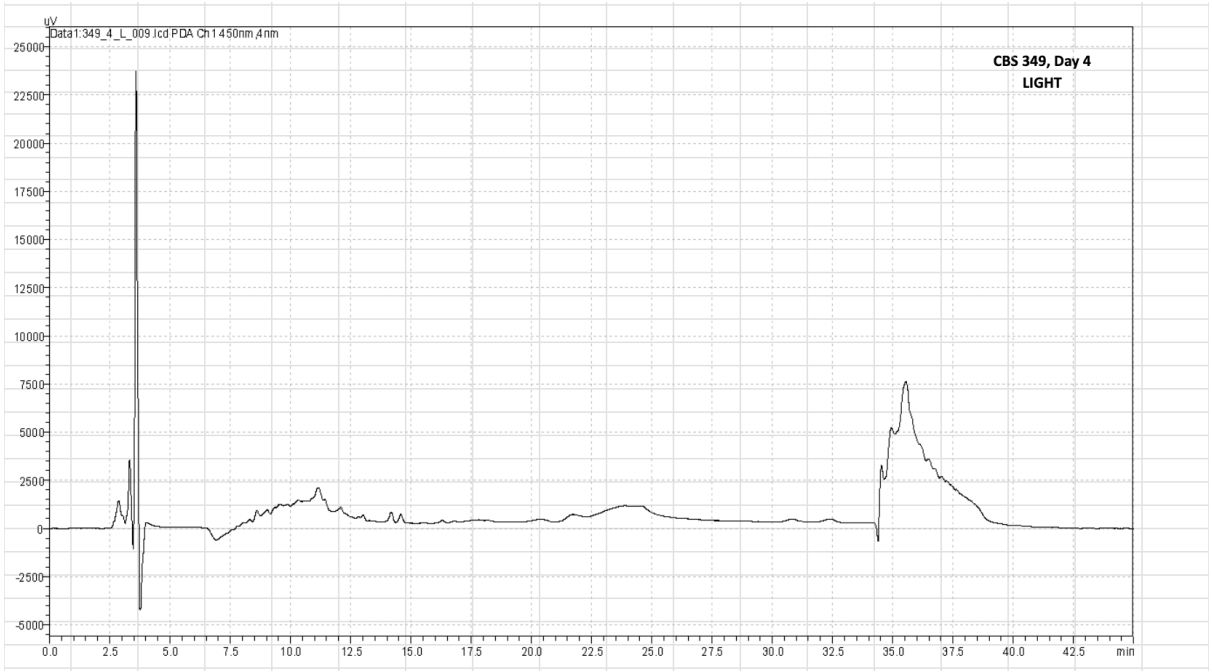

Figure 18: Chromatograph of CBS 349, Day 4, Light