Chapter 15

Lycopene Production by Mated Fermentation of *Blakeslea trispora*

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Abstract

Lycopene is a carotenoid mainly present in red-colored fruits and vegetables. Its value in the pharmaceutical and food industry is linked to its benefits for the human health, including properties against cancer and cardiovascular diseases, and its use as a food colorant. Lycopene can be produced either by synthetic or natural means, but there is a preference for the second, since it is considered a more eco-friendly and less harmful process. Among natural methods for obtaining lycopene, microbial fermentation is a good alternative to extraction from plants that naturally contain lycopene, since it implies obtaining higher and more specific amounts of this carotenoid. This chapter describes lycopene production by fermentation of the fungus *Blakeslea trispora*, a naturally carotenoid producer, at 30 L scale. This procedure involves separated growth of the two sexual mating types of *B. trispora* during the vegetative stages and the use of a lycopene cyclase inhibitor to achieve lycopene accumulation during the production stage.

Key words Lycopene, Carotenoids, Pilot scale, *Blakeslea trispora*, Fermentation

1 Introduction

Carotenoids are lipophilic pigments with many applications, ranging from feed supplements, colorants, and nutraceuticals to medicine and cosmetics. They are synthesized by plants, bacteria, and fungi and are responsible for some of the vivid colors present in nature, such as the yellow, red, and orange colors observed in fruits, flowers, and vegetables. In addition, they have a large and varied number of biological activities such as provitamin A function, antioxidant activity, blue light filtering, intracellular communication, and immune function [1].

Some of the carotenoids with a greater demand in the market are lycopene, β-carotene, astaxanthin, lutein, canthaxanthin, and annatto. They are mostly commercialized as food pigments,

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animal feeding additives, coloring agents in the pharmaceutical and cosmetic industries, and as dietary supplements [2].

Lycopene is an intermediate of the β-carotene biosynthetic pathway (Fig. 1) mainly present in red-colored fruits and vegetables such as tomato, papaya, peach, or watermelon. This carotenoid is a 40-atom open chain containing 11 conjugated and 2 non-conjugated double bonds. It lacks the terminal β-ionic ring, which means that it is not a vitamin A precursor. Given lycopene's large number of conjugated double bonds, it has the highest singlet oxygen quenching capacity among all carotenoids. This implies that lycopene is also a powerful antioxidant that could have many health-benefiting properties [3].

Lycopene has many uses as a pigment in the food industry. As other colorants, it can be used for several purposes, such as enhancing an already existing color, restoring it after processing, or just to give color to uncolored food. It is used, for example, in beverages, sauces, soups, cereals, bread, dairy products, dietary products, and dietary supplements [4, 5]. But the growing interest in lycopene is not only due to its role as an efficient pigment in the food industry but also in human health as a valuable compound that could prevent diseases such as cancer or cardiovascular diseases. Several studies suggest that it decreases cholesterol levels and can be used as a treatment against oxidative stress. It also inhibits cell growth in various human cancer cell lines, such as prostate cancer; it lowers the risk of cardiovascular diseases, including atherosclerosis, myocardial infarction, and stroke; and it has anti-inflammatory activity [6, 7]. According to BBC Research, lycopene market accounted for $60 million in 2010, expecting to reach $84 million in the next few years [8].

Commercial lycopene can be obtained by chemical synthesis, by extraction from natural sources, and by fermentation. Synthetic lycopene is established by the double Wittig condensation of a symmetrical dialdehyde with two identical phosphonium salts [9]. However, there is a preference in using natural ingredients rather than synthetic compounds in food, cosmetics, and pharmaceuticals, so natural methods to obtain lycopene are given more attention nowadays. To date, the most common method to obtain lycopene is extracting it from plants, mainly tomato, but this involves variable and relatively low extraction yields, as lycopene levels in the fruit are low and depend on variety, maturity stage, and agrochemical practices of cultivation. There are also some complications regarding the purification process due to the presence of other related carotenoids in plants [10, 11].

All these difficulties regarding lycopene production have triggered the extensive research into an effective way of producing lycopene. Several microorganisms have been studied for their ability to produce lycopene, such as mucoral fungi of the genera

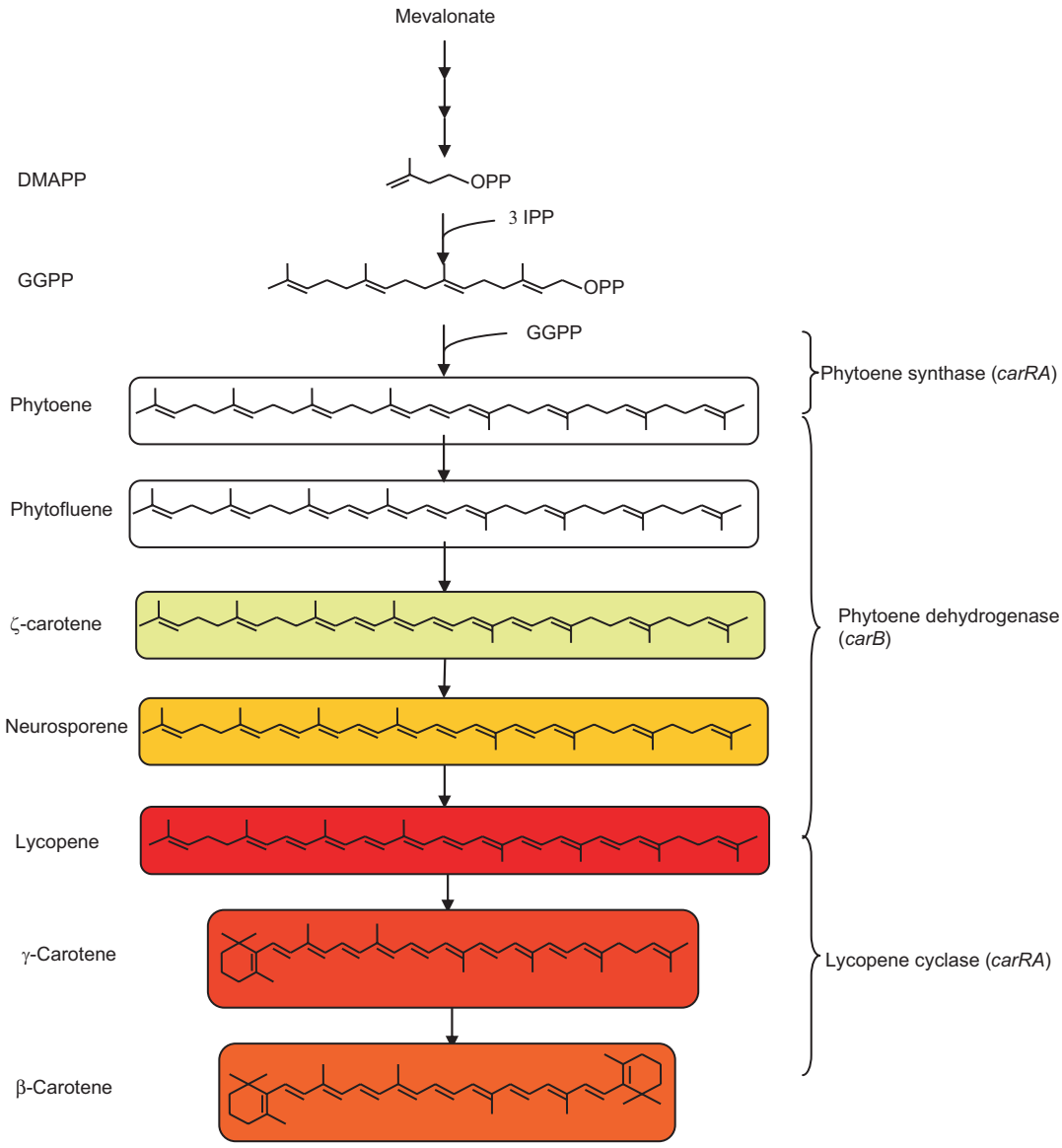

Fig. 1 β-Carotene biosynthetic pathway in *B. trispora*. One molecule of dimethylallyl pyrophosphate (DMAPP) and three molecules of isopentenyl pyrophosphate (IPP) are transformed into geranylgeranyl pyrophosphate (GGPP). Afterwards, phytoene synthase, encoded by the A domain of the *carRA* gene, catalyzes the junction of two molecules of GGPP to form phytoene. Subsequently, phytoene dehydrogenase, encoded by the *carB* gene, introduces four double bonds to yield sequentially phytofluene, ζ-carotene, neurosporene, and lycopene. Finally, lycopene cyclase, encoded by the R domain of the *carRA* gene, sequentially modifies each of the acyclic ends to form γ-carotene and finally β-carotene. The colored box framing each molecule indicates approximately their color

Blakeslea [12] and *Phycomyces* [13], *Flavobacterium* sp. [14], a mutant strain of *Streptomyces chrestomyceticus* [15], or genetically engineered strains of *Escherichia coli* [16, 17], *Pichia pastoris* [18], *Yarrowia lipolytica* [19], *Saccharomyces cerevisiae* [20, 21], and *Candida utilis* [22] among others.

To date, only lycopene produced by fermentation of *Blakeslea trispora* is accepted by the European Commission for commercial use [5]. This microorganism is a fungus with two mating types (+) and (−) that produces β-carotene by the cyclization of lycopene. Mated fermentation of both mating types (+) and (−) has proved to be successful by increasing carotenoid production [23, 24]. To produce lycopene using this microorganism, it is necessary to use a strain lacking lycopene cyclase or feed it with cyclase inhibitors such as imidazole or pyridine derivatives, in order to avoid formation of β-carotene and therefore accumulate lycopene [12, 25, 26]. Moreover, there are other factors, including strain improvement, culture media, and process optimization, that increase lycopene production [27, 28].

Pilot plant fermentation is an indispensable step previous to scaling up the laboratory work to industrial production. It is the best way to confirm that all the parameters and the fermentation conditions defined during the fermentation development at laboratory scale are successful at industrial scale. A pilot bioreactor of about 30 L mimics an industrial fermentor—and therefore an industrial process. This is a powerful tool to study critical fermentation parameters that cannot be tested at flask scale such as vessel design, type of fermentation (batch, semi-batch, or continuous), vegetative stages, aeration, or agitation. With pilot bioreactors it is also possible to identify potential fermentation limitations during the scaling-up and to optimize fermentation conditions being at the same time an environmentally friendly process and time- and cost-effective.

In this chapter we describe a protocol for lycopene production by fermentation of *B. trispora* at 30 L scale, emphasizing the key aspects of the fermentation process.

2 Materials

2.1 Vegetative Stages

2.1.1 First Vegetative Stage (Flask Scale)

1. *Blakeslea trispora* F-816 (+) and *Blakeslea trispora* F-744 (−) obtained from the Russian National Collection of Industrial Microorganisms (VKPM).
2. VM medium: Soybean meal 23 g/L, corn meal 47 g/L, KH_2PO_4 0.5 g/L, and thiamine hydrochloride 2 mg/L. Adjust to pH 6.3.
3. 500 mL Erlenmeyer flasks.
4. Flow laminar cabinet.
5. Orbital shaker.

2.1.2 Second Vegetative Stage (Bioreactor Scale)

1. Fermentor BIOSTAT® CPlus (Sartorius), or equivalent, with 10 L operating volume.
2. VM medium (*see* Subheading 2.1.1) with antifoam agent 1 g/L.
3. Inoculation flask: Sterile Erlenmeyer 500 mL flask with silicone tube and stainless steel needle.

2.2 Production Stage

1. Fermentor BIOSTAT® CPlus (Sartorius), or equivalent, with 30 L operating volume.
2. FM medium: Soybean meal 44 g/L, corn meal 19 g/L, KH_2PO_4 0.5 g/L, Na_2HPO_4 3.5 g/L, NaH_2PO_4 0.2 g/L, thiamine hydrochloride 2 mg/L, soybean oil 100 g/L, and antifoam agent 0.175 g/L. Adjust to pH 7.5.
3. Imidazole 20% (*see* **Note 1**).
4. Peristaltic pump.

2.3 Analytical Methods

1. Graduate tubes.
2. Swinging-head centrifuge.

2.3.1 Cell Growth

2.3.2 Viscosity

1. Viscometer Brookfield, model DV-II, or equivalent.
2. Spindle S-63.

2.3.3 Glucose Quantification

For glucose quantification, use the Megazyme D-Glucose (glucose oxidase/peroxidase, GOPOD) Assay Kit or equivalent.

1. Glucose determination reagent (Megazyme D-Glucose Assay Kit): Dilute the content of bottle I (GOPOD reagent buffer) to 1 L with distilled water. Dissolve bottle II (GOPOD reagent enzymes) in approximately 20 mL of the previous solution, and transfer this to the bottle containing the remainder solution I. Mix thoroughly and store at 4 °C.

2.3.4 Phosphate Quantification

1. Phosphate solution 50 ppm in water (prepared with $Na_2HPO_4 \cdot 12H_2O$).
2. Ammonium molybdate solution: $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 25 g/L and H_2SO_4 28 mL/L, dissolved in water.
3. Tin chloride II solution ($SnCl_2$ 12.5 g/L dissolved in glycerol:H_2O 50:50).

2.3.5 Carotenoid Analysis

1. Laboratory vibration mill.
2. Glass beads (0.5 mm diameter).
3. Lycopene 85% purity (Sigma-Aldrich).
4. Carotene 95% purity (Sigma-Aldrich).
5. Methanol HPLC grade.

6. Dichloromethane.
7. Acetone.
8. Analytical balance.
9. HPLC equipment. Waters Alliance e2695 HPLC system with Waters 2998 Photodiode Array (PDA) Detector or equivalent.
10. Column: Hypersil ODS column (5 μm, 100 × 4.6 mm) or equivalent.
11. Mobile phase: Acetonitrile/methanol/chloroform (47/47/6).

2.3.6 General Equipment

1. Micropipette P200.
2. Micropipette P1000.
3. Desktop centrifuge.
4. Incubator 25–50 °C.
5. Spectrophotometer UV/visible.
6. pH meter.
7. Autoclave.
8. Microscope.
9. Magnetic stirrer.

3 Methods

3.1 Vegetative Stages

Vegetative stages are necessary to grow the microorganism and get enough biomass for the following steps of the fermentation process. Number and time of vegetative stages depend on the microorganism speed rate of growth and the volume of the production broth to be inoculated. In this case, the 30 L lycopene fermentation needs two vegetative stages to obtain enough biomass to inoculate the production tank.

Fermentation of *B. trispora* to produce lycopene requires separated growth during the vegetative stages for (+) and (−) strains to then inoculate the production media with a mixture of both strains.

3.1.1 First Vegetative Stage (Flask Scale)

1. Seed spores from (+) and (−) strains in separate vegetative flasks with 100 mL of VM culture medium (*see* **Notes 2** and **3**).
2. Incubate in an orbital shaker at 25 °C and 250 rpm for 48 h.

3.1.2 Second Vegetative Stage (Bioreactor Scale)

1. Set up the fermentors, calibrate the pH probes (*see* **Note 4**), and place pH and DO_2 probes in their corresponding inputs of the vessel.
2. Weigh the raw materials, and dissolve them sequentially in approximately 5 L of distilled water. Adjust to pH 6.3 using 20% NaOH.

3. Pour the previously prepared medium into the fermentors and add distilled water to get a final volume of 10 L.
4. Close all the ports of the fermentation vessels, and start the sterilization cycle according to the following sterilization parameters: temperature 121 °C, agitation 100 rpm, and sterilization time 30 min (*see* **Note 5**).
5. Cool down the fermentors, and set up the following fermentation conditions: 25 °C, 0.7–1.5 vol/vol/min airflow, 250 rpm, and 0.5 kg/cm^2 of back pressure.
6. Calibrate the DO_2 probe assigning a theoretical value of 100% before inoculating. This is the maximal oxygen supply at the initial aeration, pressure and agitation conditions (*see* **Note 6**).
7. Transfer each first vegetative flask independently (+ and − strains) to 10 L second vegetative tanks (0.1% seeding) (*see* **Note 3**) and incubate for 48 h.
8. Take a sample each 8 h to check lack of microbial contamination, cell growth, pH, viscosity, glucose and phosphate (*see* Subheading 3.3). At 48 h, microorganism should be well-grown and should have consumed most of the nutrients, so glucose and phosphate levels should be near zero (*see* **Note 7**).
9. After 48 h of incubation, for (−) strain pH should be around 5.5, PCV value around 30%, glucose levels less than 2 g/L and phosphate concentration in broth between 0 and 2 mg/L. For (+) strain, pH values should reach pH 8, PCV value around 35% approximately, glucose levels should be below 0.5 g/L and phosphate concentration in broth between 0 and 2 mg/L.

3.2 Production Stage

1. Set up the fermentor, calibrate the pH probe (*see* **Note 4**) and place pH and DO_2 probes in their corresponding inputs of the vessel.
2. Weigh the raw materials, except the oil, and dissolve them sequentially in approximately 5–10 L of distilled water. Adjust to pH 7.5 using 20% NaOH.
3. Pour the previously prepared medium into the fermentor, add the oil and distilled water to get a final volume of 25 L.
4. Close all the ports of the fermentation vessel and start the sterilization cycle according to the following sterilization parameters: temperature 121 °C, agitation 100 rpm, sterilization time 30 min (*see* **Note 5**).
5. Cool down the fermentor and set up the following fermentation conditions: 25 °C, airflow rate 1.0 vol/vol/min, agitation rate 250 rpm, and back pressure 0.5 kg/cm^2.
6. Calibrate the DO_2 probe assigning a theoretical value of 100% before inoculating. This is the maximal oxygen supply at the initial aeration, pressure, and agitation conditions (*see* **Note 6**).

7. Transfer 5 L of a mix of both second vegetative cultures (20%) to inoculate the production fermentor (*see* **Note 8**). Taking into account the poorer growth of the (−) strain, a proportion of 1 (+)/10 (−) ratio is used for the seeding (*see* **Note 9**).
8. During incubation, it is advisable to set a dissolved oxygen control over 30% to keep an optimal oxygen supply for the microbial growth (*see* **Note 10**). Keep agitation between 250 and 600 rpm and airflow rate between 1.0 and 1.2 vol/vol/min.
9. After 25–35 h of fermentation, add 120 mL of a sterile 20% imidazole solution using a peristaltic pump to get a final concentration of imidazole of 0.8 g/L in the fermentation broth (*see* **Note 11**).
10. Take a sample of the broth each 12–24 h to check lack of contamination, cell growth, pH, viscosity, and lycopene and β-γ-carotene production (*see* Subheading 3.3).
11. Lycopene production is mostly associated with secondary metabolism of *B. trispora*: maximum production rate is found during the second half of the fermentation when the metabolism of the microorganism decreases and nutrients of the broth are finishing. Fermentation lasts approximately 144 h, when lycopene production reaches about 1 g/L.

3.3 Analytical Methods

There are some critical parameters that need to be measured during the vegetative and/or productive stages of this fermentation process: cell growth, viscosity of broth, glucose and phosphate concentration in the fermentation broth, and carotenoid production. In this section, we have selected one among the various analytical methods available to measure each parameter, but an alternative method can be chosen.

3.3.1 Cell Growth (Packed Cell Volume, PCV)

The biomass content can be expressed through the packed cell volume (PCV).

1. Fill a graduated 10 mL glass tube with culture broth.
2. Centrifuge in a swinging-head centrifuge at 2000 × *g* for 10 min.
3. Express the biomass content as a percentage of the centrifuged broth.

3.3.2 Viscosity

1. Deep the spindle of the viscometer in a sample of 100 mL of fermentation broth, and set the run speed at 60 rpm for 20 s at room temperature.

3.3.3 Glucose Quantification

1. Mix 3 mL of the glucose determination reagent with 0.1 mL of the sample supernatant and incubate at 40–50 °C for 20 min.

2. Prepare as well a mix for the reagent blank and the D-glucose standard (*see* Subheading 2.3.3 for reagent preparation).
3. Read the absorbance at 510 nm. The absorbance results of this colorimetric assay are proportional to the concentration of D-glucose in the sample (*see* **Note 12**).

3.3.4 Phosphate Quantification

Standard Curve

1. Prepare samples of 1, 5, 10, and 20 ppm phosphate concentration from a 50 ppm phosphate stock solution.
2. Prepare a 2.2 mL microtube for each phosphate concentration with the following reagents: 840 μL distilled water, 50 μL ammonium molybdate solution, 10 μL tin chloride II solution, and 100 μL of each phosphate concentration (*see* Subheading 2.3.4 for reagent preparation).
3. Prepare a blank following the procedure described above but adding 100 μL of distilled water instead of phosphate solution.
4. Incubate at 25 °C for 15 min.
5. Read absorbance at 700 nm.
6. Represent the results of absorbance at 700 nm against phosphate concentration to obtain the regression equation (*see* **Note 13**).
7. Use this regression equation to calculate the phosphate concentration of the sample from the measured absorbance at 700 nm.

Phosphate Quantification Procedure

1. Add to a 2.2 mL microtube the following reagents: 840 μL distilled water, 50 μL ammonium molybdate solution, 10 μL tin chloride II solution, and 100 μL of the sample supernatant (*see* Subheading 2.3.4 for reagent preparation) (*see* **Note 14**).
2. Prepare a blank following the procedure described above but adding 100 μL of distilled water instead of sample.
3. Incubate at 25 °C for 15 min.
4. Read absorbance at 700 nm.

3.3.5 Carotenoid Analysis

Sample Extraction

1. Take 1–2 mL of the fermentation broth, and homogenize it with a laboratory vibration mill for 15 min using glass beads and 4 mL of methanol/dichloromethane (50/50).
2. Take 100 μL of the homogenized broth and mix with 900 μL of methanol/dichloromethane (50/50).
3. Stir at 50 °C for 20 min.
4. Centrifuge at 10,000 × *g* for 10 min.
5. Take 100 μL of supernatant, evaporate, and redissolve in 1 mL of acetone.

Carotenoid Quantification

1. Analyze 10 μL of the sample according to the following HPLC conditions: temperature 50 °C, flow 1 mL/min, and wavelength 470 nm (*see* **Note 15**).

4 Notes

1. Sterilize imidazole dissolved in water (20%) using a filter of nylon 0.2 μm.
2. Both strains, *B. trispora* F-816 (+) and *B. trispora* F-744 (−), can be maintained either in potato dextrose agar slants at room temperature for short-term maintenance or at −80 °C as a glycerol stock solution (40% v/v) for long-term maintenance.
3. Seed a TSA Petri plate and incubate at 31 °C for 24–48 h to detect potential microbial contaminations.
4. Use 7.02 and 4.02 buffer standard solutions for calibration.
5. It is advisable to do a pressure test before sterilization to check the tank is hermetically sealed. To do it, close the air output, open the air inlet until a pressure of 1 bar is reached inside the tank, and stop the airflow inlet. The 1 bar pressure should be maintained at least for 5 min. Before starting with the sterilization cycle, do not forget to open the air output to release the pressure.
6. A value of 0% can be obtained by briefly disconnecting the lead cable from the DO_2 electrode and setting the zero.
7. The (+) strain shows a higher growth rate than the (−) strain. This means that (+) strain may finish glucose before 48 h of incubation, PCV values of the (+) strain vegetative tank are higher than those for the (−) strain, and pH rises earlier due to the consumption of nitrogen sources from the culture medium for growth.
8. Higher seeding volume could lead to uncontrolled growth, with high viscosity and poor oxygenation, affecting to lycopene production.
9. Extreme proportions of both strains in the seeding can lead to a significant decrease in lycopene production, probably due to the inability of growth of one of the strains, thus avoiding mating stimulation for lycopene synthesis.
10. The most useful DO_2 control is the agitation cascade: when DO_2 decreases, the agitation increases to keep DO_2 level at about 30%. Another option is using a combined DO_2 control with agitation and aeration. Increasing aeration will ease DO_2 control without increasing too much agitation, which could affect negatively the microorganism growth during the first hours of growth.

11. Using lower imidazole concentrations, lycopene cyclase activity is not completely inhibited, leading to accumulation of β- and γ-carotene. On the other hand, concentrations of imidazole higher than 0.8 g/L affect biomass growth and reduce lycopene production.
12. The absorbance of the samples must be in the range of 0–1, so you may need to prepare a dilution of the sample.
13. The regression curve is a linear equation.
14. You may need to dilute the samples taking into account their expected phosphate content.
15. Prepare lycopene, β-carotene, and γ-carotene standards of the desired concentration in acetone to quantify lycopene and carotene production.

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