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Temperature Effects on Carotenoid Concentrations in a Pink-Pigmented Thermophile

A Thesis

Presented to

The Faculty of the Department of Chemical Engineering

San Jose State University

In Partial Fulfillment

Of the Requirements for the Degree

Masters of Science

By

William Dewhirst

December 2002

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ABSTRACT

Effects of Growth Temperature on Carotenoid

Concentrations of a Pink-Pigmented Thermophile

by William E. Dewhirst

This thesis examines growth temperature effects on carotenoid concentrations in a bacteria designated as S119. S119 is a member of the genus *Meiothermus*, and is described as a pink-pigmented thermophile that can grow at temperatures as high as 65°C. Carotenoid concentrations were measured from samples grown at 40, 45, 50, 55, 60 and 65°C using a 1090 HPLC with at C18 reverse phase column and DAD detector. This revealed that S119 produces at least four different types of carotenoids designated as Carotenoids I, II, III and IV. Carotenoid IV is the only carotenoid that is dramatically affected by a change in temperature. The concentration of Carotenoid IV is 37.6 times higher at 40°C then at 65°C. This decrease in concentration of Carotenoid IV correlates with a decrease in the pink pigmentation of S119, which suggests that Carotenoid IV is responsible for the pink color of S119.

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INTRODUCTION

Biosynthetic processes in industry are overwhelmingly dominated by the use of mesophilic bacteria such as *E. coli* because the genetics and physiology of these organisms are much better understood than for any other types of bacteria [1]. The broad knowledge base for mesophilic bacteria facilitates their incorporation into a biosynthetic process; however, a disadvantage of using mesophiles is that the process design becomes limited by the capabilities of these microorgansims. For example, *E. coli* is capable of optimum growth only at a temperature of 30 - 37°C and at a neutral pH [2]. Therefore, processes that use *E. coli* must maintain the microorganism within these environmental conditions. Many microorganisms are fully able to survive outside of mesophilic conditions, and there is a need to extend the level of understanding we have for bacteria beyond *E. coli* to other organisms so that we can take advantage of the full spectra of capabilities that bacteria have to offer.

The microbial kingdom enjoys a breadth of diversity, including bacteria that thrive in extreme temperatures as well as in acidic, caustic and high salt solutions. Thermophiles are bacteria that can grow at temperatures greater then 50°C and can have optimum growth temperatures that can range as from 50 to 75°C or even higher [2]. This thesis will focus on a thermophilic, pink-pigmented bacterium, designated as MTU Strain S119. The pink pigment is believed due to the production of carotenoids. Carotenoids are commercially important chemicals sold around the world [3].

S119 was isolated in a hot spring in Thailand by a graduate student in the research group of Susan Bagley at Michigan Technological University. S119 grows best at about

55°C, but will grow in temperatures up to nearly 70°C. Using 16S rRNA testing Dr. Bagely determined S119 belongs to a group of thermophilic bacteria known as *Meiothermus* [4]. 16S rRNA testing is used for identification and phylogenetic characterization of a microorganism based on the comparison of a 16S rRNA molecular sample from that bacteria to a database of 16S rNA sequences retrieved from known bacteria. Similarity values above 95% should be regarded as good evidence of the organisms belonging to the same species, but rRNA sequence information alone should not be used to split or lump strains into species [5]. Figure 1 shows a distance matrix phylogenetic tree based on 16S rRNA testing showing how S119 relates to other *Meiothermus* strains. Figure 1 was by provided by Susan Bagely [4].

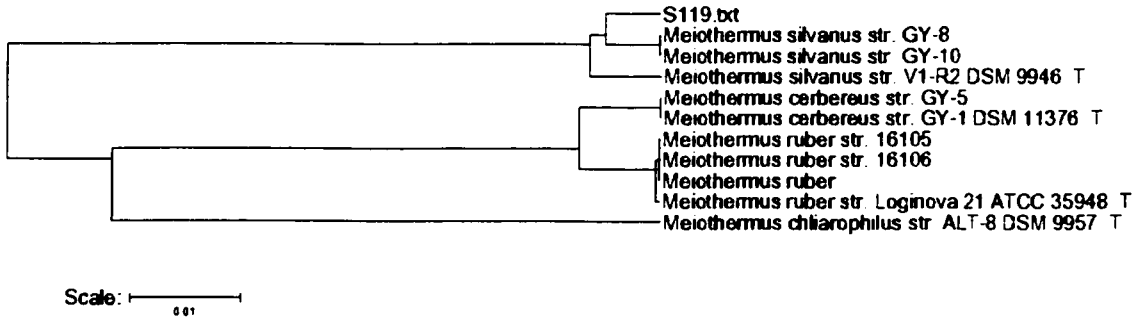

FIGURE 1. Phylogenetic tree provided by Susan Bagely based on 16S rRNA sequence data relating S119 to other *Meiothermus* strains [4]. Scale bar represents 0.01 fixed point mutations per sequence position.

Carotenoids are important to both humans and animals and are commonly found in fruits and vegetables with orange-red color such as oranges, tomatoes and carrots and also in flowers. Carotenoids are also synthesized in nature by plants and microorganisms

such as bacteria, fungi and algae. Animals use carotenoids for coloration and are found in the bright colors of many birds and fish. Although animals can adsorb and metabolize carotenoids they do not have the ability to synthesize these chemicals and depend on the consumption of plants and microorganisms as a carotenoid source [6]. Plants require carotenoids during photosynthesis and also for protection against destructive photooxidation. Carotenoids are also important factors in the health of animals. The most common source of vitamin A in humans is through ingestion and metabolism of carotenoids that are converted into vitamin A. Vitamin A is required for healthy vision, normal growth and development. There are about 50 types of carotenoids that can be converted into vitamin A, and they are also known as provitamin A [7].

The first carotenoid to be made commercially available was β-carotene in 1954 and since then the carotenoid market has continued to increase every year [8]. Commercial use of carotenoids includes additives as colorants to manufactured food. Carotenoids have also become increasingly important to the pharmaceutical market because of their ability to act as anti-oxidants and as the main dietary source of vitamin A. The first carotenoid to be isolated was β-carotene in 1831 by Wackenroder from carrots, and since then great effort has been placed into the synthesis of carotenoids. Currently six synthetic carotenoids are commercially important: beta-apo-8'-carotenal, beta-apo-8'carotenoic acid and ethyl ester, citranaxanthin, β-carotene, canthaxanthin and astaxanthin [8]. Other health benefits of carotenoids that have been recognized include protective effects against cancer, heart disease and degenerative eye disease [9].

Synthesis of β-carotene was first reported in 1950 independently by Karrer & Eugster [10], Inhoffen and co-workers [10], and Milas [12].

The structure and UV-Vis spectrum of β-carotene in acetone and acetone:water (1:3) is shown in Figure 1 [13]. The UV-Vis spectrum of β-carotene shows peaks between 400 and 500 nm. Almost all carotenoids absorb light in the range between 400 and 500 nm [14].

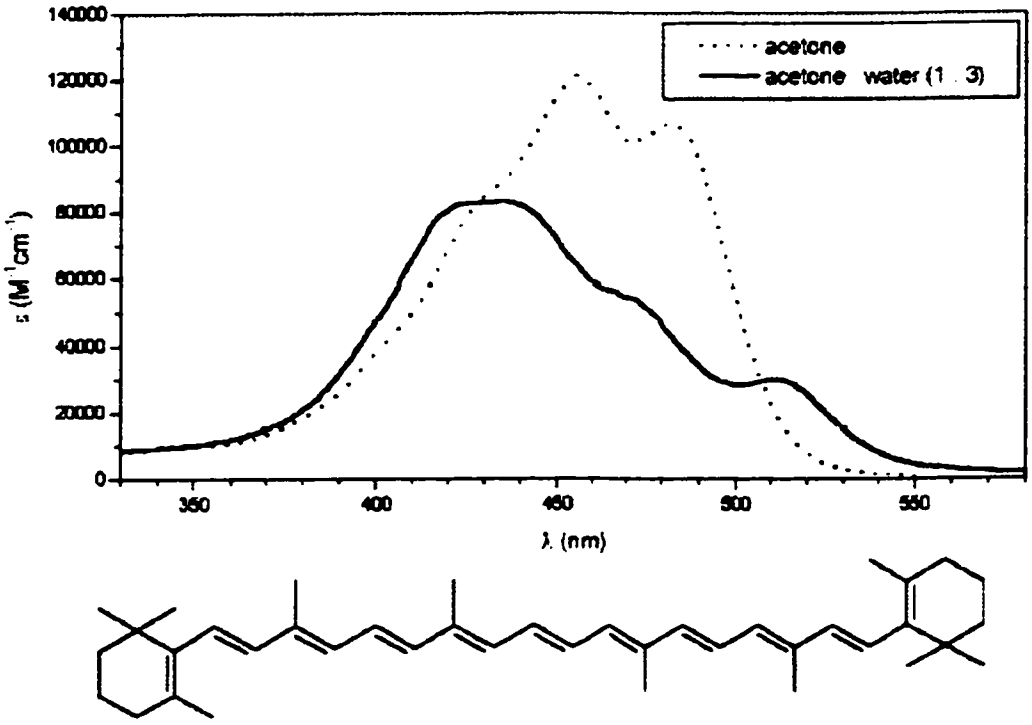

FIGURE 2. Structure and UV-Vis spectrum of β-carotene in acetone and acetone:water (1:3) [13].

An industrial process for the synthesis of β-carotene was later developed using the Inhoffen synthesis and since 1954 β-carotene has been commercially produced. Today the production of β-carotene and other commercially important carotenoids is dominated

by chemical synthesis [3]. Currently, there is a great effort to develop a biosynthetic process using carotenoid producing bacteria, such as β-carotene production by the halotolerant genus *Duniela* [15].

Carotenoids are used mostly as ingredients in the food, pharmaceutical, animal feed and industrial chemical markets. According to Business Communications Co., Inc [3] the global market for carotenoids was estimated at $786 million in 1999 and is expected to exceed $935 million by 2005. The animal feed market will account for the largest application segment valued at $462 million in 1999 and expected to reach $527 million by 2005. The food and pharmaceutical markets were valued at $209 million and $115 million in 1999 and are expected to reach $236 and $173 million by 2005. Table 1 shows the estimated global market for carotenoids for 1999 and 2005.

TABLE 1. Global market for carotenoids, 1999-2005 [3].

	($ Millions)		
	1999	**2005**	**AAGR**
Food	209	235	2.0
Animal feed	462	527	2.2
Pharmaceutical	115	173	7.0
Total	786	935	2.9

Business Communications Co., Inc reported the two most industrially produced carotenoids as astaxanthin and β-carotene, each of which accounted for about 28% of total sales in 1999. Public interest in the health benefits of carotenoids are reflected by the 7% average annual growth rate (AAGR) of the pharmaceutical market in 1999-2000 as compared to 2.0% and 2.2% growth rate in the animal feed market and the food market during the same period [3].

Carotenoid production in genetically engineered *E. coli* has been demonstrated [16]. However, it would be more efficient to produce carotenoids at higher temperatures, such as could be done with thermophilic bacteria. Heat must be removed from bioreactors due to energy generated by microbial growth in order to prevent over-heating of the fermentation broth. To determine the amount of heat to be removed, the two relevant heat equations needed to make energy calculations are Newton's law shown in Equation 1

$$Q = hA(T_s - T_\infty) \qquad (1)$$

and an equation to express the amount of heat generated by microbial growth shown by Equation 2

$$Q_{generated} = V_L \mu X \frac{1}{Y_H} \qquad (2)$$

These equations are used in the following section to calculate the heat balance in a large bioreactor.

An additional benefit of using thermotolerant bacteria in fermentors is that such systems are less likely to become contaminated because the reactors are maintained at high temperatures where many bacteria cannot survive. Most microorganisms responsible for contamination of bioreactors come from the soil or are found living on higher organisms. This includes both yeasts and bacteria. Growing S119 at elevated temperatures reduces the chance of contamination to the growth culture.

CALCULATIONS

The following calculations model heat generation in a large bioreactor due to microbial growth and demonstrate how using a thermophilic bacteria will make a process more efficient. These calculations are based on a fermentor of 10,000 L with a height equal to four times the length of its radius. The reactor is treated as vertical cylinder with assumed to have uniform temperature due to complete mixing. Heat transfer to the surrounding environment is assumed to occur only through natural convection expressed by Newton's law in Equation 1

$$Q = hA(T_s - T_\infty) \qquad (1)$$

where

A = the surface area of the reactor, m^2

T_s = the surface temperature of the reactor, °C

T_∞ = the ambient temperature of the surrounding fluid, °C

h = the average heat transfer coefficient at the surface, $W/m^2{}^\circ C$

h is calculated by first determining the Rayleigh number Ra with Equation 3

$$Ra = \frac{g\beta(T_s - T_\infty)L^3}{\nu^2}\Pr \qquad (3)$$

where

g = gravitational acceleration, m/s^2

β = coefficient of volume expansion, 1/K (β = 1/T for ideal gases)

ν = kinematic viscosity of the fluid, m^2/s

L = height of the reactor

and then determining the Nusselt number Nu for natural convection over surfaces by Equation 4. In this the reactor can be treated as a vertical plate and heat conduction through the top and bottom surfaces is ignored [17].

$$Nu = \left(0.825 + \frac{0.387 Ra^{1/6}}{(1 + (0.492/\mathrm{Pr})^{9/16})^{8/27}} \right)^2 \qquad (4)$$

and then using the following equation to calculate h

$$h = \frac{Nuk}{L} \qquad (5)$$

where k is thermal conductivity in W/m°C.

Q is calculated assuming a T_s of 35 and 55°C, which represents the operating temperature for a mesophile and a thermophile. T_∞ is assumed to be 25°C and all gases are treated as ideal. All fluid properties in equation 3 are evaluated using a film temperature $T_f = 1/2(T_s + T_\infty)$ and 1 atm pressure.

The heat generated during microbial growth can be calculated with Equation 2

$$Q_{generated} = V_L \mu X \frac{1}{Y_H} \qquad (2)$$

where

V_L = liquid volume of reactor, l

μ = specific growth rate, 1/s

X = cell concentration, g/l

$1/Y_H$ = metabolic heat evolved per gram of cell mass produced (J/g cells)

The values of μ, X, and $1/Y_H$ vary depending on the bacteria, conditions during the bacteria's growth, and the substrate used to feed the bacteria.

Heat from microbial growth is usually removed through cooling coils in large fermentors. Equation 6 is used to calculate the amount of heat that must be removed

$$Q_{remove} = Q_{generated} - Q \qquad (6)$$

The case described above compared growing a mesophile and a thermophile in a 10,000 liter fermenter at 37 and 55°C. At 37°C, $\beta = 0.0033$ 1/K, $\nu = 1.64 \times 10^{-5}$ m^2/s, Pr = 0.7113, and k = 0.0264 W/m°C. At 55°C, $\beta = 0.0032$ 1/K, $\nu = 1.70 \times 10^{-5}$ m^2/s, Pr = 0.7107, and k = 0.0270 W/m°C. The following constants are used to calculate heat generated by bacterial growth: $\mu = 0.44$ 1/hr, $Y_h = 2.87 \times 10^{-5}$ g/J [18] and X = 4.4 g/L. Details of the calculations are shown in the Appendix.

The amount of heat required to be removed from the reactor run at 37°C is calculated to be 186.6 kW. When the same reactor is run at 55°C, the amount of heat needed to remove is 184.4 kW. Running a reactor at 37°C requires removing 2.2 more kW of energy then a reactor ran at 55°C. Cooling coils within the reactors can be used to remove excess heat generated by bacterial growth. Equation 7 shows a heat transfer equation for heat exchangers that incorporates effectiveness. Equation 7 is used to compare the amount of cooling water required for the reactors run at 37 and 55°C.

$$Q = \varepsilon \cdot mC_v(T_{Hi} - T_{Ci}) \qquad (7)$$

where

ε = heat-exchanger effectiveness

m = mass flow of cooling water, kg/s

C_v = specific heat of water, 4187 J/kg K

T_{Hi} = Temperature of reactor, K

T_{Ci} = Temperature of cooling water into reactor, K

T_{Ci} is assumed to be 20°C and the heat exchanger effectiveness, ε, is assumed to be constant. With this assumption the amount of cooling water required at 55°C is calculated to be 52% less then the amount of cooling water required at 37°.

LITERATURE REVIEW

Dr. Susan Bagely from Michigan Technoligical University used 16S rRNA analyses to determine that the bacteria designated as S119 belongs to the genus *Meiothermus* [4]. Loginova, Egrova, Golovacheva, and Serigena [19] reported the first species from this genus after they discovered pink-pigmented, thermophilic gram-negative rods growing in hot springs in the Kamchatka Peninsula of what was then the USSR. The bacteria was originally given the name *Thermus ruber*. Loginova and associates reported *T. ruber* cells as gram-negative nonmotile rods that are 3 to 6 by 0.5 to 0.8 μm that have rounded ends and are nonsporeforming. The bacteria was said to be obligately aerobic and thermophilic with a temperature range for growth between 40 and 70°C with the optimum temperature of growth at 60°C. Loginova *et. al.* reported *T. ruber* as having a bright red (or occasionally bright orange) intracellular pigment due to the production of a carotenoid. The absorption spectra of this carotenoid in acetone, methanol-acetone (1:1), and hexane extracts showed three maxima at 455, 483 and 513 nm. The procedure used to study the intracellular pigment was identical to the method

used by Jackson *et. al.* [20] in which cells were lysed with an organic solvent and then the pigment, along with all other intracellular chemicals, were dissolved into the organic chemical and the absorption spectra of this extract was recorded.

When Longinova and associates first reported *Thermus ruber*, the bacteria was very different from all other *Thermus* species. *Thermus* were known to produce yellow-pigmented colonies believed due to the production of carotenoids, or non-pigmented colonies, with an optimum growth temperature between 70 and 75°C [21]. *Thermus ruber* was the first so-called low temperature *Thermus* species, but not the only one. Tenreiro, Nobre and Costa [22] later reported two other low temperature *Thermus* species; *Thermus silvanus* and *Thermus chliarophilus*. The strains of *T. silvanus* produce orange-red-pigmented colonies and have an optimum growth temperature of about 55°C, while the strains of *T. chliarophilus* produce yellow-pigmented colonies and have an optimum growth temperature of about 50°C.

After reports of three low temperature *Thermus* species, Nobre, Truper and Costa suggested that *Thermus ruber*, *Thermus silvanus*, and *Thermus chliarophilus*, should be reclassified in the genus *Meiothermus* [23]. Nobre and associates based their proposal on the results of phylogenetic studies, as well as the differences in growth temperatures, distinctive polar lipid patterns, and the hydroxy fatty acid compositions of the low and high temperature *Thermus* species. Gene sequence analysis showed the level of 16S rRNA sequence similarity is about 86% between the two groups that suggests two distinct phylogenetic lines [23]. The phenotypic and chemotaxonomic differences between high- and low-temperature *Thermus* species, along with the phylogenetic

analysis results, support the argument that there are two groups in the genus. The high-temperature group has been given the name *Thermus* and the low-temperature group has been given the name *Meiothermus*.

Meiothermus were described by Nobre *et. al.* [23] as cells 0.5 to 0.8 μm in diamater with variable cell length. The cells are gram negative and not motile. Red- or yellow-pigmented colonies are produced. The optimum temperature of growth varies between 50 and 65°C. None of the species grow at 70°C. The optimum pH is about 8.0. All known strains of *Meiothermus* are pigmented, which reportedly is due to the production of carotenoids. Burges, Barrow, Gao, Heard and Glenn [24] identified the major carotenoid of a *Meiothermus ruber* as 1'-β-glucopyranosyl-3,4,3',4'-tetradehydro-1',2'-dihydro-β,ψ-caroten-2-one. The sample analyzed by Burges and associates was taken from a strain of *M. ruber* that was grown at 60°C. The pigment was extracted using methanol and preliminary separation was done with HPLC using an isocratic elution of MeOH-CH_3CN-H_2O (86:7:7 v/v/v) on a Waters Symmetry C18 column. This was followed by isocratic elution using MeOH-H_2O (19:1 v/v) on an Active GoldPak C18 column. The structure of the carotenoid was identified by spectral means, including ^1H and ^{13}C NMR.

The purpose of carotenoid synthesis by thermophilic bacteria has been suggested to be related to protection against photochemically induced oxidation. Krinsky [25] noted that in photosynthetic bacteria, carotenoids could serve as protective agents against photosensitized oxidation. The ability to protect cells appears to depend on the polyene chromophore length. Carotenoids with nine or more conjugated double bonds are

capable of offering protection against photosensitized damage, while carotenoids with seven or less conjugated bonds are not as effective [25]. 1'-β-glucopyranosyl-3,4,3',4'-tetradehydro-1',2'-dihydro-β,ψ-caroten-2-one, the major carotenoid identified by Burges *et. al.* in the pigment of *M. ruber* contains 11 conjugated double bonds [24]. Hoshino, Guevarra, Ishida, Hiruta, Fujii and Nakahara [26] demonstrated the protective effects of carotenoids against photochemically induced oxidations for thermophilic bacteria. In their study, a carotenoid overproducing mutant of *Thermus thermophilus* HB27 was found to be highly resistant to UV irradiation compared to the parent strain.

Carotenoid production by thermophiles has also been used to try to explain how such bacteria can grow at elevated temperatures. Thermophiles are thought to possess special mechanisms for membrane stabilization that allows growth at high temperatures. Ourisson [27] reported that membrane reinforcement is one of the biological functions of bacterial carotenoids, which is based on the fact that the lengths of carotenoid molecules are similar to the depth of a lipid bylayer. Thermophilic bacteria are known to utilize ether lipids for the stabilization of the membrane [28]. Yokoyama, Sandmann, Hoshino, Adachi, Sakai and Shizuri [29] suggested that carotenoid glycoside esters may span the lipid bilayer and act as stabilizers of the membranes of thermophiles at high temperature.

Experimental evidence has been inconclusive with regards to the role of carotenoids in membrane stabilization of thermophiles. Ray, White and Brock [30] measured carotenoid concentration in *Thermus aquaticus* by monitoring the absorbance of the total carotenoid extract at 460 nm in methanol-toluene (1:1), and reported that when the growth temperature of *T. aquaticus* was increased from 50 to 75°C, there was a

progressive increase in the total lipid content, including a 1.8-fold increase in the carotenoid content. This increase suggested carotenoids play a role in the molecular mechanism of thermophilic growth. On the other hand, Hoshino and associates [26] reported that a carotenoid overproducing mutant of *T. thermophilus* demonstrated poorer growth at 80°C when compared to the parent strain or underproducing mutants of *T. thermophilus*. Growth curves were made from optical density measurements at 580 nm. The overproducing mutants were also found to be more resistant to UV irradiation when compared to the parent strain or underproducing mutants. From these results, Hoshino *et. al.* concluded that carotenoids are secondary metabolites which are not essential for the growth of *T. thermophilus* and that the overproduction of carotenoids at higher temperatures could be a burden to its growth. According to Hoshino *et. al.*, carotenoids play no role in membrane stabilization whose primary function is protection against photochemically induced oxidation [26].

RESEARCH OBJECTIVES

S119 is a member of the genus *Meiothermus*, and is described as a pink-pigmented thermophile that can grow at temperatures as high as 65°C. The pink pigment of S119 is likely due to intracellular production of carotenoids by the bacteria. The research objective here has been to separate and purify carotenoids produced by S119 for analyses and to quantitatively measure carotenoid production at different temperatures.

RESEARCH APPROACH

MTU Strain S119 can be grown on R2A Agar solid medium and in R2A Broth liquid medium. R2A Agar and broth are undefined media and can be purchased from DIFCO and other media companies. S119 was grown at temperatures of 40, 45, 50, 55, 60, and 65°C on R2A agar plates and in R2A broth. An image of S119 grown at different temperatures on R2A agar plates was taken using a Kodak DC260 digital zoom camera. The OD at 600 nm was monitored using a UV-Vis spectrum at 50, 55 and 65°C during the first 24 hours of growth to measure the rate. The dry weight of S119 grown in R2A broth was measured to determine the dry wt./ml·OD at 600nm ratio. The S119 samples grown in R2A broth were also used to measure the bacteria's level of carotenoid production. After 2 days of growth at the desired temperature in R2A broth, the OD of the culture was measured at 600 nm along with the culture volume and the procedure described below was followed to measure carotenoid levels in the cell cultures using a 1090 HPLC with a diode array detector.

The procedure used here for the extraction of carotenoids from S119 is essentially the same method described by Schiedt & Liaaen-Jensen [31] for the extraction and isolation of carotenoids from biological material. Cells were pelleted by centrifugation at 6000 RPM for 5 minutes in VWR 50 ml centrifuge tubes. The supernatant was discarded and each pellet was washed with 10 ml methanol to lyse the cells and dissolve carotenoids. The methanol mixture was centrifuged, collected and filtered. Then the methanol was evaporated using a rotovap and the precipitate was flushed with nitrogen to complete dryness. The precipitate was dissolved in 10 ml of ether. A solution of 25%

KOH/ethanol was added 1:1 with the ether to cause a saponification reaction. The mixture was allowed to sit for 2 hours under nitrogen in the dark. After saponification, the ether layer was collected and again evaporated to dryness using a rotovap. The remaining residue was dissolved in 1 ml of MeOH. 100 µl of this sample was run on a 1090 HPLC with a Supelco Discovery C-18 reverse phase column using HP Chemstation software. The column was equilibrated with 7:7:86 (v/v/v) water-acetonitrile-methanol. After injection, the elution was held at 7:7:86 for 35 minutes. From 35 minutes to 40 minutes the gradient was shifted to 0:100:0 and held there until 60 minutes. From 60 minutes to 65 the gradient was shifted back to 7:7:86 and held there until 70 minutes. The UV-Vis signal was monitored at 280 nm and at a pre-determined wavelength of the carotenoid. To determine the wavelength at which to monitor the carotenoids, the UV-Vis spectrum of a single methanol sample was measured prior to running on the HPLC. The HP Chemstation software saves absorbance data over the range of 200 to 600 nm for the entire sample run. This is used to report the UV-Vis absorbance spectrum of each recorded peak.

RESULTS

S119 was grown at temperatures of 40,45, 50, 55, 60, and 65°C in shaker flasks filled with 250 ml of R2A broth. The bacteria did not grow at 70°C. The cultures were allowed to grow for two days after inoculation to insure that stationary phase was reached. Each broth was inoculated with 2.5 ml of an overnight culture grown from a

single colony. The dry wt./ml·OD at 600nm ratio was determined to be 0.31 ± 0.06 mg/ml·OD at 600nm. The data from this experiment is shown in Table 2.

After 2 days of growth at the desired temperature, the OD of the culture was measured at 600 nm and the volume of the culture was also measured. OD and volume of the culture differed for each temperature. The data is shown below in Table 3.

The OD at 600 nm was monitored at 50, 55 and 65°C during the first 24 hours of growth. The growth curves for these temperatures are shown in Figure 1. In each case it appears that the bacteria's growth has reached stationary phase after 24 hours.

TABLE 2. Dry weight measuremetns of S119.

Sample	Temperature of growth (°C)	Volume of culture (ml)	OD @ 600 nm	Dry weight (mg)	dry wt./ ml·OD at 600nm
1	60	420	0.4934	59.5	0.29
2	60	467	0.4883	71.4	0.31
3	60	217	0.5605	41	0.34
4	55	219	1.0731	54.7	0.23
5	45	238	0.5113	46.5	0.38

TABLE 3. Calculated dry weight of S119 at different temperatures.

Temperature (°C)	Volume of culture (ml)	OD @ 600nm	Dry weight (±0.06 mg)
40	242	0.4758	36
45	236	0.5958	44
50	235	0.6648	48
55	228	0.6689	47
60	224	0.4300	30
65	215	0.4887	33

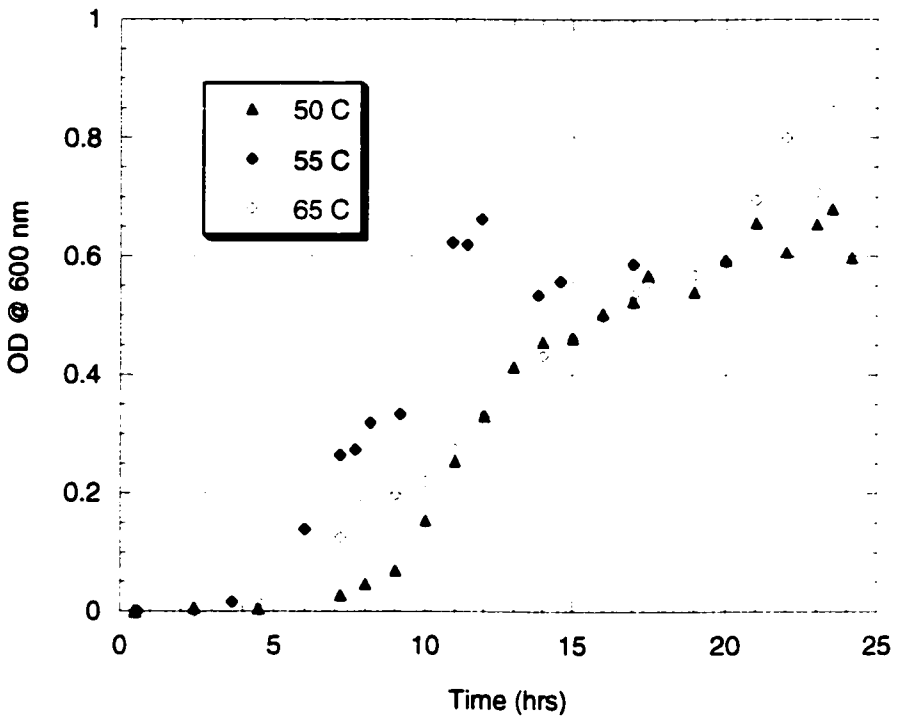

FIGURE 3. Growth of S119 in 250 ml of R2A broth at 50, 55, and 65 C.

Carotenoids samples from S119 grown at 40, 45, 50, 55, 60 and 65°C and samples were run on a 1090 HPLC with a C-18 reverse phase column. In order to determine an appropriate wavelength to monitor the carotenoids on the HPLC, a sample of S119 was grown at 55°C and the pigment of the bacteria was dissolved in methanol. The UV-Vis spectrum of the sample was measured and is shown in Figure 3. A wavelength of 480 nm was determined to be appropriate for monitoring the carotenoids.

The UV-Vis signal of samples ran on the HPLC were monitored at 480 and 280 nm. The chromatogram below in Figure 3 is from a sample grown at 50°C. The crhomatogram shows four distinct peaks that are attributed to four unknown pigments labeled as Carotenoid I, II, III and IV. Figure 6-11 show the chromatograms monitored at 480 nm of carotenoid samples taken from S119 grown at 40, 45, 50, 55, 60 and 65°C.

The HP Chemstation software used to monitor the UV-Vis signal at 480 and 280 saves the absorbance data over the spectral range of 200 to 600 nm for the entire sample run. This data was used to plot the UV-Vis spectrum of each carotenoid peak from each chromatogram shown in Figure 6-11. The plots of all the UV-Vis spectrums measured for Carotenoid I, II, III and IV at each different temperature are shown in Figures 12-15.

The similarities in the peak retention times of the chromatograms shown in Figures 6-11 and the similarities of the UV-Vis spectrums of those peaks shown in Figures 12-15 suggest that there are four major carotenoids being produced by S119. These carotenoids have retention times of about 19, 27, 29, and 53 minutes. They have been designated as Carotenoid I, II, III and IV. The retention time and peak area of each carotenoid is shown below in Tables 4, 5, 6 and 7 along with a calculated value for the peak area/dry weight ratio. The peak area/dry weight value is plotted versus temperature for Carotenoid I, II, III and IV in Figure 16. A photograph of S119 grown on R2A agar at 40, 45, 50, 55, 60 and 65°C is shown in Figure 17.

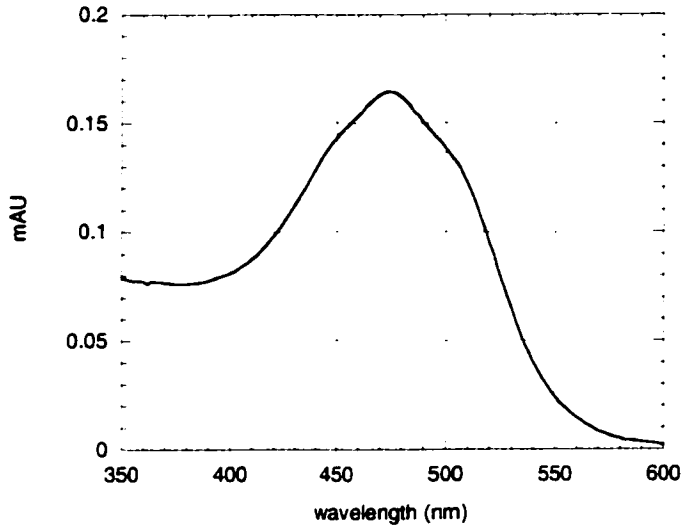

FIGURE 4. UV-Vis spectrum of S119 pigment dissolved in methanol. Bacteria was grown at 55 C.

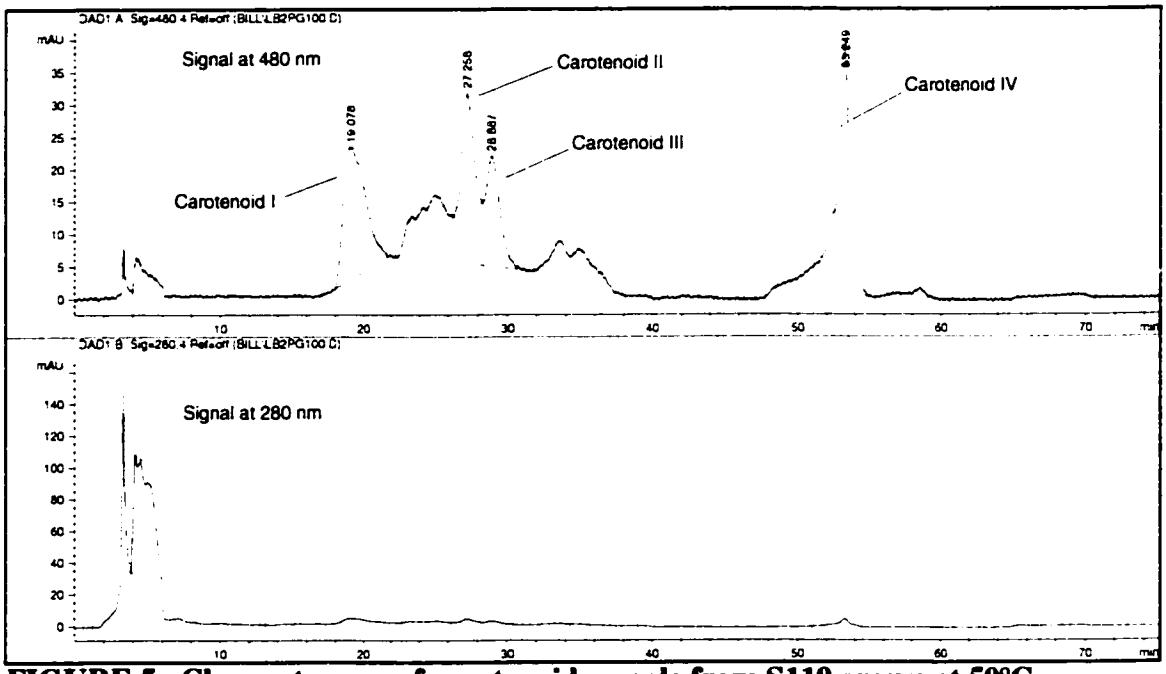

FIGURE 5. Chromatogram of carotenoid sample from S119 grown at 50°C.

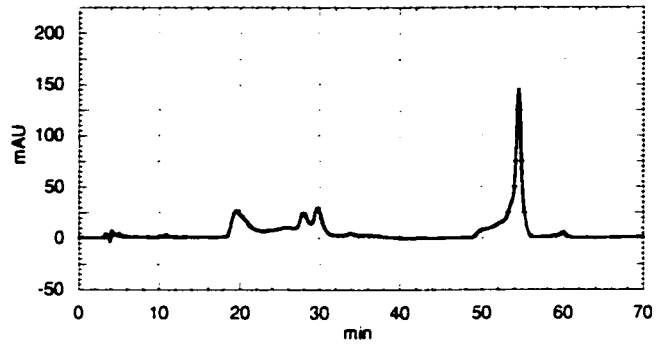

FIGURE 6. Chromatogram monitored at 480 nm of carotenoid sample from S119 grown at 40 C that had an estimated dry weight of 36 ± 0.06 mg.

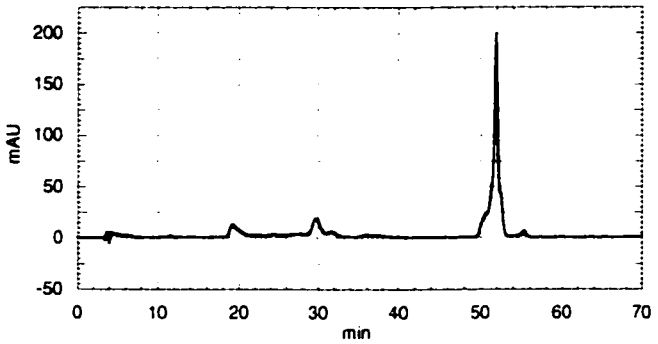

FIGURE 7. Chromatogram monitored at 480 nm of carotenoid sample from S119 grown at 45 C that had an estimated dry weight of 44 ± 0.06 mg.

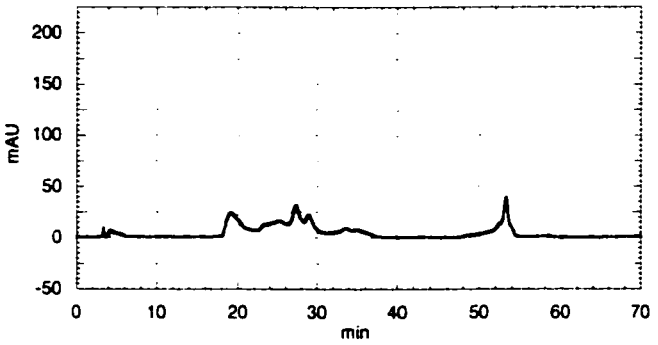

FIGURE 8. Chromatogram monitored at 480 nm of carotenoid sample from S119 grown at 50 C that had an estimated dry weight of 48 ± 0.06 mg.

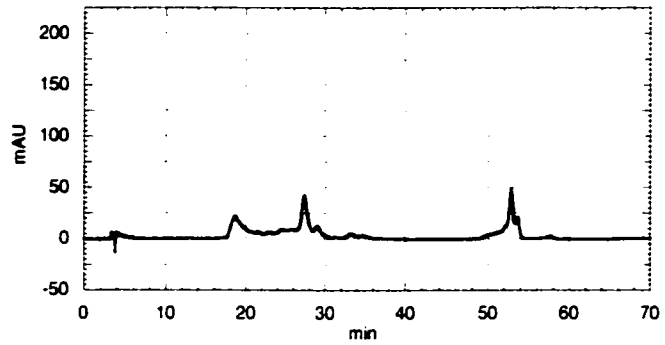

FIGURE 9. Chromatogram monitored at 480 nm of carotenoid sample from S119 grown at 55 C that had an estimated dry weight of 47 ± 0.06 mg.

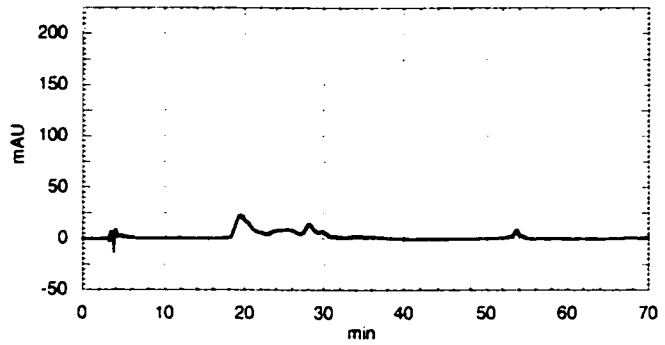

FIGURE 10. Chromatogram monitored at 480 nm of carotenoid sample from S119 grown at 60 C that had an estimated dry weight of 30 ± 0.06 mg.

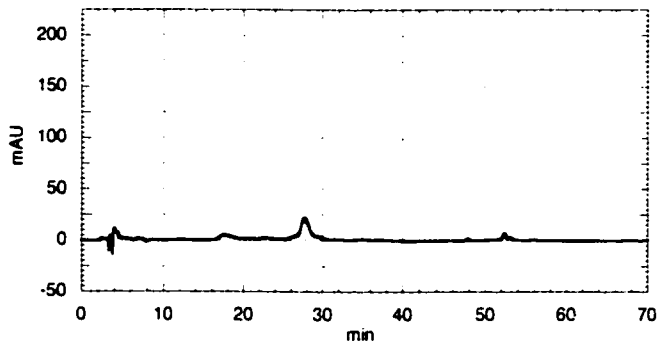

FIGURE 11. Chromatogram monitored at 480 nm of carotenoid sample from S119 grown at 65 C that had an estimated dry weight of 33 ± 0.06 mg.

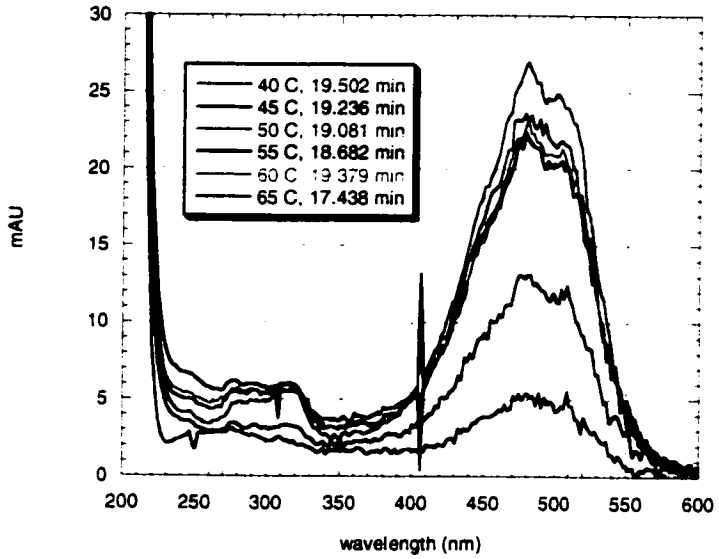

FIGURE 12. UV-Vis spectrum of Carotenoid I which has a retention time of about 19 minutes. Caroteniods were extracted from S119 samples grown at different temperatures that varied in weight.

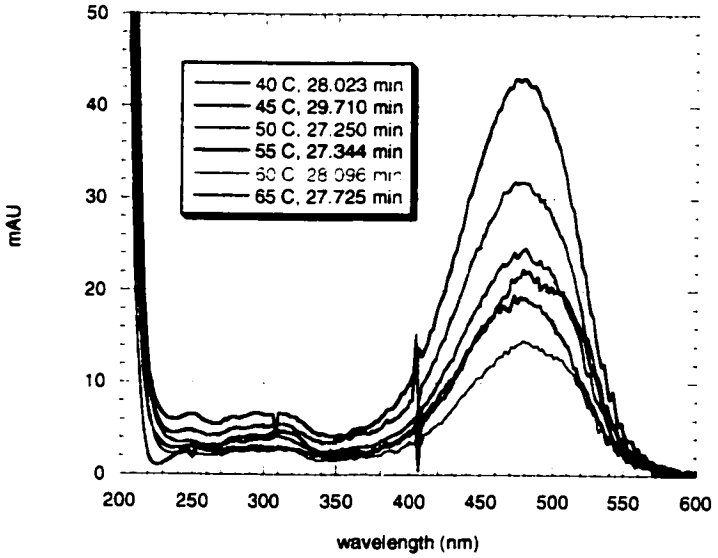

FIGURE 13. UV-Vis spectrum of Carotenoid II which has a retention time of about 27 minutes. Caroteniods were extracted from S119 samples grown at different temperatures that varied in weight.

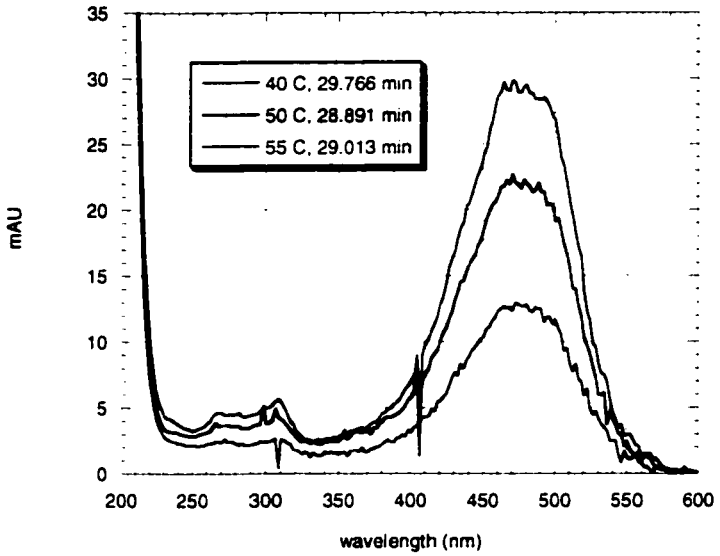

FIGURE 14. UV-Vis spectrum of Carotenoid III which has a retention time of about 29 minutes. Caroteniods were extracted from S119 samples grown at different temperatures that varied in weight.

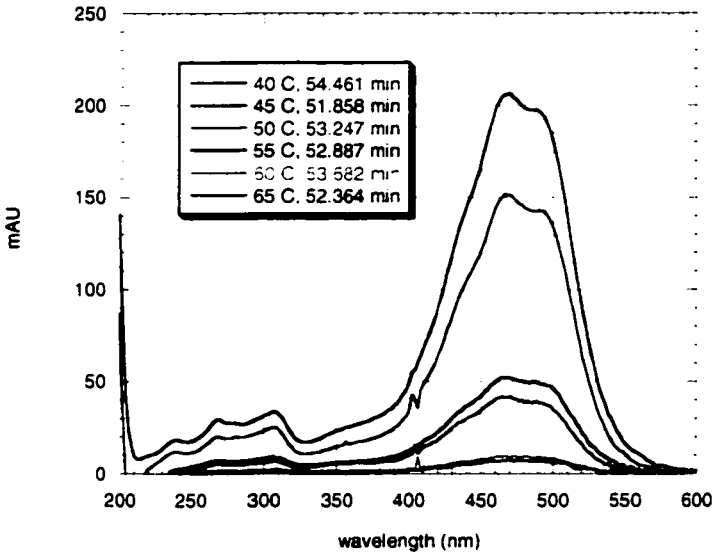

FIGURE 15. UV-Vis spectrum of Carotenoid IV which has a retention time of about 53 minutes. Caroteniods were extracted from S119 samples grown at different temperatures that varied in weight.

TABLE 4. Carotenoid I peak area measurements at different temperatures

Temperature (°C)	Ret. Time (min)	Peak Area (mAU*s)	Peak area per dry weight (mAU*s/mg dry wt.)
40	19.51	2762.59	77.4
45	19.23	725.37	16.6
50	19.08	2212.56	45.7
55	18.68	1714.65	36.3
60	19.38	2211.84	74.1
65	17.42	398.20	12.2

TABLE 5. Carotenoid II peak area measurements at different temperatures

Temperature (°C)	Ret. Time (min)	Peak Area (mAU*s)	Peak area per dry weight (mAU*s/mg dry wt.)
40	27.96	1183.81	33.2
45	29.71	996.19	22.9
50	27.26	1907.98	39.4
55	27.34	1747.17	37.0
60	28.09	620.60	20.8
65	27.70	1327.98	40.8

TABLE 6. Carotenoid III peak area measurements at different temperatures

Temperature (°C)	Ret. Time (min)	Peak Area (mAU*s)	Peak area per dry weight (mAU*s/mg dry wt.)
40	29.739	1658.11	46.5
50	28.887	1155.30	23.9
55	29.003	404.30	8.6

TABLE 7. Carotenoid IV peak area measurements at different temperatures

Temperature (°C)	Ret. Time (min)	Peak Area (mAU*s)	Peak area per dry weight (mAU*s/mg dry wt.)
40	54.49	9784.18	274.1
45	51.86	9437.05	216.5
50	53.25	2492.56	51.5
55	52.88	1980.40	41.9
60	53.68	284.14	9.5
65	52.36	237.99	7.3

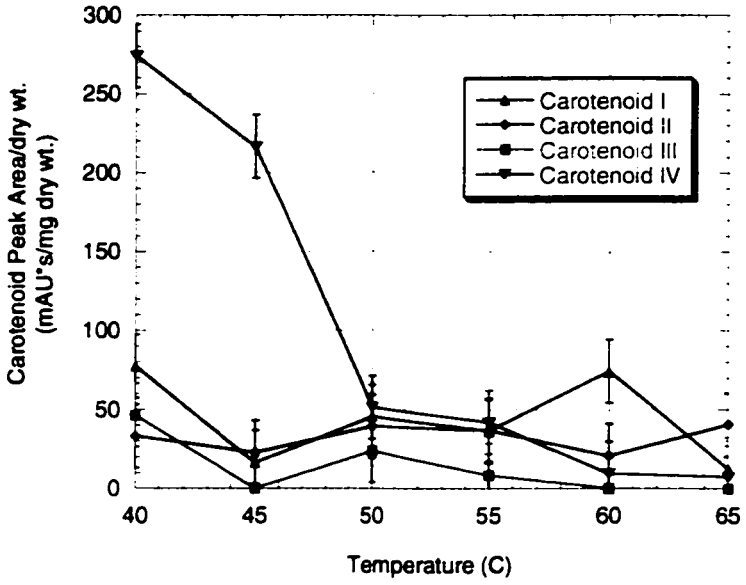

FIGURE 16. Carotenoid peak area per dry weight of S119 at different temperatures.

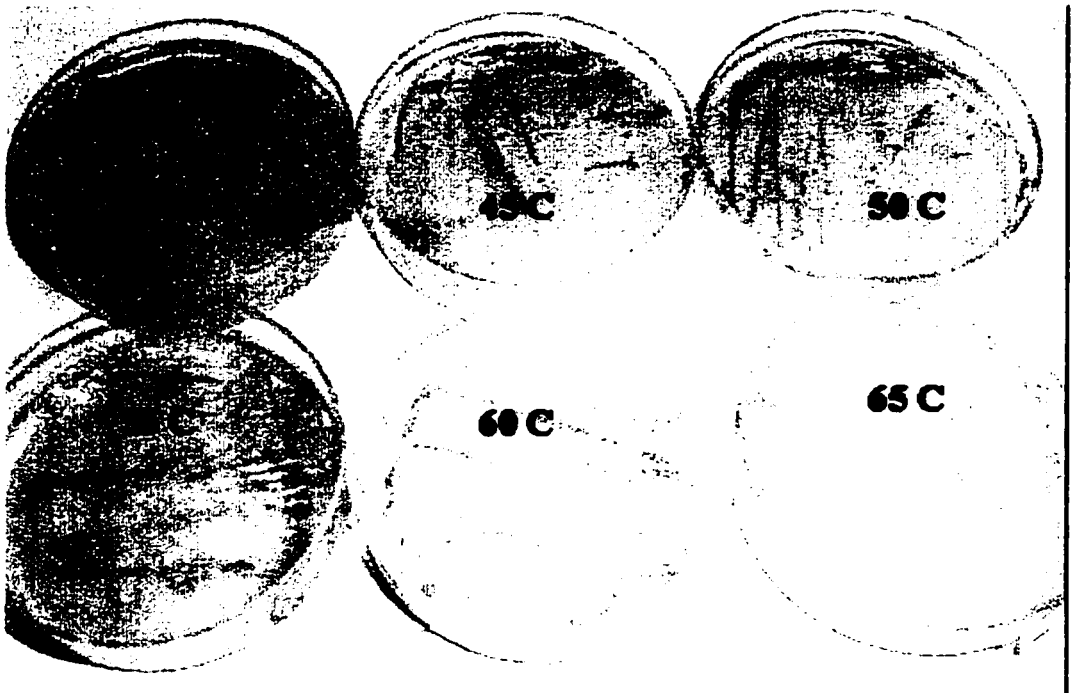

FIGURE 17. S119 grown on R2A agar plates at 40, 45, 50, 55, 60 and 65°C.

DISCUSSION

Figure 5 shows the chromatograms of a carotenoid sample from S119 grown at 50°C monitored at 280 and 480 nm. These chromatograms are typical of the samples from S119 grown at 40, 45, 55, 60 and 65°C. The chromatogram at 280 nm showed an intense peak signal starting at about 3 minutes and going to six minutes. No other peaks appear after 6 minutes. This suggests that all of the large bio-molecules in the sample, except for any carotenoids, are being eluted from the column at the very beginning of the run. The chromatograms at 480 nm show four distinguishable peaks, which have been attributed to four different types of carotenoids based on the shape of the UV-Vis spectrum from these peaks and the retention time of the peaks. The four unknown carotenoids have been labeled Carotenoid I, II, III and IV, respectively, and have retention times of about 19, 27, 29 and 53 minutes. The UV-Vis spectra of Carotenoids I, II, III and IV are shown in Figures 12-15 and the plots show that each of these carotenoids has its own unique UV-Vis spectrum that distinguishes it from the others. Carotenoid I has a spectrum with peaks at 480 and 505 nm. Carotenoid II's spectrum shows a peak 475 nm. Carotenoid III's spectrum has peaks at 308 and 470 nm. Carotenoid IV's spectrum shows peaks at 306, 465 and 490 nm. The UV-Vis data is summarized in Table 8. Based on the differences in retention time and UV-Vis data, it is clear that S119 produces at least four different kinds of carotenoids.

TABLE 8. Major peaks in carotenoid UV-Vis spectrum.

Carotenoid	Major peaks in spectrum (nm)
I	480, 505
II	475
III	308, 470
IV	306, 465, 490

The peak area measurements for Carotenoids I, II, III and IV are listed in Tables 4, 5, 6 and 7. The size of the peak area is proportional to the amount of carotenoid in the sample. Since the carotenoids studied in this experiment have not been characterized, all quantitative data here is reported in terms of peak area per dry weight. Tables 4, 5, 6 and 7 list the peak area/dry weight ratio of each carotenoid determined at the different temperatures. Figure 16 is a plot of this data showing how temperature affects the peak area/dry weight ratio of each of the carotenoids. This figure shows that the concentration of Carotenoid IV decreases as the temperature increases. At 40 and 45°C the peak area/dry weight ratio of Carotenoid IV was measured to be 283.2 and 223.7 mAU·s/mg dry wt. However, as the temperature is increased to 50 and 55°C, the peak area/dry weight ratio of Carotenoid IV drops to 53.2 and 43.3. When the temperature of growth reaches 60 and 65°C, the peak area/dry weight ratio drops to 9.8 and 7.6, respectively. Carotenoids I, II and III were relatively unaffected by the change in temperature.

Conversion from peak area/dry weight ratio to concentration values is done with the Beer-Lambert Law as written in Equation 8

$$A = \varepsilon bc \qquad (8)$$

Where

A = absorbance

ε = extinction coefficient, L/mol·cm

b = path length, cm

c = concentration of compound, mol/L

To calculate the concentrations of Carotenoids I, II, III and IV would require knowing their extinction coefficients. However, since the extinction coefficient is a constant for each compound, it can be removed from the calculation by normalizing the data. In this experiment, the concentration of Carotenoid IV is normalized in terms of the peak area/dry weight ratio calculated at 65°C and the results are shown in Figure 18. Table 9 lists the normalized concentration values of Carotenoid IV at different temperatures. This plot shows that at 40 and 45°C the concentration of Carotenoid IV is 37.6 and 29.7 times higher then at 65°C, respectively.

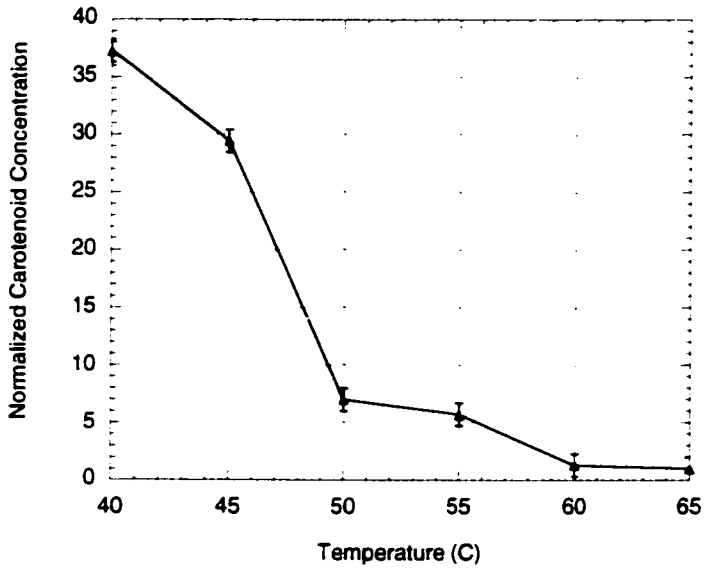

FIGURE 18. Concentration of Carotenoid IV normalized to carotenoid levels measured at 65 C.

TABLE 9. Normalized concentration of Carotenoid IV at different temperatures

Temperature (°C)	Carotenoid IV normalized production
40	37.6
45	29.7
50	7.1
55	5.7
60	1.3
65	1

Figure 17 shows S119 grown at different temperatures and the color of the bacteria's pigmentation appears brighter and more concentrated at 40°C then at any other temperature. The pigment is still quite apparent at 45, 50, and 55°C, but at 60 and 65°C the color is very dull and is hardly noticeable. Figure 18 suggests that the change in pigmentation shown in S119 is due to a change in concentration of Carotenoid IV. The concentration of Carotenoid IV is 37.6 and 29.7 times higher at 40 and 45 C then at 65°C, and the decrease in concentration of Carotenoid IV with increasing temperature correlates with the change in pigmentation. As the concentration of Carotenoid IV in S119 is decreased, the pigmentation of the bacteria becomes duller. This would explain why S119 grown at 40 and 45°C, where the concentration of Carotenoid IV is the highest, has such a bright color. While the bacteria grown at 60 and 65°C, where the concentration is the lowest, shows hardly any coloration at all. Apparently, Carotenoid IV is responsible for the pink pigmentation shown in S119.

The major pigment of a *Meiothermus ruber* strain was determined by Burges and associates to be 1'-β-glucopyranosyl-3,4,3',4'-tetradehydro-1',2'-dihydro-β,ψ-caroten-2-one [23]. However, it is not likely that this carotenoid is the same as Carotenoid IV. This conclusion is based on the differences between elution gradients used to separate the carotenoids during purification on HPLC systems with C_{18} columns. The carotenoid

sample reported by Burges *et. al.* was separated by an isocratic elution using MeOH-CH_3CN-H_2O (86:7:7 v/v/v) followed by isocratic elution using MeOH-H_2O (19:1 v/v). Caroternoid IV has a very high affinity for C_{18} packing, and an elution of MeOH-CH_3CN-H_2O (86:7:7 v/v/v) is not enough to remove the carotenoid from this column. The carotenoid only comes off the column after about 20 minutes of elution with pure CH_3CN. The differences in affinity for C_{18} indicate that Carotenoid IV is quite different from the carotenoid reported by Burges and co-workers.

Bacterial carotenoids have been shown to protect against photochemically induced oxidation [24], but the role that carotenoids play in the molecular mechanism of thermophilic growth has been inconclusive. It has been proposed that carotenoids assist in membrane stabilization during growth at high temperatures [26], but experimental evidence has not been supportive of this idea [25]. The results of temperature studies on S119 presented in this work also do not support the mechanism proposed by Ourisson [26]. If carotenoids produced by S119 assisted in membrane stabilization during thermophilic growth by spanning the lipid bilayer, then carotenoid concentration would be expected to increase as the growth temperature increased. In the case of S119 the carotenoid concentration actually decreased as the growth temperature was increased from 40 to 65°C. In particular, concentration of Carotenoid IV decreased 37.6-fold from 40 to 65°C. If Carotenoid IV produced by S119 does play a role in membrane stabilization, then it must be through a mechanism different from that proposed by Ourrison. Such a mechanism has yet to be proposed in literature.

Investigation of carotenoid producing bacteria such as S119 can provide valuable information about a method for directing carbon and energy to the carotenoid pathway, which for most carotenoid-producing bacteria is similar [16]. To make S119 a viable candidate for industrial production of carotenoids, it would have to be modified through metabolic engineering to produce commercially important carotenoids such as β-carotene and astaxanthin. This has already been done with strains of *E. coli*. A metabolically engineered strain of *E. coli* was made to produce astaxanthin 50 times higher then any previously reported value by manipulation of the carotenoid pathway [16]. This was done by introducing an *E. coli idi* gene, an *Archaeoglobus fulgidus gps* gene and the gene cluster *crtBIYZW* of the marine bacterium *Agrobacterium aurantiacum* into *E. coli* to produce astaxanthin at elevated levels.

CONCLUSION

S119 is a pink pigmented thermophile that produces at least four different types of carotenoids. The growth temperature of S119 affects the concentration of one of the carotenoids, designated as Carotenoid IV. Concentration of Carotenoid IV decreases as growth temperature increases. The concentration of Carotenoid IV is 37.6-fold higher at 40 C then at 65°C. The change in concentration of Carotenoid IV correlates with a change in the pink pigmentation of S119. As the growth temperature of S119 is increased, the concentration of Carotenoid IV decreases and the pigmentation lightens until there is almost no pink color at 65°C. This suggests that Carotenoid IV is responsible for the pink color in the pigmentation of S119.

FUTURE EXPERIMENTS

An experiment should be performed to determine if the carotenoids produced by S119 are secondary metabolites. The experiment would involve growing S119 in 3 L of R2A broth using a bioreactor of suitable size. Growth of the bacteria would be monitored at 600 nm. When the bacteria starts to enter the exponential growth phase, a large sample of the culture (100 to 500 ml) should be removed from the reactor and used to measure carotenoid concentration in the culture. The carotenoid levels should be measured about every two hours and compared with the growth of the bacteria to determine if carotenoid production begins during the exponential growth phase or the stationary phase of the bacteria. Secondary metabolites are formed during the stationary phase.

There is also a need to perform more growth experiments on S119. Growth of S119 in 250 ml of R2A broth was measured in this experiment at 50, 55 and 65°C by measuring the absorbance of the samples at 600 nm. Growth of S119 should be measured in a similar way at 40, 45, and 60°C in a future experiment. This growth information could be used to compare the specific growth rate of the bacteria at different temperatures.

Future experiments should also include efforts to determine the structure of Carotenoid IV. A carotenoid sample from S119 grown at 40 or 45°C should be used because Carotenoid IV is produced at the highest levels at these temperatures. Running a carotenoid sample from S119 on an 1100 series LCMS would be the first step in characterizing Carotenoid IV. The 1100 series LCMS would give both UV-Vis and mass spectrometry data, which may be enough to determine the structure of the carotenoid. If

further analysis is needed to characterize the structure, then ^{1}H NMR spectroscopy should next be performed on a purified sample of Carotenoid IV. The combination of UV-Vis, mass spectrometry and ^{1}H NMR spectroscopy data is sufficient to identify the structure of any carotenoid (Pfander pg. 102-103).

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APPENDIX

The amount of heat generated by bacteria growth is estimated using Equation 2

$$Q_{generated} = 1000L \times 0.000122\frac{1}{s} \times 4.4\frac{g}{L} \times \frac{1}{0.0000287\frac{g}{J}} = 187600 \text{ W}$$

Calculating Q in a reactor ran at 37°C first requires calculating the Rayleigh number using Equation 3

$$Ra = \frac{9.8\frac{m}{s^2} \times 0.00329\frac{1}{K} \times 12K \times (3.71m)^3}{\left(0.0000164\frac{m^2}{s}\right)^2} \times 0.7113 = 5.23 \times 10^{10}$$

and then calculating the Nusselt number with Equaion 4

$$Nu = \left(0.825 + \frac{0.387 \times (5.23 \times 10^{10})^{1/6}}{(1 + (0.492/0.7113)^{9/16})^{8/27}}\right)^2 = 427$$

Equation 5 is then used to calculate h

$$h = \frac{427 \times 0.02638\frac{W}{m \cdot C}}{3.71m} = 3.04 \text{ W/m}^{2*}\text{°C}$$

and finally Equation 1 is used to estimate Q

$$Q = 3.04\frac{W}{m^2 \cdot C} \times 26.98m^2 \times 12C = 984 \text{ W}$$

A similar approach is used to estimate Q for a reactor grown at 55°C. Ra is first calculated using Equation 3

$$Ra = \frac{9.8\frac{m}{s^2} \times 0.00319\frac{1}{K} \times 30K \times (3.71m)^3}{\left(0.0000171\frac{m^2}{s}\right)^2} \times 0.7107 = 1.16 \times 10^{11}$$

Nu is then calculated

$$Nu = \left(0.825 + \frac{0.387 \times (1.16 \times 10^{11})^{1/6}}{(1 + (0.492/0.7107)^{9/16})^{8/27}}\right)^2 = 551$$

and then Equation 5 is used to calculate h

$$h = \frac{551 \times 0.02702\frac{W}{m \cdot C}}{3.71m} = 4.01\,\mathrm{W/m^{2*}{}^{\circ}C}$$

finally, Equation 1 is used to calculate Q

$$Q = 4.01\frac{W}{m^2 \cdot C} \times 26.98m^2 \times 30C = 3248\ \mathrm{W}$$

Equation 6 is then used to calculate Q_{remove} at 35 and 55°C. Q_{remove} is first calculated for the reactor ran at 35°C

Q_{remove} = 187600 W – 984 W = 186616 W = 186.6 kW for reactor ran at 35°C

Now Q_{remove} is calculated for the reactor ran at 55°C

Q_{remove} = 187600W – 3248W = 184352W = 184.4 kW for reactor ran at 55°C

These calculations show that 2.2 fewer kW from the 55°C reactor then the 35°C reactor.

Equation 7 is used to compare the amount of cooling water required for the reactors ran at 35 and 55°C, assuming that T_{Ci} is 20°C and ε is constant

$$m_{55C} = \frac{Q_{55C}}{Q_{37C}} \times \frac{(T_{Hi} - T_{Ci})_{55C}}{(T_{Hi} - T_{Ci})_{37C}} \times m_{37C} = \frac{184.4kW}{186.6kW} \times \frac{37-20}{55-20} m_{37C} = 0.48 \times m_{37C}$$

which shows that 52% less cooling water would be needed for the reactor ran at 55°C.