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Analysis of carotenoids produced by strains isolated in Calistoga, CA with LC/MS

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ANALYSIS OF CAROTENOIDS PRODUCED BY STRAINS ISOLATED IN CALISTOGA, CA WITH LC/MS

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ABSTRACT

ANALYSIS OF CAROTENOIDS PRODUCED BY STRAINS ISOLATED IN CALISTOGA, CA WITH LC/MS

By Philip Lo

Cartenoids are long-chain hydrocarbons with conjugated double bonds. There are more than 700 naturally produced carotenoids, with most of them being produced by algae and bacteria. Some carotenoids have been reported to have anti-oxidant and cancer-prevention characteristics, and are considered commercially valuable. A few strains of *Meiothermus* bacteria were isolated from the hot springs in Calistoga, California, in order to analyze the pigments they produced, since a few past reports indicated that they possibly produce zeaxanthin-like carotenoid glycoside. The bacteria were grown in shake-flask cultures under controlled conditions, before the biosynthetic carotenoids were separated and analyzed. An HPLC-APCI-MS was used for the analysis with HPLC-DAD for structural elucidation and APCI-MS for structural confirmation.

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CHAPTER 1.0 INTRODUCTION

1.1 Carotenoids

Carotenoids are organic, lipid soluble pigments that are widely distributed in nature. Most natural carotenoids consist of 35~40 carbon atoms with long carbon chain configurations, where the long chain consists of eight isoprenoid units and the center of the molecule is the point of mirror symmetry or asymmetry. The terminals of the chains may be either open or cyclic. All carotenoids contain conjugated, oxidized (xanthophylls) or unoxidixed (carotenes) double bonds. It is the conjugated double bond system that gives carotenoids high wavelength colors. Most naturally produced carotenoids are found in microorganisms or plants such as fungi, bacteria, and algae [1]. There are approximately 700 carotenoids discovered presently [2]. For plants, carotenoids are essential to their growth and photosynthesis. Carotenoids serve as singlet oxygen quenchers for triplet chlorophyll [3]. This prevents deleterious effects of singlet oxygen on plant cells and therefore prolongs plant lives. Since most carotenoids are lipid-soluble, animals that consume carotenoid-producing microbes exhibit colors on their skin.

1.2 Health Benefits of Carotenoids

Carotenoid-related health benefits have been asserted by a number of organizations, and in some cases, research has demonstrated the veracity of the claims. For example, lycopene has been proposed to reduce the incidence of lung, prostate, and

digestive cancers [4]. The biochemistry of lycopene isomers in plasma is still unknown [4]; however, the anticarcinogenic effect of carotenoids has been widely recognized. Although the FDA has not yet endorsed the health benefit of lycopene [5] in June 2005, more long-term research may change the decision eventually [6]. In addition to lycopene, other carotenoids like β-carotene and lutein are also important for their anti-oxidant and photo-resistant functions [7].

1.3 Carotenoid Markets – the Present and Future Potential

Among all, the most popular carotenoids are astaxanthin and β-carotene, which are supplied by two European giants, BASF and DSM, worth about $138 million during 2003 [5]. The largest demand still remains in feed industry. For example, astaxanthin was the second largest demanded commercial carotenoid in 2004 (Figure 1). The future of the carotenoid market, although difficult to predict, is very bright. The global carotenoid market was $786 million in 1999 [8]. The latest research indicated that the market reached $887 million in 2004 [9]. Regardless of the prediction, Roche profits due to carotenoid and vitamin sales were $2.4 billion in 2001 before selling the division to DSM, which already exceeded the conservative estimation. Since the carotenoids market is experiencing such dramatic average annual growth rate increase, there will likely be more companies joining the competition.

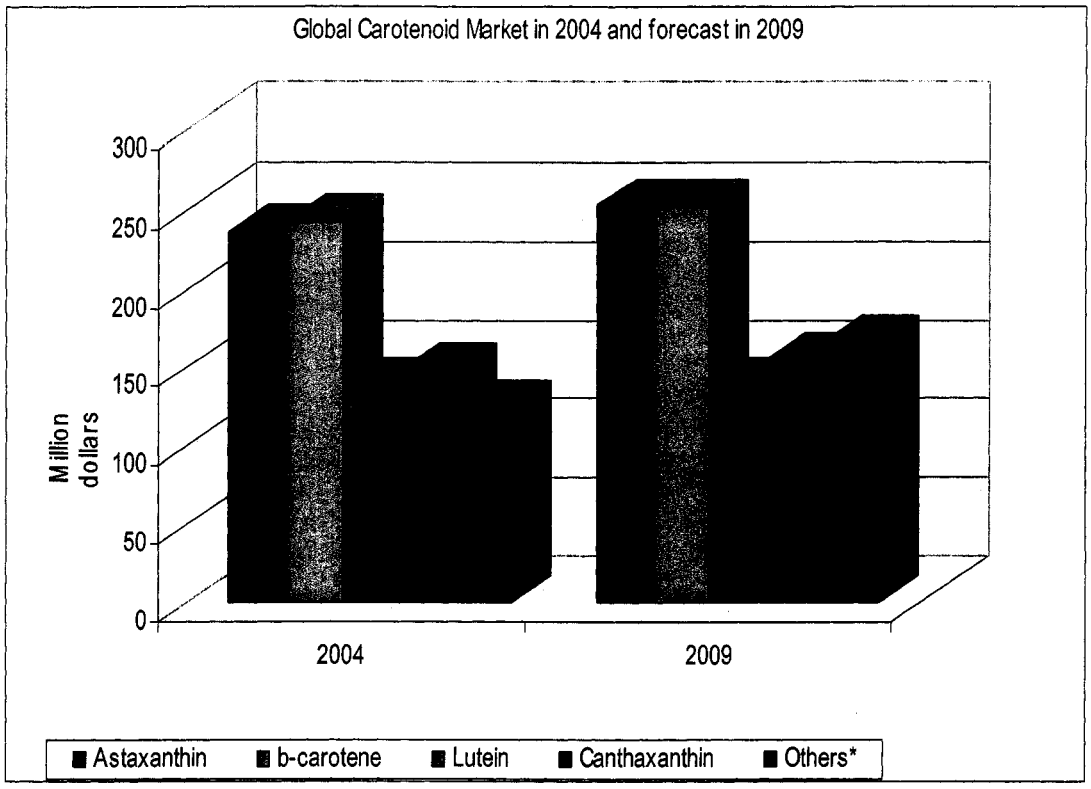

Figure 1: The market of major carotenoids in 2004 and its forecast in 2009 [9].

1.4 Microbial Carotenoids

Due to the availability of chemical synthesis technology, very few carotenoids are produced commercially using biochemical methods. However, as the requirements for supplement production become more demanding, biosynthetic carotenoids may become the alternatives of their chemical counterparts due to less environmental concerns and production costs. For example, DSM uses co-fermentation of sexually reproducing *Blakeslea trispora* to produce its β-carotene. For naturally carotenoid-producing microbes like *Blakeslea trispora* can produce up to 0.18g per g dry cell [10]. In addition

to wild-type carotenoid-producing microbes, scientists also attempted to produce carotenoids using GMO (Genetically Modified Organisms). Some researchers used yeast [11, 12] and bacteria [13, 14]. Although the GMO's usually have substantially lower yields than natural carotenoid-producing microbes, scientists and engineers designed methods to improve yields. For example, J. Liao used a dynamic controller to regulate the flux to the engineered pathway so that the production of lycopene increased to 0.16 mg/mL/h [14]. Many scientists utilized Rhodobacter derived carotenogenic genes [15]. Although not carotenoid producing, *E. coli* were also popular experimental species tested by scientists and engineers. C. Schmidt-Dannert [16] used recombinant *E. coli* DNA to produce several terpenoid pathway products such as lycopene and β-carotene.

1.5 Carotenoid Purification

Many carotenoids are soluble in organic solvents and insoluble in water. This makes them extractable from fruits and vegetables using organic solvents. However, because fruits or vegetables containing carotenoids have more than one carotenoid, extracting chemically similar carotenoids from each other is a challenging task. Traditionally, chemical separation uses acetone, methanol, ethanol, benzene, petroleum ether, carbon disulphide, and chloroform [17]. The majority of these chemicals are either lethal or carcinogenic. Because different carotenoids function differently in the human body, and because the isomerization of carotenoids may change their biochemistry, characterization and quantification is very important.

1.6 Carotenoid Separation, Elucidation, and Analysis

Chromatography has been an effective tool for carotenoid analysis due to its sensitivity, adaptability and accuracy. Chromatographic determination of carotenoids began with open column chromatography. The method was used to determine the amount of carotenes and xanthophylls in plants. As the technology advanced, carotenoids could be individually identified and purified. HPLC provides very accurate quantitative and qualitative carotenoid analysis. For carotenoid separations, the mobile phase plays an important role. The solvents for carotenoids with polar functional groups should be polar, and their acidity should be low to avoid reactions of the analytes and the solvents. Isocratic elution is frequently employed but secondary or even ternary gradient elution [18] may also be utilized if a good separation is required. For lycopene and highly non-polar carotenoids, non-polar solvents should be used. Reverse-phase chromatography may be used for nonpolar carotenoid detection. An octadecyl (C18) column is a typical reverse-phase stationary phase for carotenoid separations since it has sufficiently long hydrophobic ligands on its matrix.

Carotenoids can be detected by a number of detection methods. Photodiode array detectors (DAD) are frequently used by researchers because each carotenoid has a unique absorption maximum when dissolved in different solvents. The mass spectrometer detector is also useful for carotenoid characterization. A mass spectrometer has an ion source that fragments molecules into ions. The fragments then travel through the analyzer, where the ions are separated based on mass-to-charge ratios. Finally, the separated ions hit the detector and a signal is generated. The most typical ion sources are

electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption ionization (MALDI). These ionizors may be coupled with one or more of time-of-flight (TOF) or quadrupole analyzers. The introduction of HPLC and a wide range of column packing, together with a DAD, or an MS [19, 20] made carotenoid determination feasible.

1.7 Characterization of Carotenoids Produced by the Bacteria Used for This Study

Several thermo-tolerant bacteria isolated from the hot spring in Calistoga, CA, were analyzed in this study. The strains were grown on both liquid and solid medium. Similar to many mesophiles or thermophiles, these strains display different bright colors (from deep to light red, and even orange). For this reason, the bacteria are suspected to be carotenoid-producing, similar to the *meiothermus* species studied previously. The advantage of producing carotenoids by thermo-tolerant bacteria is reduced cooling water requirements in large-scale production as compared with mesophilic bacteria. An example of a thermotolerant species that grows on methanol, a low cost substrate, is *Bacillus methanolicus* [21]. After the fermentation process, carotenoids produced by the bacteria can be extracted by solvent extraction methods, separated by HPLC under fixed conditions such as temperature, mobile phase flow rate and column, and characterized by LC-MS.

CHAPTER 2.0 LITERATURE REVIEW

2.1 *Thermus / Meiothermus* Bacteria

2.1.1 Characteristics of *Thermus / Meiothermus* Bacteria

Although the species of bacteria to be used in this study are not yet characterized, given the physical appearance of the bacteria and the similarity of growth conditions there is reason to suspect they are they are *Thermus* sp. Many *Thermus* bacteria are gram-negative, obligately thermophilic, colored, and have an optimal growth temperature range between 60°-75 °C [22], with some at slightly lower temperatures (50 °C) [23, 24, 25]. Each bacterium utilizes different carbon and nitrogen sources. Some species such as *T. silvanus* and *T. chliarophilus* can thrive at a lower temperature [23]. Eight validly described thermophilic bacterial species were assigned to the genus *Thermus*: *T. aquaticus*, *T. filiformis*, *T. brockianus*, and *T. thermophilus* form yellow or nonpigmented colonies and have an optimum growth temperature below 80 °C; *T. aquaticus/flavus/thermophilus* are extremely thermophilic, while *T. ruber* is obligately thermophilic (35 °-70 °C in potato yeast extract), gram-negative, nonmotile, rod shaped, and are 3 to 6 by 0.5 to 0.8 μm, with rounded ends [26]. The generation time of *T. thermophilus* is about 18 to 20 minutes [26], which is much shorter than the generation time of *T. ruber*. *T. ruber* is obligately aerobic and the growth is sensitive to the amount of D-glucose. The species has four main cellular fatty acids: isopentadecanoic acid, 30.6%; isoheptadecanoic acid, 15.8%; anteisopentadecanoic acid, 8.3%; and anteisoheptadecanoic acid, 7.3%. *Thermus* species all have a characteristic corrugated

outer layer with regularly spaced channel-like structures connected to a thin peptidoglycan layer [27].

Thermus ruber/silvanus/chliarophilus were reclassified as *Meiothermus* genus in 1996 for their obligately thermophilic nature [28]. Their physiological characteristics are listed in Table 1:

Table 1: Physiological characteristics of Meiothermus species.

Species	Pigment	Optimal temperature (°C)	Catalase
M. rubber	Red	60~65 (< 70)	positive
M. Silvanus	Red	55 (< 65)	negative
M. chliarophilus	Yellow	50 (< 60)	negative

2.1.2 *Thermus / Meiothermus* Characterization

It is now possible to characterize *Thermus* species using DNA homology data (e.g., 16S rRNA gene sequences) [25], biochemical and tolerance characteristics, microscopic and HPLC analysis (or CHEMTAX) provide useful information for taxonomy of *Thermus* bacteria to genus/species level. The biochemical and tolerance characteristics can be carried out [25] in a *Thermus* medium or on a *Thermus* agar incubated at 50 °C for up to 5 days. Catalase activity can be examined, with a pH range for growth being from 5 to 10.5 [25].

All *Thermus* species have close sequence alignment (~ 91%) to 16S rRNA. It is the phenotypic and chemotaxonomic [27] (growth temperature, the polar lipid patterns, and the hydroxyl fatty acid compositions) differences that make up the differences between *Thermus* and *Meiothermus*.

The difference in major phospholipids and major glycolipids in their polar lipid patterns differentiates low-temperature *Thermus* strains from the high-temperature ones [27]. Most strains have a minor glycolipid and a minor aminophospholipid. Chemotaxonomy data show that the polar lipid pattern of low-temperature species consists of a phospholipid and two glycolipids.

2.1.3 Meiothermus Bacteria and Carotenoids

There are several wild types of *Thermus* species that produce carotenoids, and these carotenoids tend to be stored in the lipid bilayer of the cellular membrane. Ramaley [22] reported absorption spectra of a species of *Thermus* in 1975. He showed that all bacteria in his research yielded an absorption spectrum with a wavelength range between 450 and 503 nm. This absorption spectrum is also that of many carotenoids. Other species such as *T. chliarophilus* produced yellow-pigments. For *T. ruber*, a bright-red pigment with absorption spectra of acetone, methanol-acetone, and hexane extracts showed three maxima at 455, 483, and 513 nm. One strain contains a bright yellow pigment resembling neurosporaxanthanthine in its spectral properties. The role of carotenoids in bacteria may vary. According to Burgess *et al.* [23], carotenoid production may serve to protect the bacteria from extreme heat. Ever since Ramaley reported the absorption spectra of *Thermus*, researchers began to study carotenoids produced by the bacteria. *T. thermophilus* and *T. aquaticus* produced the same carotenoids [23, 24]; *T. silvanus* produced orange-red-pigments.

2.2 Carotenoid Extraction

Being highly unsaturated, carotenoids are prone to isomerization and oxidation. Isomerization to the *cis*-isomers is promoted by contact with acids, heat treatment, and exposure to light. Cell disruption by cutting or shredding allows substrate/enzyme interactions and makes these products more susceptible to physiological/biochemical changes.

The extraction procedures depend on the type of biological material [29, 30]. Solvents for extraction should be able to extract all of the desired carotenoids contained in the matrices. The length of the exposure time to the solvent for the dissolution of carotenoids requires optimization so that maximal carotenoid extraction may be achieved without resulting in the isomerization of the carotenoids [30]. In addition, complete dryness should be avoided, since some carotenoids such as lycopene degrade easily under low humidity [31].

2.3 Saponification

The carotenoids are frequently esterified by fatty acids before the saponification step. The extent of esterification is directly related to the number of hydroxyls (esterification sites) in the xanthophylls. The purpose of saponification is to remove chlorophylls, unwanted lipids, hydrolyzing carotenoid esters, and other impurities. These impurities may interfere with the chromatographic separation and shorten a column's life. Carotenoid esters frequently form mixtures that are more difficult to separate than

carotenoids. Based on the results published by Scott *et al.* and Sharpless *et al.* [32, 33], the relative uncertainties caused by saponification-induced isomerization or losses can range from 13 to 47%. On the contrary, over-exposure to solvents or other extraction conditions can decrease the throughput as well as increase the processing costs. Saponification has been shown to result in substantial isomerization. Indeed, most carotenoid isomerization is attributed to saponification conditions. The combination of KOH wash/removal and organic phase pH adjustment causes the major carotenoid losses. For fatty samples, saponification should be carried out under a nitrogen atmosphere in the dark [35]. After saponification, the carotenoids were usually further extracted with ethyl ether and the alkali was washed with water before drying [30]. If antioxidant such as butylated hydroxytoluene were added to the carotenoid samples, they can be saponified overnight with minor carotenoid losses [34].

2.4 Separation and Identification of Carotenoids

2.4.1 C_{18} Stationary phases

C18 columns are the most common chromatographic columns available commercially for carotenoid analysis. Reversed-phase HPLC on C_{18} stationary phase generally has weak hydrophobic interactions with the analytes. C_{18} columns are available commercially with a variety of carbon loading, end capping, and the polymerization (mono/poly) of the bond phases. The majority of nonpolar carotenoid analysis has been carried out with 5μm C_{18} spherical particles packed in a 250 x 4.6 mm column. Polymeric C18 columns have been found to have excellent results for geometric isomers

of β-carotene [36]. Despite the advantages of polymeric C_{18} columns, the total carbon load is lower in the wide-pore polymeric phases and therefore the carotenoid retention is weaker in the polymeric column [37]. Polymeric ODS tend to elute broader peaks than monomeric columns. Using a reversed-phase C_{18} column, Oliver *et al.* [35] were able to classify 44 peaks that belonged to three categories: (1) carotenoid peaks present in both saponified and non-saponified extracts (simple carotenoids), (b) carotenoid peaks present only in non-saponified extracts, and (c) carotenoid peaks present only in saponified extracts. The retention characteristics of carotenoid esters depend on their acid moieties.

The general order of retention time of commercial carotenoids with a polymeric C_{18} column is:

Astaxanthin < lutein < zeaxanthin < canthaxanthin < β-cryptoxanthin < echininone < α-carotene < β-carotene < δ-carotene < lycopene (from polar to nonpolar)

2.4.2 C_{30} Stationary phases and isomer separation

Because carotenoids generally have several isomeric forms, and only certain isomers may have health effects in the human body, proper carotenoid separation and characterization are important issues. For example, most plant-produced lycopene is predominantly *trans*, whereas *cis*-lycopene consists of 50% of human plasma lycopenes [4]. More advanced chromatographic columns may achieve better carotenoid isomer separations. C_{30} stationary phases, introduced in 1987, showed their first application in carotenoid analysis in 1994 [38]. In 1997, Pesek *et al.* [39] provided a non-traditional method for the synthesis of alkyl stationary phases. The method is also applicable to C_{30} phases. In a study conducted by Emenhise *et al.* [40], a C_{30} reversed-phase column

(developed by NIST) was utilized to resolve *cis-trans* isomers. C_{30} columns proved to be the most effective column for isomer separation of extremely nonpolar carotenoids such as lycopene [41].

2.4.3 Column Factors

The stationary phase of a column determines shape selectivity in carotenoid separations. But, reproducibility of carotenoid separation is temperature dependent. Column capacity factor and the solute-stationary absorption characteristics are temperature sensitive. Since the capacity factor can be calculated using the van't Hoff equation, the enthalpic and the entropic effects (both are temperature-dependent) can be verified:

$$\ln k' = - \Delta H/RT + \Delta S/R + \ln \phi$$

At low temperatures, the enthalpy term is smaller, and the retention is more entropy driven. On the other hand, if the temperature is higher, the enthalpic effect is more negative, and the capacity factor has more enthalpy effect. The changes of these factors may possibly affect the rigidity of the ligand chains on the stationary phases, and hence the retention mechanism. Although only a few reports of the temperature variation of carotenoid separation exist, Bell *et al.* [42] reported the effect of temperature on carotenoid retention on C_{18}, C_{30}, and C_{34} columns with isocratic elution. Based on the results reported by Bell, the impact of low temperature on the order and the rigidity of the ligand chains may prevent the analytes (especially the polar carotenoids) from penetrating through the chains and interacting with the silanol surfaces directly.

Temperature effects are also less obvious for all-*trans*, non-polar carotenoids (the deviations from linearity of the van't Hoff plot are very minute). Higher solute-stationary phase interaction was observed above 308K for bent carotenoids. Bell correlates the retention of carotenoid isomers with the rigidity and the mobility of alkyl phases. A later study conducted by Albert [43] proved that stereoisomerism of alkyl chains of the stationary phases was caused by high temperature (315K), which determined the elution of carotenoid isomers. At a low temperature (298K), the chains exist in *trans* conformation, which leads to better selectivity. As temperature increases, interconversion between *trans* and gauche conformation become more frequent, and the resolution is lost.

2.4.4 Solvents and mobile phases

The most important properties to be considered in selecting the mobile phase are polarity, viscosity, volatility, toxicity, and inertness. Acetonitrile has been widely used because of its low viscosity and good selectivity for xanthophylls when a monomeric C_{18} column is used [44]. Nevertheless, acetonitrile does not recover polar carotenoids as well as methanol-based solvents in a reverse-phase column. Therefore, methanol is frequently added to acetonitrile as a solvent modifier, according to Epler *et al.* [45] after 65 columns were tested. Besides methanol, triethylamine was found to promote carotenoid recovery in acetonitrile-based solvent [46]. Oliver *et al.* [35] prepared gradient (acetone-water (100:50) and acetone-water (100:5) HPLC to avoid problem caused by esterification (esterified and unesterified carotenoids usually have different polarities). The method is suitable for analyzing the carotenoid content in any fatty sample.

The following table lists the compositions of isocratic solvent systems and the flowrates for carotenoid analysis using C_{18} columns:

Table 2: Isocratic Mobile Phase Systems Used for RP-18 Polymeric Columns.

Column	Solvent (v/v/v)	Flowrate	Carotenoids separated	Reference
5 μm Sphrisorb-ODS	MeCN/MeOH/H2O (85/10/5)	0.8	Lycopene Astaxanthin	[47]
5 μm Vydac 201 TP54	MeOH/MeCN (9:1)	1.0	Some 9-*cis*-β-carotene	[48]
5 μm Sphrisorb-ODS	MeCN/MeOH (85/15)	1.0	β-carotene	[49]
5 μm Vydac 201 TP54	MeOH/THF (95/5)	1.0	β-carotene	[50]
	MeOH based (3-5% THF)		β-carotene lycopene	[51]
5 μm Suplex PKB 100	MeCN/MeOH/DCM (75/15/10) Or MeCN/MeOH (9/1 or 5/95)		β-carotene isomers	[52]

2.4.5 Gradient elutions

Gradient elution has the advantages of greater resolving power, improved sensitivity, and elution of strongly retained compounds, and has been used for separating complex carotenoid mixtures. However, it has several disadvantages: (a) increased complexity, (b) need for column re-equilibration between runs, (c) greater differential detector response, and (d) poor reproducibility. 10 to 30 minutes equilibration is required to bring the column back to the starting solvent. Good solvent miscibility is required to prevent baseline disturbance due to outgassing and refractive index effects [37].

2.4.6 Carotenoid Detection Using UV/Vis

UV/Vis spectroscopy has always been recognized as the most common method in carotenoid detection because carotenoids absorb visible light. Both the wavelengths of maximum absorption (λ_{max}) and the shape of the spectrum are characteristics of the chromophore. Most carotenoids absorb maximally at three wavelengths, resulting in a three-peak spectrum. As the number of conjugated double bonds increases, the λ_{max} shifts to longer wavelengths. Table 3 summarizes the absorption maxima of the two most commercially valuable carotenoids:

Table 3. Ultraviolet and Visible Absorption Data for Carotenoids. [30]

Carotenoid	Solvent	λmax			%III/II
Astaxanthin	Acetone		480		0
	Benzene, chloroform		485		0
	Ethanol		478		0
	Petroleum ether		468		0
β-carotene	Acetone	(429)	452	478	15
	Chloroform	(435)	461	485	
	Ethanol	(425)	450	478	25
	Hexane, Petro ether	(425)	450	477	25

Because most carotenoids have their absorption range between 380 and 500 nm, the cutoff ranges of solvents must not exceed the absorbency ranges of carotenoids. For alkyl alcohols, the cutoff range is 205-220 nm [53]. Acetonitrile and water are frequently used solvent, due to the fact that their cutoff ranges are less than 190 nm. Powerful solvents such as ethyl and butyl acetates have their UV cutoff ranges between 250-260 nm. These solvents are all suitable for carotenoid analysis.

2.4.7 Carotenoid Detection Using LC-MS

In order to properly identify a carotenoid using UV/Vis, at least three solvents must be used and all the absorption maxima must match that of the literature values of a known carotenoid. Due to their thermal instability, carotenoids cannot be analyzed by simpler GC/MS. A number of LC-MS techniques for analyzing carotenoids have been reported. LC-MS involves the separation of compounds by HPLC, coupled to detection of compounds by a mass spectrometer (MS). The species entering the MS encounter two mechanisms. First, the stream is ionized by a number of techniques, followed by analysis in a mass analyzer. For carotenoid analysis, ionization methods such as electron impact (EI), chemical ionization (CI), fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) were published. In general, EI and CI are not suitable for carotenoid determination due to their high vaporization temperatures. Other ionization methods such as continuous-flow FAB cannot be used for high-sample volume (only < 10 μL/min) analysis. For ESI-quadrupole mass spectrometer, the addition of post-column halogenated solvents improves detection limits of carotenoids [54]. An alternative method is to introduce adduct ions to carotenoid samples so that nonpolar carotenoids can be charged and then ionized. Unlike ESI, APCI does not require adduct ions to charge nonpolar molecules. Hence, it was also used for nonpolar carotenoid detection [55]. Despite its advantage in sample preparation, APCI-MS is a "molecular weight" detector. The APCI mass spectrum is "cleaner" than other types of mass spectra, it is difficult to determine the

structure from an APCI mass spectrum. Therefore, it is an excellent instrument to confirm carotenoids in a known sample.

2.5 Errors in Carotenoid Analysis

Introduction of error is common in carotenoid analysis because the compounds are extremely sensitive to humidity, temperature, light, acidity, enzymes, and other chemicals. Other sources of errors can be found in all steps of bioprocesses, from sampling to data processing / interpretation.

Sample preparation error is attributed to any man-made error introduced during bioprocessing. There are many error factors in addition to heat and light. For instance, carotenoid extraction should be immediately executed after cell disruption, otherwise liberated enzymes such as lipoxygenase (release acids for *cis-trans* isomerizations) or lycopene cyclase (changes lycopene into β-carotene) may have sufficient time to distort the analytical data.

Analysis error caused by an inappropriate injection volume in HPLC can have a detrimental effect on the analytical results. It has been proven by Khachik *et al.* [56] that the 5 to 10 μL of injection volume eliminated HPLC peak distortion. Metal surfaces and stainless steel frits in columns can interact with the π-orbital of the polyenes [57] can also alter the analytical results.

CHAPTER 3.0 RESEARCH HYPOTHESIS AND OBJECTIVE

3.1 Research Hypothesis

Pigments from bacterial strains screened from Calistoga Hot Springs will be purified from the bacteria and consequently analyzed by LC/MS. The pigments can be separated by HPLC and some of the pigments can be identified by Mass spectrometry.

3.2 Research Objectives

The purpose of this project was to identify color pigments produced by some uncharacterized bacteria. Five pigmented strains were previously isolated from the hot springs in Calistoga, California, USA. They were grown in shaker flasks and after the cell cultures reached stationary phase, the cells were lysed and the color pigments were harvested. The color pigments will be extracted and saponified before they are analyzed by HPLC-MS.

Standards of astaxanthin and β-carotene were purchased. These standards were used to determine whether astaxanthin or β-carotene were present in the bacteria.

CHAPTER 4.0 MATERIALS AND METHODS

4.1 Bacteria Culture

Strains isolated from the hot springs in Calistoga were cultured both in liquid and on solid medium. R2A Broth and R2A agar were prepared and used in this experiment.

4.1.1 Solid Growth Medium

The bacteria isolated from Calistoga springs were incubated on solid R2A agar. The composition of R2A agar is listed in Table 4:

Table 4: Composition of R2A Agar.

Nutrient	Mass (g/L)
Yeast extract	0.5
Proteose Pepton No.3	0.5
Casamino Acids	0.5
Dextrose	0.5
Soluble Starch	0.5
Sodium Pyruvate	0.3
Dipotassium Phosphate	0.3
Magnesium Sulfate	0.05
Agar	15.0

R2A agar is in powder form upon receipt (Becton Dickinson, catalog number 218263). The powder was dissolved in DI water and the solution heated (with agitation) for complete dissolution of the agar. After the powder was fully dissolved, the agar was autoclaved at 121°C for 15 minutes. When the sterilization was completed, the agar solution was removed from the autoclave and transferred onto agar plates before it was cooled to the solidifying temperature (45°C). Agar plates were prepared according to

standard methods. The plates were incubated at 55°C for at least 24 hours or until well-developed colonies could be observed.

4.1.2 Liquid Growth Medium

The same bacteria were cultivated using a liquid growth medium in shaker flasks. R2A broth was prepared according to the composition given in Table 5:

Table 5: Composition of R2A Broth.

Nutrient	Supplier	Item number	Per Liter purified water (g)
Yeast extract	BD	212730	0.5
Peptone	BD	211830	0.5
Casein	Sigma chemical	C-0626	0.5
Dextrose	Fisher Sci.	D16-500	0.5
Soluble Starch	Difco	217820	0.5
Dipotassium Phosphate	Mallinckrodt	886101-1	0.3
Magnesium Sulfate (anhydrous)	Fisher Sci.	M63-500	0.024
Sodium Pyruvate	National Chem.	N/A	0.3

R2A broth was prepared by mixing the ingredients listed in Table 5 with DI water. Sterilization of R2A broth is identical to section 4.1.1. Once cooled, the flasks were inoculated with a bacteria colony from the agar plates. The flasks were placed on a rotary shaker (Innova 2100, New Brunswick Scientific) and shaken at 200 rpm; the process temperature was maintained at 55°C and an initial pH of 7.0. This process was continued until the stationary phase had been reached. Samples were removed periodically to monitor the growth rate with UV-Vis (HP 8452A).

4.1.3 Optical Density, Cell Mass, and Colony Count

Samples of cells were taken regularly from shaker flasks during the production of cells. Based on the optical density results, the growth rates (plot OD vs. time) of the microbes were monitored. Dry cell masses were calculated by the equation:

Dry cell mass = conversion factor x OD

(where OD is optical density)

Since cell mass is the function of OD, the derivative of *f*(cell mass) is the conversion factor.

4.2 Harvesting Carotenoids

4.2.1 Centrifugation

At the end of cell culture, the bacteria were transferred to 50 mL centrifuge tubes (Corning 25325-50) and centrifuged at 6000 rpm for 5 minutes, using a Model 5678 centrifuge from Forma Scientific, Inc. The supernatant was discarded and the cell pellet was lysed with 10 mL of methanol (ACROS 26828-0025) or isopropanol (Fisher A41604). The alcohol-cell mixtures were centrifuged again, and the supernatants were collected [58].

4.2.2 Extraction

The extraction scheme was developed from procedures stated in *Carotenoids Volume 1A: Isolation and Analysis* [30]; and the procedures developed by William Dewhirst [58]. After lysis, the cell pellets were repeatedly extracted using methanol, solvents such as isopropyl alcohol were also used as long as the cell pellets remained

colored. Similar to the first extract, the second, and the third extracts were filtered, and collected.

The isopropanol-cell debris mixture was poured through a sub-micron filter (Falcon 7105 bottle top filter, 0.22 um). The filtrate was washed with cold isopropanol until all the pigments were washed off the filter, before transferring the initial extract into a rotovap for concentration. An equal-volume of diethyl ether (Fisher chemicals E134-1) was used to separate carotenoids from alcohol solutions. The procedure was repeated until all the initial extract had been dissolved in the ether.

4.2.3 Saponification

Saponification was carried out after transferring the carotenoids to ethyl ether, and then adding an equal volume of 25% ethanolic KOH (Sigma P-1767). The resulting mixture was left at least two hours at room temperature in the dark, and then the carotenoid solution was washed with water to remove the alkali. In order to prevent the loss of polar carotenoids, the hypophase was re-extracted with ethyl ether until it became colorless. Unless absolutely necessary, saponification is the step to be avoided in carotenoid analysis. Due to the fact that saponification introduces isomerization of carotenoids, therefore, both saponified and unsaponified extracts were analyzed for comparison.

4.2.4 Concentration

Since the carotenoid extracts were dissolved in large amounts of diethyl ether, it was necessary to concentrate the extracts for chromatographic analysis. To do this, the extracts were transferred into the round-bottomed evaporation flask of rotovap Model RE

121 (Buchi). The system was filled with inert gas or kept under vacuum during the entire extraction process to avoid oxidation of the polyenes. With only a trace amount of water in the flask, low boiling-point ethyl ether was added into the evaporation flask and evaporating the solvent continued until the solution was nearly dry (10-20mL).

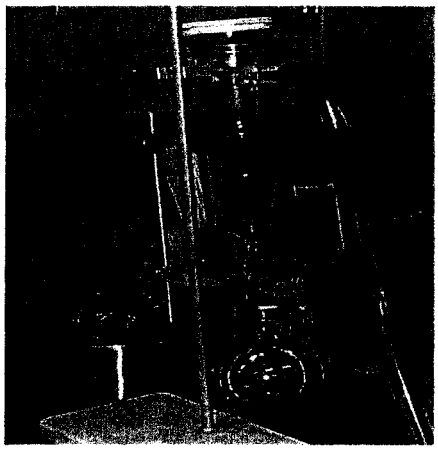

Figure 2: Buchi RE 121 Rotovap.

All carotenoid extracts were stored in covered flasks when processed; and they were stored in covered test tubes for long term storage.

The final extracts were loaded into a 10 mL syringe and filtered by 0.2 μm HPLC syringe filters (Pall 4905) to ensure optimum removal of particles before analysis.

4.3 Carotenoid Separation and Analysis

Agilent HP series 1100 HPLC-MSD module includes a G1946D mass spectrometer detector with an APCI source, a G1315A DAD, binary pump, auto sampler, automatic solvent degasser, and automatic temperature control column compartment.

Although guard columns could increase the flow path and cause band broadening, it was used to protect the column from cellular debris and proteins.

4.4 HPLC Separations

4.4.1 Using Pre-Determined Solvent Systems

The mobile phase system for the initial runs followed the framework determined previously [58]: equilibration of the column with 7:7:86 (v/v/v) water (Alfa Aesa 22934)-acetonitrile-methanol, which is the mobile phase A, followed by an isocratic flow with the same composition for the first 35 minutes after the injection. The solvent proportion was changed to pure acetonitrile (Alfa Aesa 61001-0040), which is the mobile phase B, from the 35th to the 40th minute and maintained the setting till the 60th minutes. After the 60^{th} minute to the 65^{th} minute, the solvent composition was returned to 7:7:86 (v/v/v) water-acetonitrile-methanol until the 70^{th} minute to re-equilibrate for the next sample. The sample injected was 10 μL, and the UV-Vis signal was monitored at 280nm for proteins, 450nm for β-carotene, and 478nm for astaxanthin and carotenoid glycosides. The mobile phase flow rate was fixed at 1 mL/min.

4.4.2 HPLC Column

From the retention order listed in 2.4.1, astaxanthin is expected to elute much earlier than β-carotene in reverse phase liquid chromatography. A 4.6 mm x 250 mm Supelco C18 column with 5 μm packing (Supelco 504971) was used for carotenoid separation.

Based on the results described by Albert [59] and Bell *et al.* [60], the column temperature was fixed at 298 K for optimal selectivity of isomers.

4.4.3 Carotenoid Elucidation Using LC/MS

An Agilent G1946D LC-MS mass spectrometer with atmospheric pressure chemical ionization (APCI) was used in this experiment. The MSD parameters depend on the solvents and the type of instrument used. The capillary voltage must be measured to find the optimized discharge voltage. Therefore, the testing voltage values were set from 2500V to 4000V (maximal voltage). Since 5 μA is sufficient to ionize most molecules, this parameter will remain unchanged throughout the experiment. The vaporization temperatures were also tested. Again, based on the values obtained from the literature, the vaporization temperatures were adjusted from 325° to 400°C. Nebulizer pressure can be fixed at 45 psi, and the drying nitrogen gas can also be fixed at 7 L/min [55].

Table 6: Predetermined Parameters for Agilent G1946D MSD.

Capillary voltage	Vaporization temp.
2500	325
3000	350
3500	375
4000	400

CAPTER 5.0 ERROR ANALYSIS

5.1 Error Prevention

Errors can be introduced in carotenoid analysis due to the polyene's reactive properties. Exposure to light and heat must be avoided once carotenoids have been extracted from the bacteria. Cell debris must be removed promptly to prevent further enzymatic reactions. In addition, all measurement devices / instruments were calibrated to obtain proper measurements if any astaxanthin and β-carotene were discovered from the bacteria samples. At least three sets of LC/MS results were obtained to ensure the stability of the instruments.

5.2 Optical Density Measurements

The optical density for bacteria samples were monitored using the UV-Vis spectrophotometer mentioned in section 4.1.2. Three optical density values were taken to ensure the precision of the measurements.

CHAPTER 6.0 RESULTS AND DISCUSSION

6.1 Frozen Cultures

Five strains of bacteria previously isolated from Calistoga Hot Springs were used for this work (coded a3, EB01, EDB1, FlP02, FDP01). 250 mL of R2A broth were inoculated with 2 mL of frozen culture of each bacteria. The growth temperature was controlled according to section 4.1.2, and the optical density was measured at 600nm. The growth patterns of the five bacterial strains are shown in Figure 3.

Three of the strains have sigmoidal growth patterns typical of bacteria grown in a closed system, or batch culture. From the fifth to the tenth hour, as shown in Figure 3, which is the exponential (log) phase, the optical densities increased rapidly. After 10 hours, the growth rate slowed down.

6.2 Bacterial Growth Using Single Colony

After the initial selection, the three strains of bacteria were grown in 50 mL shaker flasks and on agar plates. As with the frozen cultures, the growth temperature remained unchanged. The average lag phase was ten hours, and the average exponential phase was approximately seven hours. The maximal absorbance rarely reached 0.6 AU. Sigmoidal patterns were again observed for these runs. The results of the bacterial growth experiments are illustrated in Figures 4 to 6:

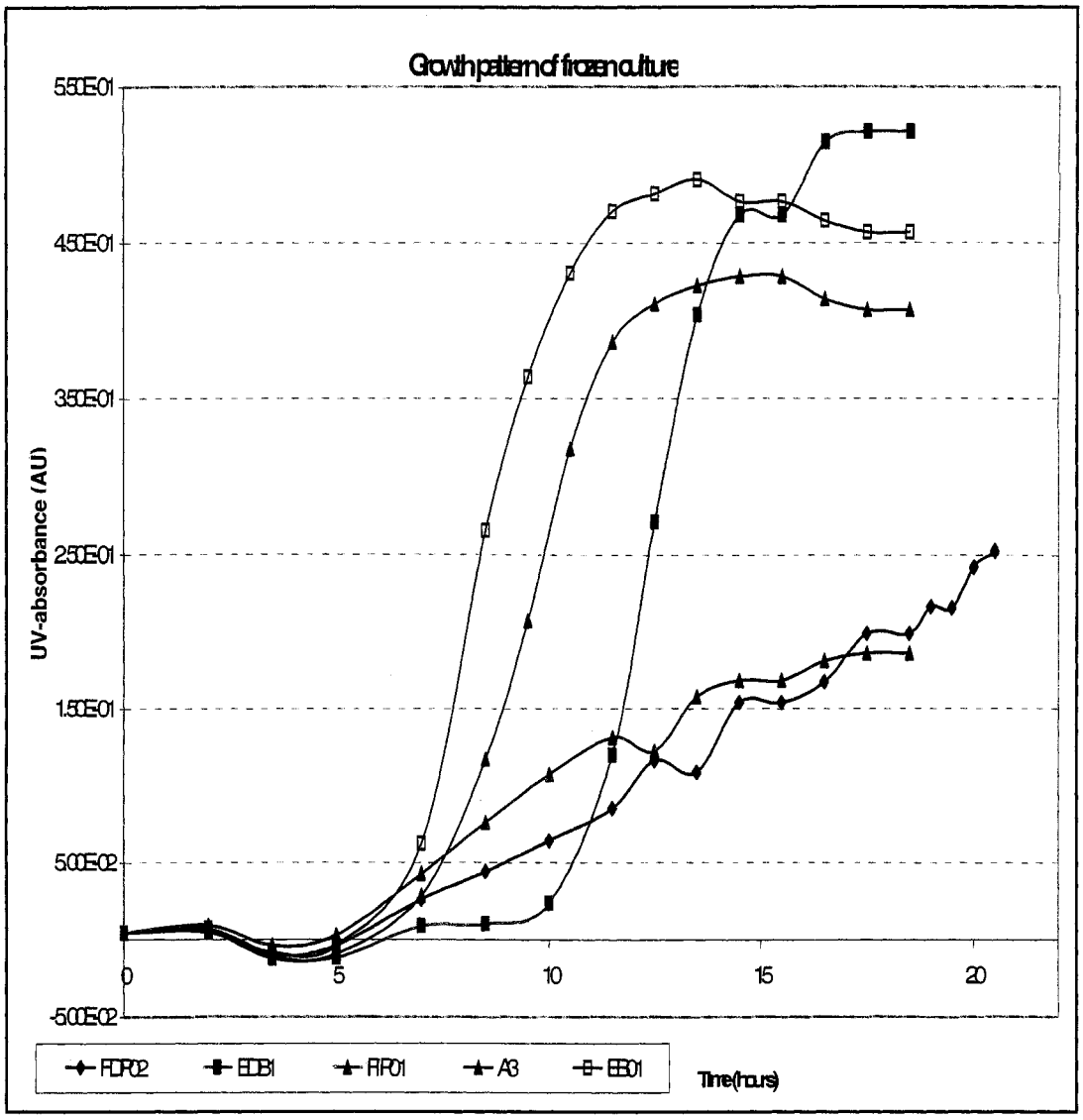

Figure 3: The growth pattern of frozen cultures.

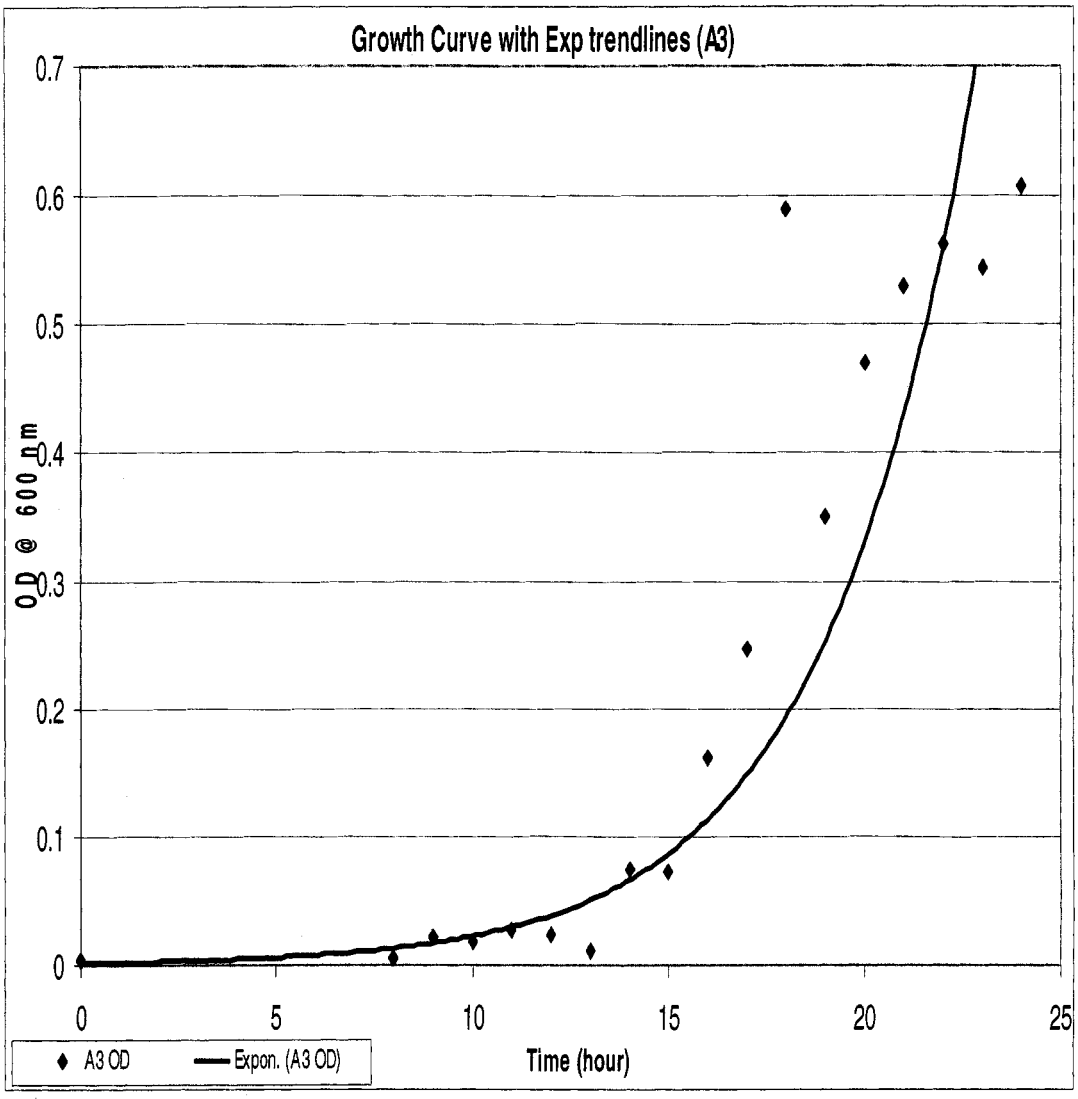

Figure 4: The growth pattern of a3 in 50 mL of R2A broth from a single colony.

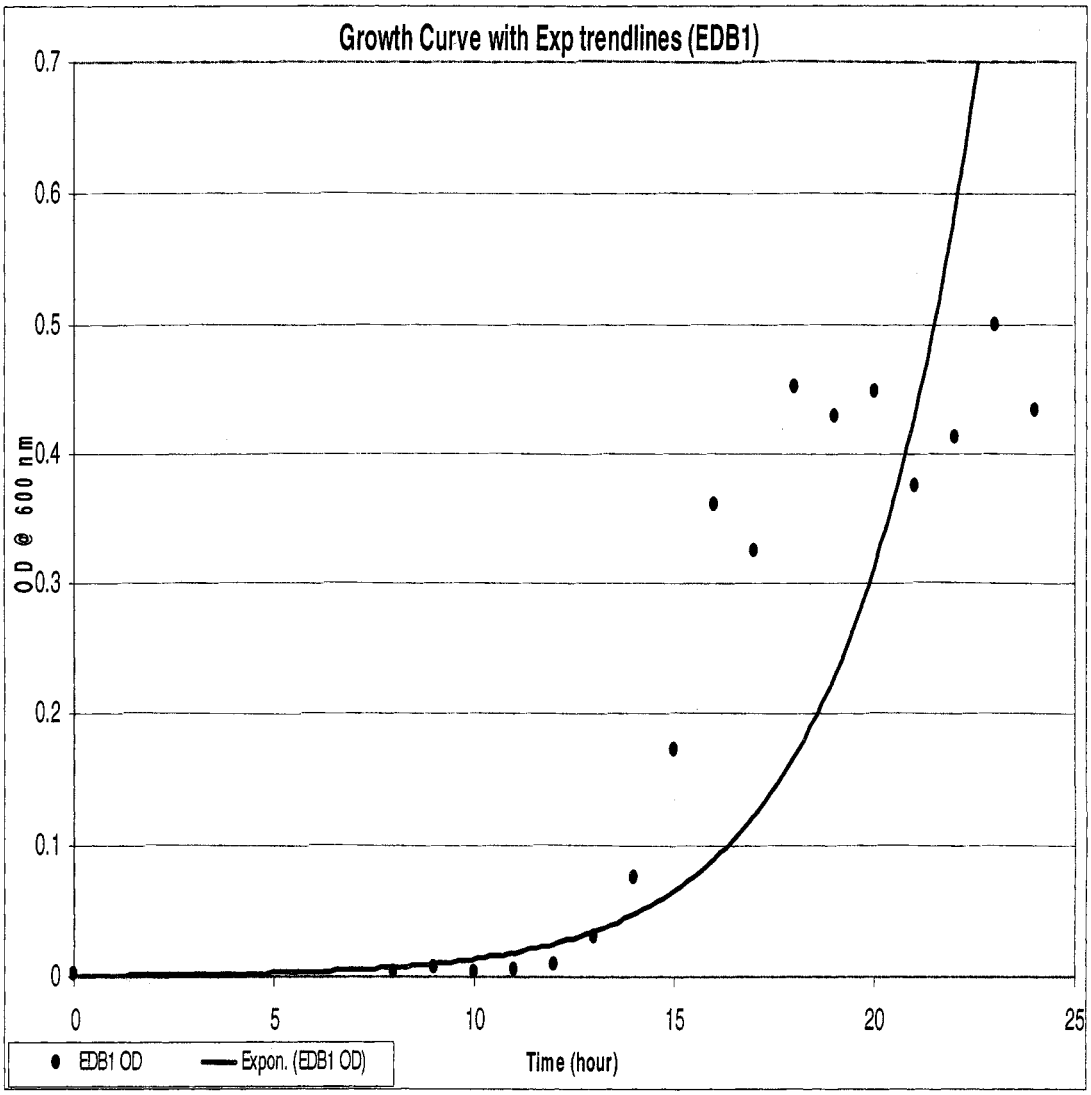

Figure 5: The growth pattern of EDB1 in 50 mL of R2A broth from a single colony.

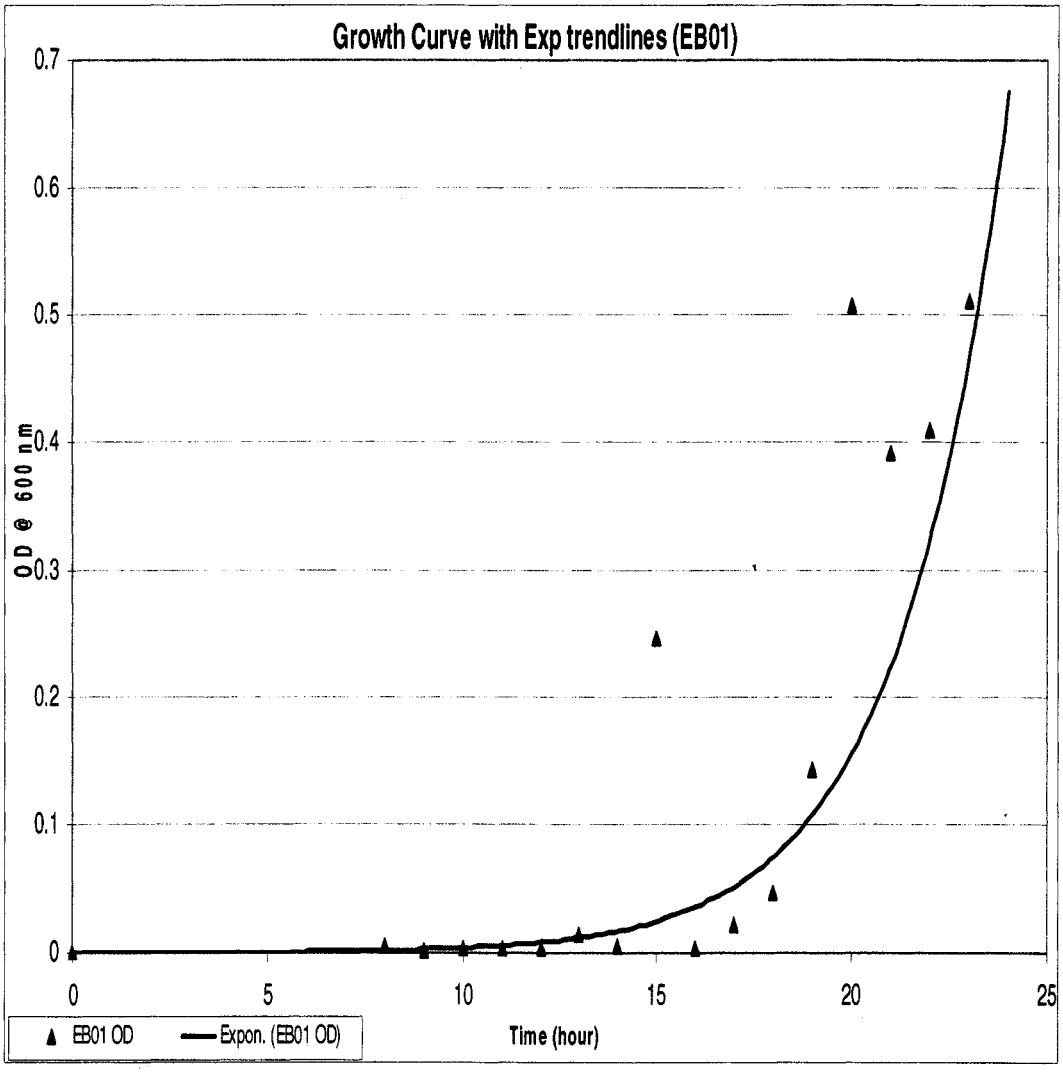

Figure 6: The growth pattern of EB01 in 50 mL of R2A broth from a single colony.

6.3 Liquid Bacteria Cultures

A liquid inoculum was prepared for a larger-scale liquid culture. The bacteria in a 250 mL liquid culture started to grow five hours after inoculation (Figure 7). If the seed was inoculated between 0.1 to 0.3 AU, final optical densities of 1.9 – 2.3 were achieved, as can be seen in Table 7.

Table 7: The Maximal Growth Rates of Liquid Cultures.

	A3	EB01	EDB1
Exp. Phase (hours)	6	7	8
Abs. Max (AU)	2.3	2.3	1.9
Inoculum OD at Transfer	0.2 ~ 0.3	0.1 ~ 0.2	0.2
Color Observed	Red	Orange	Pink-Red

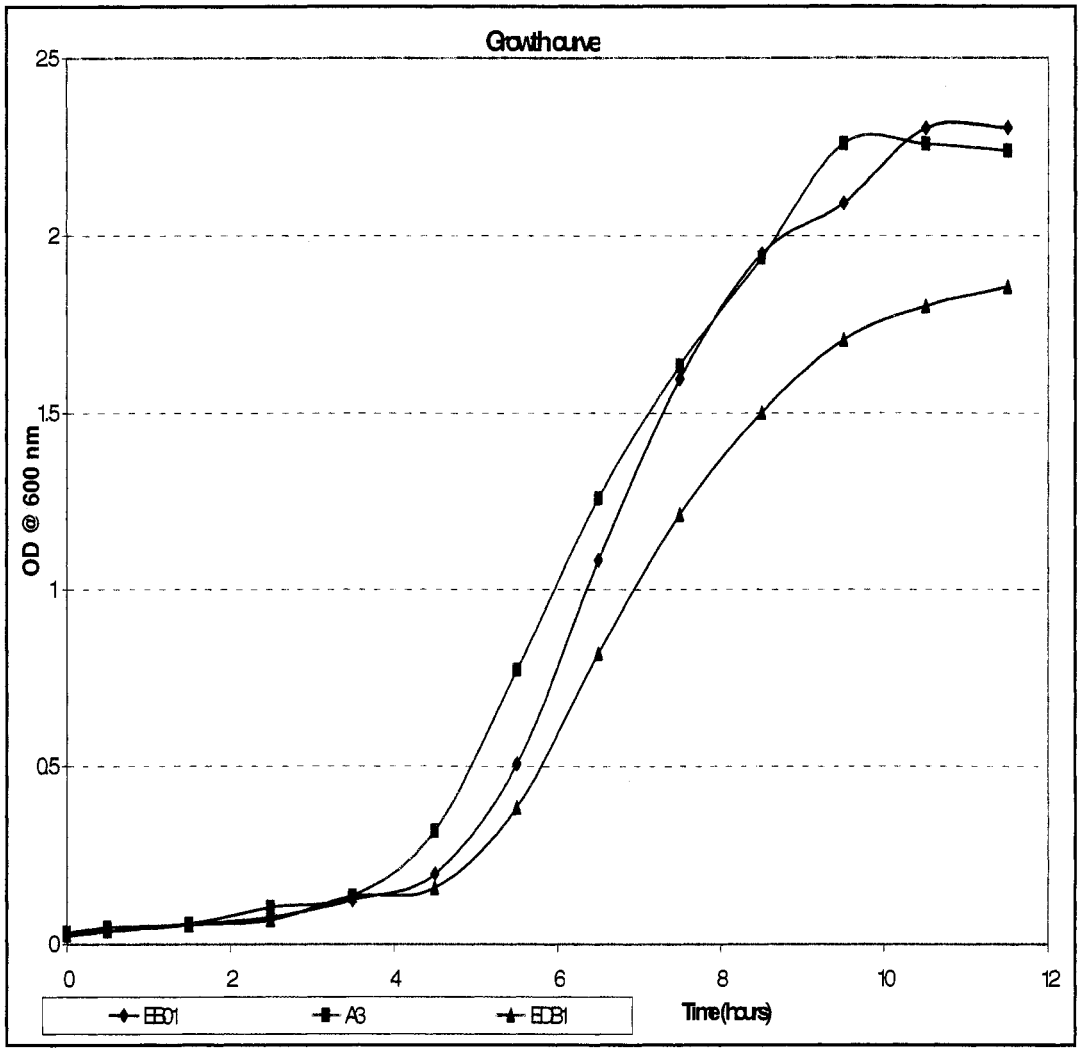

Figure 7: The Growth Curves of Liquid Bacteria Cultures (250 mL).

6.4 Bacteria Cultures on Solid Media

Solid bacterial cultures were developed on R2A agar by transferring bacteria from the liquid cultures during the exponential phase. The solid cultures required 20 to 24 hours to develop. The colors of the bacterial colonies were consistent for more than five generations. The colors of the bacteria colonies were red or orange (Figure 8).

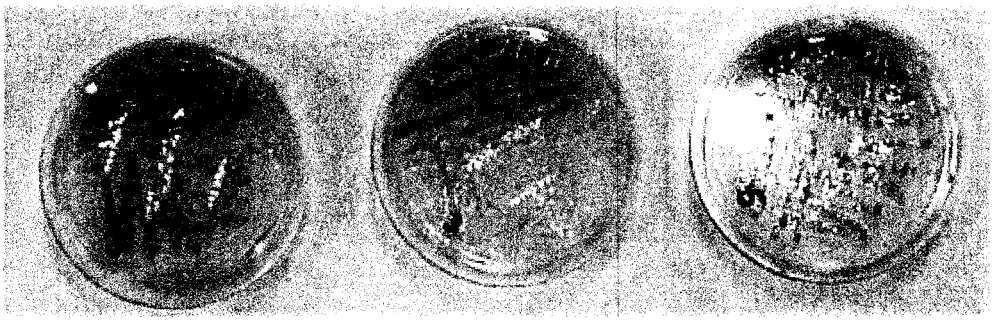

Figure 8: Solid Cultures Developed on R2a Agar.

6.5 Optical Density and Cell Mass

Dry cell weights from liquid bacterial cultures were determined. To do this, the cultures' optical densities, volumes, and the dry weights were measured. The conversion factors for each species were determined from the slopes of the dry weight graphs, with the cell densities (g/L) on the ordinates and the optical density readings at 600 nm on the

abscissas. Figures 9 – 11 illustrate how the conversion factors were obtained. The summarized conversion factors for each species are tabulated in Table 8.

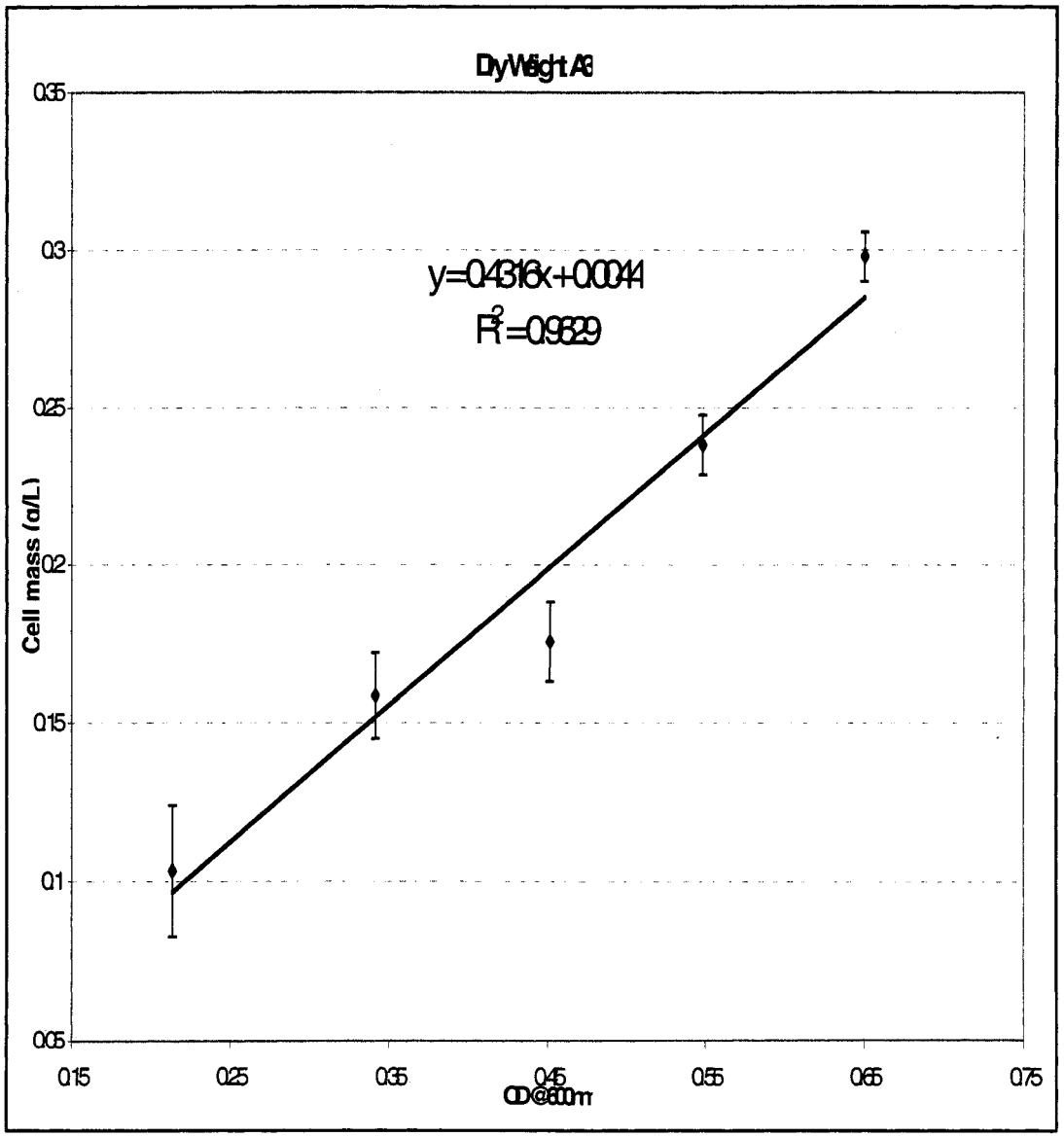

Figure 9: The dry weight measurements and the conversion factor of bacteria A3.

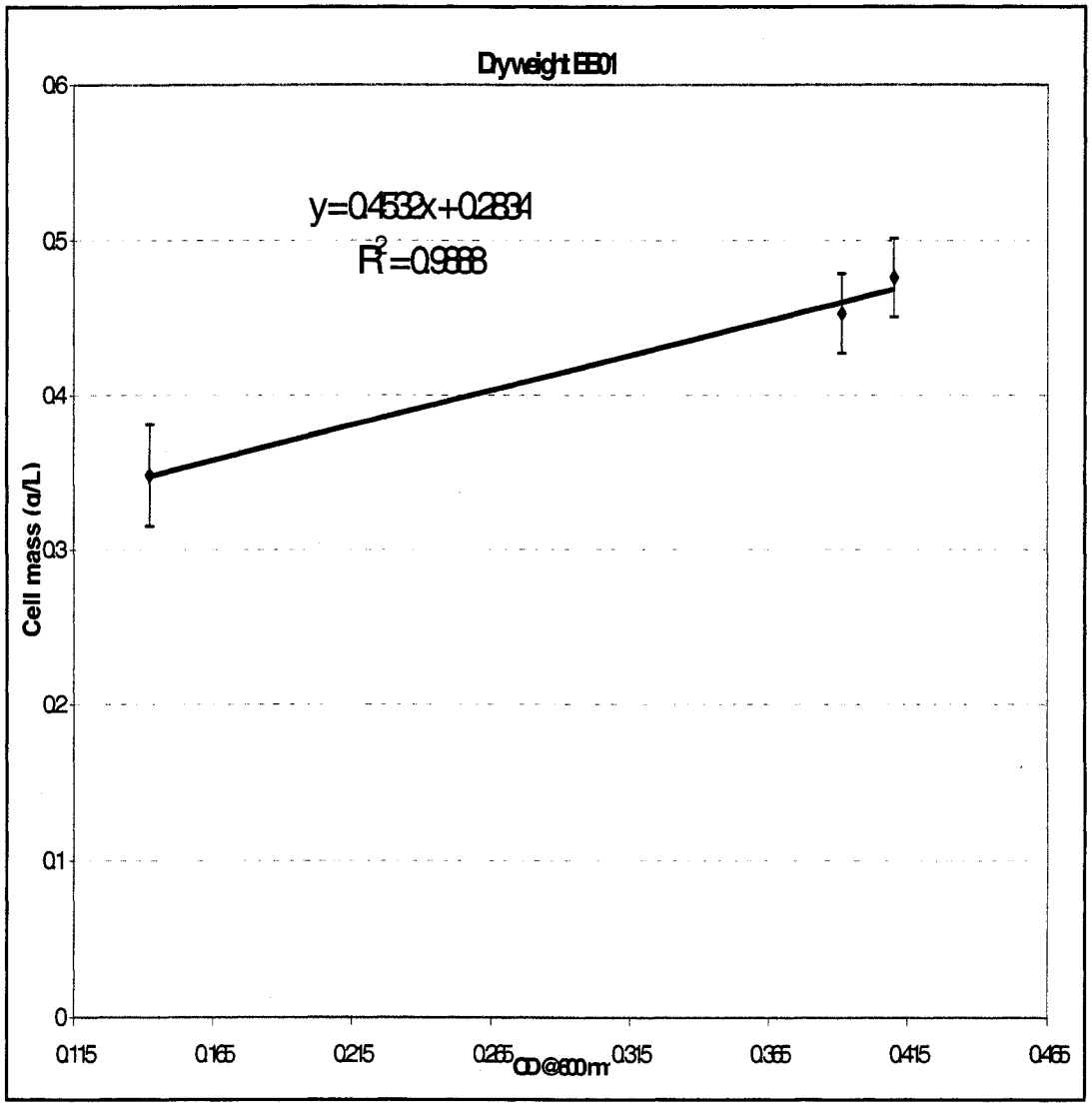

Figure 10: The dry weight measurements and the conversion factor of bacteria EB01.

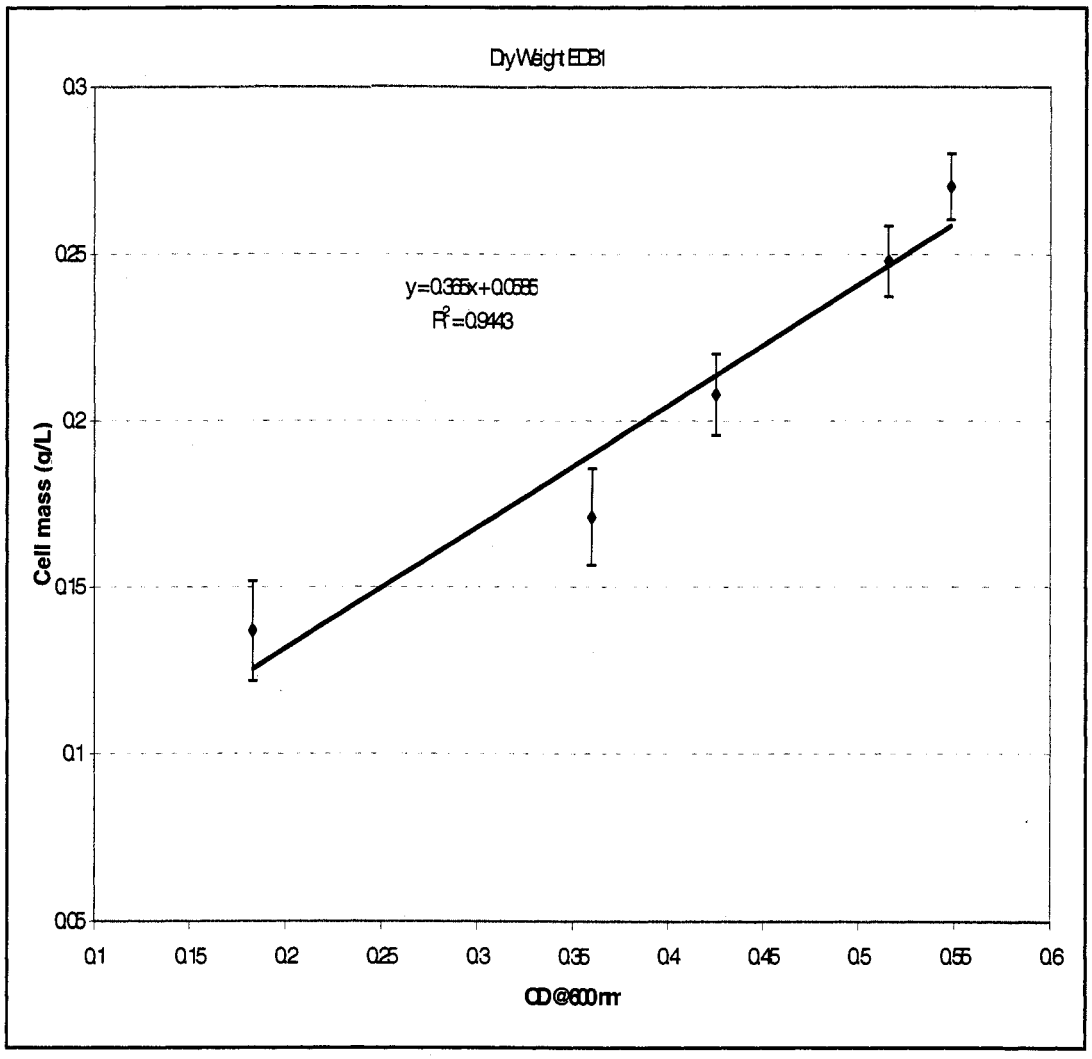

Figure 11: The dry weight measurements and the conversion factor of bacteria EDB1.

Table 8: Summarized Conversion Factors for the Species.

	A3	EB01	EDB1
Conversion factor	0.43	0.45	0.37
Residuals	0.96	0.99	0.94

The dry weight curves for all bacteria are linear with small residuals. The results suggest that the dry weights of each bacteria culture can be interpolated with reasonable accuracy.

With the conversion factors determined, the dry weights of the bacterial cells can be calculated using Equation 1:

$$\text{Dry Cell Mass} = \text{Conversion Factor} \times \text{OD in g/L} \qquad \text{Equation 1}$$

Using equation 1, the maximum dry weights for the strains were calculated and are listed in Table 9.

Table 9. The Calculated Dry Weights.

	A3	EB01	EDB1
Maximum D.W (g/L)	1.4	1.5	0.99

6.6 Carotenoid Extraction

6.6.1 Centrifugation and Extraction

The procedure of centrifugation was outlined in section 4.2.1. It is a repetitive process for separating cellular debris and large molecular weight compounds from the bacteria carotenoids.

The bacteria were harvested in 50 mL tubes and centrifuged at the end of the bacteria culture. The cell pellets displayed either orange or red colors after centrifugation. The supernatants were slightly turbid, but almost colorless, and they were filtered and discarded.

The extraction was carried out according to the procedures indicated in section 4.2.2. When isopropanol was added to the cell pellets, the supernatants became deep orange after centrifugation. These colored supernatants were filtered and stored for concentration / saponification. The extraction with isopropanol was repeated until the supernatant became colorless. At that point, the cell pellets changed from their original colors to white.

The supernatants were filtered by submicron filters according to the methods proposed in section 4.2.2. The pigments stayed on the filters after filtration, but they could be easily washed with isopropanol. The colors of filtered extracts were deep orange.

6.6.2 Concentration and Saponification

The extracts were dried in a rotovap to a dry film on the wall of the flask. The pigments were soluble in ether, ethanol, and isopropanol. It was observed that the

pigments did not partition significantly into a second hexane phase if they were dissolved in ethyl ether first. Likewise, the concentrates had a higher affinity to water if hexane was used as the epiphasic (the organic layer of the extraction solvents) solvent. In contrast, the pigments were only slightly soluble in water if ether was the epiphasic solvent. These observations indicated that the carotenoid extracts could be moderately polar. Five milliliters of ether was used to recover the residual pigments in 20 mL of hypophase (the aqueous layer in a two-solvent extraction).

Saponification was carried out since the initial chromatographic data were noisy, suggesting that lipids, proteins, or other contaminants were present. The process was implemented according to section 4.2.3. After two hours of reaction, the alkali solutions were decanted, and the organic layers were washed with water. At least 50 mL of water was used to lower the pH of each extract to seven. Because ether is not a stable solvent, the epiphases were dried again and dissolved in minimal amounts of ethanol for analysis. The colors remained deep orange after concentration and saponification.

6.7 Carotenoid Separation and Analysis

6.7.1 Carotenoid Detection Using UV/Vis

Concentrated carotenoids were first identified by UV/Vis. In order to gain some initial information about the carotenoids, the extracts were dissolved in hexane so that their UV/Vis spectra could be compared with that of astaxanthin and β-carotene. When dissolved in hexane, β-carotene has a UV/Vis absorption maximum at 450 nm, and astaxanthin at 470nm. The carotenoids produced by the bacteria had a broad absorption

maximum at 478nm in hexane or ethanol (Figure 12), with poorly defined shoulders. The general shape and location of the peaks appeared between 400 ~ 550 nm suggest that carotenoid pigments are present in the bacterial extract.

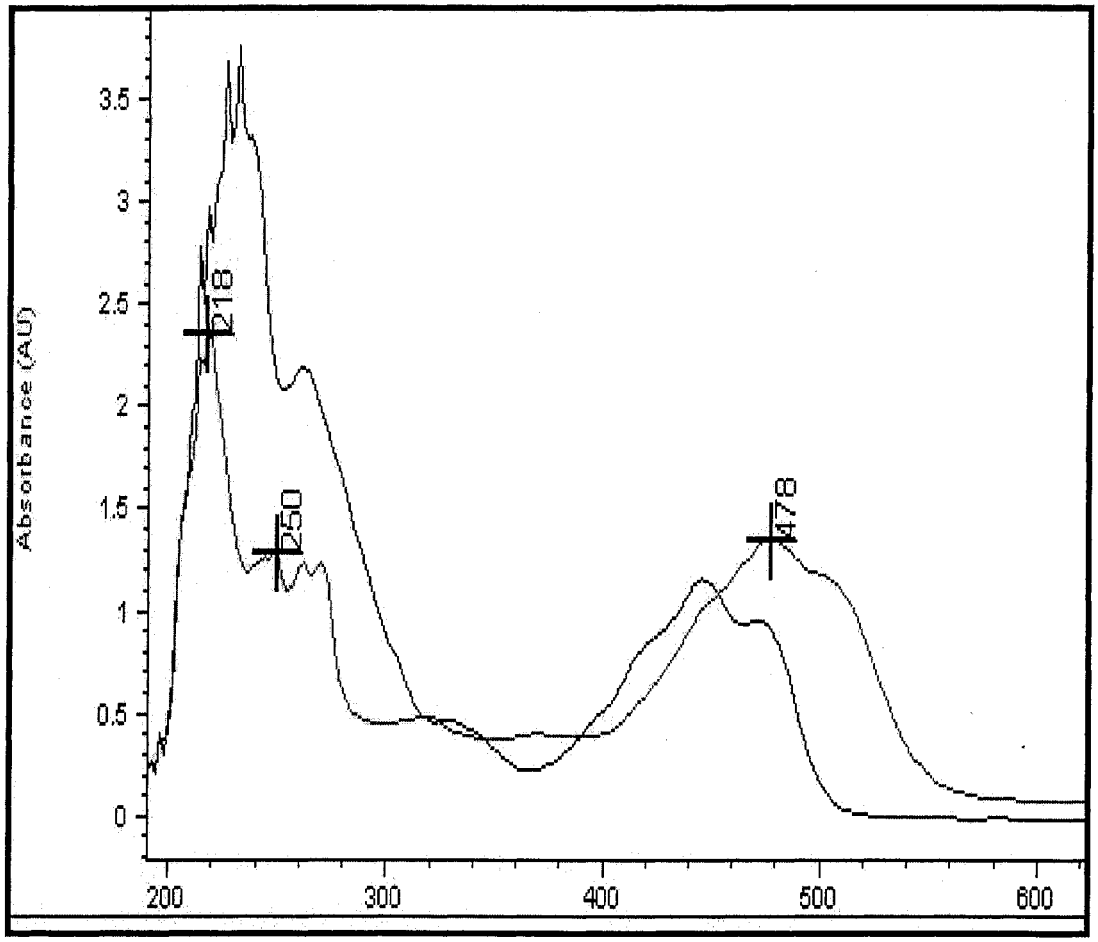

Figure 12: The UV/Vis Spectrum of the Carotenoids Produced by the Bacteria. The spectrum of bacterial carotenoids has a marked absorption maximum at 478nm. The spectrum in the background is β-carotene.

6.7.2 LC/MS Determination of β-Carotene and Astaxanthin

In order to determine if astaxanthin or β-carotene were presented in the bacteria, a mixture of pure astaxanthin (ACROS) and β-carotene (Nature Made) were prepared as the standard solution for analysis. To produce an astaxanthin standard with absorbance of 0.2 AU, 17mg of astaxanthin were dissolved in 30mL of ethanol. The absorbance value at 450 nm of 50μL of β-carotene in 15mL of ethanol was 0.93 AU.

The HPLC method was followed as described in section 4.4.1. The flow rate was 1 mL/min, which is common for a 4.6 mm diameter column with 5 μm packing. The column temperature was maintained at 298K for all analysis. The mobile phase, described in section 4.4.1 was unable to elute highly nonpolar carotenoids such as β-carotene. As a result of this, 68%/32% of methanol/ethyl acetate was used as the mobile phase B to correct the elution problem [30]. Although pure ethyl acetate and water are not miscible, test mixtures of 10% in 90% of mobile phase A in B suggested that the two chemicals were miscible with each other in the presence of co-solvents. The modified mobile phase system provided a reasonably good resolution to both polar and less polar carotenoids using one reverse-phase column.

With the modified mobile phase system, astaxanthin eluted at approximately 8.8 minutes after injection, while the less polar β-carotenes eluted after the 49th minute (Figure 13). Because astaxanthin was separated on a reverse phase column, which has been reported to be less effective in xanthophyll isomer separations [30], no astaxanthin geometric isomers were observed.

Figure 14 shows the HPLC-MS chromatogram of β-carotene and astaxanthin. A sharp peak at 8.9 minutes after the injection was observed on the astaxanthin mass spectrum: 597.3 (Figure 14c), which corresponds to the molecular weight of the compound. Similar for β-carotene, the APCI provided a clean spectrum with a major peak at 537 Da, as shown by Figure 14b.

Due to a less polar character of β-carotene, its elution time appeared to be much longer than that of astaxanthin. In addition, β-carotene was not soluble in any polar solvents; it did not elute until the column was equilibrated with a 100% nonpolar mobile phase.

The analysis showed that all-E-β-carotene eluted first; 9-Z and 13-Z were the last to elute (Figure 15).

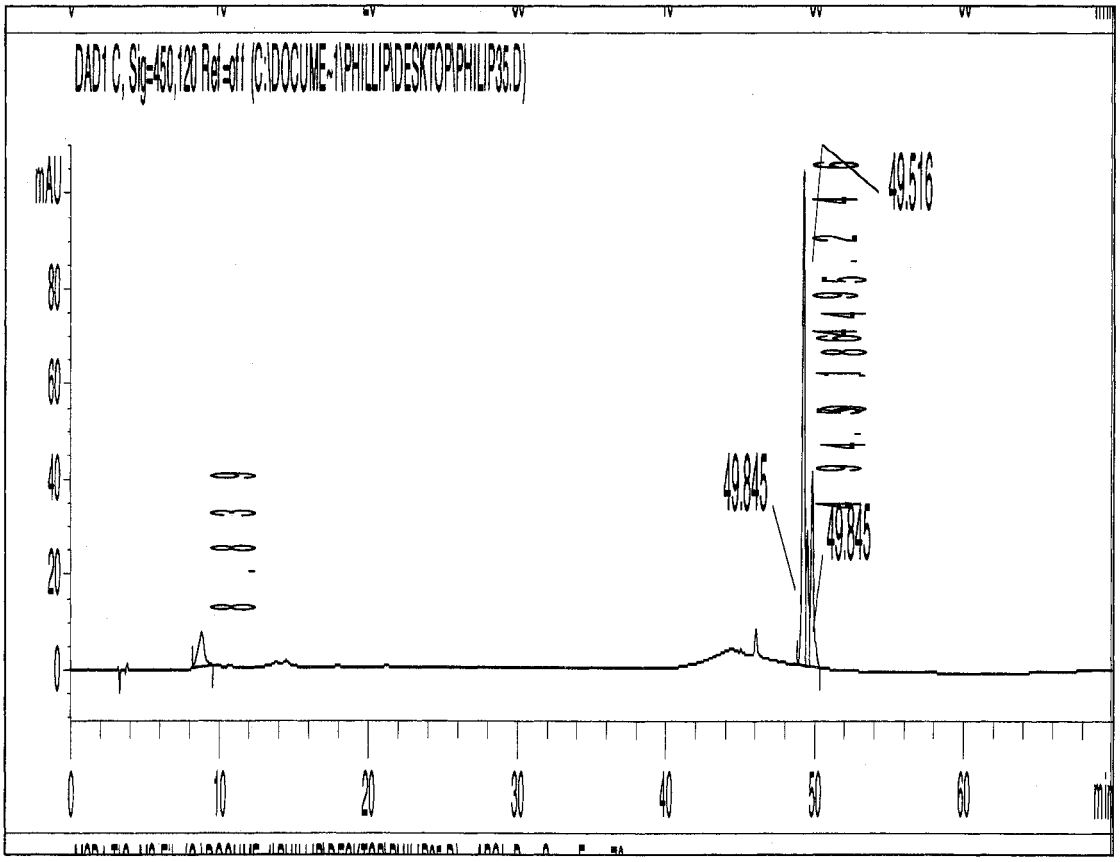

Figure 13: The chromatogram of astaxanthin and β-carotene at 450nm. Peak (1) astaxanthin (8.839 minutes); (2) isomers of β-carotene (49 minutes).

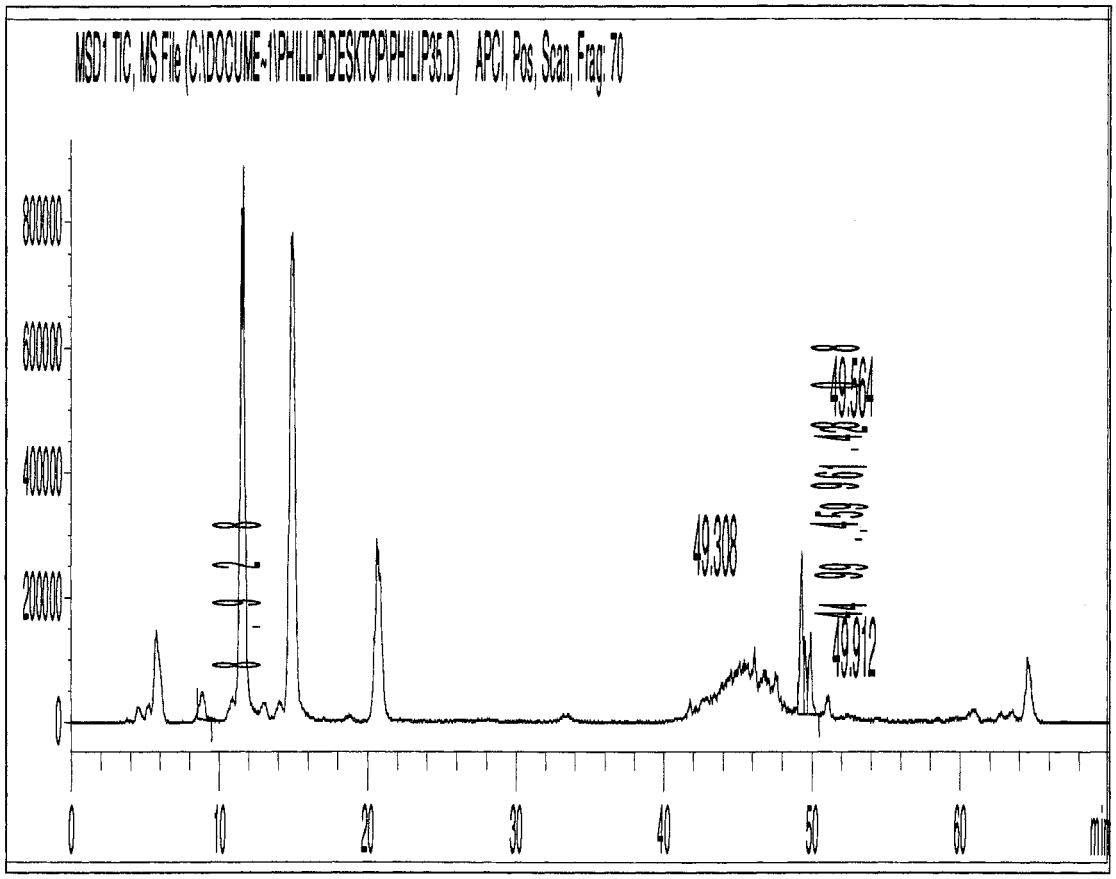

Figure 14a: The mass chromatogram of astaxanthin and β-carotene. Peak (1) astaxanthin (8.928 minutes); (2) isomers of β-carotene (49 minutes).

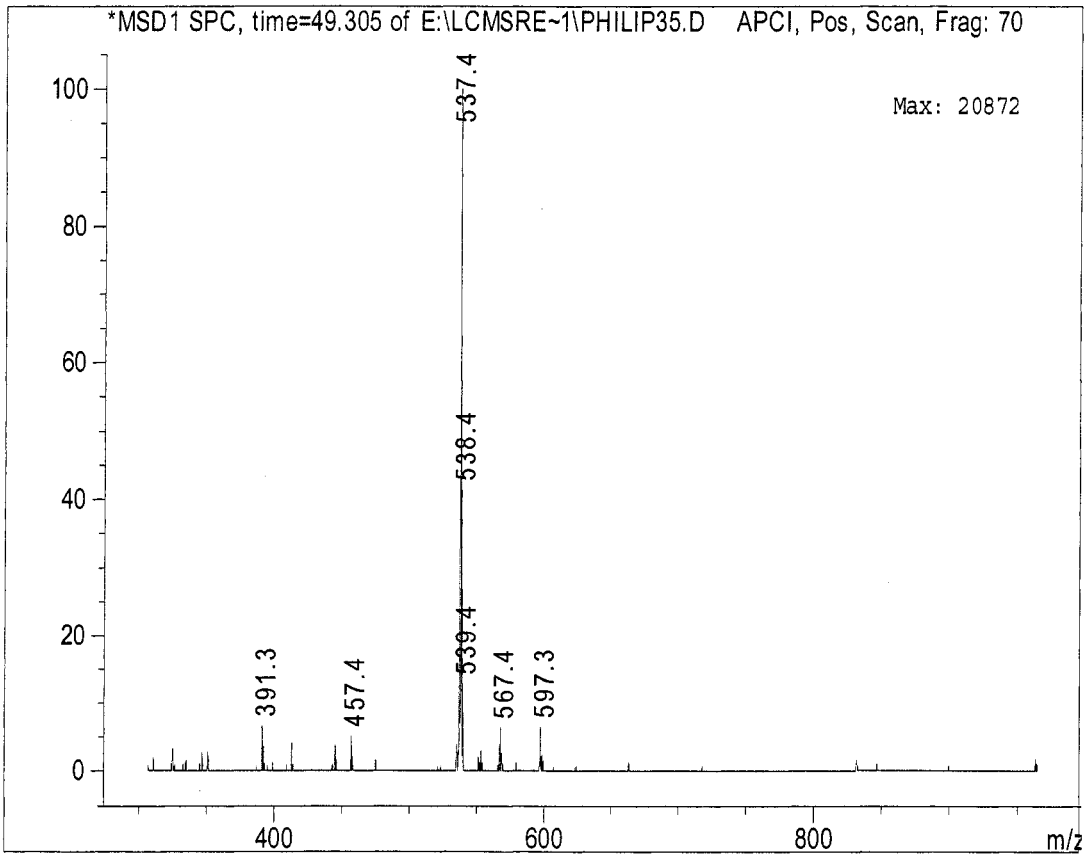

Figure 14b: The mass spectrum of β-carotene.

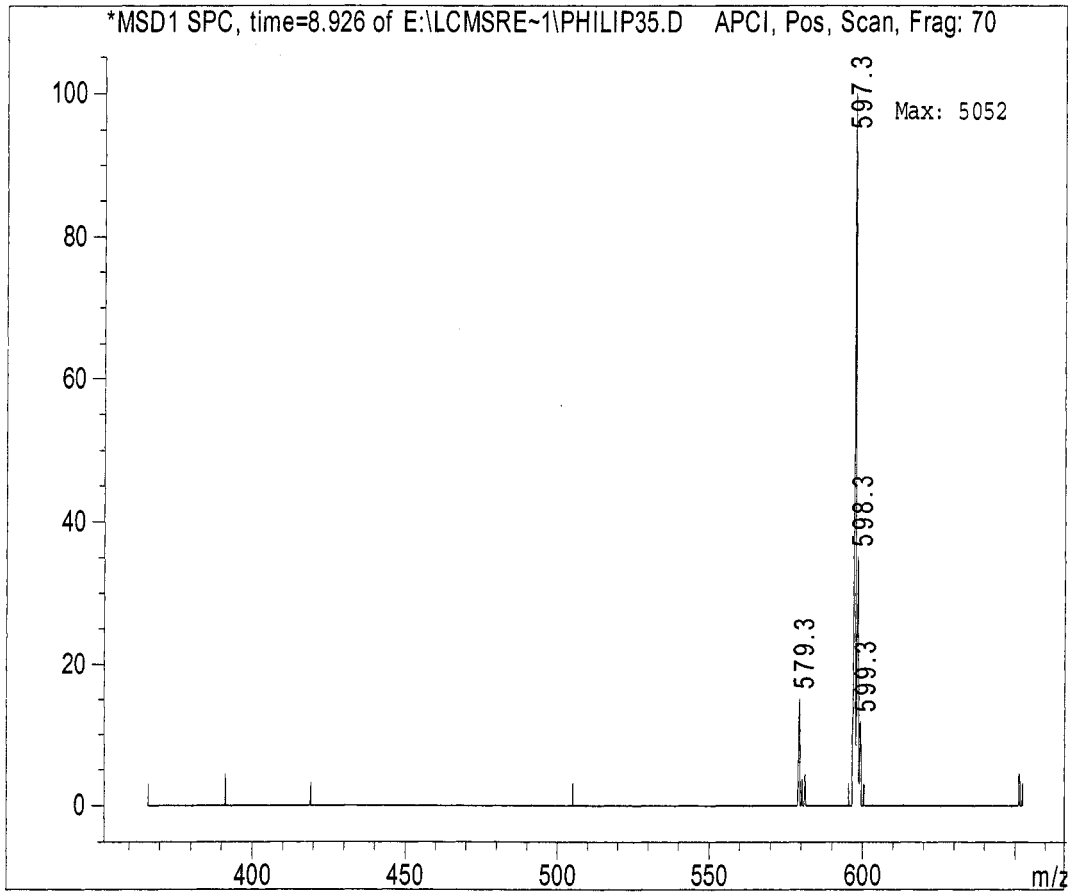

Figure 14c: The mass spectrum of astaxanthin

The UV/Vis spectra of the isomers match the literature values [30]: the all E-β-carotene had an absorption maximum of 452nm with no absorption maximum observed in the 300nm–350nm range; the 9-Z-β-carotene had an absorption maximum of 446nm with a less pronounce but visible Z-peak in the 300nm–350nm range; and the 13-Z-β-carotene had an absorption maximum of 444nm, with an apparent Z-peak. The mobile

phase did not have a bathochromic (an up shift of spectrum position to a higher wavelength due to the effect of solvents) effect on the carotenes. Figure 15 shows the UV/Vis spectra of all β-carotene isomers. The retention times for the three detected β-carotene isomers were 49.2, 49.5, and 49.8 minutes. All of them eluted after the column was equilibrated with 100% of mobile phase B.

The astaxanthin had a substantially different UV/Vis spectrum than did the β-carotene, with an absorption maximum of 470nm in hexane. When the xanthophyll was dissolved in ethanol, a bathochromic effect was observed, specifically, the absorption maximum increased by 8nm (Figure 16). The effects were the same for both ethanol and methanol (mobile phase A is 86% methanol).

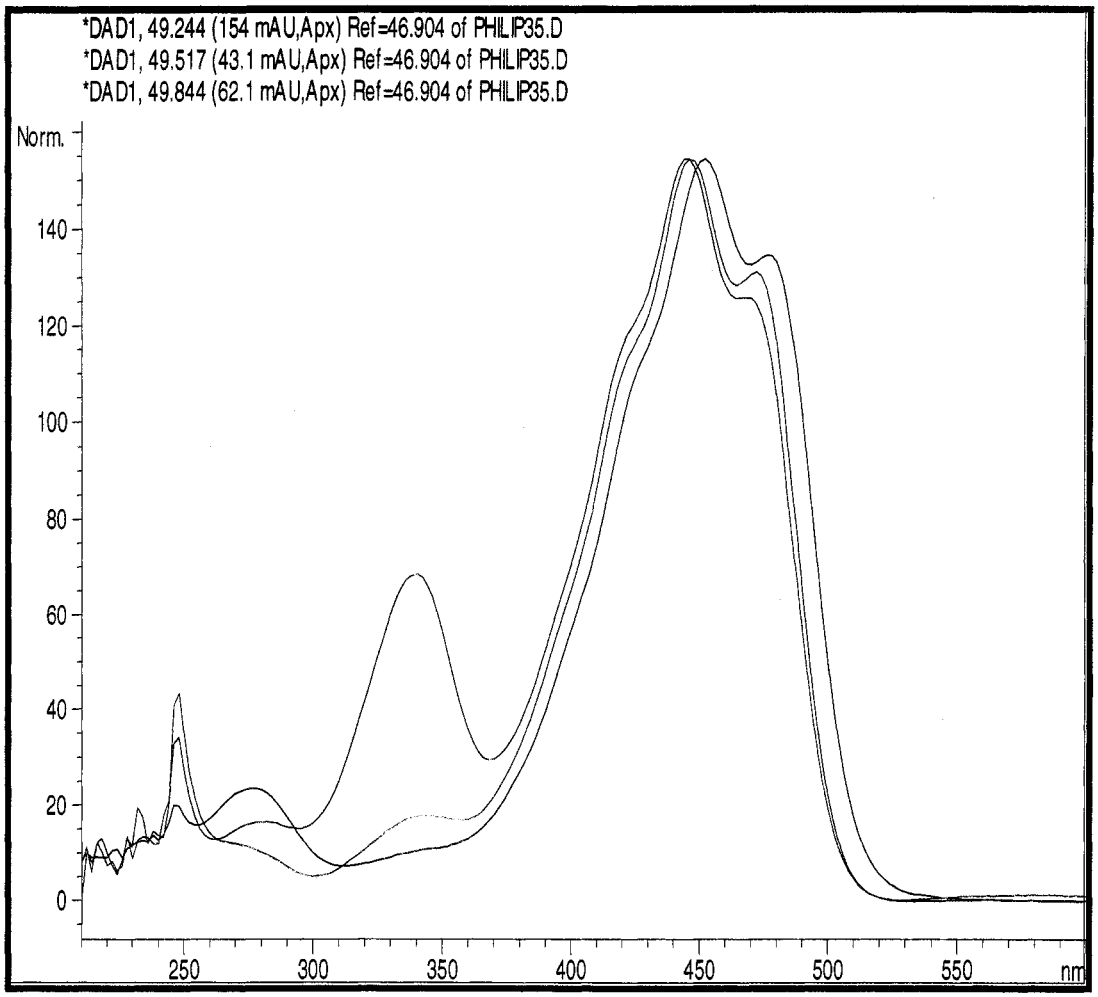

Figure 15: The UV/Vis Spectra of β-Carotene Isomers. The blue line represents all-E-β-carotene; the red line represents 9-Z-β-carotene; the green line represents 13-Z-β-carotene.

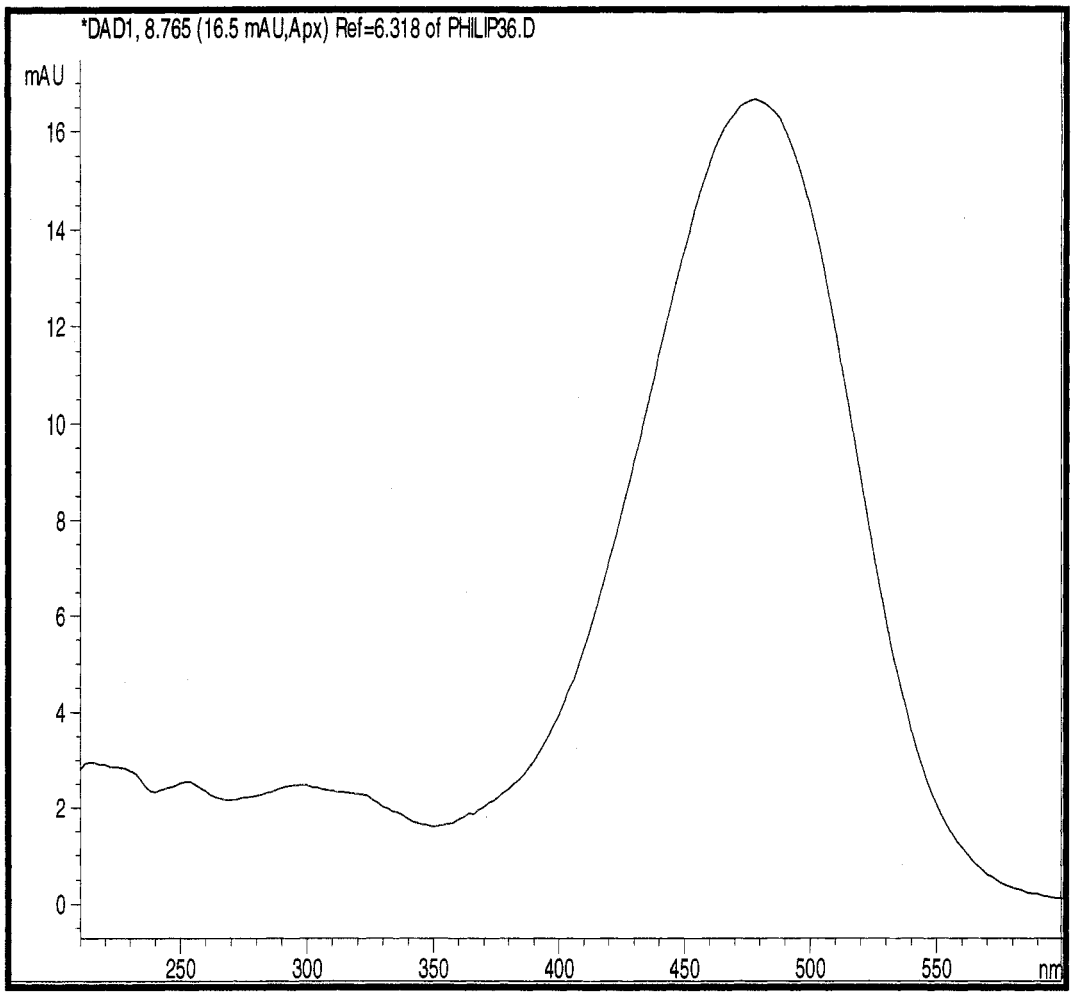

Figure 16: The UV/Vis Spectrum of Astaxanthin in 86/7/7 of Methanol/Acetonitrile/Water.

6.7.3 LCMS Analysis of Unsaponified Bacterial Carotenoids

The carotenoid extracts were first separated by HPLC without saponification. The mobile phases used were the same as those for the β-carotene separation. Both the red and the orange strains contained at least one nonpolar carotenoid.

There is a significant chromatographic difference between the carotenoids produced by the orange-pigmented bacteria and the red-pigmented bacteria. The

chromatograms of orange-pigmented bacteria show a far smaller amount of nonpolar carotenoids than the red-pigmented bacteria. Figures 17 – 19 are 3-D chromatograms that indicate the differences between the carotenoids produced by each species. EB01, the orange-pigmented bacterium, lacks a major nonpolar carotenoid, as the red bacteria do.

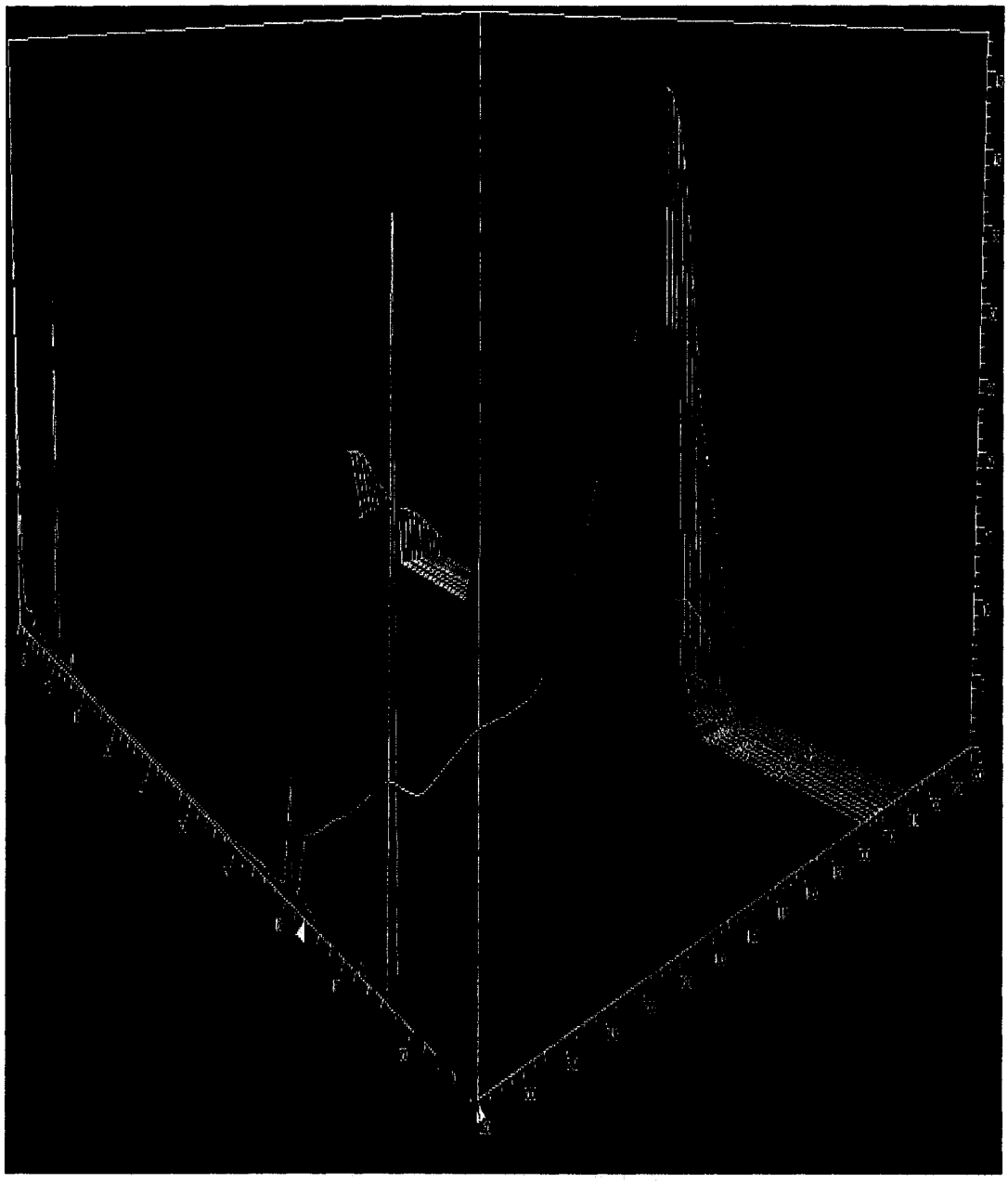

Figure 17: Unsaponified Carotenoids Produced by Bacteria A3.

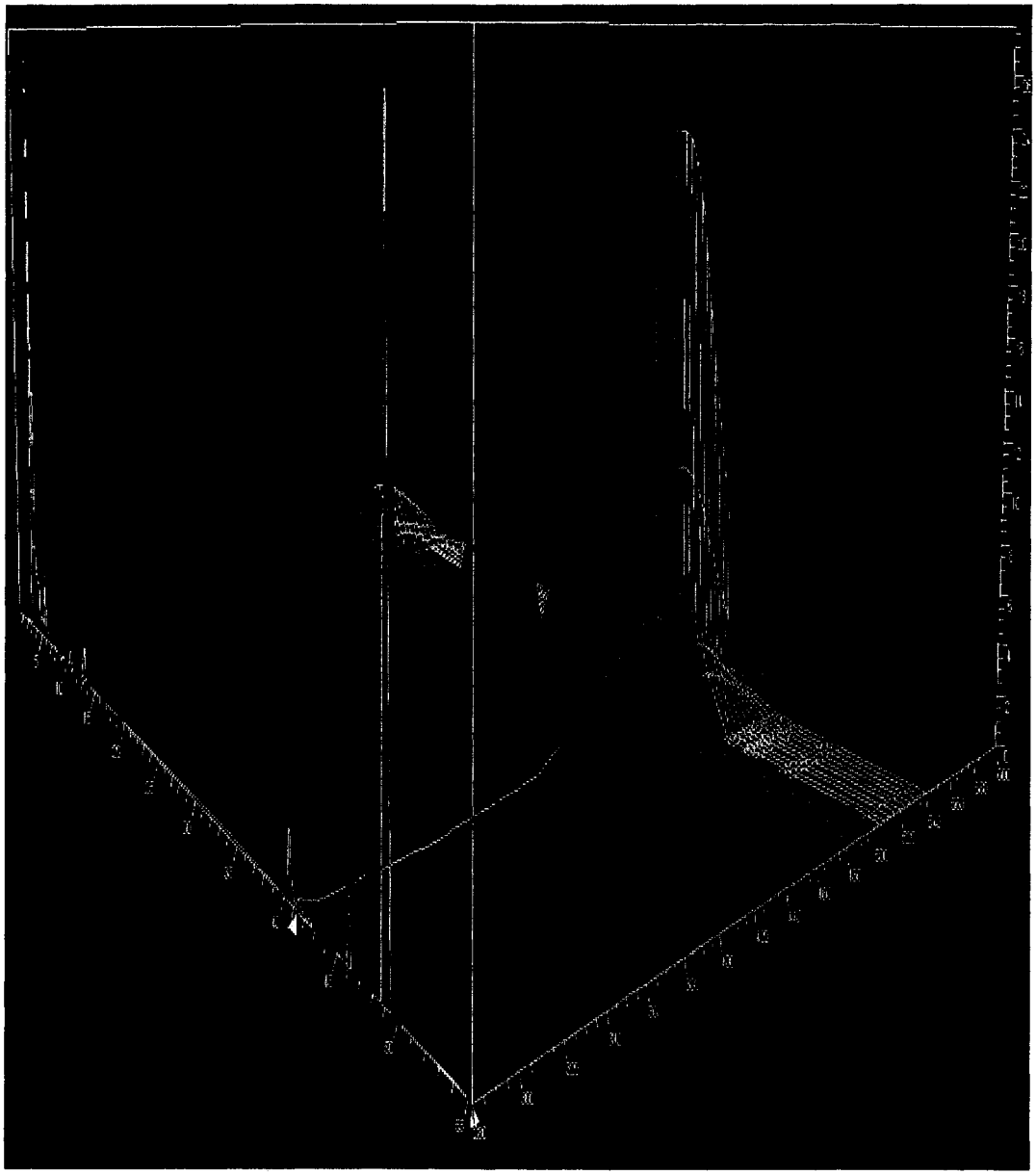

Figure 18: Unsaponified Carotenoids Produced by Bacteria EDB1.

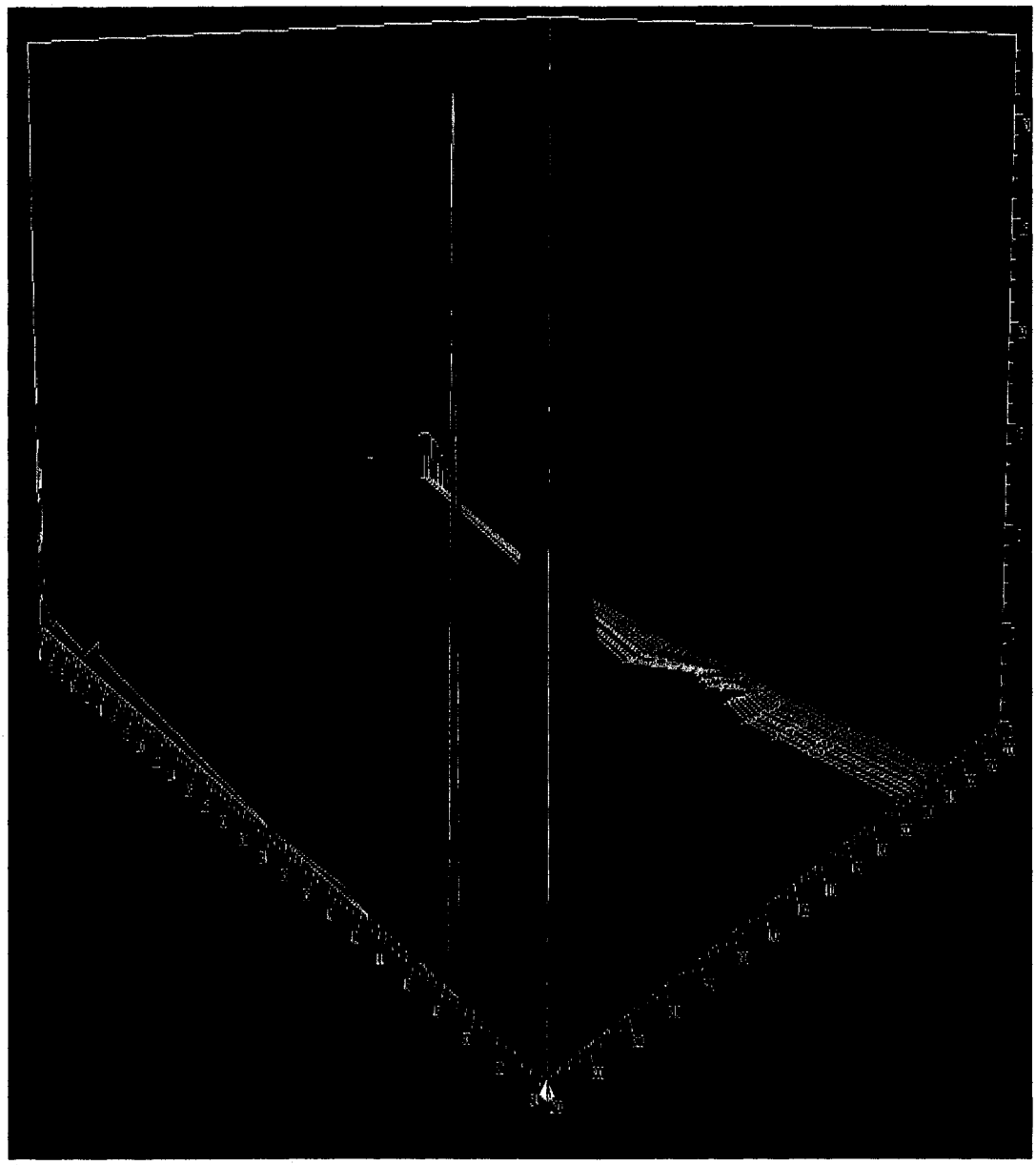

Figure 19: Unsaponified Carotenoids Produced by Bacteria EB01.

The retention times and the mass values of the carotenoids are listed in table 10:

Table 10. Characteristics of the Carotenoids Analyzed (Unsaponified Extracts).

Bacteria	A3 (red)	EDB1 (red)	EB01 (orange)
Retention time (min)	40.5	40.4	40.4
m/z	547	547	547
Retention time (min)	41.7	41.6	41.4
m/z	537	537	537
Retention time (min)	42.7	42.7	---
m/z	547	547	---
Retention time (min)	38.6	38.4	---
m/z	547	547	---
Retention time (min)	---	12.9	13.6
m/z	---	729	729
Retention time (min)	9.5	---	---
m/z	565	---	---
Retention time (min)	6.1	6.2	6.1
m/z	420	420	420

The chromatograms of all carotenoids produced by unsaponified extracts are shown in Figures 20 –22:

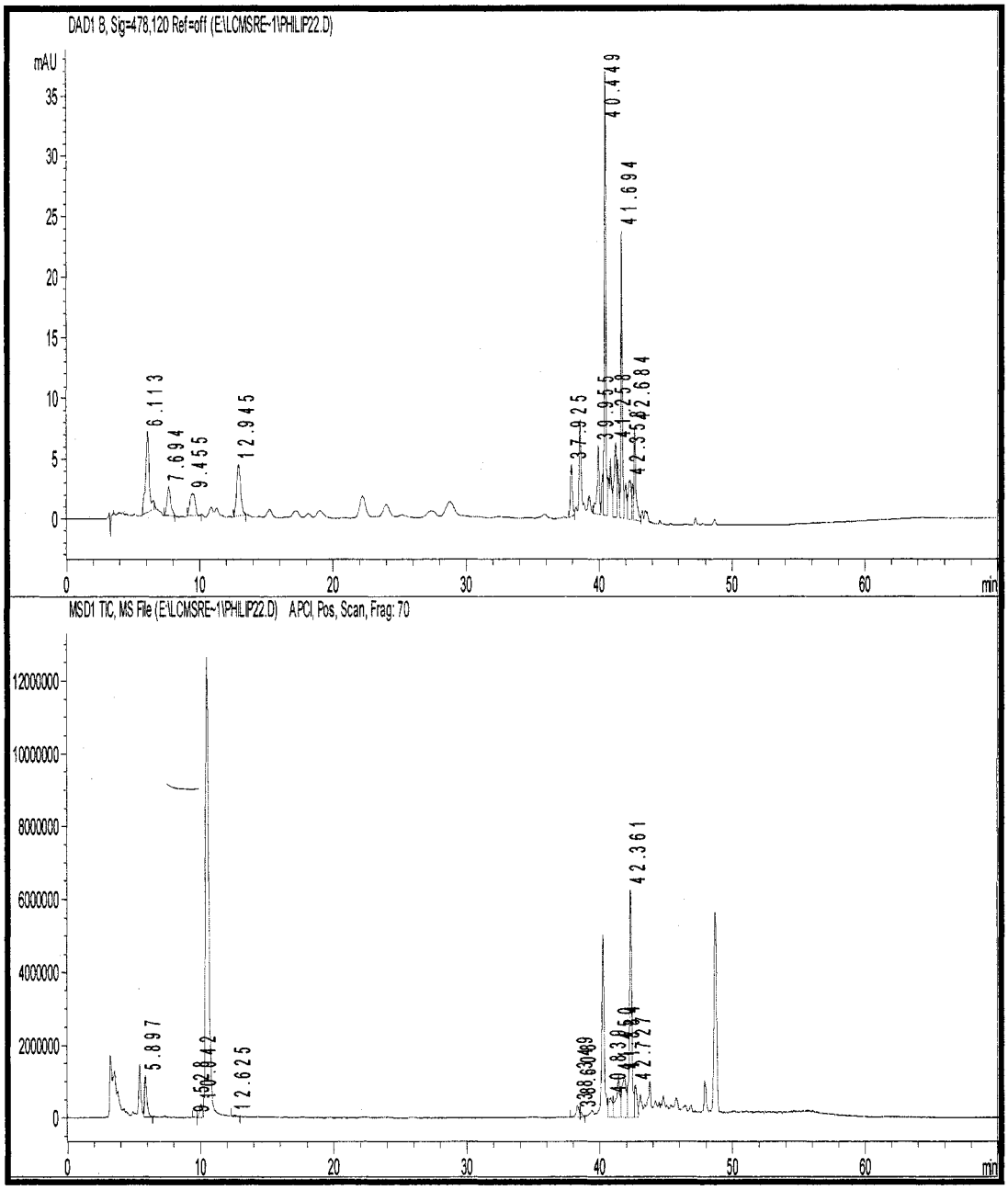

Figure 20: The HPLC-DAD and the HPLC-MS Chromatograms of Unsaponified A3.

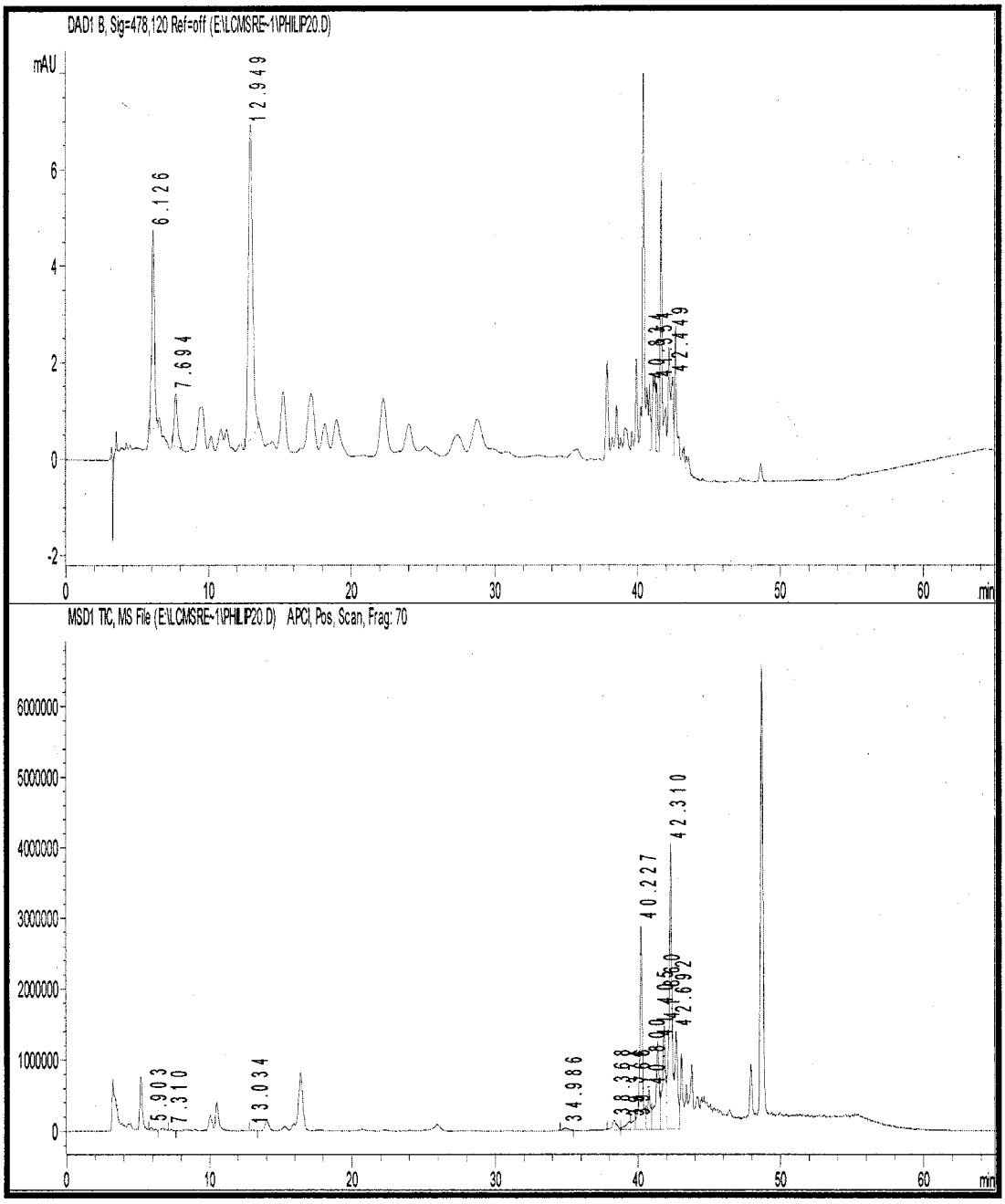

Figure 21: The HPLC-DAD and the HPLC-MS Chromatograms of Unsaponified EB01.

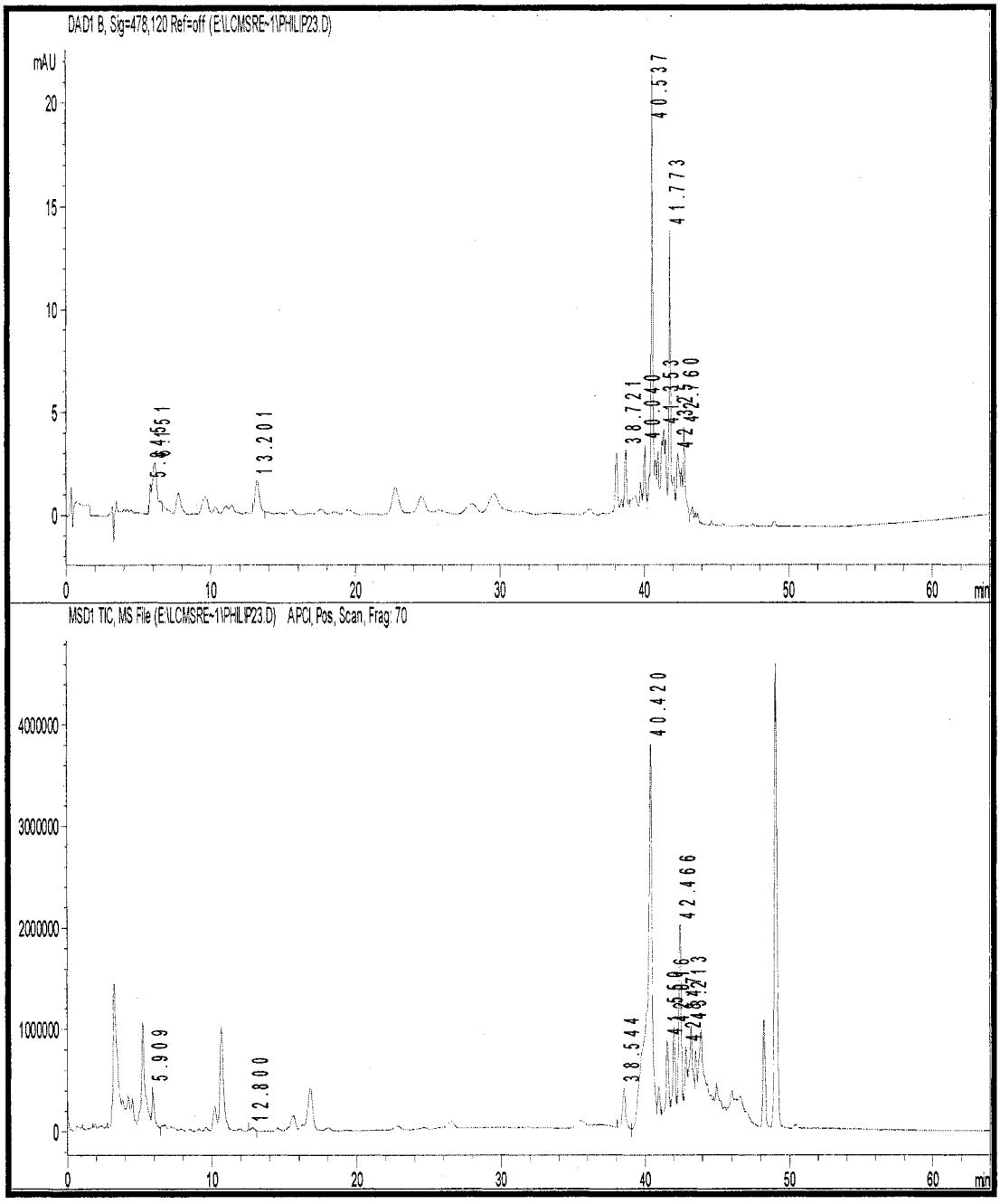

Figure 22: The HPLC-DAD and the HPLC-MS Chromatograms of Unsaponified EDB1.

For A3, a nonpolar carotenoid with a molecular weight of 547 was found. The carotenoid had absorption maxima of 480nm (III) and 505nm (II), and the ratio of II/III was approximately 20%. In addition, the carotenoids with this molecular weight showed peaks that were possibly isomers: one with an elution time of 40.5 minutes, and the other with an elution time of 41.6 minutes. It is possible that the two peaks were isomers since they had the same mass values, and their UV-Vis spectra displayed isomeric relationships. There was a Z-peak appeared at 350nm, which lead to minor hypochromic (an upshift of absorption max as the result of the presence of the Z-peak) shifts for the second (474nm–480nm) and the third (498nm–504nm) absorption maxima. The carotenoid had very weak Z-peaks (Figure 23).

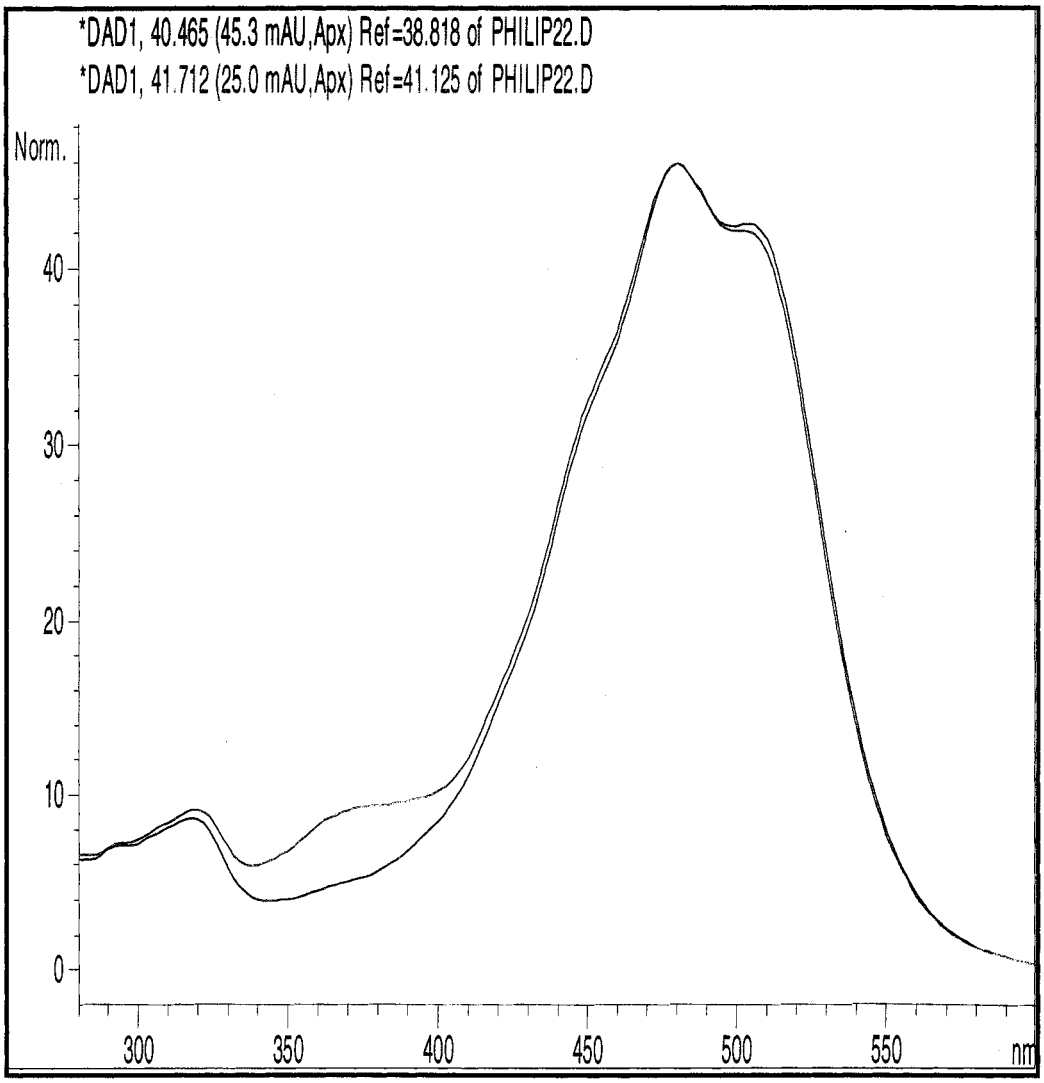

Figure 23: The UV/Vis Spectrum of the nonpolar carotenoid produced by A3.

A minor nonpolar carotenoid with a molecular weight of 537 was observed to have eluted between the two isomers mentioned previously. The compound was not β-carotene, since its absorption maximum was 470nm, and it had a well-defined shoulder. A large Z-peak indicates that the eluent was a Z carotenoid. (Figure 24).

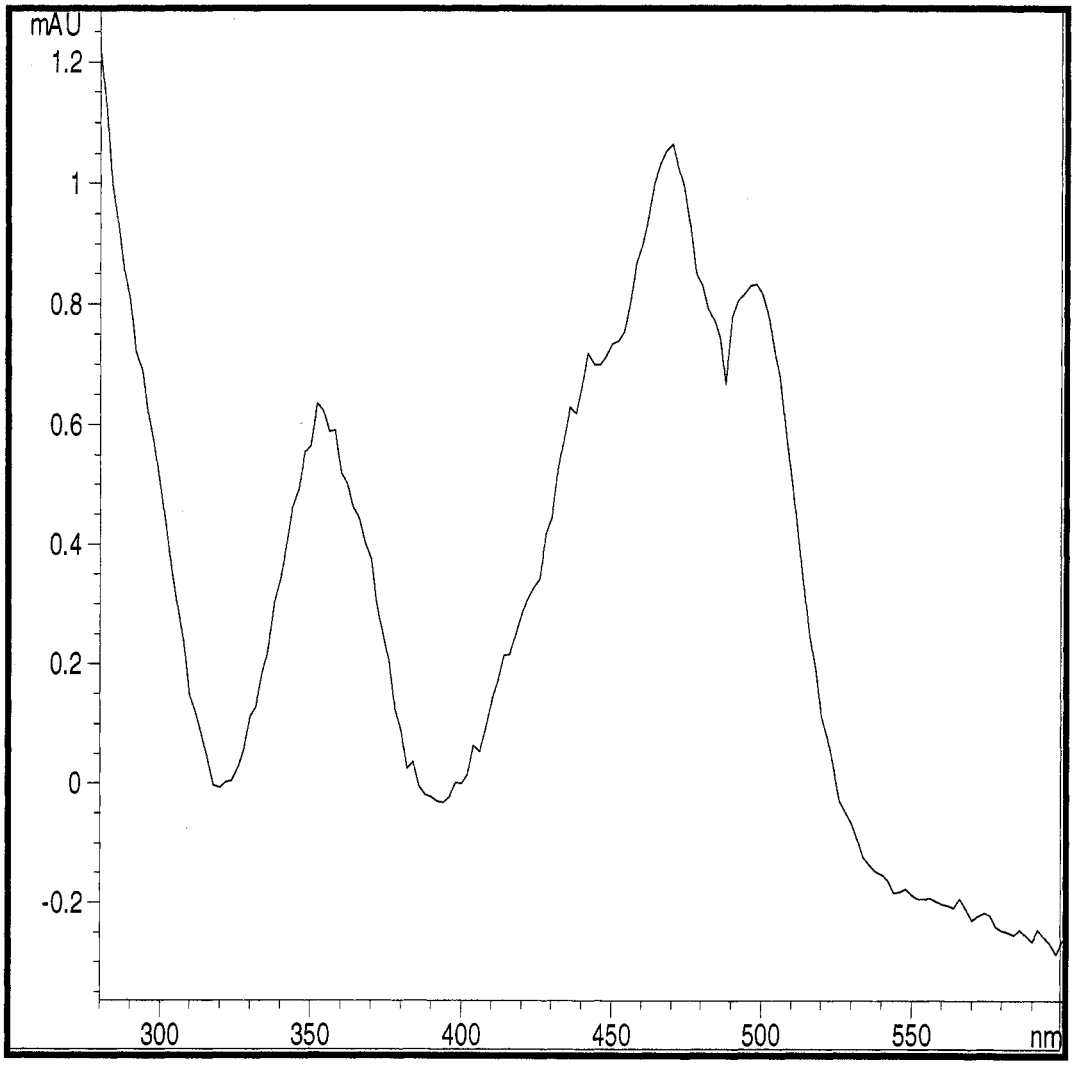

Figure 24: The UV/Vis Spectrum of the Carotenoid with m/z of 537.

One polar carotenoid was observed for the unsaponified A3 extract. It eluted at 6.1 minutes and had three absorption maxima: 320nm, 478nm, and 504nm. Because it had a shorter retention time than astaxanthin, it apparently had a higher affinity for the

mobile phase than astaxanthin. Its molecular weight of 420 was considerably lower than astaxanthin's 597.

Like A3, the other red bacterium, EDB1, had the same LC/MS chromatogram. It also had the same major polar and nonpolar carotenoids. The HPLC-DAD and the HPLC-MS chromatograms were almost identical for the two bacteria. This suggests that the two bacteria probably have nearly identical metabolic pathways.

The orange bacterium (EB01) had a slightly different HPLC-DAD-MS chromatogram. A nonpolar carotenoid was produced, which had a molecular weight of 537. However, it was not β-carotene, for its retention time was a lot shorter than that of β-carotene. Furthermore, there were two major polar carotenoids detected by both DAD and MS. Both carotenoids were found in red-pigmented bacteria. The first one had mass values of 420. The second carotenoid had a more complex mass spectrum. It has a molecular weight of 729.

6.7.4 LCMS Analysis of Saponified Bacterial Carotenoids

There were trace amounts of nonpolar carotenoids remained after saponification. It is possible that the bacterial carotenoids were reactive to the saponification reagents. Between the 14th and the 18th minutes, there existed a carotenoid with an m/z value of 729. The good resolution of the isomers indicated that they had moderate affinity for the C18 stationary phase (Figure 25).

The major nonpolar carotenoid was greatly reduced after saponification, as can be seen in figures 26 – 28.

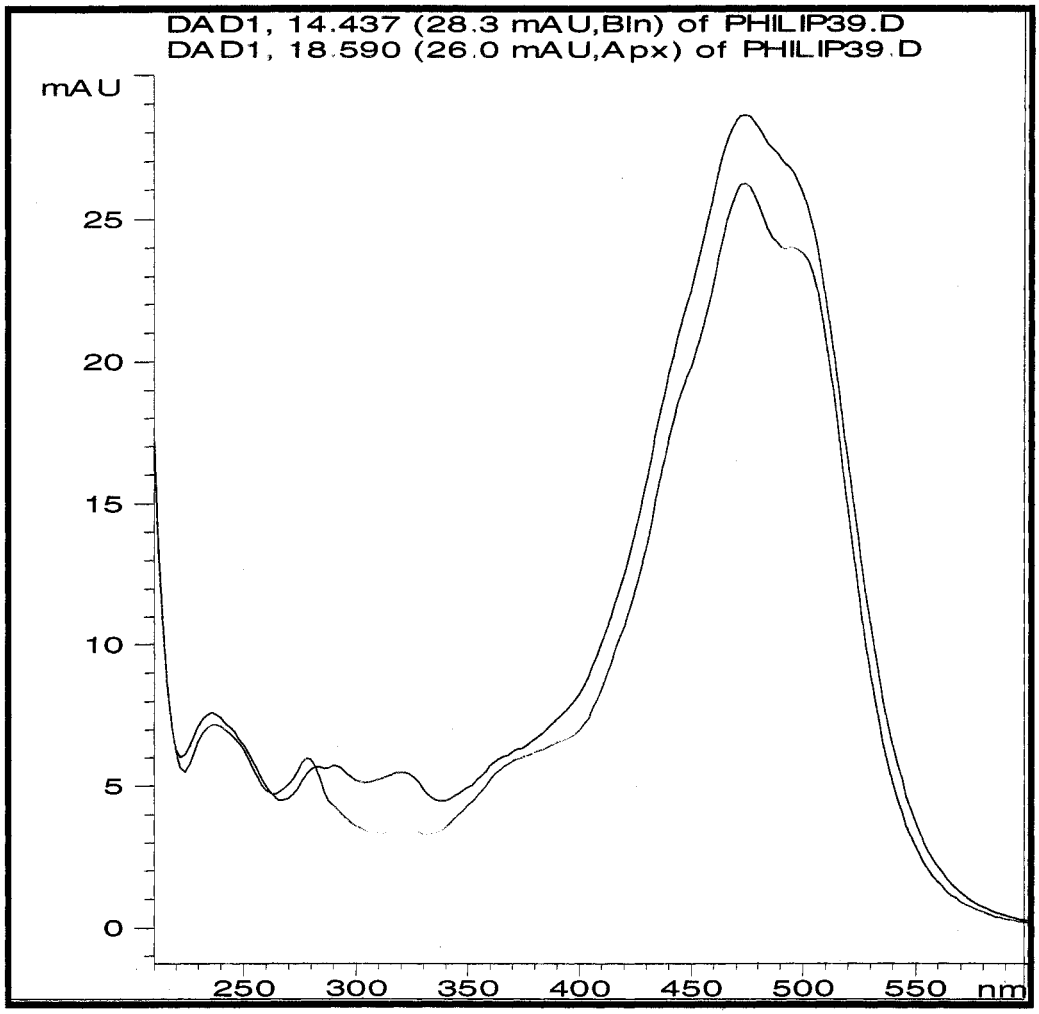

Figure 25: The UV/Vis Spectrum of Polar Carotenoid Isomers Produced by a Red-Pigmented Bacterium.

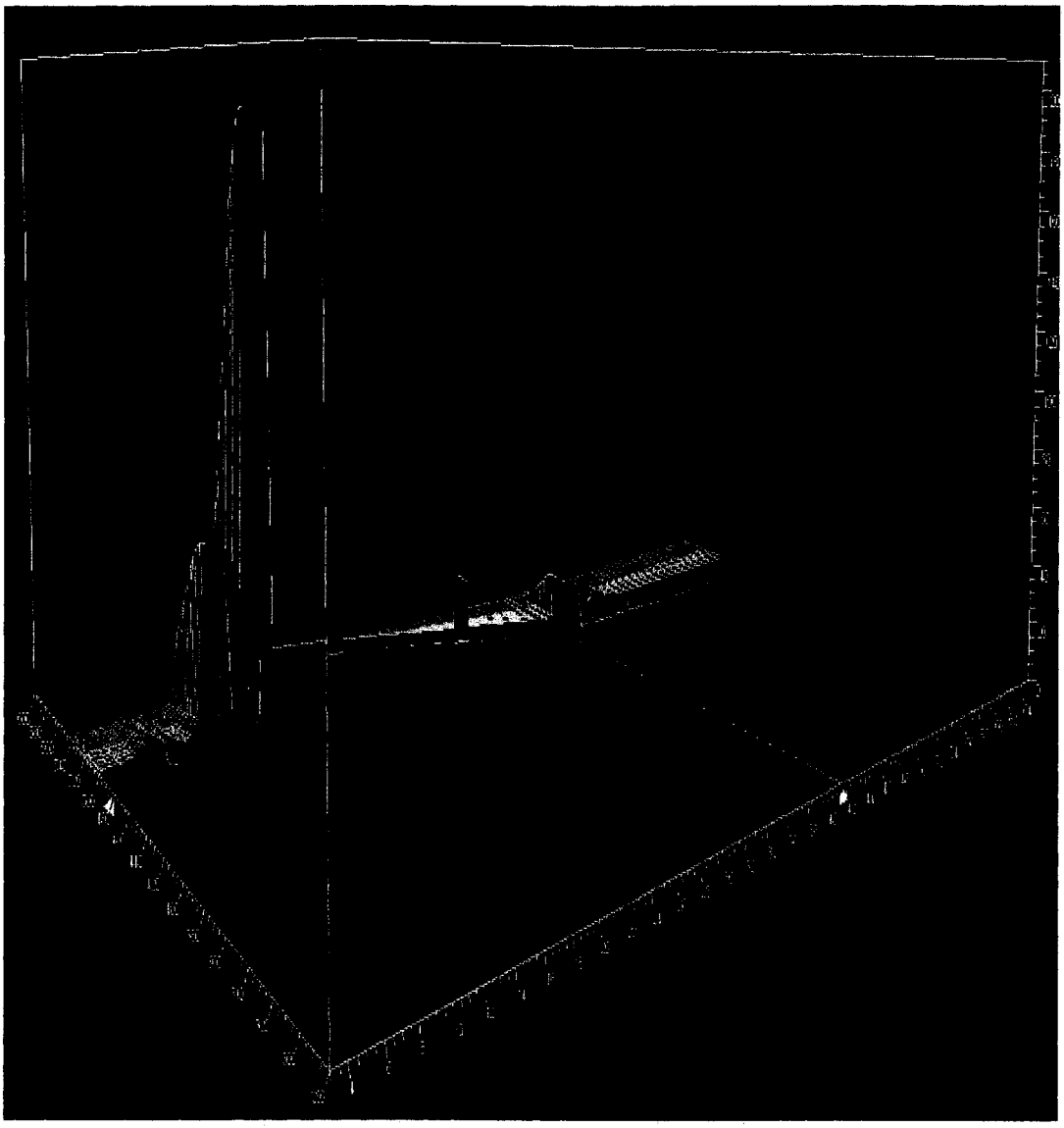

Figure 26: Saponified Carotenoids Produced by Bacteria A3. The nonpolar carotenoid decreased after saponification.

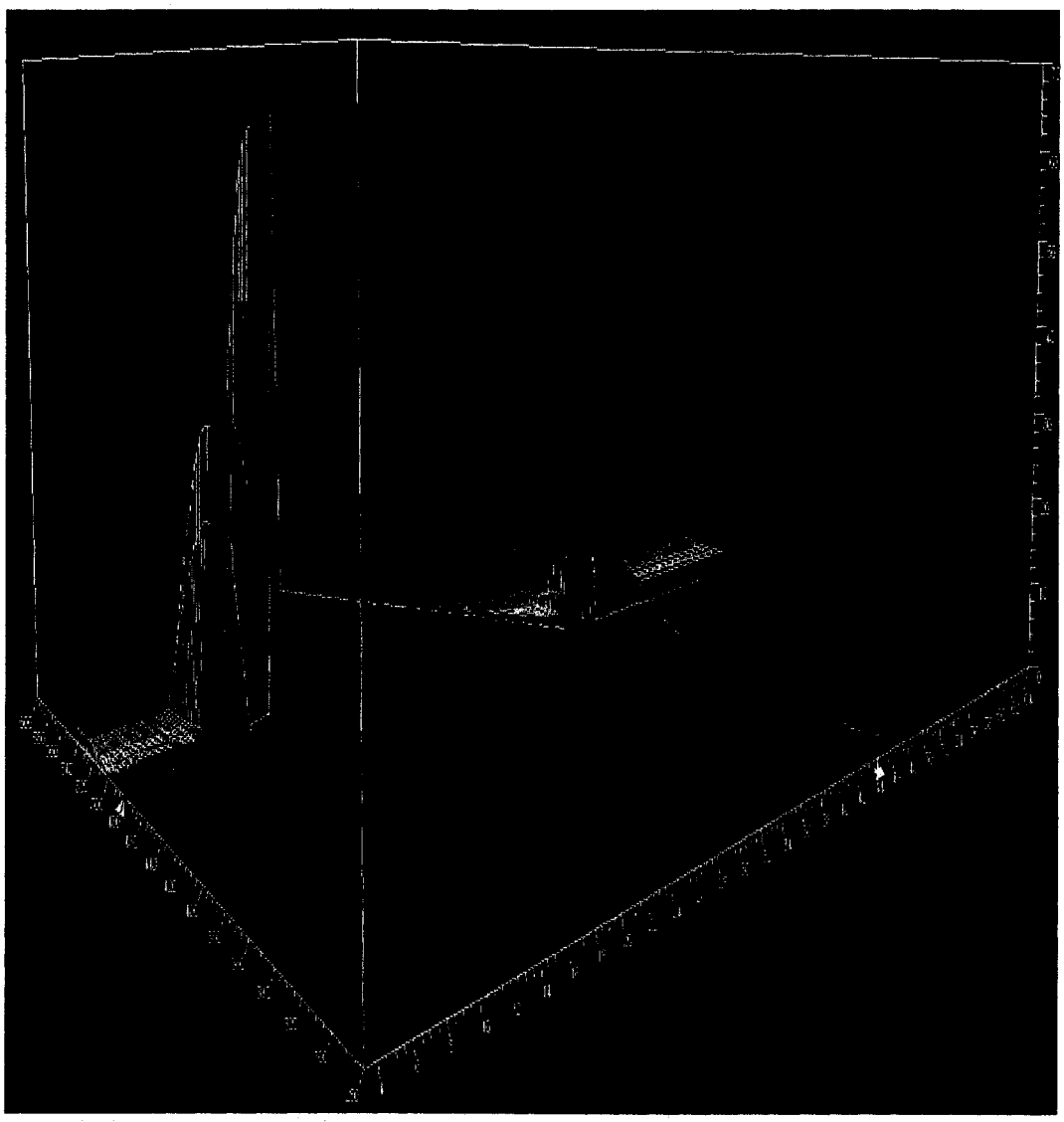

Figure 27: Saponified Carotenoids Produced by Bacteria EDB1. As with A3, the nonpolar carotenoid decreased after saponification.

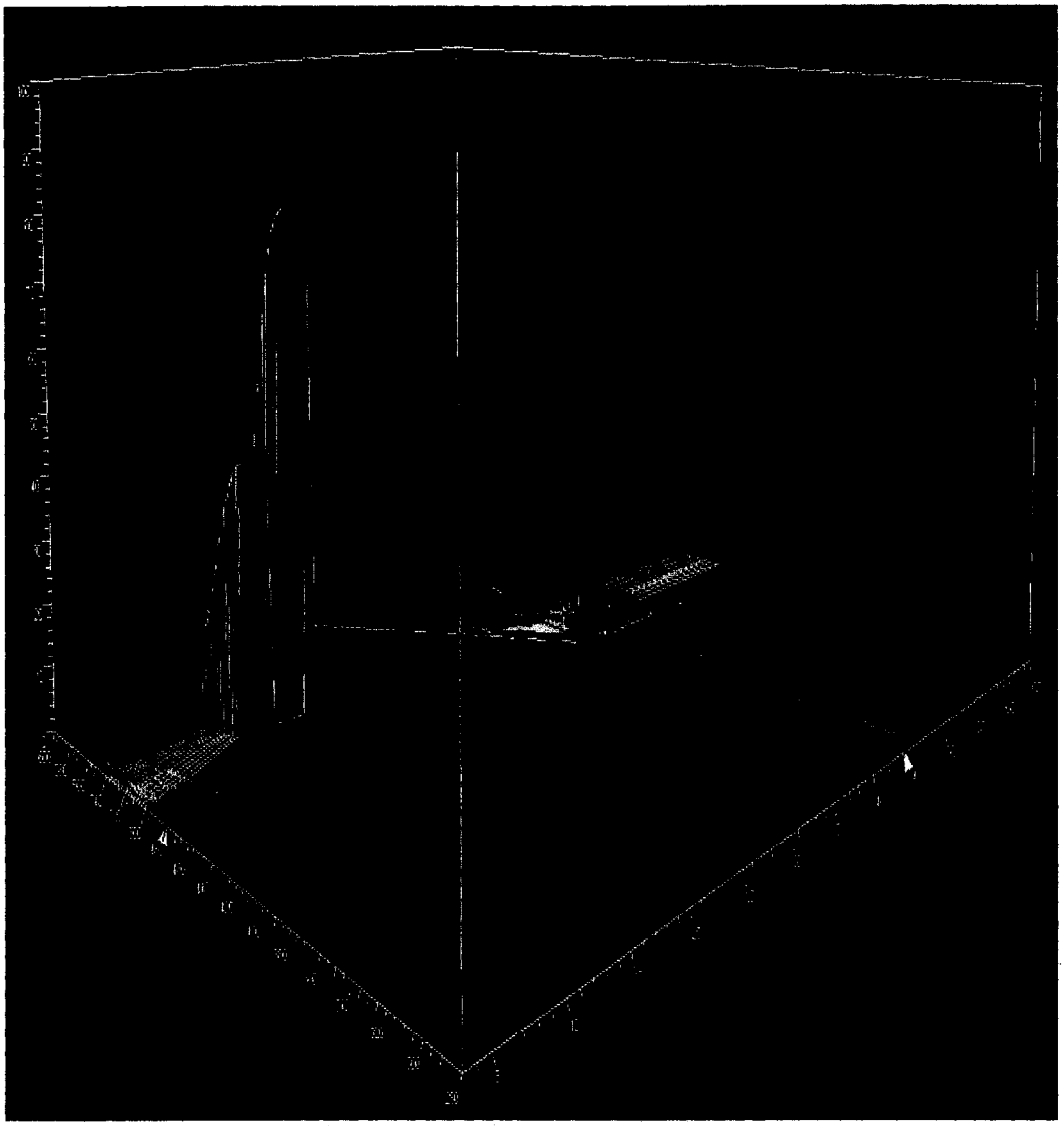

Figure 28: Saponified Carotenoids Produced by Bacteria EB01. The lipids were vastly reduced, but the chromatogram remains virtually unchanged.

Unlike A3, the major polar carotenoid with a molecular weight of 420 disappeared completely from EDB1's chromatograms after saponification. A major polar carotenoid with a molecular weight of 344 (absorption maxima of 481nm and 505 nm)

appeared after saponification. Its Z-isomer eluted at 12.6 minutes after injection. As with A3, the most abundant polar carotenoid had an m/z of 727 (absorption maxima of 480nm and 504 nm), and it eluted between the 14th and the 18th minutes. Also as with A3, no major nonpolar carotenoid was observed. Although a nonpolar carotenoid that eluted at 43.1 minutes was found, only trace amounts were detected. The carotenoid had a molecular weight of 537 and three absorption maxima: 448nm, 470nm, and 502 nm. The ratio of III/II was 0.55. The carotenoid was not β-carotene, since it eluted much earlier than the β-carotene (49 minutes). The UV/Vis spectrum of the non-polar carotenoid can be seen in Figure 28.

This trend was the same for the orange-pigmented bacteria. A polar carotenoid with a retention time at the 12th minute was detected. It had m/z values of 727. Again, β-carotene and astaxanthin were not found in the saponified samples.

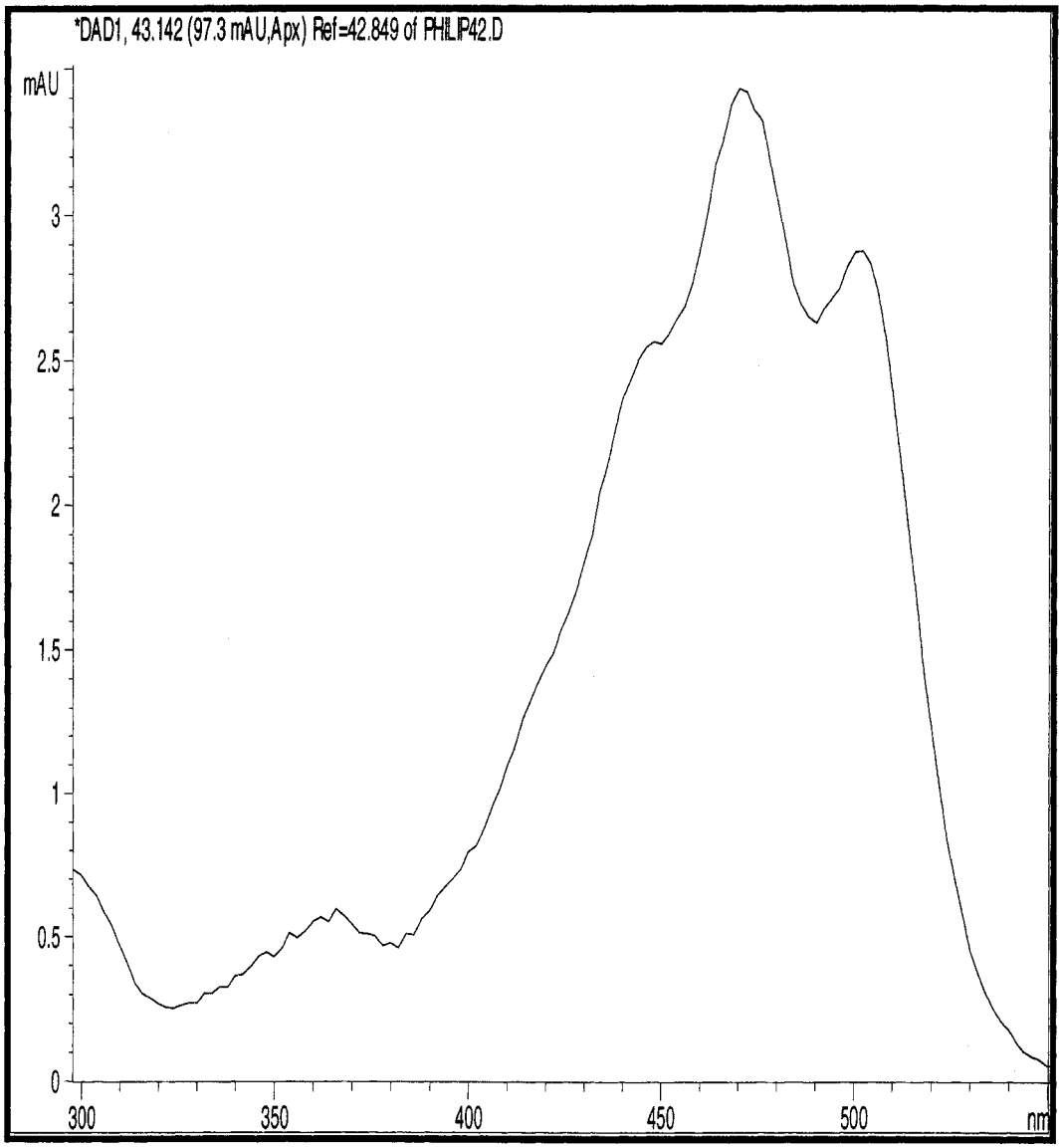

Figure 29. The UV/Vis Spectrum of Nonpolar Carotenoid Produced by a Red-Pigmented Bacterium.

6.7.5 Instrumentation

The instrumental setup for HPLC was based on the predetermined parameters prepared by William Dewhirst [58] except a mixture of ethyl acetate and methanol was

used for the nonpolar mobile phase. Poor resolution of polar carotenoids was observed when an aqueous mobile phase with 85/15 methanol / acetonitrile [30] was used. Therefore, it was necessary to include 7% of water in mobile phase A to enhance the resolution of polar carotenoids.

The vaporization temperature was less influential to the analytical results. The responses were the same at 623K and 673K. In addition to the vaporization temperature, the drying gas flowrates were also tested. Kurilich [55], who used an Agilent 1946A MSD for carotenoid analysis, proposed a low drying gas flowrate of 7L/min. However, the drying gas flowrate was increased to 12L/min for better signal response and less noise.

6.7.6 Carotenoid Production

Because the bacterial samples did not contain any astaxanthin or β-carotene, there is no immediate commercial value for the bacteria carotenoids. Normalized data shown in Figure 29 estimated the relative quantities of the carotenoids produced by the bacteria.

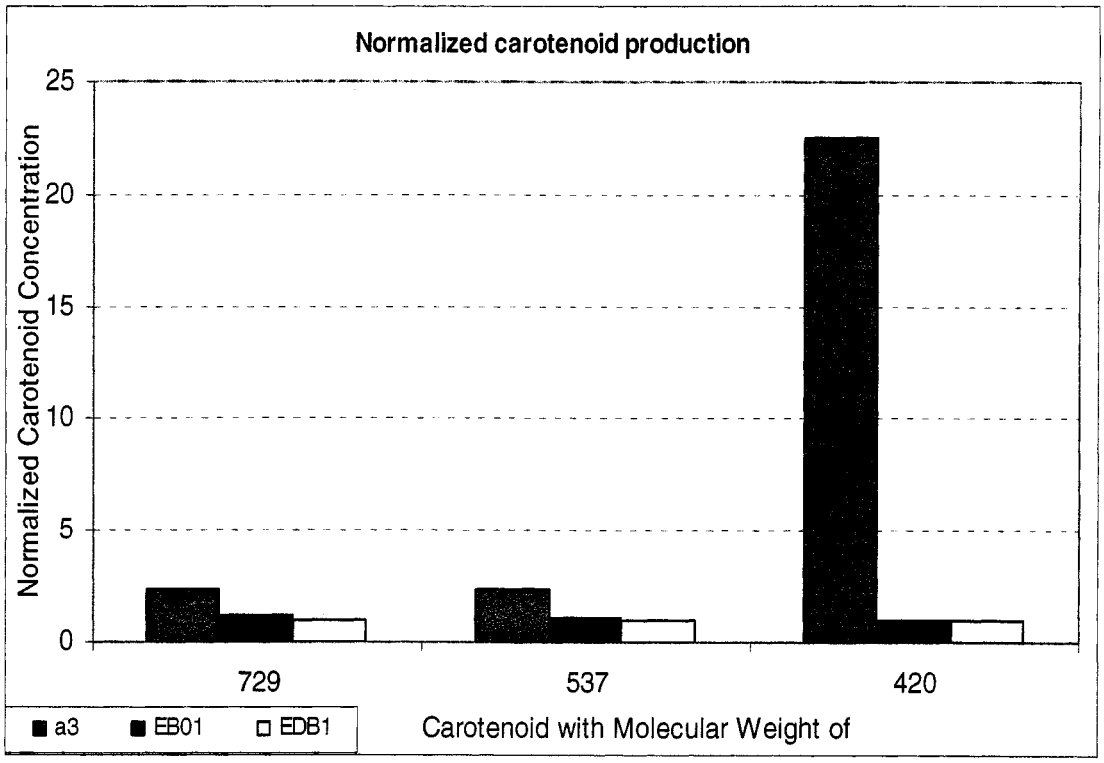

Figure 29: Normalized Bacterial Carotenoid Production.

Figure 29 shows all the common carotenoids that were produced by the tested bacteria. The normalized values were calculated from the carotenoid responses of the photodiode array detector divided by the total dry cells of the bacteria. There is no obvious trend to indicate which type of carotenoid (polar or nonpolar) was produced in the greatest quantities. Compared to the other two bacteria, A3 was the most carotenoid-produced bacterium, producing 20+ times more polar carotenoid (420 Da) than the other species. In addition, A3 produced twice as much polar and nonpolar carotenoids as the other two bacteria.

7.0 CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

7.1 Conclusions

Like many other bacteria living in extreme conditions, the bacteria examined here contained more than one carotenoid. However, the carotenoids produced by these bacteria do not have immediate commercial values the way astaxanthin, β-carotene, and lycopene do. The main carotenoid produced by the strains tested here was a nonpolar carotenoid, for it eluted after the mobile phase gradient. This carotenoid was vulnerable to saponification, since it diminished in amount after saponification. Furthermore, this carotenoid distinguished between the red-pigmented bacteria and their orange-pigmented counterparts.

The tested bacteria had similar growth patterns. When a single colony was developed in 50mL of R2A broth, the patterns were sigmoidal with a lag phase of 10 hours or more. The liquid cultures would have higher titers if the inoculum was transferred in the early stage of the exponential phase.

7.2 Suggestions for Future Research

A purification step is necessary to reduce the noises caused by non-carotenoid components in the bacterial samples. Liquid chromatography or preparative HPLC with sample collection programs can provide more purified carotenoid samples. A C_{18} HPLC column is adequate to separate the isomers of the bacterial carotenoids. It is not necessary to use a C_{30} column for final purification.

Because the carotenoids were produced for identification purpose, only sufficient amounts of carotenoids were produced for analysis. If the carotenoids are to be produced for other purpose, using a fermentor can improve the production.

Due to the nature of ionization, APCI could not produce enough fragments for detailed structure elucidation. The polar carotenoids can be analyzed using an electrospray ionizor to produce sufficient fragments for structure determination. Since the nonpolar carotenoid produced by the bacteria does not have strong nonpolar characteristics like those of β-carotene or lycopene, it can be ionized by an ESI. A ^{1}H-NMR may be used after HPLC purification for precise structural identification.

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