Studies on Microbial Utilization of Petroleum. I. Separation and Characterization of Carotenoids Produced by a Species of *Brevibacterium* in Hydrocarbon Media

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Ten pigment fractions were separated from a *Brevibacterium* sp. KY-4313 in hydrocarbon fermentation. The pigments were extracted from cell with a mixture of petroleum ether and acetone, and purified by column and thin-layer chromatography. The principal carotenoid of this organism was identified as canthaxanthin (4,4′-dioxo-β-carotene) by using visible, IR, NMR, and mass spectra of the pigment. Three other pigments were characterized as β-carotene, echinenone (4-oxo-β-carotene), and 15-*cis*-4,4′-dioxo-β-carotene. Three carboxylic carotenoids and three other carotenoids were also separated but their structures were left unidentified. Quantitation of the pigments showed that canthaxanthin represented 53% and its *cis*-isomer 20% of the pigments separated from extracts. β-Carotene derivatives comprise more than 80% of the pigments. Canthaxanthin and echinenone have not previously been reported as bacterial pigments in hydrocarbon fermentation.

Studies on carotenoid production by hydrocarbon fermentation have been reported regarding *Mycobacterium lacticola*,[1,2] *Nocardia lutea*,[3] *N. corallina*,[3] *Pseudomonas methanica*,[4] *Rhodotorula aurantiace*,[5] *R. glutinis*,[5] *Mycobacterium smegmatis*,[6] and *M. lacticolum*.[7]

Recently Iizuka and Nishimura[8] have reported that *Brevibacterium* strain No. 103 essentially produces astacin and astaxanthin when cultured on several *n*-alkanes.

Kato *et al.*[9] reported the cultural conditions for carotenoid production by a *Brevibacterium* sp. KY-4313 in *n*-alkane media and suggested that the bacterium seemed to produce xanthophylls extractive with petroelum ether.

The present authors reexamined the carotenoid pigments produced by *Brevibacterium* sp. KY-4313 cultivated on the different hydrocarbon medium, and established the presence of canthaxanthin among other carotenoids, as the main component.

Canthaxanthin has been isolated from mushroom *Cantharellus cinnabarinus*,[10] the feathers and the skin of the lesser flamingo *Phoeniconias minor*,[11] *Cyclops strenuus*,[12] and *Micrococcus roseus*[13] and *Corynebacterium michiganense*[14] in non hydrocarbon media.

Experimental

Measurements. Hitachi EPS-3, Perkin Elmer 225, Varian HA 100D, and Hitachi RMU-6 spectrometer were used for the measurements of visible, IR, NMR, and mass spectrum, respectively.

Reduction of Carbonyl Group. Carbonyl groups in carotenoids were reduced by sodium borohydride in 95% ethanol solution.

Detection Test for Carboxylic Group. Carboxylic groups were detected by the formation of ferric hydroxamates (brown).

Microorganism. The microorganism used was a *Brevibacterium* sp. KY-4313. The organism was maintained on a meat extract agar slant.

Media and Cultural Conditions. A meat extract agar slant was used as an inoculum. For sub-inoculum a portion of 100 m*l* conventional natural nutrient medium in a 500 m*l* Erlenmeyer flask was used. Mass cultures were grown for 7 days in fifty 500 m*l* Erlenmeyer flasks containing 200 m*l* of medium shown in Table 1 at 30°C on a rotary shaker running at 220 rpm describing a circle of 70 mm diameter.

Harvesting of Cells. The pale yellow culture was poured into a separatory funnel, allowed to stand for 1 hr, and the green aqueous layer was discarded. The yellow supernatant was centrifuged at 7000 rpm at 0°C for 10 min.

Extraction of Pigments from the Medium. Experiments

TABLE 1. COMPOSITION OF HYDROCARBON MEDIUM

$NH_4H_2PO_4$	2.5 g
KH_2PO_4	2.0 g
$Na_2HPO_4 \cdot 12H_2O$	3.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.01 g
$FeSO_4 \cdot 7H_2O$	0.005 g
$MnSO_4 \cdot 4\text{–}6H_2O$	0.005 g
Vitamin B_{12}	100 γ
n-C_{14-16} mixture	20 m*l*
Distd. water	1000 m*l*
pH	7.0

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TABLE 2. CHROMATOGRAPHIC SEPARATION OF THE CAROTENOIDS AND THEIR CONTENTS

Column chromatog.[a]		Thin-layer chromatography				Per cent[b] content
Fraction	System of solvents	Layer	System of solvents	Fraction	R_f value	
1	PE-Ac (9.5+0.5, v/v)	Silica gel	PE-Bz (85+15)	1-1	0.67	6.2
				1-2	0.53	
				1-3	0.49	
				1-4	0	
2	(98+2)	$MgHPO_4$	CCl_4	2	0.57	7.4
3	(50+50)	Silica gel-$Ca(OH)_2$ (1+2)	Bz-Me (98+2)	3-1[c]	0.21	52.8
				3-2	0.12	20.2
4	Ac	Silica gel	PE-Ac (30+70)	4-1	0.71	7.0
				4-2	0.49	4.1
				4-3	0	2.3

a) Silica gel (200 mesh, 0.8×48 cm) was used for adsorbent.
b) The content was determined spectrophotometrically as β-carotene ($E^{1\%}_{1cm}$: 2580 in PE).
c) The main pigment was also isolated from Fr. 3 in PE at −20°C in the form of a fine deep red plate, which was found pure by thin-layer chromatography.
PE: petroleum ether, Ac: acetone, Bz: benzene, Me: methanol.

were carried out throughout under dim light and N_2 atmosphere. A portion of 2.5 *l* of petroleum ether and the same volume of acetone were added to about 200 g of orange yellow harvest. The hole mixture was washed with 5 *l* of distd. water. The procedure was repeated twice. The collected orange petroleum ether layer was evaporated to dryness in a vacuum and pigments were then dissolved in acetone. The carotenoid solution in acetone was allowed to stand at −20°C overnight, and the waxy substances separated were removed by filtration. The pigments were again transferred from the acetone solution into petroleum ether. The solution was passed through a silica gel column (0.8×48 cm, 200 mesh) to remove residual hydrocarbon, the column being washed with petroleum ether, and the adsorbed pigments were eluted with acetone.

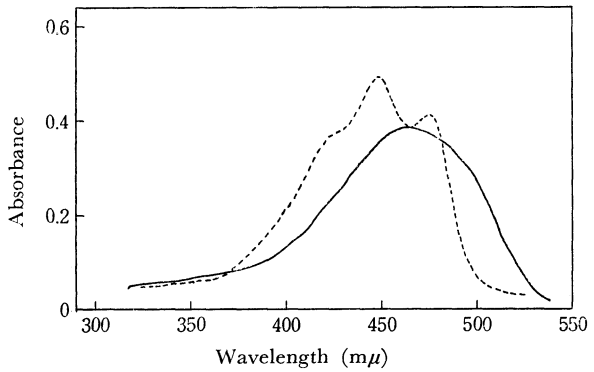

Fig. 1. The change of the absorption spectrum of Fr. 3-1 by $NaBH_4$ reduction.
Solvent: *n*-Hexane, (——): Fr. 3-1, (- - -): Reduced Fr. 3-1.

Saponification of the pigments was carried out in a 10% methanolic potassium hydroxide solution for 30 min at 40°C with continuous stirring in N_2 atmosphere, and the unsaponifiable matters were extracted with petroleum ether.

Separation of Pigments. Separation procedures are summarized in Table 2. Thin-layer chromatographic purification by different adsorbents was carried out.

Results and Discussion

The content of the pigments and their absorption maxima are shown in Tables 2 and 3, respectively.

The main carotenoid (Fr. 3-1, Fr. is used for fraction instead hereinafter) was identified with canthaxanthin. Three carotenoids were characterized as β-carotene, echinenone, and canthaxanthin *cis*-isomer. Hydroxy-carotenoid, a presumable precursor in oxocarotenoid biosynthesis, was not obtained.

From the fact that reduction of the pigment makes a 17 mμ hypsochromic shift in the visible spectra (Table 3, Fig. 1), the main pigment is considered to have two conjugated carbonyl groups on its ionone

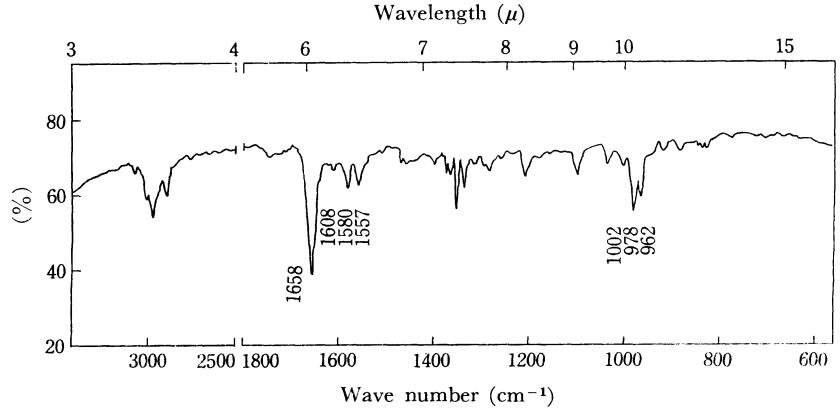

Fig. 2. IR spectrum of Fr. 3-1 (KBr: 0.3 g, Sample: 0.5 mg).

Table 3. Absorption maxima and identification of carotenoids

Carotenoid	Absorption maxima (mμ)	Solvent	R_f Value	Identification
Fr. 1-1	425 446 473	PE	0.67[g]	β-Carotene[a]
Fr. 1-3	439 466 496	PE		Unknown[b]
Fr. 2	458	*n*-Hexane	0.85[h]	Echinenone[c]
Reduced Fr. 2	426 450 478	*n*-Hexane	0.76[h]	
Fr. 3-1	466	*n*-Hexane	0.83[h]	Canthaxanthin[d]
Reduced Fr. 3-1	426 449 476	*n*-Hexane	0.51[h]	
Fr. 3-2	356 460	*n*-Hexane		*cis*-Isomer of canthaxanthin[e]
Reduced Fr. 3-2	332 422 445 472	*n*-Hexane		
Fr. 4-1	348 435 457 483	Chloroform		Carboxylic carotenoids[f]
Fr. 4-2	435 459 482	Chloroform		
Fr. 4-3	433 455 482	Chloroform		
β-Carotene[i]	425 447 475	PE	0.67[g]	
Echinenone[15]	458	PE		
Isocryptoxanthin[16]	426 451 479	*n*-Hexane		
Canthaxanthin[15]	467	*n*-Hexane		
Isozeaxanthin[15]	426 450 478	*n*-Hexane		

a) Identified with β-carotene from the R_f values and spectrum.
b) This pigment is neither carbonyl, hydroxy, nor carboxylic carotenoid from TLC behavior and result of $NaBH_4$-reduction.
c) Identified with echinenone from results of the maximum absorption shift and the change of R_f value on reduction, and by agrrement with the absorption maxima given in literature (Fig. 4).
d) The main pigment was identified with canthaxanthin from the results of visible absorption, IR, NMR and mass spectrum.
e) Identified with canthaxanthin *cis*-isomer (15-*cis*-) from the position of *cis*-peak[17] (Fig. 5).
f) Positive to the detection test of carboxylic group.
g) Layer: Silica gel, Solvent system: PE-Bz (85+15).
h) Layer; Silica gel, Solvent system: CH_2Cl_2-AcOEt (90+10).
i) β-Carotene (E. Merck, for biochemistry grade) used after being purified on TLC.

Table 4. IR absorption of Fr. 3-1 and canthaxanthin

Carotenoid	Frequencies in cm^{-1}						
	Conjug. C=O	Conjug. C=C			CH out-of-plane of conjug. *trans*-CH=CH		
Fr. 3-1	1658 vs	1608 w	1580 m	1557 m	1002 w	978 s	962 m
Canthaxanthin[18]	1658 vs	1608 w	1581 m	1555 s		998 m	966 s
Canthaxanthin[19]	1640 vs		broad (m)			*ca.* 960 s broad	

vs: very strong, s: strong, m: medium, w: weak. KBr discs were used.

Table 5. NMR absorption of Fr. 3-1 and canthaxanthin

Carotenoid	Solvent	*gem*-Me	Me on C adjacent to C=O	Me on polyene chain	*Vicinal* CH_2					
					α position to C=O			β position to C=O		
Fr. 3-1	C_6D_6-$CDCl_3$ (3+1)	8.99	7.98	8.12 8.14[a]	7.53	7.60	7.67	8.36	8.42	8.49[b]
Fr. 3-1	$CDCl_3$	8.79	8.12	7.99	7.42	7.48	7.55	8.08	8.15	8.22
Canthaxanthin[19]	CCl_4	8.81	8.21	7.89 7.99						
Relative intensity[c]		6	3	6		2			2	

a) The bands at 8.12 and 8.14 arise from 9,9'-Me and 13,13'-Me groups, respectively.
b) These two sets of triplet due to the vicinal CH_2 groups were confirmed by spin-spin decoupling technique.
c) The ratio was calculated from the spectrum measured in C_6D_6-$CDCl_3$.

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rings. As shown in Table 4 and Fig. 2, the IR spectrum gives more information than the spectra reported heretofore, that is, we may conclude that the splitting of the region (960—1002 cm^{-1}) is due to the conjugation of ω,ω'-carbonyl groups through the trans polyene chain.

The NMR spectrum measured in a C_6D_6-$CDCl_3$ solution provides more useful information for complete assignment of bands than the ones measured in $CDCl_3$, or in CCl_4 (Table 5, Fig. 3). In the measurement in a C_6D_6-$CDCl_3$ solution, a band at 7.98 (τ value, Me on C adjacent to C=O) and a triplet at 8.42 (*vicinal* CH_2) can be completely separated by magnetic anisotropy effect of C_6D_6, while it is observed as an overlapping spectrum in $CDCl_3$.

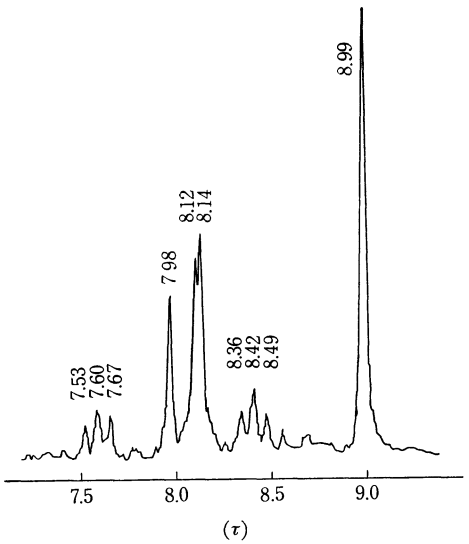
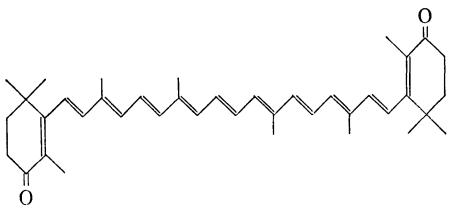


Fig. 3. NMR spectrum of Fr. 3-1. 0.5 mg/0.01 m*l* C_6D_6-$CDCl_3$ (3 : 1) at 100 MHz.

In the mass spectrum of Fr. 3-1, M^+ shows 564 mu (Calcd for $C_{40}H_{52}O_2$: mol wt, 564.4). The intensity ratio of M^+-92 to M^+-106 is 2.6, which agrees well with that of canthaxanthin (2.56) reported by Enzell *et al.*[21]

From the results, we identify Fr. 3-1 with canthaxanthin (4,4'-dioxo-β-carotene) (I).



(I) Canthaxanthin

Fr. 3–1 exposed to sunlight shows the same absorption spectrum as that of Fr. 3–2. Canthaxanthin is considered to have been converted to its *cis*-isomer during the cultivation of bacterium.

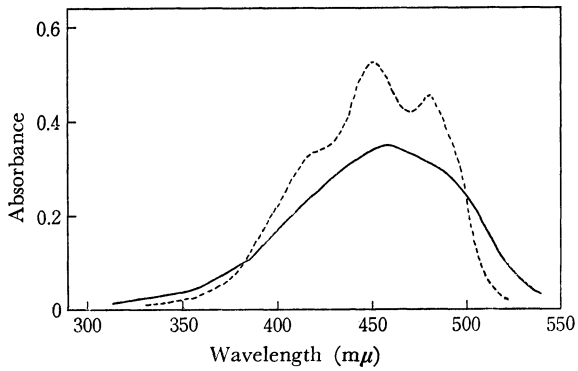


Fig. 4. The change of the absorption spectrum of Fr. 2 by $NaBH_4$ reduction. Solvent: *n*-Hexane. (——): Fr. 2, (- - -): Reduced Fr. 2.

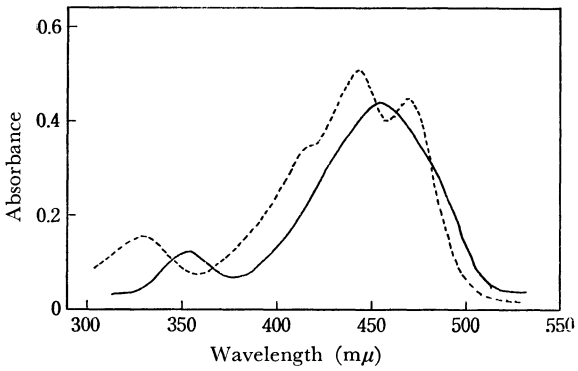


Fig. 5. The change of the absorption spectrum of Fr. 3-2 by $NaBH_4$ reduction. Solvent: Petroleum ether. (——): Reduced Fr. 3-2, (- - -): Reduced Fr. 3-2.

Brevibact. sp. KY-4313 and *B.* strain No. 103[8] differ in the carotenoid formation. While the former produces mainly mono- and dioxo-carotenoids, the latter gives dihydroxy-dioxo- and tetraoxo-carotenoids almost exclusively.

Mycobact. smegmatis[6] was reported to produce oxo- and hydroxy-oxo-carotenoids as main components in hydrocarbon media, but no hydroxy-carotenoid has been isolated so far.

In non-hydrocarbon medium *Micrococcus roseus*[13] has been found to produce a trace of isozeaxanthin as a precursor for canthaxanthin which is the main component.

From these facts, it seems that the organisms such as *Brevibact.* sp. KY-4313, *N.* strain No. 103, *Mycobact. smegmatis*, and *Micrococcus roseus* readily oxidize the hydroxyls of precursors to ketones.

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