

Microbiological Production of Carotenoids

VIII. Influence of Hydrocarbon on Carotenogenesis by Mated Cultures of *Blakeslea trispora*

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ABSTRACT

CIEGLER, ALEX (U. S. Department of Agriculture, Peoria, Ill.), GEORGE E. N. NELSON, AND HARLOW H. HALL. Microbiological production of carotenoids. VIII. Influence of hydrocarbon on carotenogenesis by mated cultures of *Blakeslea trispora*. Appl. Microbiol. **10**:132-136. 1962.—Synthesis of β -carotene by mated strains of *Blakeslea trispora* in shaken-flask culture was considerably enhanced by adding either 5% kerosene after 2 days of fermentation or acid-refined kerosene at the start of fermentation to a grain-based medium that also contained a natural lipid, nonionic detergent, and β -ionone; average yields of 17,500 μ g per g of dry fermentation solids (86,000 μ g per 100 ml of medium) were attained when refined kerosene was used. Almost all of the carotene was retained within the mycelium. Peak yields were achieved in 5 days.

Addition of simple lipids, such as oils and greases, to fermentation media considerably enhance β -carotene production by mated strains of *Blakeslea trispora* (Anderson et al., 1957, 1958; Ciegler, Arnold, and Anderson, 1959a, b). Usually after 24 hr of incubation in shaken-flask culture, lipids in the medium had a tendency to coalesce, even in the presence of a detergent, and to form small, stable agglomerates. These agglomerates were not readily metabolized by the mold and were often present at the end of the fermentation. The weight of lipid in these agglomerates ranged from 0.05 to 0.6 g per 100 ml of medium. Pan, Bonanno, and Wagman (1959) also noted the formation of these lipid masses when they used fatty oils as an energy source in penicillin fermentations.

We attempted to prevent lipid-agglomerate formation, as did Pan and his co-workers, by addition of hydrocarbon solvents to the fermentation medium. A second reason for adding hydrocarbon to the medium was to leach carotene from the mycelium during the course of fermentation. Since almost all carotene is produced and stored intracellularly, continuous excretion of the provitamin into the medium during fermentation could conceivably

increase the total yield per unit volume of fermentation medium. Maximal yields previously reported were 42,000 μ g per 100 ml of medium (Ciegler et al., 1959b).

MATERIALS AND METHODS

Two strains of *B. trispora*, NRRL 2456 (+ mating type) and NRRL 2457 (- mating type), were used in our previously reported work. Unfortunately, the results given by these two strains were difficult to reproduce consistently because of culture degeneration.

Two new strains subsequently acquired, NRRL9216(+) and NRRL9159(-), proved superior to the earlier cultures used. The minus strain (NRRL9159) grew at the same rate as the plus strain (NRRL9216) in both liquid and agar slant cultures but did not show the prolonged lag given by NRRL2457(-). The plus strain (NRRL9159) sporulated heavily at 28 C on potato dextrose agar (PDA), which had been supplemented with 1 mg of thiamine per liter of medium, whereas the minus strain (NRRL9159) produced either only a few or no sporangiospores under these conditions. Stock cultures of both strains were maintained at room temperature; serial transfers were made once a week.

Inocula for fermentation purposes were produced in 500-ml Erlenmeyer flasks containing 150 ml of the following medium: acid-hydrolyzed corn, 2.3%; acid-hydrolyzed soybean meal, 4.7%;² thiamine-HCl, 0.2 mg per 100 ml; NaOH to pH 6.5. Flasks were sterilized 30 min at 121 C and, after cooling, were inoculated with pieces of agar containing mycelium from 5- to 6-day-old PDA slants; incubation was for 2 days on a rotary shaker operating at 200 rev/min. Two flasks of culture, one of each mating type, were combined and macerated 5 to 10 sec in a sterile micro-Waring Blendor cup to give a homogeneous mycelial suspension. An 8- to 10-ml fraction of the homogenate was used to inoculate each 100 ml of fermentation medium.

The fermentation medium had the same composition as that used for the inocula but, in addition, contained

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² To prepare 1 liter of medium, 47 g of soybean meal and 23 g of ground corn were added to 500 ml of 0.2 N H₂SO₄ in a 2.8-liter Fernbach flask. The mash was autoclaved for 1½ hr at 121 C 15 psi, and cooled, then the remaining ingredients were added

0.12% nonionic detergent (Triton X-100),³ 5% white grease, and 0.1% β -ionone. One-hundred milliliters of medium were dispensed into 500-ml Erlenmeyer flasks and sterilized for 30 min at 121 C. The β -ionone was added 2 days after initiation of fermentation. Fermentations were run for a total of 6 days. Procedures for harvesting and analyzing the fermentation have been described by Anderson et al. (1958). Solvents were sterilized by autoclaving at 121 C for 10 min, except for those too volatile for this procedure; they were sterilized by Seitz filtration.

RESULTS

Following the report of Pan et al. (1959), we determined, in preliminary experiments, the effect of adding mineral oils of various viscosities (11.6 to 135.2 centipoises) to the fermentation medium. Several ratios and levels of fats and mineral oils were also tested. Representative data are shown in Table 1. Although addition of mineral oils of various viscosities to fermentation media generally results in decreased carotene yields, the oils did prevent formation of the lipid agglomerates. In addition, mineral oil at certain levels, in the absence of detergent, prevented clumping of the mycelial growth; dispersed mycelia are necessary for good carotene yields. The mineral oils had no apparent toxicity since the yield of solids in the presence of the hydrocarbons was as high or higher than in control flasks. The lower carotene yields in the presence of mineral oil were believed to result from a decreased availability of oxygen, for the mycelia were thickly coated with the oil.

Kerosene, a hydrocarbon solvent with very low viscosity, was substituted for mineral oil. Addition of kerosene and detergent at the start of the fermentation usually proved

³ Rohm and Haas Company, Philadelphia, Pa.

TABLE 1. *Effect of the ratio of lipid to mineral oil and of detergent on carotene production**

Lipid	Mineral oil†	Detergent (0.12%)	Dry mycelium	Carotene yield in solids	Carotene yield
ml/100 ml	ml/100 ml		g/100 ml	μ g/g	μ g/100 ml
3	0	+	4.45	7,425	33,000
5	0	+	6.22	7,450	46,400
3	3	+	5.14	6,500	33,400
3	6	+	4.61	6,975	32,100
5	5	+	7.61	5,950	45,300
5	10	+	6.67	3,975	26,400
3	3	—	Clumped‡		
3	6	—	4.83	4,375	21,100
5	5	—	6.15	6,050	37,200
5	10	—	5.92	5,225	30,900

* All fermentations were conducted in 500-ml Erlenmeyer flasks containing 100 ml of medium.

† Light mineral oil of 30-centipoise viscosity as determined at 28 C with a Brookfield model LVT equipped with a no. 1 spindle rotating in 400-ml oil at 30 rev/min.

‡ In the absence of detergent, 3% mineral oil did not prevent clumping of the mycelium in three replicate experiments. Analyses were not run on clumped growth.

inimical to carotene synthesis. Without any detergent and with kerosene in a 1:1 ratio to lipid, carotene yields based on the dried recoverable solids (Table 2) increased considerably. Also, considerable amounts of carotene were in the supernatant although the lipid content of the mycelium grown under both conditions remained at about 50%. In addition, kerosene prevented the formation of the lipid agglomerates. Unfortunately, the total carotene yield per flask was usually the same as that produced by the controls because of the decreased amount of mycelial growth.

Delaying kerosene addition until growth was essentially completed, usually in 2 days, eliminated the toxic effect of the hydrocarbon, increased the yield of carotene per 100 ml of medium, but resulted in very little free carotene in the supernatant (Table 3). No further advantage accrued from further delay in solvent addition.

The promising results obtained with kerosene, coupled with certain undesirable characteristics of this hydrocarbon, e.g., odor and toxicity, made it desirable to investigate other hydrocarbons. The most promising of those tested was a commercial deodorized kerosene,

TABLE 2. *Effect of the ratio of lipid to kerosene on carotene production**

Lipid	Kerosene*	Detergent (0.12%)	Dry mycelium	Carotene yield in solids	Carotene in supernatant	Carotene yield
ml/100 ml	ml/100 ml		g/100 ml	μ g/g	μ g	μ g/100 ml
4	0	+	4.44	9,300	1,100	42,550
5	0	+	5.76	8,500	2,100	51,050
6	0	+	6.76	6,200	2,600	44,600
4	4	—	3.37	12,900	3,450	47,950
4	8	—	2.56	10,700	10,800	38,700
4	12	—	2.82	9,400	5,600	31,500
5	5	—	4.00	10,150	9,600	50,200
5	10	—	3.81	11,500	11,200	54,950
5	15	—	3.45	8,200	5,400	33,700
6	6	—	5.15	7,600	10,300	49,100
6	9	—	3.01	6,400	2,150	21,350
6	12	—	4.34	1,450	1,650	7,850

* Kerosene was added to flasks at the start of the fermentation.

TABLE 3. *Effect of kerosene, time of solvent addition, and of detergent on carotene production**

Kerosene	Time of hydrocarbon addition	Detergent (0.12%)	Dry mycelium	Carotene yield in solids	Carotene in supernatant	Carotene yield
ml/100 ml	day		g/100 ml	μ g/g	μ g	μ g/100 ml
0		—	Clumped			
0		+	5.76	8,500		49,500
5	0	—	4.39	9,470	10,870	51,760
5	0	+	4.67	6,150		28,900
5	1	+	3.97	11,800	4,100	50,950
5	2	+	5.87	9,800	3,300	60,300
5	3	+	5.65	10,000		59,400

* All flasks contained 5% lipid (white grease).

Deobase,⁴ treated with sulfuric acid to remove unsaturated compounds normally present in kerosene. The treated solvent had an iodine value of zero and an initial boiling point of 197 C.

When Deobase was used, it was again necessary to add 0.12% detergent to the fermentation medium to obtain optimal pigment synthesis. In the presence of detergent and 5% lipid, the optimal concentration of Deobase was

⁴ Deobase is a product of Sonneborne Chemical and Refining Corporation, 300 Park Avenue South, New York, N. Y. The use of trade names in this article is for identification only and does not imply endorsement by the U. S. Department of Agriculture over other products of similar quality.

TABLE 4. Effect of varying concentrations of hydrocarbon (Deobase) on carotene production*

Hydrocarbon	Detergent (0.12%)	Dry mycelium	Carotene yield in solids	Carotene yield
ml/100 ml		g/100 ml	μg/g	μg/100 ml
0	+	5.04	8,100	40,525
1	+	5.02	12,660	62,400
3	+	4.64	15,125	69,700
5	+	4.64	18,950	87,875
7	+	4.77	17,375	83,500
10	+	5.21	19,075	99,250
5	-	3.91	11,000	43,000
5†	-	4.75	10,300	49,000

* All flasks contained 5% lipid (white grease).

† Solvent added after 2 days of fermentation.

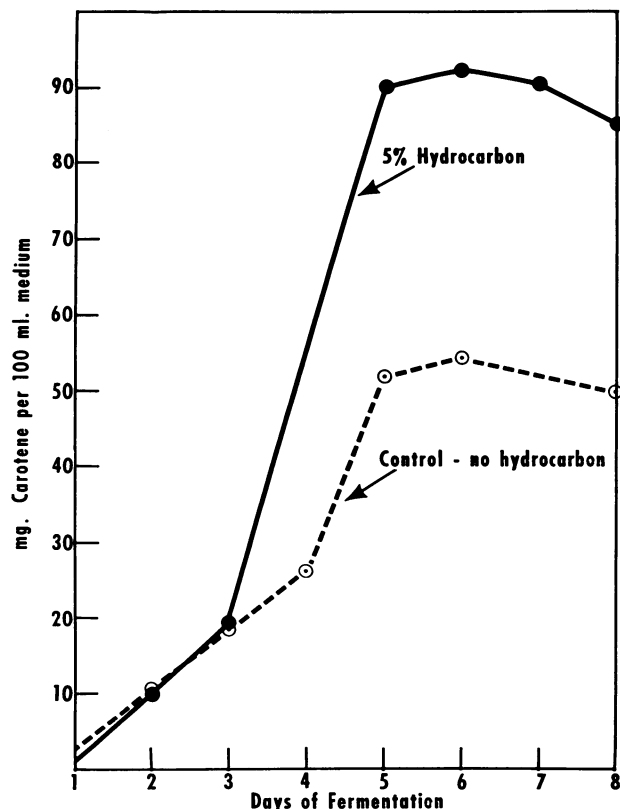


FIG. 1. Effect of hydrocarbon (Deobase) on rate of carotene production.

between 5 and 10% (Table 4). Unlike crude kerosene, the refined solvent neither prevented the formation of the lipid agglomerates nor resulted in free carotene in the supernatant; all carotene produced remained intracellular.

Maximal carotene yields were attained after 5 to 6 days of fermentation in both controls and flasks containing 5% refined kerosene (Fig. 1). The presence of solvent did not appreciably affect the rate of carbohydrate or nitrogen utilization; about two-thirds of the available carbohydrate and one-half of the available nitrogen were consumed during the first 24 hr; thereafter, consumption of these nutrients decreased markedly. Maximal mycelial growth was attained in about 48 hr; the mycelium contained approximately 50% lipid (dry weight basis) in both control and hydrocarbon-containing flasks. Addition of 5% Deobase did not markedly influence the concentrations of either lipid or β -ionone found to be optimal for carotene production in control flasks (Tables 5 and 6).

The influence of other organic solvents on carotene synthesis by mated cultures of *B. trispora* was also investigated. Almost all the hydrocarbons or derivatives tested, e.g., pentane, hexane, cyclohexane, benzene, xylene, and chloroform, either inhibited growth when added at

TABLE 5. Effect of lipid and hydrocarbon on carotene production

Lipid*	Hydrocarbon†	Dry mycelium	Carotene yield in solids	Carotene yield
ml/100 ml	ml/100 ml	g/100 ml	μg/g	μg/100 ml
3	0	3.95	5,750	22,700
4	0	4.77	6,800	33,700
5	0	5.97	9,000	53,850
6	0	6.54	6,670	43,700
7	0	7.25	5,720	41,470
8	0	8.31	4,750	39,800
3	5	3.27	13,100	42,800
4	5	3.93	15,300	61,570
5	5	4.95	17,000	84,000
6	5	5.64	15,500	87,200
7	5	6.42	10,750	69,200
8	5	5.97	9,300	56,570

* White grease.

† Deobase.

TABLE 6. Effect of β -ionone and hydrocarbon on carotene production

β -Ionone	Hydrocarbon*	Dry mycelium	Carotene yield in solids	Carotene yield
ml/100 ml	ml/100 ml	g/100 ml	μg/g	μg/100 ml
0	0	5.19	7,375	38,150
0.05	0	5.31	9,875	52,500
0.1	0	5.50	9,500	52,300
0.2	0	5.66	5,600	31,650
0.3	0	5.71	5,100	29,100
0	5	3.73	11,650	43,500
0.05	5	3.88	17,000	65,750
0.1	5	3.92	21,750	85,250
0.2	5	3.85	15,700	60,000
0.3	5	3.91	14,000	54,700

* Deobase.

the start of fermentation or caused lysis of the mycelium when added after 2 days of incubation. Solvents that proved comparatively nontoxic to growth, e.g., methyl Cellosolve, did not enhance carotene synthesis. Only Sonobase,⁵ a hydrocarbon similar to Deobase and also derived from acid treatment of kerosene, gave stimulation comparable to Deobase (Table 7). Two solvents that were either miscible or partially miscible in both lipid and aqueous phases, Solketal⁶ and dimethyl formamide, were inimical to carotene production (Table 7).

Addition of Deobase to shaken-flask cultures of various strains of *Choanephoraceae* did not increase carotene synthesis by mated strains that were initially poor producers (Table 8). Some stimulation resulted when hydro-

⁵ Sonneborne Chemical and Refining Corporation, 300 Park Avenue South, New York, N. Y.

⁶ Solketal is 2,2-dimethyl-1,3-dioxolane-4-methanol, a product of Aldrich Chemical Company, Milwaukee, Wis.

TABLE 7. *Effect of various solvents on carotene production*

Solvent*	Dry mycelium	Carotene yield in solids	Carotene yield
	g/100 ml	μg/g	μg/100 ml
None	5.67	8,000	45,100
Sonobase	5.26	14,475	75,950
Frankobase†	5.64	10,380	58,700
Lobase†	4.03	10,825	43,700
Solketal	5.82	2,900	16,900
Methyl Cellosolve	5.15	1,360	6,950
Dimethyl formamide	4.42	362	1,610

* All solvents, except Sonobase, added after 2 days of fermentation because of varying degrees of toxicity.

† L. Sonneborne Sons, Inc., New York, N. Y.

TABLE 8. *Effect of hydrocarbon on carotene production by various strains of Choanephoraceae*

Organism	NRRL no.	Hydrocarbon*	Dry mycelium	Carotene yield in solids	Carotene yield
			g/100 ml	μg/g	μg/100 ml
<i>Blakeslea trispora</i>	9216 × 9159	—	5.36	9,250	49,500
<i>B. trispora</i>	9216	—	5.26	575	3,020
<i>B. trispora</i>	9216	+	Clumped		
<i>B. trispora</i>	9159	—	5.37	190	1,020
<i>B. trispora</i>	9159	+	3.11	630	1,950
<i>B. trispora</i>	2456 × 2457	—	5.97	5,700	34,000
		+	4.37	11,170	48,800
<i>B. trispora</i>	2456 × 9159	—	5.36	7,575	39,750
		+	4.28	17,000	72,800
<i>B. trispora</i>	2457 × 9216	—	5.83	4,200	24,550
		+	4.77	9,375	44,650
<i>B. circinans</i>	2546 × 2548	—	6.01	785	4,725
		+	4.65	767	3,600
<i>Choanephora conjuncta</i>	2560 × 2561	—	4.87	1,050	5,100
		+	4.21	1,320	5,550
<i>C. cucurbitarum</i>	6097 × 6098	—	4.95	930	4,600
		+	3.76	780	2,930

* Five per cent Deobase.

carbon was added to the mated pair of *B. trispora*, NRRL-2456(+) and NRRL2457(-). Interchanging plus and minus strains of NRRL9216 and NRRL9159 with NRRL-2456 and NRRL2457 resulted in good carotene production only by NRRL2456(+) × NRRL9159(-) in the presence of hydrocarbon. Added hydrocarbon did not enhance production by nonmated cultures of *B. trispora* (Table 8).

DISCUSSION

Data presented in this paper demonstrate that addition of hydrocarbons, such as kerosene, or modified kerosenes, to fermentation media markedly stimulates carotenogenesis by mated cultures of *B. trispora*. Although the function of these solvents in stimulating carotene synthesis is not at present known, the data obtained encourage some speculation.

Stimulation is probably not caused by a potential precursor in the solvent as indicated by experiments using fractionated solvent;⁷ all fractions gave some stimulation. In addition, adding hydrocarbon in the absence of β -ionone does not sufficiently enhance yields to suggest precursor activity or to account for the considerable increase of carotene in the presence of β -ionone. Evidence also indicates that the primary action of the hydrocarbon is not emulsification of the natural lipid for the following reasons: hydrocarbon stimulates carotene synthesis in the absence of lipid; acid-purified kerosene (Deobase) does not prevent formation of lipid agglomerates; crude kerosene prevents agglomerate formation of lipid but does not give yields as high as those produced in the presence of purified kerosene; ratios of solvent to lipid higher than 1:1 did not increase yields, although unused lipid still remained in the medium; and addition of hydrocarbon after most lipid was consumed still resulted in enhanced yields.

When purified hydrocarbon is used, almost all of the carotene accumulates within the mycelium. This effect suggests that the hydrocarbon may function as a reservoir within the mycelium and make possible storage of greater amounts of the fat-soluble pigment. This second possibility would be difficult to prove experimentally and would be outside the scope of our present investigation.

Added hydrocarbon did not qualitatively influence the pigments produced as determined by column chromatography using MgO: Celite (1:1); all-*trans*- β -carotene remained the predominant pigment. In addition, there was no apparent morphological influence of the hydrocarbon on the mycelium as determined by microscopic observation.

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⁷ Two-hundred milliliters of Deobase were vacuum distilled, and the distillate was collected in five aliquots of 40 ml each.

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