

Enhancement of β -Carotene Synthesis by Citrus Products

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Received for publication 12 October 1962

ABSTRACT

CIEGLER, ALEX (U.S. Department of Agriculture, Peoria, Ill.), GEORGE E. N. NELSON, AND HARLOW H. HALL. Enhancement of β -carotene synthesis by citrus products. *Appl. Microbiol.* **11**:128-131. 1963.— β -Ionone, a stimulatory compound in the microbiological production of β -carotene by mated cultures of *Blakeslea trispora*, could be replaced with low-cost agricultural by-products (citrus oils, citrus pulp, or citrus molasses) with as good or better carotene yields. Peak yields (81 to 129 mg of carotene per g of dry solids) were achieved in 5 days. The various citrus products tested did not change the pigments produced; all *trans*- β -carotene remained the predominant pigment. The acid-hydrolyzed soybean meal and corn used in previous production media could be replaced with unhydrolyzed cottonseed embryo meal and corn in a medium that also contained a natural lipid, deodorized kerosene, nonionic detergent, and a precursor.

β -Ionone stimulates carotenogenesis by mated cultures of *Blakeslea trispora* (Anderson et al., 1958; Ciegler, Arnold, and Anderson, 1959b). However, this compound is comparatively expensive, and it was desirable to find a lower-cost substitute for use in large-scale commercial fermentations. Because carotenoid compounds are found to occur naturally in many plants and plant products, there appeared a possibility that the precursors to these pigments would also be present. Such intermediates should have potential in the microbiological production of β -carotene. Hence, efforts were made to find suitable low-cost agricultural products or by-products.

In addition, it was desirable, prior to scaled-up operations, to eliminate the necessity for acid hydrolysis of soybean meal in the medium (Ciegler, Arnold, and Anderson, 1959a).

MATERIALS AND METHODS

Cultures. Two mating strains of *Blakeslea trispora*, NRRL 9216(+) and NRRL 9159(-), were used for all fermentations. Stock cultures of these strains, maintained at room temperature, were transferred once a week.

Fermentation. Inocula were produced as previously described by Ciegler, Nelson, and Hall (1962). In preliminary experiments, the fermentation medium had the following composition: acid-hydrolyzed ground whole

corn, 2.3%; acid-hydrolyzed soybean meal, 4.7%; deodorized kerosene (Deo-Base; Sonneborne Chemical and Refining Corp., New York, N. Y.) 5%; soybean or cottonseed oil, 5%; nonionic detergent (Triton X-100; Rohm and Haas Co., Philadelphia, Pa.), 0.12%; thiamine HCl, 0.1%; NaOH to pH 6.5. Amounts (100-ml) of medium were dispensed into 500-ml Erlenmeyer flasks and sterilized for 30 min at 121 C. After the flasks were incubated for 2 days on a rotary shaker operating at 200 rev/min, 0.1% β -ionone was added to those flasks indicated in the tables. Fermentations were run for a total of 6 days. The above medium was subsequently modified, as described later in the text, by replacement of the acid-hydrolyzed soybean meal and corn with nonhydrolyzed cottonseed embryo meal and corn at concentrations of 5 and 2.5%, respectively. This modified fermentation medium was used in subsequent experiments.

Mycelium containing the β -carotene was recovered at completion of fermentation without blanching by filtration through cheesecloth discs on Büchner funnels. Procedures for analyzing the fermentation have been described by Anderson et al. (1958).

Citrus-pulp fractionation. Portions of approximately 33 g of commercially dried citrus pulp were extracted in a Soxhlet apparatus with 200 to 300 ml of pentane-hexane (bp 33 to 57 C) for 3 to 4 hr; about 3% of the pulp was extracted. (The citrus pulp was obtained from Minute Maid Co., Orlando, Fla., and from U.S. Fruit and Vegetable Products Laboratory, U.S. Department of Agriculture, Winter Haven, Fla. The pulp used also contained seeds, rag, and pelleted fines.) The residue was treated in a similar manner with acetone, which removed 6% more solids. The two solvent extracts were separately concentrated by flash evaporation at 35 C until no solvent remained, and then sufficient Deo-Base was added so that 1 ml of each of the final volumes was equivalent to material found in 1 g of original dried pulp. The pulp was air-dried after the last solvent extraction and suspended in approximately 250 ml of water; the mixture was heated in an autoclave for 10 min at 121 C. Solids were recovered by filtration and repeatedly washed with boiling water until the effluent was comparatively clear. The aqueous extract and washings were combined and carefully concentrated at 35 C, so that 1 ml of concentrate was equivalent to 1 g of dried pulp. About 36% of the organic solvent-extracted pulp was water-soluble. The solids remaining after organic

solvent and aqueous extractions were dried in an air oven at 60 C. These solids constituted 55% of the original citrus pulp.

RESULTS

Various agricultural products were tested as a replacement for acid-hydrolyzed soybean meal, but best results were obtained with cottonseed embryo meal (Pharmamedia; Traders Oil Mill, Fort Worth, Texas) as shown in Table 1. Optimal yields were attained with 5% unhydrolyzed cottonseed meal; little further advantage accrued from higher concentrations. Further work revealed that Fermatein (Traders Oil Mill, Fort Worth, Texas), a crude and low cost cottonseed meal, served almost as well as Pharmamedia. Acid treatment of the cottonseed embryo meal did not result in substantially increased yields. Hence, in all subsequent experiments, 5% unhydrolyzed cottonseed embryo meal and 2.5% unhydrolyzed corn were substituted for the previously acid-treated medium components.

In preliminary experiments to find a low-cost substitute for the stimulatory compound β -ionone, various terpenoid compounds were investigated. These proved toxic to both growth and carotene synthesis when added at the beginning of the fermentation, although they considerably enhanced synthesis when added after 48 hr of incubation. However, even under conditions of delayed addition, the maximal effective concentration for most terpenes was only 0.05%; lower yields resulted from higher concentrations except for geraniol (Fritzche Brothers, Inc., New York, N.Y.) and *d*-limonene (U.S. Fruit and Vegetable Products Laboratory, Orlando, Fla.), which were effective at a concentration of 0.1% (Table 2).

Encouraging results obtained from use of *d*-limonene, a cyclic monoterpene found in citrus and other oils, led to investigation of citrus pulp, a low-cost waste by-product of the citrus industry which contains trace quantities of *d*-limonene. Citrus pulp, as sold commercially, is composed of dried alkali-treated peel, seeds, rag, and fre-

quently pelleted citrus fines. For our work, the pulp was ground in a Wiley mill equipped with a 20-mesh screen prior to its use in fermentation. Varying concentrations of citrus pulp were added at the start of fermentation; no advantage accrued from later addition. Citrus pulp at a 3 to 5% concentration stimulated carotene production as well as or better than 0.1% β -ionone (Table 3). At pulp concentrations higher than 5%, the medium became very viscous and difficult to handle during recovery procedures. In addition, excessive viscosity probably limited oxygen transfer, resulting in lowered carotene yields.

The time course of carotene production in which the adjuvant is β -ionone or citrus pulp is seen in Fig. 1. Carotene synthesis was initiated earlier in flasks containing citrus pulp than in flasks containing β -ionone. However, maximal yields were attained between 5 and 6 days under both conditions. Thereafter, rapid carotene destruction occurred.

Because a small amount of oil-soluble material present in dried citrus pulp (approximately 1 ml per 30 g of pulp as determined by extraction with pentane-hexane) may have constituted the active fraction, crude essential oils as commercially derived from various citrus products were tested to determine their effect on carotenogenesis. Grapefruit, tangerine, and Valencia orange essential oils, at concentrations of 0.1 to 0.5%, were found to be effective

TABLE 2. Stimulation of carotene synthesis by terpenes and terpene derivatives

Adjuvant*	Adjuvant concn	Dry solids	Carotene yield in solids	Carotene yield
	%			
None	—	5.21	3.6	18.7
β -Ionone	0.1	5.00	19.5	97.5
α -Pinene	0.05	5.55	8.5	47.1
Carveol	0.05	5.78	9.0	52.0
Pimenta oil†	0.05	5.63	7.3	41.0
Isoborneol	0.05	5.89	10.7	63.0
Terpineol	0.05	5.89	7.2	41.7
Geraniol	0.1	5.77	10.7	61.6
<i>d</i> -Limonene	0.1	6.09	10.5	64.0

* Production medium: as noted in Table 1 plus 5% cottonseed embryo meal.

† Pimenta oil is derived from allspice (*Pimenta officinalis*) and is 70% eugenol plus miscellaneous compounds.

TABLE 1. Effect of varying concentrations of unhydrolyzed cottonseed embryo meal on carotene synthesis

Meal substrate*	Meal concn	Hydrolysis	Dry solids	Carotene yield in solids	Carotene yield
	%				
Soybean	5	+	6.39	16.3	104.0
Soybean	5	—	5.31	6.3	33.4
Cottonseed	1	—	5.23	6.0	31.4
Cottonseed	3	—	6.11	10.5	64.2
Cottonseed	4	—	5.98	14.0	83.7
Cottonseed	5	—	6.10	15.9	97.0
Cottonseed	6	—	7.19	14.7	105.5
Cottonseed	7	—	7.06	14.3	100.1
Cottonseed	5	+	6.54	14.3	93.5

* Production medium: corn, 2.3%; cottonseed oil, 5%; Deo-Base, 5%; nonionic detergent, 0.12%; thiamine HCl, 0.2 mg/100 ml (pH 6.5); 0.1 ml of β -ionone added to each flask after 2 days of fermentation.

TABLE 3. Stimulation of carotene synthesis by citrus pulp

Adjuvant*	Adjuvant concn	Dry solids	Carotene yield in solids	Carotene yield
	%			
None	—	6.26	5.0	31.3
β -Ionone	0.1	6.40	15.9	101.8
Citrus pulp	0.5	5.36	6.3	33.8
Citrus pulp	1.0	6.33	10.4	65.8
Citrus pulp	3.0	6.76	16.0	108.0
Citrus pulp	5.0	7.68	16.8	129.0
Citrus pulp	7.0	9.32	8.3	77.4

* Production medium: as noted in Table 2 plus appropriate adjuvant.

in stimulating carotene synthesis. Representative data for grapefruit oil are shown in Table 4.

The citrus pulp subsequently was extracted with various solvents in an attempt to obtain a crude fraction con-

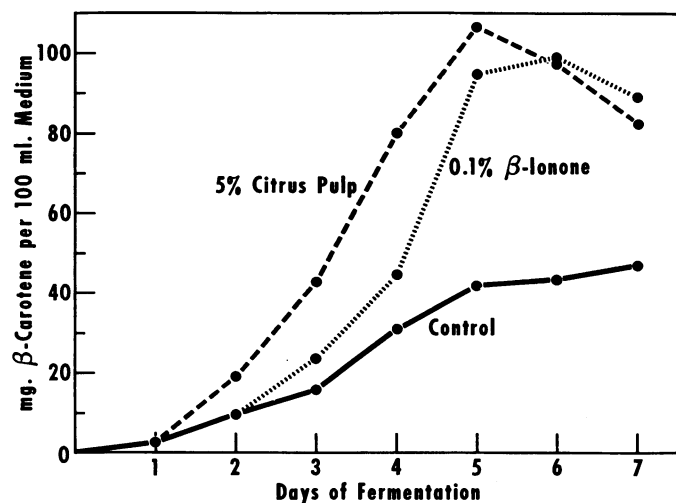


FIG. 1. Effect of citrus pulp and β -ionone on time course of carotene production.

TABLE 4. Stimulation of carotene production by grapefruit oil

Adjuvant*	Adjuvant concn	Time of adjuvant addition	Dry solids	Carotene yield in solids	Carotene yield
	%	day	g/100 ml	mg/g	mg/100 ml
None.....	—	—	5.96	6.3	43.5
Citrus meal.....	5	0	7.95	12.8	101.8
Grapefruit oil....	0.1	0	6.17	11.3	69.8
Grapefruit oil....	0.5	0	5.75	14.3	82.0
Grapefruit oil....	0.1	2	5.00	18.6	93.0
Grapefruit oil....	0.5	2	5.83	18.0	105.0

* Production medium: as noted in Table 2 plus appropriate adjuvant.

TABLE 5. Effect of various extracts of citrus pulp on carotene synthesis

Adjuvant*	Adjuvant concn	Dry solids	Carotene yield in solids	Carotene yield
	%	g/100 ml	mg/g	mg/100 ml
None.....	—	5.63	3.9	22.0
Citrus pulp.....	5	7.78	10.4	80.9
Acetone-extracted pulp...	5	8.92	9.4	83.4
Acetone extract†.....	2.5	5.59	6.5	36.4
Acetone extract.....	5	5.01	3.8	19.0
Acetone extract.....	10	3.32	1.6	5.4
Pentane-hexane‡ extract...	5	5.28	2.3	12.1
Pentane-hexane extract...	10	4.84	2.2	10.6
Water extract.....	5	5.89	13.5	79.4
Water extract.....	10	6.06	14.8	89.4
Extracted pulp‡.....	5	7.72	4.1	31.6

* Production medium: as noted in Table 2 plus appropriate adjuvant.

† The solvent was removed by flash evaporation, and the remaining material was dissolved in Deo-Base so that 1 ml of the solution contained the extractables found in 1 g of citrus pulp.

‡ Citrus pulp extracted with organic solvents and water.

taining the active material (Table 5). At concentrations representing 5 g of citrus pulp per 100 ml of medium, organic-solvent fractions gave little or no stimulation and actually reduced yields at higher concentrations. However, the stimulation given by the water-soluble fraction was approximately equivalent to that given by the whole dried pulp. Addition of the extracted residual solids to the fermentation medium gave slight stimulation, possibly because of the resulting increased viscosity (Zajic, 1960).

Upon retesting of the pulp, we found a considerable portion of the enhancing activity to be associated with the pelleted fines. These fines are produced from the fine material screened from the dried citrus pulp to prevent a dusty feed product. The fines were put through a press, extruded as pellets, and then added to the citrus pulp. Table 6 shows that the stimulation given by commercial pulp is, in considerable part, due to the pelleted material. Maximal yields (120 mg of carotene per 100 ml of medium) were attained at a 3% concentration of added fines. The same yields were attained from a water-soluble extract representing 3% citrus fines. At higher concentrations, the medium became very viscous with a concomitant decrease in yield. Pulp minus the fines portion still enhanced carotene synthesis but not to the same extent as when the fines portion was present. The reason for the higher activity of the fines is unknown.

The unexpected stimulation given by the water-soluble

TABLE 6. Stimulation of carotene production by citrus fines

Adjuvant*	Adjuvant concn	Dry solids	Carotene yield in solids	Carotene yield
	%	g/100 ml	mg/g	mg/100 ml
None.....	—	4.94	8.3	41.0
Citrus pulp.....	5	8.98	10.3	92.5
Pulp minus fines.....	5	8.82	7.0	61.7
Citrus fines.....	1.5	5.82	15.0	87.3
Citrus fines.....	2.5	7.21	15.8	113.7
Citrus fines.....	3.0	7.99	15.0	119.9
Citrus fines.....	5.0	8.67	7.8	67.6
Water extract of fines....	3.0†	6.43	17.9	115.0

* Production medium: as noted in Table 2 plus appropriate adjuvants.

† A 3-ml amount of the aqueous extract was equivalent to 3 g of unextracted citrus fines.

TABLE 7. Stimulation of carotene production by citrus molasses

Citrus molasses*	Dry solids	Carotene yield in solids	Carotene yield
	%	g/100 ml	mg/100 ml
None	6.93	9.7	67.2
0.1	5.79	14.3	82.6
0.3	5.91	16.2	95.8
0.5	5.94	16.8	99.9
1.0	6.27	17.2	107.9
3.0	6.48	17.5	103.3
5.0	7.62	13.8	105.0

* Production medium: as noted in Table 2 plus appropriate adjuvant.

fraction led us also to test the molasses derived from limed wet citrus pulp. Citrus molasses enhanced carotenogenesis at concentrations from 0.1% to 1.0%; at higher concentrations there was no further yield increase (Table 7).

DISCUSSION

β -Ionone has been effectively replaced in the fermentative production of β -carotene with low-cost agricultural products. This should make the process of considerable industrial interest. Addition of these products 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.

The data indicate that enhanced yields resulting from addition of citrus pulp or citrus molasses are not a result of adding carotenoid compounds already present in the citrus products. Our analyses show only trace amounts of carotenoid compounds to be present, i.e., in the range of 100 μ g per g of dry citrus material. Addition of these carotenoid compounds to the fermentation did not enhance carotene yields. Addition of increasing concentrations of the organic solvent-extractable portions of citrus pulp results in both decreased growth and decreased yields, suggesting the presence of an inhibitor. Removal of the oil-soluble material by solvent extraction did not cause any appreciable loss of stimulation by the remaining citrus pulp.

The presence of oil-soluble material in the citrus pulp does not appear to interfere with the activity of the water-soluble portion, which, as indicated by the data, contains the enhancing factor. The nature of the compound in the latter fraction is not yet known but is under investigation. The factor is, however, apparently not the same as that found in grapefruit, tangerine, and orange oils, because of solubility differences.

Enhanced carotene synthesis derived from addition of citrus oils may result from the presence of terpenoid compounds, e.g., *d*-limonene. *d*-Limonene is found in citrus oils and has been shown, as well as other terpenes, to be stimulatory to carotenogenesis.

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