Stabilization of Crude Carotene-Containing Mycelium

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Carotene in dry crude mycelia produced by fermentation was stabilized by blending with polymers, frequently in the presence of dextrose. Starch, gelatin, gum arabic, carboxymethyl cellulose, and several bacterial polysaccharides stabilized the carotene effectively. In a typical preparation of a stabilized product, the dry mycelium was blended with polymer and dextrose dispersed in water, the pastry mixture was extruded, and the extruded filaments were air-dried and powdered. Several stabilized products showed 80% or more of the original carotene content after storage at 41° C. for one year. If the powder was too fine, stability was poor. As humidity increased, storage stability decreased. Blends of the stabilized products in three commercial poultry feeds also showed good stability.

Extensive investigations were carried out between 1958 and 1963 to develop a process for producing β-carotene by fermentation (Ciegler, 1965). Ciegler noted that poor storage stability of β-carotene in the fermentation solids was an important factor in considering this process for commercial development. The present report is concerned with stabilization of this product.

The primary application projected for stabilized crude mycelium was in animal and poultry feeds as a vitamin A source. This application required that the stabilized product be in particle form and that the particles have a size range suitable for blending with feeds to give stable mixtures. Also, the stabilized product should be readily adaptable for pelletizing. In addition to the requirements related to particle form, materials used to stabilize the mycelium must be both readily digestible and nontoxic. Stabilized particles should also be acceptable by farm animals without discrimination. The present report, however, is restricted to studies on the stabilization of β-carotene in dried mycelium, and to establishing that the provitamin retained its stability in blends with animal feed.

Ciegler *et al.* (1961) reported the stability of blends of crude mycelium with different antioxidants. Santoquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, Monsanto Chemical Co.) significantly decreased the rate of disappearance of β-carotene from the samples. Other antioxidants were relatively ineffective. The author confirmed both of these observations.

Encapsulation of the fermentation solids was considered a promising approach to a stabilized product. Before preparation and evaluation of encapsulated mycelium particles, storage stability of mycelia embedded in several polymer films was determined.

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MATERIALS

Mycelium. The methods described by Ciegler (1965) were used to obtain mycelium. The fermentation solids were filtered, blended with 0.25% Santoquin, dried in a vacuum below 50° C., ground to approximately 20-mesh, and stored in a deep freeze at −10° C. for stabilization experiments. Mixture 6, a dispersion of Santoquin on an inert solid, was found equally satisfactory and more convenient than the liquid form. Many stabilization experiments were done using mycelium free of Santoquin. However, the antioxidant served to minimize losses of carotene during processing; hence, its use is advantageous. Crude dry mycelium containing over 8 mg. of β-carotene per gram was used in the stabilization work.

Poultry Mash. Poultry mashes were obtained from Ralston Purina Co. (St. Louis, Mo.), Farm Bureau Assoc. (Waltham, Mass.), and Agway, Inc. (Syracuse, N. Y.) for blending with stabilized mycelium and storage of the mixtures.

METHODS

Embedded Mycelium Mixtures. Twenty milligrams of mycelium of known carotene content were placed in an aluminum dish approximately 2 inches in diameter and 1 inch deep. A measured volume (generally 5 ml.) of an aqueous solution containing a known weight of polymer was poured into the dish. The mycelium was not readily wetted by the solution, therefore the dish was placed in a desiccator; vacuum was applied until the solution boiled

and was then released. The mycelium settled to the bottom of the solution. Particles frequently remained on the surface, so application and release of the vacuum were repeated. As the vacuum was applied, moisture evaporated from the mixture, and its temperature decreased. If the viscosity of the solution increased unduly, the mixture was warmed to about 50° C. before vacuum was applied. The aluminum dishes were placed in storage in an oven. Moisture evaporating from the dishes passed through a vent in the top of the oven. The contents of the dishes were dry within one or two days.

In one experiment, a dispersion of 1 gram of dry mycelium in 100 ml. of a solution containing 5% anhydrous dextrose and 5% thin boiling starch (Crown XH, Penick and Ford Co., Cedar Rapids, Iowa) was prepared. Mycelium was impregnated with the solution under vacuum as described. Several of the mycelium particles were withdrawn carefully with a spatula from the suspension and were air-dried individually.

Extruded Mycelium Mixtures. Crude mycelium was combined with aqueous solutions of different polymers, frequently in the presence of dextrose. The polymer dispersion was prepared by the usual methods. Six parts of water were used per part of carboxymethyl cellulose (Grade 12 HP, Hercules, Inc.), five parts of water per part of starch, and 0.67 part of water per part of gum arabic. The aqueous solution was passed through a 3-roll mill 10 times. A weighed amount of mycelium was added to the mixture, which was passed through the rolls again 10 times. The orange mass was very smooth, and entirely free of lumps.

Dispersions in gelatin were prepared using a different technique. A weighed amount of dry mycelium was dropped into a hot (60° to 70° C.) solution of gelatin in a vacuum flask. Vacuum was applied until the solution boiled and was then released. This process was repeated three times. During the treatment, the temperature of the mixture was held at about 50° C. to maintain a relatively low viscosity. This treatment is believed to eliminate a high proportion of air from the mycelium particles and replace it with solution. The pasty mixtures were loaded into the extruder (Figure 1) with a spatula and by advancing the piston were extruded through a hole in the nozzle, $^1/_{16}$ inch in diameter. Extruded filaments were collected on a sheet of aluminum foil and allowed to air-dry. The dry product was cut with a razor blade or ground. Yield of extruded mixtures generally amounted to over 90% of the input solids. Average yields of β-carotene were slightly lower.

Blends of Stabilized Mycelium with Poultry Mash. Poultry mash and stabilized mycelium were mixed by shaking. Fifty-gram samples of the mixture were stored in petri dishes, forming a layer about $^1/_2$ inch deep. Ten-gram samples were stored in aluminum dishes, forming a layer about $^3/_8$ inch deep. Similar samples of feed alone were stored with the blends, and both were analyzed on the same day.

Analysis of Mycelium and Stabilized Mycelium. Solids of both of these types were analyzed as follows. A weighed sample (50 to 100 mg.) was ground under petroleum ether in a mortar and pestle. The solids were allowed to settle, and the supernatant solution was decanted. New

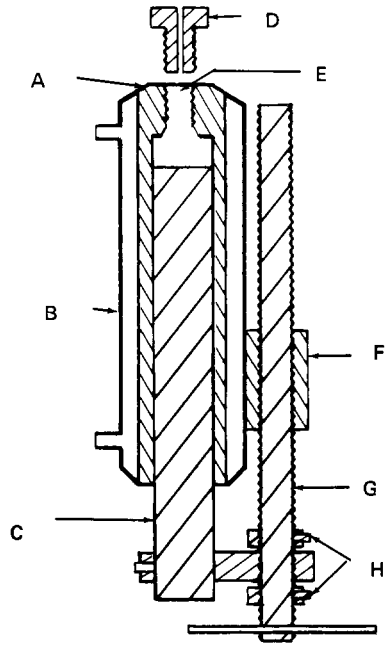

Figure 1. Experimental extruder

A. Brass cylinder
B. Copper steam jacket
C. Steel piston
D. Nozzle (1/32-, 1/16-, or 3/32-inch opening)
E. Threaded opening
F. Threaded brass nut soldered to steam jacket
G. Brass screw
H. Set screws

portions of petroleum ether were used until the extract was colorless. The combined extracts were made up to a suitable volume. Intensity of color was determined at 445 to 450 mμ with a Coleman or Beckman spectrophotometer. Both instruments gave identical results. The concentration of carotene was determined by use of the extinction coefficient, $E^{1\%}_{1\,cm.} = 2450$ (Bunnell and Bauernfeind, 1962). This coefficient was checked several times by comparison with a standard absorbance curve from pure trans-β-carotene dissolved in petroleum ether.

Analysis of Mixtures of Stabilized Mycelium and Poultry Mash. The following procedure was developed after many experiments and gave satisfactory results.

A mixture of 50.0 grams of feed and 50.0 mg. of stabilized mycelium was placed in a 16-ounce bottle. To this was added 30 cc. of white sand and 35 cc. of deoxygenated water. The head space of the bottle was flushed with nitrogen; the bottle was sealed and shaken on a paint shaker for 30 minutes. The bottle was opened; 10 cc. of acetone was added followed by 100 cc. of deodorized kerosene (Deo-base), and the head space was again flushed with nitrogen. The mixture was shaken on the paint mixer for 15 minutes. About 20 cc. of the mixture was decanted and centrifuged. Optical transmittance of the upper layer at 445 mμ was measured using a spectrophotometer. The acetone–Deo-base mixture was used in the reference cell in the same proportion used in the analysis.

In this procedure, the white sand served to break down the particles of stabilized mycelium In the absence of sand and/or water, recovery of β-carotene was poor.

Air was displaced from the water and from the bottle to prevent carotene decomposition. Acetone appeared to enhance transfer of carotene to the Deo-base, but its exact role is complex. Recovery of β-carotene increased and then decreased as more acetone was used. The amount given in the above procedure was suitable for the feeds which were examined but might have to be changed for a different brand of feed. The amount of water used is important in the analytical procedure. With 35 cc. of water, the Deo-base was easily decanted, but with 50 cc., the entire mass formed a viscous emulsion that was difficult to separate. Deo-base was used because it could be handled easily without loss owing to evaporation. Many tests showed that it gave results identical with petroleum ether in analysis for β-carotene.

The above procedure was satisfactory for the three feeds used in this work. Animal feeds vary considerably, and a brand of feed other than those used in this work might require modification of the above procedure.

Storage Test Conditions. Samples of stabilized mycelium were stored in thin layers in petri dishes so that all of the particles were exposed to air. The dishes were kept in an oven maintained at 41 ± 1° C. This temperature, selected arbitrarily, is more severe than average summer conditions. The oven was open to the atmosphere via a small orifice in the top of the cabinet. The humidity in the oven therefore varied with atmospheric conditions, but was consistently low, probably less than 20%.

Samples also were stored at relative humidities of 23, 53, and 75%, all at 41° C. For these tests, the samples were dried for three days in the 41° C. oven and then weighed amounts in petri dishes were stacked with appropriate spacers over saturated salt solutions in 1-gallon cans which were sealed during storage. Uniformity of the atmosphere throughout the cans was checked by analysis of samples stored near the bottom as well as near the top. Oxygen in these vessels was replenished when the cans were opened for analyses; however, the container volume compared with the mycelium mass was large, and this alone probably assured an ample supply of oxygen.

Table I. Stability of Embedded Mycelium Stored in Air at 41 ± 1° C.

Embedding Mixture	Storage Period, Days				
	0	3	42	56	98
	β-Carotene Content, Mg./Gram of Mycelium				
None	14.3	1.9			
2% Gelatin[a]	12.0		4.6		5.1
5% Gelatin 0.5% Dextrose[b]	29.1		24.9		19.6
5% Gum arabic	27.0				17.3
2% Carboxymethyl cellulose[c]	12.0		8.2		8.4
5% Corn starch[d] 0.5% Dextrose	27.0				14.3
5% Corn flour, cooked 0.5% Dextrose 0.1% Santoquin	14.6			0.2	1.2
5% Wheat flour, cooked	14.4			4.2	4.0

[a] 125 bloom (Atlantic Gelatin Co., Woburn, Mass.).
[b] Anhydrous.
[c] Grade 12HP (Hercules, Inc., Wilmington, Del.).
[d] Douglas Crown XH fluidity (Penick and Ford Co., Cedar Rapids, Iowa).

RESULTS AND DISCUSSION

Stabilized Crude Mycelium. Table I shows that several embedding materials provided significant protection for carotene in mycelium. Since these experiments were performed with mycelium particles of a fairly broad size range, and since the major objective of these experiments was to determine in a general way the merit of coating mycelium, no attempt was made to compare the effectiveness of the different embedding materials. Gelatin with dextrose, gum arabic, starch, and carboxylmethyl cellulose all have good results. Both corn flour and wheat flour gave relatively poor results.

In these experiments, high loss of β-carotene was frequently observed within the first few days' storage and was attributed to incomplete coating of the mycelium. Embedded mixtures did not provide a perfect barrier for the mycelium particles. When several of the embedded mixtures were contacted with petroleum ether (a good solvent for carotene) for a few minutes, the solvent became yellow, indicating that some of the mycelium was incompletely coated. However, most of the mycelium was well coated.

These data encouraged the author to proceed with a study of methods for preparing coated mycelium in particle form. In an initial attempt, embedded particles were dried individually as described above. In this experiment, there was no control of the proportion of the coating material to mycelium. The dry particles showed no loss of β-carotene during 80 days' storage.

Subsequently, mycelium particles were spray-coated with a solution of starch and dextrose in a laboratory Wurster apparatus (Caldwell and Rosen, 1964). The resulting product contained 50% coating (w./w.). This material retained 60% of its original β-carotene content after storage for 450 days at 41° C. No further work was done using this method. This result is presented because it demonstrates the effectiveness of the method which should merit attention in any work conducted on a large scale.

Table II shows stability of stabilized extruded particles of different mesh size. As particles were made finer, there was a consistent trend toward lower β-carotene content after seven days' storage. During subsequent storage, stability was generally good over periods up to 364 days. These tests were run on a limited number of mixtures, but the results appear to be generally valid.

Cut and ground products of the same particle size were equally stable. As a result of these experiments, extruded products were generally ground and a 16- to 30-mesh fraction was sieved for storage tests. Many blended products were prepared containing different proportions of mycelium with the ingredients shown in Table I. The objective was to prepare mixtures showing good storage stability and containing minimum amounts of stabilizers.

Table III shows storage stability of selected mixtures. All of the products listed contained one third by weight

Table II. Stability of Extruded Mixtures of Mycelium and Other Components in Air at 41 ± 1° C. and Comparison of Cut and Ground Products

Run No.	Composition: Components	Composition: Ratio	Mesh Size	Storage Period, Days: 7	14	28	49	168	364
				β-Carotene Content, % of Initial					
13850-14	Starch XH	1	Cut (~16)	87	87	81	81	82	103
	Dextrose	1	Ground on 16	93	84	94	100	76	100
	Mycelium	1	16–30	90	81	94	97	79	91
			through 30	75	66	73	73	58	67
13850-29	Gum arabic	1	Cut (~16)	90	76	79	90	80	98
	Mycelium	1	Ground on 16	92	75	83	90	83	96
			16–30	71		75	79	72	73
			through 30	71	55	52	56	49	48
13850-40	Gum arabic	3	Cut (~16)	97	85	91	94	96	100
	Dextrose	1	Ground on 16	94	85	97	106	96	100
	Mycelium	2	16–30	76	85	97	94	91	94
			30–60	76	79	82	91	80	85
			60–100	58	55	65	71		
			through 100	44	29				

Table III. Stability of Extruded Mixtures of Mycelium and Other Components in Air at 41 ± 1° C. and Summary of Best Results

Run No.	Components	Ratio	Storage Period, Days: 14	28	91	196	364
			β-Carotene Content, % of Initial				
13599-38	Starch XH	1	119	103	106	...	90
	Dextrose	1					
	Mycelium	1					
13850-26	Gelatin	1	100	91	83	79	100
	Dextrose	3					
	Mycelium	2					
13321-46	Gum arabic	1	72	72	79	58	68
	Dextrose	1					
	Mycelium	1					
13599-34	CMC 12 HP	1	131	95	104	94	93
	Mycelium	1					
14049-1	Polymer[a] Y-2448	1	81	100	100	84	84
	Dextrose	1					
	Mycelium	1					

[a] Bacterial polysaccharide (Northern Regional Research Laboratory, USDA, Peoria, Ill.).

of mycelium. Mixtures containing more mycelium showed poorer stability. The data show that over 80% of the β-carotene was retained during storage for one year.

Dextrose was found to be advantageous with gelatin and starch. Since one of the first products prepared from CMC and mycelium alone showed excellent stability, no attempts were made to incorporate dextrose with this polymer. Blends containing dextrose might substantially reduce costs of mixtures with CMC. The proportion of starch to dextrose could be changed over a wide range with no significant effect on stability. At a dextrose to starch ratio of 4 to 1, stability was poor; in contrast, stability was good in a mixture containing 10 parts of starch and 1 part of dextrose. The film-forming capacity of the stabilizers may play a role in the effectiveness of stabilization.

Table IV shows stability of mixtures of starch, dextrose, and mycelium (1:1:1) at 23, 53, and 75% relative humidity, all at 41° C. As humidity increased, storage stability decreased. Samples stored at 75% relative humidity turned black within the first week of storage, and over half of the original β-carotene content was destroyed. Considering the stability observed at 41° C. and 53% relative humidity, storage at this average humidity, or perhaps at slightly higher humidities, probably will prove satisfactory.

Stabilized Crude Mycelium with Animal Feed. Storage stability data for mixtures of stabilized mycelium with different poultry mashes are shown in Table V. Absorbance at 445 mμ of extracted carotene from the feed decreased rapidly during storage. Absorbance of the extract of the mycelium mixture alone was constant up to 140 days for two samples (14201-10 and 14201-21); thereafter, it decreased. For the other two samples, which were stored for only 91 days, absorbance of the extract of the mycelium mixture was constant. Absorbance of the extract of the feed-mycelium mixture decreased during storage. These data indicate that stabilized

Table IV. Stability of Mixtures of Mycelium, Starch, and Dextrose in Air at 41 ± 1° C. and Different Relative Humidities

Run No.	Composition: Components	Composition: Ratio	% Relative Humidity[a]	Initial Analysis, Mg./G.	Storage Period, Days: 7	28	70	112	196	224	252
					β-Carotene Content, % of Initial						
14201-27	Starch XH	1									
	Dextrose	1	75	3.3	41	21	6				
	Mycelium	1									
14201-34	Starch XH	1									
	Dextrose	1	53	3.3	97	89	85	64	60	35	54
	Mycelium										
14201-40	Starch XH	1									
	Dextrose	1	23	3.3	97	106	90	93	112	85	
	Mycelium										

[a] 75% R.H.—saturated sodium chloride solution. 53% R.H.—saturated sodium dichromate solution. 23% R.H.—saturated potassium fluoride solution. (See Lange, 1956.)

Table V. Recovery of β-Carotene from Blends of Feed and Mycelium Mixtures Stored in Air at 41 ± 1° C.

Run No.	Feed Mixture	Storage Period, Days: 0	14	28	49	91	140	245
		Absorbance at 445 mμ						
14201-10	10.0 grams of Ralston feed	0.46[a]	0.30	0.17	0.10	0.08	0.06	0.02
	10.0 mg. of mycelium mixture (14049-29)[b]	0.19	0.20	0.20	0.20	0.22	0.16	0.11
	Mixture of the above two	0.60	0.44	0.32	0.26	0.26	0.23	0.18
	Per cent recovery[c]	92	88	87	87	87	109	138
14201-21	10.0 grams of Ralston feed	0.21[d]	0.19	0.12	0.05	0.04	0.04	0.02
	50.0 mg. of mycelium mixture (14201-12)[e]	0.38	0.38	0.39	0.35	0.35	0.29	0.22
	Mixture of the above two	0.44	0.48	0.43	0.37	0.33	0.30	0.27
	Per cent recovery	75	85	84	93	85	91	112
14444-2	50.0 grams of Farm Bureau feed	0.52[d]	0.47	0.44	0.38	0.17		
	50.0 mg. of mycelium mixture (14049-29)[b]	0.46	0.46	0.30	0.44	0.44		
	Mixture of the above two	0.89	0.83	0.77	0.58	0.52		
	Per cent recovery	94	88	104	91	85		
14444-4	50.0 grams of Agway feed	0.92[d]	0.87	0.57	0.55	0.34		
	50.0 mg. of mycelium mixture (14049-29)[b]	0.44	0.46	0.26	0.39	0.42		
	Mixture of the above two	1.22	1.19	0.94	0.78	0.70		
	Per cent recovery	89	89	114	83	92		

[a] Absorbance figures for run number 14201-10 were made on 50 ml. of solution.
[b] 3.9 mg. of β-carotene per gram; composition: starch, dextrose, mycelium, 1:1:1.
[c] Per cent recovery equals absorbance of mixture multiplied by 100 and divided by the sum of the absorbances of the two components.
[d] Absorbance figures were made on 100 ml. of solution.
[e] 3.1 mg. of β-carotene per gram; composition: starch, dextrose, mycelium, 1:1:1

mycelium does not deteriorate significantly within at least three months in a feed environment. For the mixture containing Ralston feed, a stability of more than eight months is indicated.

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