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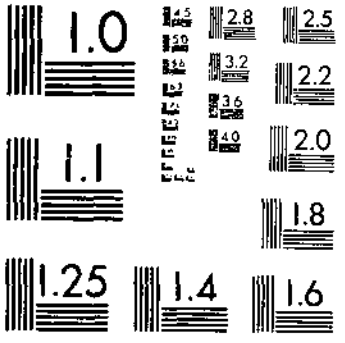

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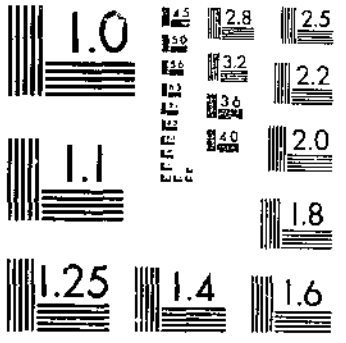

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This review was made as part of the investigations being conducted at the Northern Regional Research Laboratory on industrial utilization of cereal grains. The ARS Culture Collection, maintained there, is one of the world's largest and most complete collections of industrially important bacteria, molds, actinomycetes, and yeasts. The Collection serves as a source of authentic micro-organisms for the fermentative production of organic acids, vitamins, antibiotics, enzymes, feeds, beverages, and foods.

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CAROTENOIDS in the FUNGI MUCORALES

Special Reference to Choanephoraceae

By C. W. Hesseltine, Head, ARS Culture Collection, Fermentation Laboratory, Northern Utilization Research and Development Division, Agricultural Research Service [1]

INTRODUCTION

Mycological aspects of the fungi Mucorales and Choanephoraceae are emphasized in this review. Most of the chemical aspects of carotene and the theories of the metabolic pathways in its formation were intentionally omitted. Books about the chemistry of carotenoids have been written by Goodwin (*44*)[2] and by Karrer and Jucker (*79*). Their terminology is used throughout this review.

One characteristic of many of the Mucorales is the yellow or orange pigments within the fungus mycelium, especially the substrate mycelium. Aside from these two colors, pigments in the order are restricted to blackish ones with but few exceptions. In a few species the yellow color has been identified as carotenoid in nature, of which the major proportion is β-carotene. This material, in addition to its apparent importance to the organisms, especially in sexual reproduction, has certain practical implications. Isler (*78*), Bunnell (*21*), and Bauernfeind (*8*) and their coworkers touch on the use of carotenoids in foods. Colors are desired in some foods for better consumer acceptability. Since the fat-soluble carotenoids are yellow, orange, and red, they are excellent for this purpose.

One method of incorporating the β-carotene in fat-base foods is to micropulverize it and to suspend it in edible fat. In this form it could be used in such foods as margarine, butter, shortening, process cheese, egg yolk products (both frozen and dried), and bakery products. It might be used in other foods where color is desirable such as in macaroni, semolina, breaded food products like fish sticks, French dressings, whipped cream and toppings, and popcorn oil. Recently Bunnell and others (*21*) showed that β-carotene may be prepared in

[1] The Northern Regional Research Laboratory at Peoria, Ill., is headquarters for the Northern Utilization Research and Development Division.

[2] Italic numbers in parentheses refer to Literature Cited, p. 23.

a stable, dry, beadlet form for coloring water-base foods. In this form it could be used in such foods as citrus beverages, primary cheese, egg yolks (frozen and dried), eggnog, ice cream, dry cake mix, puddings, and gelatin desserts.

A second method of adding pigmentation to foods would be to introduce added carotenoids in a crude form into animal feeds to give more color to poultry, eggs, and beef. Another important consideration is the fact that β-carotene when ingested by many animals, including man, is converted into vitamin A.

At present two sources of carotenoids are available—natural and synthetic. They occur in natural materials, such as β-carotene in dairy products, lycopene in tomatoes, and zeaxanthin in yellow corn. Commercially, large amounts of alfalfa are used as a source of natural carotenoids. Beta-carotene is being produced in large quantities by chemical synthesis. It might be possible to utilize a third source—fungi—especially of the order Mucorales, either as a crude fermentation product for feeds or as a pure compound by extraction of the β-carotene from the mycelium.

CAROTENE IN MUCORALES

In 1890, Zopf (*111*, p. *148*) stated that a yellow fat material, probably lipochrome, was present in the sporangiophores, sporangia, and spores of *Pilobolus* and *Mucor*. He stated that none of it had been isolated and its exact nature was not known. Two years later, Zopf (*112*) studied the yellow and orange-red materials of the Mucorales in *Pilobolus*, especially *P. kleinii*. He illustrated the distribution of pigments in the mycelium and described their solvent extraction. By spectrographic means he showed also that absorption bands occurred at 484-469 and 452-439. By this and chemical means he concluded that the pigments were carotenes. The same materials were found in *P. oedipus* and *P. crystallinus*. The carotenes were present in the sporangiospores and were also associated with the fatty material in the zygospores. In Zopf's opinion the carotenes were reserve foods since they and the fatty material disappeared with the germination of spores. Kohl (*81*) accepted the same idea, although others, such as Palmer (*92*), believed that more likely the carotenoids were only associated with food reserves. Van Wisselingh (*110*) showed that carotene was present in *Mucor flavus*.

A study of carotenes in Mucorales has recently been undertaken primarily in the species *Mucor hiemalis* and *Phycomyces blakesleeanus* and even more recently at our laboratory on members of the Choanephoraceae. Earlier reviews on the occurrence of carotene in Mucorales are by Schopfer (*103*) and Goodwin (*43*, *44*).

Chodat and Schopfer (*29*) proved that the yellow pigment in *Mucor hiemalis* was a carotene. Schopfer (*102*) showed that β-carotene was present in *Phycomyces blakesleeanus* and determined that either asparagine or glycine could be used as a source of nitrogen for growing the fungus for producing carotene. Schopfer and Jung (*108*) further found that the β-carotene in this organism had vitamin A activity in feeding experiments. Castle (*25*) also reported the presence of α-carotene in *Phycomyces*. Bünning (*18–20*) reported carotene in the sporangiophores of *Pilobolus kleinii*. In a study of *Phycomyces*, Karrer and Krause-Voith (*80*) reported both β-carotene and α-carotene present in the mycelium. Bernhard and Albrecht (*9*) studied lipids of *Phycomyces blakesleeanus* and reported pigments—possibly β-carotene, α-carotene, and lycopene.

Schopfer and Grob (*104*) and Grob, Schopfer, and Poretti (*69, 70*), studying *Phycomyces blakesleeanus* and *Mucor hiemalis*, found that a medium having lactate as a sole source of carbon permitted only slight growth and no carotene formation. When acetate was added, growth and carotene formation were stimulated. Later, Schopfer and Grob (*105*) demonstrated that a medium composed of sodium acetate and ammonium nitrate supported growth of this fungus and allowed abundant formation of carotene. When acetate was the sole source of carbon, it acted as a precursor of carotene. This finding was confirmed by Friend, Goodwin, and Griffiths (*36, 37*), who stated that any member of the tricarboxylic acid cycle added to a medium containing glucose and ammonium nitrate stimulated growth equally well, but that varying degrees of β-carotene production were due to variations in the final pH values of the medium. The addition of β-ionone stimulated carotenogenesis from 200 to 300 percent in an acetate medium. The addition of β-ionone had earlier been shown by Mackinney and others (*87*) to stimulate β-carotene production. The Swiss workers (*68, 71*) had shown that labeled C_{14} was present in CH_3 and COOH groups and that these carbon atoms were incorporated equally into the β-carotene molecule. Further information on the carbon from acetate is given by Grob and Bütler (*58, 59*). Reichel and Wallis (*95*) have shown that *P. blakesleeanus* formed β-carotene from a variety of acids, including among others citric, succinic, and pyruvic, and earlier (*94*) discussed the metabolic pathway in the formation of β-carotene in *P. blakesleeanus*.

In 1951, Goodwin and his associates initiated a series of studies on carotenogenesis using *P. blakesleeanus*. The selection of this organism to study carotenogenesis was based on the following reasons: (1) The fungus is nonphotosynthetic. (2) It is easily cultivated on a simple aqueous medium with vitamin B_1 as the only micronutrient required. (3) It produces rather large amounts of β-carotene.

In the first detailed work, Garton and others (*38, 39*) showed that in this organism β-carotene was the predominant pigment although α-carotene also occurred. No xanthophylls or lycopene were found. Schopfer's medium was used; it contained: glucose, 10 percent; L-asparagine, 0.2 percent; KH_2PO_4, 0.15 percent; $MgSO_4 \cdot 7H_2O$, 0.05 percent; and thiamin, 25 μg. Schopfer's medium gave maximum growth, and lipid production occurred in 5 to 6 days, but maximum carotene production did not occur until 7 to 9 days after inoculation. The carotene content began to diminish by the 14th to 16th day. When grown in the dark, the fungus contained only about half as much β-carotene as when grown in the light. These investigators also found that the — strain produced twice as much β-carotene as did the + strain.

Goodwin and Lijinsky (*50*) showed that the L-valine or L-leucine, when used alone or with glycine or L-asparagine, stimulated carotenogenesis sometimes as much as four times. The minor polyene components of *P. blakesleeanus* were also studied by Goodwin (*41, 42*). Besides α- and β-carotene the following materials were found: phytofluene, 2.0 percent; γ-carotene, 0.85 percent; ζ-carotene, 0.35 percent; neurosporene, 0.6 percent; and lycopene, 0.6 percent. The percentages represent the total polyenes present. Several other materials were present that were not positively identified. When diphenylamine (1/40,000) was added to cultures, it almost completely inhibited production of the most unsaturated carotenes (α-, β-, γ-carotenes and lycopene) while stimulating production of the more saturated components, phytofluene, ζ-carotene, neurosporene, and possibly phytoene.

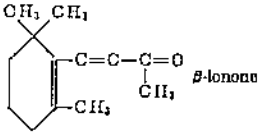
The nitrogen metabolism of *P. blakesleeanus* was further studied by Goodwin and Willmer (*53*) and Goodwin, Lijinsky, and Willmer (*51*), who showed that carotene was made only after the mycelial mat was fully formed. Furthermore, well-formed mats of mycelium dissimilating glucose could synthesize relatively more carotene in the absence of assimilable nitrogen than in its presence.

The effects of possible carotene precursors on growth, lipogenesis, and carotenogenesis were studied by Goodwin, Lijinsky, and Willmer (*52*) and by Glover, Goodwin, and Lijinsky (*40*), but no substance tested gave enhanced carotene production. Friend and Goodwin (*35*) showed that in *P. blakesleeanus* temperature did not affect the types of carotene produced in the range from 5° to 30° C. The optimum temperature for carotene production was 25° C.

Goodwin (*42*) showed that diphenylamine inhibited the synthesis of the least saturated polyenes in *P. blakesleeanus* with an increased synthesis of the most saturated polyenes, such as phytofluene. Additional study of this aspect was reported by Goodwin and others (*47*) and Goodwin, Jamikorn, and Willmer (*49*), who showed that in

this mold the saturated polyenes were produced much more rapidly than β-carotene. Thus, such materials as phytofluene reached their maximum in 3 to 4 days while β-carotene reached its maximum in 5 to 7 days of incubation. The increase of phytofluene and the decrease in β-carotene were directly proportional to amounts of diphenylamine added. When mycelial pads of the mold were transferred to non-diphenylamine medium containing large amounts of phytofluene, they did not convert phytofluene into β-carotene.

Recently, Varma and others (*109*) studied the effect of starvation and of diphenylamine addition on the production of various carotenoids in *P. blakesleeanus*. In 1952 the California group (*87*) began studying β-carotene in the same organism. They demonstrated that the addition of β-ionone to cultures of *P. blakesleeanus* at the rate of 2 μl per 20 ml. of medium, added at 72 hours, increased the β-carotene from 91.2 to 218 μg per gram of dry mycelium. However, adding β-ionone resulted in depressed culture development.



β-Ionone

Later work by Mackinney and others (*84*) showed that methylheptenone brought about a reduction in β-carotene formation in this organism, a marked increase in phytofluene, and the appearance of ζ-carotene. Mackinney and others (*88*) postulated that the β-ionone, or a large portion of it, is incorporated into the carotene molecule. According to Chichester and others (*26*), when β-ionone was added to *P. blakesleeanus* cultures grown in the dark, yields of 678 μg per gram were obtained while those in light produced 869 μg per gram. However, β-ionone was more effective in stimulating carotene formation than was light. Furthermore, these authors demonstrated that when labeled sugar was incorporated into the medium, the organism preferentially used nonsugar carbon for the carotene molecule. Chichester and others (*27*) showed that both asparagine- and leucine-containing culture media with β-ionone gave high yields of β-carotene. Using labeled glycine, Mackinney and others (*86*) stated that there appeared to be incorporation of labeled C into β-carotene from glycine. Mixtures of β-ionone and methylheptenone act independently on carotenoid production in *P. blakesleeanus* according to Mackinney and others (*85*).

Nakayama and others (*91*) proved that methylheptenone acted on *P. blakesleeanus* as a powerful stimulent for phytoene production. Thus the asparagine control medium gave 314 μg of phytoene while the

asparagine medium and methylheptenone gave a yield of 2180 μg. Chichester and others (*28*) reported that carotene produced on a glucose-glycine medium had 38 of its C atoms derived from glucose and the remaining 2 from the methyl carbon of glycine.

Grob, Bein, and Schopfer (*57*) found that *P. blakesleeanus*, when grown on a medium containing 0.2 percent ammonium lactate to 1 percent sodium acetate as sole N and C sources, gave the usual amounts of fat, sterols, and carotenoid pigments. Grob and Von Beust (*71*) have shown that three non-carotenoid pigments are present in *Mucor hiemalis*. Grob and others (*67*) showed that pantothenic acid, pantothine, and phosphorylated pantothine increased carotenogenesis in *M. hiemalis*. The role of pyruvic acid in the biosynthesis of carotene was studied by Grob and others (*66*). Grob, and Grob and Butler have published a series of papers on the metabolic pathway of carotene formation in *M. hiemalis* (*54, 55, 59–66*).

The effect of antibiotics on carotene formation in *Phycomyces blakesleeanus* has been reported by Goodwin and Griffiths (*45*). Streptomycin inhibited carotenogenesis when the organism was grown under normal conditions in light. The reduction in carotene with any amount of streptomycin was never more than 60 percent. The same year Schopfer and others (*106*) confirmed this work. Chloromycetin also inhibited carotenogenesis, but tetronic acid and penicillin did not inhibit carotenogenesis in *P. blakesleeanus* (*107*). Brucker (*17*) compared phenol and carotene formation in *P. blakesleeanus*.

Later, Goodwin, Griffiths, and Modi (*48*) reported that high concentrations of streptomycin did not inhibit growth of *P. blakesleeanus* in a glucose-asparagine medium but that it did inhibit carotenogenesis. When asparagine was replaced by an ammonium salt, inhibition did not occur. When β-ionone was used to stimulate carotene production, the addition of streptomycin also inhibited carotene production. These authors also found that chloramphenicol, penicillic acid, and terrestric acid inhibited carotenogenesis to a greater extent than they inhibited growth.

Recently, using *Mucor hiemalis*, Grob (*56*) has shown that mevalonic acid at levels of 0.5, 1.0, and 2.0 mg per 25 cc. of nutrient solution increased carotene formation. Furthermore, mevalonic acid was incorporated into the β-carotene molecule. About the same time Braithwaite and Goodwin (*15*) showed that mevalonic acid (β:δ-dihydroxy-β-methylvaleric acid) was as efficient as leucine in diluting out labeled acetate incorporated in β-carotene from *Phycomyces blakesleeanus*. Using the same organism and carrot slices, these authors (*16*) have shown that mevalonic acid was incorporated in the β-carotene molecule. Ritter (*97*) reported the presence of a yellow pigment in *Mucor adventitius* which he believed was a carotenoid.

Mönch (*89*) proved that this pigment in *M. adventitius* was indeed a carotenoid.

Reichel and Wallis (*96*) reported that, when + and — strains of *Phycomyces* were mated under the conditions used by Barnett, Lilly, and Krause (*7*), no increase in β-carotene was encountered.

So far as is known, no one has attempted to increase carotene in any of the Mucorales by means of induced mutations. Blakeslee (*12*) obtained both stable and unstable natural mutants of *Mucor genevensis*, and he reported that Burgeff obtained mutants of *Phycomyces* but cited no reference. Bonner and others (*14*) showed with ultraviolet-induced mutants that the amounts of β-carotene produced could be markedly altered in another group of micro-organisms. Of the seven mutants of *Rhodotorula rubra* two had lost all ability to form β-carotene, two remained approximately the same as their parents, and three had increased in their ability to form β-carotene as much as threefold over that of the original strain. In studying carotene in *Phycomyces* and the associated polyenes, Goodwin and Griffiths (*45*, *46*) deemed it desirable to study these compounds in morphological mutants of *P. blakesleeanus* and, in a second species, *P. nitens*. All cultures studied produced the same polyenes in the same relative amounts. Carotene synthesis was similar in all — strains; whereas the + strains produced only about one-half as much as the — strains, except for one strain, which gave yields equal to that of the — strains.

FUNCTION OF CAROTENE IN SEXUALITY OF MUCORALES

In the order Mucorales two conditions exist for sexual reproduction. In some species, cultures started from a single asexual spore will always result in cultures that produce asexual spores and, in addition, form sexual spores (zygospores). These species are designated as homothallic. In the majority of species, each will be made up of two forms and only two forms that must be brought together under appropriate conditions in order to form zygospores. Such species are said to be heterothallic.

The two forms were discovered in 1904 by Blakeslee (*10*), who designated them as + or —. He designated the strains that grew less luxuriantly as — and the more luxuriant growing strains as +. In 1929, Satina and Blakeslee (*99*) stated that the female strains were + and the male strains were —. Blakeslee (*11*) noted that when + and — strains of different species in the Mucorales were placed together, an imperfect sexual reaction took place. In this phenomenon zygospores are not matured, for the imperfect reaction usually stops at the progametangial stage, or may even progress to fusion of gametes.

This behavior in heterothallic strains made it possible for Blakeslee (*13*) to assign corresponding + and — signs to strains of other species. It also led him to the conclusion that there must be something fundamental and common to all races of + strains and likewise for all — strains of members of the Mucorales. In other words, all — strains of Mucorales must have one or more chemicals in common which are characteristic of that mating type. Although a number of conditions were investigated, no definite compound or chemical reaction was found to be characteristic of either + or — strains.

Burgeff (*22*) demonstrated that sex hormones were present in the Mucorales, and recently Plempel (*93*) and Burgeff and Plempel (*23*) published further work on the hormones of *Mucor* and *Phycomyces*. For a general discussion of the physiology of reproduction, see Hawker (*72*).

Lendner (*82*) believed that the yellow pigment was always regularly stored in larger amounts in the progametangia of + strains than in the progametangia of — strains. Satina and Blakeslee (*98*) agreed after a study of numerous mating pairs in the Mucoraceae. They believed that the more pigmented strains (+ reactors) were female and the — reactors were male. With our best β-carotene-producing strains the + strain shows more pigment than the — strain. The same conclusion was held by Chodat and Schopfer (*29*) as a result of an intensive investigation of *Mucor hiemalis*.

Schopfer (*100, 101*) stated that the composition of the medium and the light affected carotene formation. He illustrated how the carotene was concentrated in oil droplets in the mycelium and even noted that crystals of carotene were seen in hyphae of *M. hiemalis*. He found that the + strain always had more carotene and a more rapid rate of absorption of nutrients from the medium.

In discussing the function of β-carotene in sexuality of the Mucoraceae, Burnett (*24*) stated that the evidence for a relationship between carotene and sexual reproduction was poor. The claims of the relationship between carotene and sexual reproduction are based on two facts: (1) Carotenes are found in gametangia during the sexual process. (2) Different amounts of carotene are found in the mating strains. Burnett states that the second claim was proved false as long ago as 1929 when Ling-Young (*83*) showed that in strains of *M. hiemalis* which had high, intermediate, and low carotene contents, carotene content varied independently of the mating type to which the strain belonged. The observations of others, according to Burnett, were made on studies of too few strains. Burnett studied the function of carotenes with respect to number 1 above. He showed that the number of gametangia formed were about the same when asparagine, glycine, or ammonium acetate was incorporated into the medium,

although with ammonium acetate β-carotene was reduced to a low level. However, only about half as many mature zygospores were formed as were formed with the other two compounds. The second experiment involved buffered media in which the number of gametangia remained constant but carotene production was only half as great. Diphenylamine was used in a third experiment with the following results:

	Gametangia produced	Zygotes matured	μg β-Carotene (Goodwin)
Diphenylamine (1:30,000)	284	197	25
Absent	282	182	626

One criticism of Burnett's work is that the author used data from Goodwin's experiments on carotene and compared it with progametangia and mature zygospores he obtained. The analysis for β-carotene should have been repeated with the same material.

CAROTENE IN THE FAMILY CHOANEPHORACEAE

Interestingly, Cunningham in 1895 observed the enhanced formation of yellow pigment in *Choanephora simsoni*. Pigment in Choanephoraceae was not investigated, aside from casual reference to it, until studies were made by Barnett and Lilly. Since their publications bear on production of inoculum for the β-carotene fermentation, their investigations on *Choanephora cucurbitarum* are briefly reviewed.

Barnett and Lilly (*4*) showed that the production of conidia in *C. cucurbitarum* was influenced by nutrients, by temperature, by light, and by aeration. A temperature of 31° C. prevented conidia production but permitted sporangia formation. Good aeration was especially important for the formation of conidia. In a second study they (*5*) reported that the accumulation of CO_2 in a closed vessel prevented sporulation. At a temperature of 25° C. or higher, a relative humidity near 100 percent enhanced sporangia production while lower relative humidities of 50 percent favored conidia formation. In a third paper (*6*) they described the conditions affecting the formation of zygospores in this species. Zygospores were formed from 15° to 37° C. and were not affected by light. Furthermore, zygospores would form in atmospheres containing as much as 10 percent of carbon dioxide. Starvation of mycelium brought about zygospore formation and this might occur from a pH 4.5 to 8.5. They concluded that the production of carotene was stimulated in either culture by hormone-like substances originating from the mycelium of the opposite sex.

In the same year Barnett, Lilly, and Krause (*7*) studied zygospore formation in liquid media. They reported that *C. cucurbitarum* produced zygospores in liquid media and identified the yellow pigment in this species as β-carotene. They showed that when + and − strains were mixed in liquid media, 15 to 20 times as much β-carotene was formed per gram of mycelium as was formed by either mating type grown alone. When + and − strains were grown on opposite sides of a cellophane membrane, both strains usually increased pigment formation. This increase in color indicated that a stimulating substance diffused through the membrane in both directions. They further concluded: "It seems probable that some relationship exists between the production of carotene and sexual reproduction in *C. cucurbitarum.*" In contrast, Reichel and Wallis (*96*) reported that when + and − strains of *Phycomyces* were mated under the conditions used by Barnett, Lilly, and Krause, no enhanced β-carotene yields could be obtained.

Occurrence and History of the Choanephoraceae

Since the question arises as to where members of the Choanephoraceae are isolated, it is appropriate here to discuss the records of their isolation from nature. Other Mucorales known to form carotene are each more or less ecologically restricted. *Mucor hiemalis* is most often found in forest soil; *Pilobolus* is strictly coprophilous. The occurrence of *Phycomyces* has recently been reviewed in detail by Benjamin and Hesseltine (*119*). Cultures of *Mucor hiemalis* and *Phycomyces* species are available in many culture collections. However, members of the Choanephoraceae, especially mating types, are not as readily available, probably because of the greater difficulty in obtaining material containing the organisms for isolation and in maintaining vigorous pure cultures.

The genus *Choanephora* was first described by Currey in 1873 (*132*) from fructifications on flowers of *Hibiscus rosaesinensis* furnished him by D. D. Cunningham from Calcutta, India. Currey described it first as *Cunninghamia* but later changed the generic name to *Choanephora* since the name *Cunninghamia* had already been used for a conifer. Cunningham (*130*) reported in detail on this species, *C. infundibulifera*, which he found growing in *Hibiscus* and *Zinnia* flowers. He recognized the zygosporic stage as a sexual one, described it in detail, and correctly placed it in the Mucorales. He gave the following tabular statement of the forms of reproductive bodies:

I. Sexual reproduction	Zygospores
II. Asexual fructification	Conidia, sporangial spores, chlamydospores

In a second paper, Cunningham (*131*) described a second species, *C. simsoni*, including the zygosporic stage. Here he stated in connection with zygospores:

> The colour of the contents of the conjugating processes varies from different shades of yellow to strong reddish, and not unfrequently, at a time when the contents of one process have already been completely accumulated terminally and separated by the formation of a partition, those of the other are present throughout its entire course and continuous with those of the parent mycelial filament.

Thus at this early date the accumulation of carotene during sexual reproduction in Choanephoraceae was observed although the material was not identified. This species was found on leaves of *Ipomoea rubrocaerulea* and *Zinnia elegans* at Calcutta, India.

Thaxter (*178*) named the species *Choanephora cucurbitarum* and recorded its occurrence on squash in Massachusetts and on *Hibiscus* and a wild malvaceous plant in Florida. Berkeley (*120*) reported this second species from putrid squashes sent to him from South Carolina by Ravenal. Thaxter (*178*) further reported that Morgan collected it in Ohio and that a specimen exists on squashes from North Carolina in the Curtis Herbarium at Harvard. Möller (*156*) recorded the same species under another name on *Hibiscus* from Brazil. Peck (*163, 164*) listed the same species on squashes from New York under the name *Rhopalomyces cucurbitarum*, as did Clinton (*128*) on squash from Connecticut. Rick (*166*) reported *C. infundibulifera* from Brazil. Sydow, Sydow, and Butler (*175, 176*) saw material of *Choanephora* from India.

Thaxter (*179*) described the genus *Blakeslea* and the type species, *B. trispora*. He noted that the mycelium when first cultivated had protoplasm with fat globules and was bright orange-yellow, but upon continuous cultivation on potato agar this color gradually faded to a light yellow. He found this organism as a contaminant with *Botrytis* from a larvae from cowpeas collected in Florida. According to Thaxter, it probably was growing on the cowpeas.

Saito and Naganishi (*168*) found what they considered to be a new species of *Cunninghamella*, *C. mandshurica*, from air over Manchuria, but this has been shown to be *Choanephora manshurica*.

Wolf (*187*) reported *C. cucurbitarum* from squash, flowers of cucumber, *Hibiscus syriacus*, *H. coccineus*, okra, and cotton. He also described the zygosporic stage of this species. Furthermore, he showed that certain insects transmit the disease from one flower to another and that infection in squashes occurs through the flower end. This species was found by Dastur (*138, 139*) on chillies (*Capsicum* sp.) in India and by Wolf and Lehman (*188*) on cowpeas in North Carolina.

Gandrup (*144*) and Ultee (*181*) studied *Blakeslea trispora* on freshly picked tobacco leaves in Sumatra. Palm and Jochems (*162*) reported *C. cucurbitarum* from *Amaranthus blitum* and *A. spinosus*. Burger (*122*) found a serious blossom blight of dahlias caused by *Choanephora* sp. in the Gainesville, Fla., area. In 1925, *Choanephora persicaria* was described by Eddy (*141*) on decaying peaches in the New York market. This species has never been reported again and was especially interesting because only sporangia were produced. The same year Couch (*129*) described still another new species from the flowers of *Hibiscus syriacus* in North Carolina and noted that it failed to grow on squashes. A *Choanephora* sp. was the causal agent of leaf rot of ground nut (*Arachis*) in Batavia, according to Van Hall (*182*). Jochems (*148*) reported *C. infundibulifera* on hibiscus flowers in the Dutch East Indies. *Choanephora* sp. was discovered at Buitenzorg, Java, as a secondary invader of plants of *Lochnera rosea*, which were infected with a species of *Phytophthora* according to Schwarz (*169*).

Weber and Wolf (*186*) reported that their *Blakeslea trispora* cultures from cucumber leaves were orange or yellow at the surface of the media on 2-percent potato dextrose agar. When their cultures were grown on 5-percent potato dextrose agar, the orange color was much intensified. They described the zygosporic stage of the genus. They also report that another strain (*123*) was isolated from a culture of fungus sclerotia by the senior author. According to Jochems (*149*), *Blakeslea trispora* commonly occurred in Java and Sumatra on faded leaves of tobacco, which had been attacked by the great green tobacco bug (*Nezara viridis*), and hence the fungus was considered a weak parasite. He also found it in drying sheds on new tobacco leaves. In addition, he found it on *Physalis angulata*, *Ipomoea batatas*, and probably other plants.

Dade (*134*) reported that *C. cucurbitarum* occurred on papaw leaves, cacao husks, and withered blossoms of *Hibiscus* and *Zephyranthes* collected in the Gold Coast region of Africa. Blakeslee and others (*121*) stated that *C. cucurbitarum* is commonly found on withered flowers of pumpkin and squash from gardens near Cold Spring Harbor, N.Y. Stevens (*173*) found *C. infundibulifera* on petals of *Hibiscus* in British New Guiana. Wallace (*184*) recorded a *Choanephora* sp. on sorghum from Tanganyika. Three species of *Choanephora* (*C. cucurbitarum* on leaves, stems, and flowers of *Capsicum* spp.; *C. simsoni* on *Ipomoea rubro-caerulea*, and on flowers of *Zinnia elegans*; and *C. infundibulifera* on flowers of *Hibiscus rosae-sinensis* and on inflorescence of *Tabernaemontana coronaria*) were reported by Butler and Bisby (*124, p. 8*) in their monograph of fungi in India. *C. cucur-*

bitarum occurred as a blossom blight of peppers in Florida according to Weber (*185*).

Tai (*177*) apparently found the same organism in China, although he called it *C. manshurica*, growing on the flowers and pods of *Dolichros lablab*. Miyake and Ito (*155*) reported a disease of squashes caused by what appeared to be *Choanephora*, although they called it *Choanephoroidea cucurbitae* and stated it was an Oomycete. Asuyama (*116*) also reported the same fungus in Japan as causing a soft rot of squash. Deighton (*140*) found *Choanephora* sp. attacking flowers of *Crotalaria retusa* and causing a die-back at the seat of infection. In Malaya the lower leaves of cassava were attacked during wet weather by *C. cucurbitarum* according to Thompson (*180*), and Su (*174*) reported the same fungus on chilli in Burma.

Singh (*170*) isolated a strain of *Choanephora* sp. from Punjab soil, which he stated had not been previously reported from soil. Christenberry (*126*) found *C. cucurbitarum* on old flowers of *Hibiscus* sp., *Cucumis* sp., *Gossypium* sp., and *Capsicum* sp. and on the flowers and young fruit of *Cucurbita pepo* and *Solanum melongena* var. *esculentum*. It was the first report of this fungus on eggplant.

Lefebvre and Weimer (*152*) found *C. cucurbitarum* growing on cowpeas in Georgia and stated that it is a saprophyte on the leaves of various grasses in Georgia and Florida. Sinha (*171*) reported this species and *Blakeslea trispora* from *Colocasia antiquorum* and *C. cucurbitarum* (*172*) as causing a wet rot of chillies. Christenberry (*127*) isolated *B. trispora* from flies and on leaves of *Liriodendron tulipifera* and *C. cucurbitarum* on eggplant flowers and fruit in North Carolina. *Choanephora cucurbitarum* was stated to produce a necrosis of the cotyledons of marrow seedlings at Antibes (*113*). Karling (*150*) mentioned a *Choanephora* sp. collected in Brazil, which had been attacked by a chrytrid. Vestal (*183*) noted that low temperatures and humidities from June to September at Allahabad, India, in 1945 reduced leaf-spot fungi while some saprophytic fungi, such as *C. cucurbitarum*, became abundant everywhere. Liu (*153*) studying the fungi associated with soybean seeds in China isolated *Choanephora* sp.

Barnett and Lilly (*117*) reported that one of their cultures of *C. cucurbitarum* was isolated from squashes in West Virginia. Roger (*167*) recorded the occurrence of *C. infundibulifera* on the flowers of *Hibiscus esculenta*. During periods of high humidity a blossom blight of dahlias caused by *C. infundibulifera* occurred in India according to Jain and Nema (*147*). Norton (*161*) reported *C. cucurbitarum* on squash in Texas. Dantas and Robeiro (*137*) encountered *C. cucurbitarum* in Brazil, and later Dantas (*135*) reported the occurrence of *C. conjuncta* on fading flowers of *Hibiscus rosae-sinensis* in Brazil and

noted that both conidia and sporangia were produced in abundance on oatmeal agar. For a more detailed account of the hosts of *C. cucurbitarum* and of *C. infundibulifera*, especially in South America, see Dantas (*136*).

Hesseltine (*145*) monographed the family Choanephoraceae and included only the genera *Blakeslea* and *Choanephora*, excluding other genera such as *Cunninghamella*, which had been included by Zycha (*189*) in the family. This same paper reported the occurrence of *C. cucurbitarum* on *Cucurbita pepo* in Wisconsin and on *C. maxima* in New York. In 1953, Ellis (*142*) noted the occurrence of *C. cucurbitarum* on summer squash in North Carolina. The following year Ara and Mahmud (*115*) reported the same fungus on *Zanomia indica* in India where it occurred as a blossom blight.

Naganishi and Kawakami (*158*, *160*) reported the isolation of *Blakeslea trispora* from the air from Japan. In addition they described a new species, *B. circinans*, from pasture soil in Japan (*159*). In still another paper (*157*) they reported that *Choanephora cucurbitarum* and *C. infundibulifera* were isolated in Japan from faded flowers of dahlia, cosmos, squash, cucumber, canna, and morning glory; but it is not clear which species occurred on which host.

The most intensive attempt to isolate strains of *C. cucurbitarum* was made by Poitras (*165*). He obtained this species from soil samples from Illinois, Mexico, Central America, and the Island of Ponape. Chevaugeon (*125*) reported the occurrence of *C. cucurbitarum* in the basin of the Cavally River (Ivory Coast) Africa. Baudin (*118*) also reported *Choanephora* from the Ivory Coast. Hesseltine and Benjamin (*146*) studied *C. circinans* strains from soil samples from Trinidad, and from an isolate from a textile sample from the Canal Zone. They also studied a strain of *C. conjuncta* from soil in Georgia, and a strain collected from air in Venezuela. Agnihothrudu (*114*) reported isolating *C. cucurbitarum* from the rhizosphere of the pigeon pea (*Cajanus cajan*). Recently Miller and others (*154*) reported finding *C. conjuncta* in cultivated soil and in forest soil in a study of soil fungi of Georgia. In a study of fungi associated with flowers and fruit of pickling cucumbers, Etchells and others (*143*) reported isolating four strains of *C. cucurbitarum*. Recent taxonomic accounts of the family have been published by Hesseltine (*145*), Dantas (*136*), Poitras (*165*), and Hesseltine and Benjamin (*146*).

Life Cycle

To understand carotene fermentation one must know something about the organisms involved. The family Choanephoraceae is considered to contain two genera, *Choanephora* and *Blakeslea* (*74*); in each, spores, whether conidia or sporangiospores, germinate to form

germ tubes that develop into much branched mycelium. The branched mycelia grow rapidly in and on the surface of nutrient agar. Within 2 or 3 days the surface of the agar is covered. About 48 hours after spore germination, conidiophores or sporangiophores arise and become branched at their apexes. These short branches, in turn, branch a second time and form small vesicles which then bear either conidia or sporangioles over their surfaces. In the genus *Blakeslea* the vesicles bear sporangioles which contain relatively few sporangiospores within a hyaline membrane. In *Choanephora* the spores are borne singly as conidia. This difference in spore formation is the generic characteristic that distinguishes the two genera.

Both conidia and sporangiospores from the sporangioles are purple or brown and are often characterized by minute markings in the form of longitudinal striations. At first the fruiting heads of either conidia or sporangioles are white, then they become rather brown, and finally nearly black. These fructifications typically are formed next to the edge of the culture dish, probably because the nutrients are exhausted or because of better aeration there. About the same time the fruiting structures are produced, thin, slightly yellowish, aerial mycelium is formed which later may almost completely obscure the fruiting heads. Fruiting is characteristically restricted to the first 3 or 4 days of growth. Mycelium, both aerial and substrate, is coarse and without septations. The substrate mycelium is generally filled with droplets of yellow material.

In addition to the conidiophores or sporangiophores bearing only sporangioles, a second method of asexual reproduction involves true sporangia. These may be formed in variable numbers depending on the species, the temperature, or nutrient conditions. The sporangiophores, which likewise arise usually from the substrate mycelium, are hyaline but sometimes become slightly pigmented above. In all cases the sporangium is borne in a nodding or circinate fashion. The sporangial wall is characterized by being relatively resistant but, when mature, by splitting into equal halves. This characteristic is almost diagnostic of the family. Inside the sporangium are numerous sporangiospores and a typically pigmented columella, which has a more or less distinct collar attached at its base. The sporangiospores from sporangia are often pigmented and in all but one species have long, stiff, hyaline appendages in tufts at both ends and sometimes even at a third location. In addition to these types of asexual spores, hyphae in the substrate may round up into irregular cells reported as chlamydospores.

As the culture becomes older it takes on a more yellow appearance. This is particularly true of *Blakeslea trispora*. When held at room temperature the cultures remain viable for considerable periods of

time. However, cultures placed in a refrigerator show erratic survival. Lyophilized spores keep very well, provided that stocks are lyophilized within a few days after the fruiting heads have become dark in color.

Sexual reproduction is practically identical in all species of the family. All species that have been studied are heterothallic, that is, the sexes are separate. To induce the sexual stage, a + and — strain are placed a short distance from each other on nutrient agar and the colonies are allowed to come into contact with each other. As the colonies come into contact, the first noticeable gross effect is the increased intensity of yellow color on the reverse side of the colonies where they meet. As the culture becomes older, this color becomes a deeper yellow to yellow orange. With the maturation of zygospores the yellow-orange color either disappears or is masked, and in its place appears a dark zone caused by the hundreds of mature zygospores.

The development of zygospores, when studied microscopically, begins as a coiling of filaments from each of the opposite mating strains. The twisted and irregularly coiled hyphae eventually diverge and form swollen progametangia that resemble a pair of tongs or a bow. Each progametangium forms a septum that delimits a gametangium which comes into contact with its mate; the wall between the gametangia breaks down and mixing of the cytoplasm results. Thus, the fused gametangia become the zygospore, which now enlarges and assumes a more or less globose shape. The thick wall of the zygospore is minutely striate, but it never becomes roughened with long projections as is seen in the zygospores of many other genera of Mucorales. The wall becomes a deep brown. At first the zygospore contents show numerous oil droplets; these later coalesce into one, central, oil droplet which is yellow-orange.

The zygospores are always formed either on the surface of the agar or in the medium just below the surface. The yellow-orange oil droplets are numerous and concentrated in the progametangia, gametangia, and the hyphae immediately adjacent to the area where zygospores are being formed. When most zygospores have reached maturity, the adjacent hyphae and gametangia become almost empty and lose the large accumulations of yellow-orange droplets. Cutter (*133*) described the nuclear behavior in *B. trispora*. According to him, zygospores germinate after 3 months to form an extensive, submerged, vegetative mycelium. Kishore (*151*) studied the conditions affecting zygospore formation.

One interesting characteristic of the family Choanephoraceae, also common to other Mucorales, is the coenocytic condition of the mycelium (a condition in which many nuclei are present in the

cytoplasm without cell walls). Even the gametangia contain many nuclei in their cells. It is important to remember that all asexual spores contain more than one nucleus. Whether it is possible to induce mutations giving larger yields of β-carotene is not at all certain. Presumably, each spore would contain various nuclei with numerous sets of the same chromosomes. An entirely different situation is found in many successfully mutated industrial organisms, in which the conidia possess but one nucleus with one set of chromosomes.

Members of the Choanephoraceae growing in nature cause a wet rot of flowers or young fruit. Undoubtedly the conidia produced on these parts are carried by insects from one plant to another. The infection does not appear to be of any economic importance, for usually only the blossoms are attacked. In some cases the mycelium apparently enters the fruit through the blossom end and causes the fruit to turn yellow, then black and soft. However, no further invasion of the host plant occurs.

Carotene in Choanephoraceae

My first acquaintance with members of the Choanephoraceae began in 1946 (*73*) with a study of *Choanephora cucurbitarum* occurring on pumpkin in Wisconsin and of a strain of *Blakeslea trispora* obtained from the Baarn Collection. Since it is my custom to study all Mucorales on a synthetic medium containing glucose and asparagine (a medium known now to enhance carotene formation), the cultures were studied growing on it. The yellow pigment was pronounced, especially in the *B. trispora* strain. The taxonomy of the known members of the family was then developed as far as possible.

Thus matters stood until 1955, when what appeared to be a nearly nonsporulating culture of *Choanephora* was received from the Quartermaster Corps Collection. It appeared to be different from all other strains seen, so it was preserved in the ARS Culture Collection for possible future study as a new species. Sometime later we received a catalogue from one of the Japanese culture collections which listed a species called *Blakeslea circinans*. We wrote for the culture, inquiring whether the name had been published. In due time we received a reply stating that the organism had in fact been described in 1955 and that the species was based on a single strain. When the culture arrived, we were interested to find that it appeared identical with the culture from Panama which came to us from the Quartermaster Corps Collection. Both strains produced only sporangia. Since all other forms of *Blakeslea* and *Choanephora*, except one, possessed a second means of asexual reproduction, we were doubtful if either strain was typical of the organism in nature. Furthermore, when these cultures were mated nothing happened. We

therefore, again dropped our work on these forms because it appeared we had too little material with which to draw any conclusions.

In the meantime, we had assembled a number of cultures in the family, at least one of which we had not seen before, namely *C. conjuncta* Couch. The validity of this species had been questioned by Poitras (*165*). From two soil samples collected in Trinidad, we observed an abundance of sporangia of a *Choanephora* which, when isolated, appeared identical with the material of *B. circinans* and furthermore sporulated very poorly in culture. On the same soil isolation plates, numerous zygospores occurred that were typical of Choanephoraceae. After we selected and paired a number of single sporangial isolates, — and + strains were secured and these were found to mate with the Panama and Japanese strains. It seemed, therefore, highly desirable to report the occurrence of this species since we had zygospores, and to place it in *Choanephora* where another species, *C. persicaria* Eddy, had already been described with only sporangia.

At the same time, we had accumulated a number of strains that could not be determined as to known species because of their poor sporulation. Hence numerous mating experiments between *C. circinans* (*Blakeslea circinans*), *B. trispora*, and *C. cucurbitarum* were made in order to identify our strains correctly (*77*). When a member of one species was mated with a strain of the opposite mating type of a different species, the typical yellow zone appeared on the reverse side of the plate, but it failed to appear if the strains were of the same mating reaction. Furthermore, the yellow zone persisted if the strains were of different species; whereas, if they were of the same species, the yellow zone usually disappeared as zygospores matured. In imperfect reactions none or only a few abnormal zygospores were formed. Naganishi and Kawakami (*90*) had reported on the morphology of the sexual organs in imperfect reactions between species of the Choanephoraceae. We concluded that if one had mating types in one species in the family Choanephoraceae, the mating reaction of any species in either genera could be determined by the increase or lack of increase in yellow pigmentation on the reverse side of mated colonies at their point of union.

About this time we became interested in a report that zygospores could be produced in liquid media in shake cultures. This observation may be of some significance in the evolution of the Mucorales, since one of the postulated ancestors is the Saprolegniales or water molds whose sexual spore, oospore, is found in aqueous media. Accordingly, we used mating types of a number of genera of Mucorales, growing the combined mating types in synthetic liquid medium and potato dextrose broth. The latter medium with agar is especially

suitable for zygospore formation. In most of these experiments we obtained negative results, but we readily confirmed Barnett, Lilly, and Krause's report (*7*) regarding *Choanephora cucurbitarum* and showed in the two other species of *Choanephora* and the one species of *Blakeslea* that zygospores were formed readily on potato dextrose media in shaken flasks. However, no zygospores could be found even though good vegetative growth resulted on the synthetic medium composed of glucose, asparagine, salts, and thiamine.

The appearance of shaken cultures grown in the synthetic medium after 72 hours at 28° C. on a rotary shaker was very striking (*75*). The mycelium from the flasks in which the + and — strains had been inoculated appeared as large, yellow-orange-pigmented balls resembling canned yellow peaches. The actual amount of mycelium from the flasks where + and — strains had been grown was approximately the same as when either strain was grown by itself. The single strain cultures lacked pigmentation. Dr. R. F. Anderson and associates at our laboratory ran β-carotene analysis on the material from this and other experiments and showed that the mated cultures contained pigment composed of about 80 percent β-carotene according to the A.O.A.C. method. In all four species, *Blakeslea trispora*, *Choanephora circinans*, *C. conjuncta*, and *C. cucurbitarum*, the yield of β-carotene in mated fermentations was at least double that obtained with a single strain grown alone. Interestingly, the *B. trispora* strains NRRL 2457(—) and NRRL 2456(+) each gave higher yields of β-carotene than the mating types of any other species. Typical results are shown for *C. circinans* on the synthetic medium.

Strain:	*Dry weight* *g./300 ml.*	*β-Carotene* *Total μgm*	*μgm/g.*
NRRL 2546(+)	0.82	20	25
NRRL 2548(—)	.69	35	50
NRRL 2546 × 2548	.75	255	340

As noted, when strains of opposite mating types from different species or species from different genera are mated, the yellow color appears at the juncture of the colonies. When such strains are mated in shake cultures on synthetic media, typical results such as the following are obtained:

Strain:	*Dry weight* *g./300 ml.*	*β-Carotene* *Total μgm*	*μgm/g.*
B. trispora (—)	0.58	90	155
C. conjuncta (+)	.71	18	25
B. trispora (—) × *C. conjuncta* (+)	.73	212	290

Thus the same enhanced production of β-carotene can be obtained in interspecific crosses as well as in intraspecific crosses. Although

yields formed were not high as compared with yields of β-carotene from *Phycomyces* of 2,500 to 3,500 μgm/g. reported by Nakayama and others (*91*), it still looked promising to study this enhanced carotene production with various conditions and media. In this work, reported by Anderson and others (*2*, *3*), pieces of mycelium were taken from the stock cultures to start inoculum on a glucose-asparagine agar (fig. 1). Each strain was grown separately. After the inoculum slants had been grown for 5 to 6 days at 28° C., the

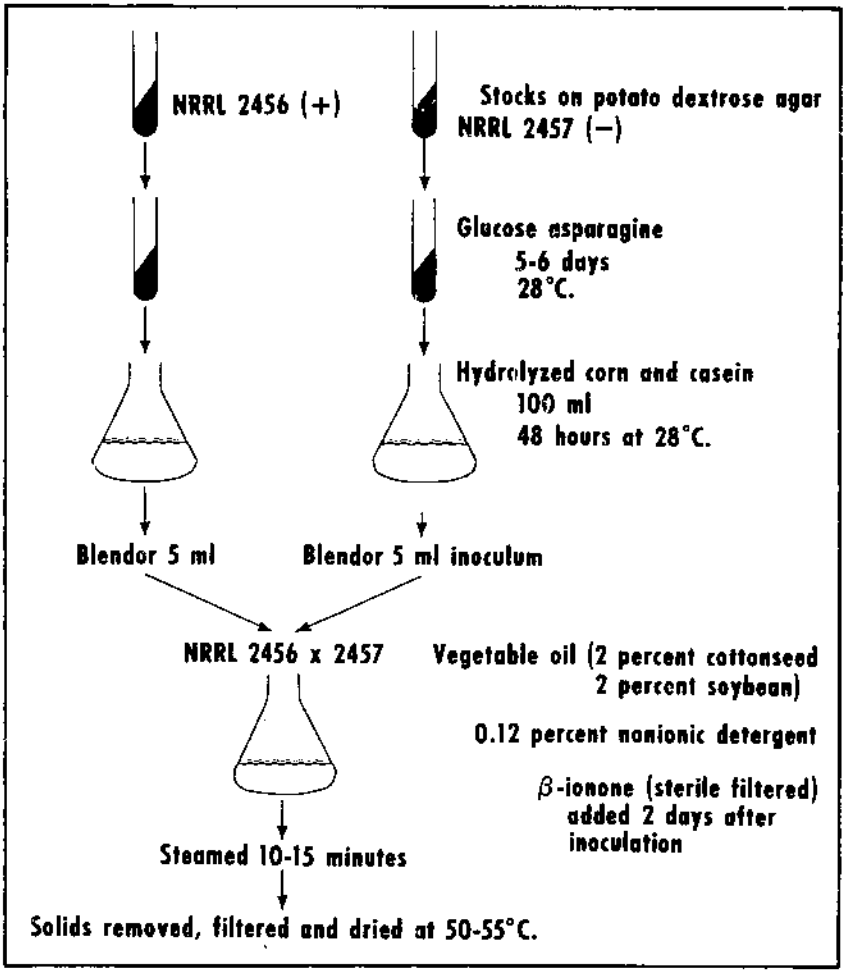


FIGURE 1.—Outline of method used for inoculating flasks for the production of β-carotene.

entire mycelium was scraped off into a 500-ml. Erlenmeyer flask containing 100 ml. of the basal medium composed of:

	Grams per liter
Corn (acid hydrolyzed)	75. 0
Casein (acid hydrolyzed)	2. 0
Corn steep liquor	5. 0
Potassium phosphate monobasic	. 5
	Milligrams per liter
Thiamine hydrochloride	1. 0

pH adjusted to 6.2 with sodium hydroxide.

The corn was hydrolyzed by autoclaving a 15-percent slurry in 0.2 N sulfuric acid at 121° C. for 90 minutes. The inoculum usually grew as large masses of mycelium which was macerated in a Waring Blendor.[3] Five ml. of this material from each mating type was then used to inoculate 100 ml. of fermentation medium. The basal fermentation medium as described contained the following additional adjuncts: 4 percent vegetable oil (2 percent cottonseed and 2 percent soybean), 0.1 percent β-ionone, and 0.12 percent nonionic detergent. The function of the detergent is to emulsify the oil, thus making it more available to the mold mycelium. The β-ionone was Seitz filtered, sterilized, and added after 2 days of fermentation. Following 6 days of fermentation, flasks were steamed for 10 to 15 minutes to prevent enzymes from breaking down the carotene. The solids were removed by filtration and were dried at 50° to 55° C. in a vacuum oven. The analytical methods employed were those described by Anderson and associates (*2,3*). When the mated cultures were grown on the basal medium, a fourfold or fivefold increase in total yield of carotene was obtained. From preliminary work, *B. trispora* appeared to be the best culture. The addition of vegetable oils increased the yield twofold. When β-ionone, representing a part of the β-carotene molecule, was added to the basal medium a fivefold increase resulted. When all three adjuncts—vegetable oil, detergent, and β-ionone were added, a sixfold to sevenfold increase over the basal medium was obtained. When other substances, such as 2-4 dinitrophenol, lipoic acid, chloroform, yeast-extract, liver-extract, and potato-extract were added, no increase in β-carotene occurred. Interspecific matings within the four species gave enhanced carotene production, but results are no better than with *B. trispora* NRRL 2456×2457.

On analysis, 75 percent of the total pigment was *trans*-β-carotene. When biologically assayed in rats, the dried solids showed the β-caro-

[3] Mention of products does not imply endorsement or recommendation by the Department of Agriculture over other products of a similar nature not mentioned.

tene was available as a precursor of vitamin A. Analysis of the dried solids gave the following:

	Percent
Moisture	5.0 – 6.2
Ash	3.0 – 3.2
Fat	52.2 –53.0
Fiber	5.2 – 5.5
Total nitrogen	5.15– 5.35
Essential amino acids were:	
Lysine	2.5
Methionine	.4

In the fermentation no zygospores were formed and pigment occurred through the mycelial growth. Anderson *et al.* state that the formation of zygospores is not necessary for the increased amounts of carotene observed.

Further papers in the series from our laboratory (*30,32*) were concerned with the effect of various grains on the production of β-carotene by mated strains of *Blakeslea trispora*. The grains and soybean products were hydrolyzed and incorporated into the fermentation medium at the rate of 7.5 percent. *B. trispora* does not appear to hydrolyze starch. Acid-hydrolyzed, hexane-extracted, soybean oil meal gave the highest yields of β-carotene, as well as the highest amounts of dry solids. This material was better than ground whole soya, which suggested that the process of extraction favorably alters the meal. Of the various treated soybean meals, the hexane-extracted soybean oil meal was best. Furthermore, there was little difference in concentration between 3 to 10 percent, although there is considerable variation in growth as measured by dry weight of solids. Use of hexane-extracted soybean oil meal with other meals or grains resulted in no increase in yields. Best yields were obtained from soybean oil meal alone.

A study of the effects of carbohydrates on the fermentation using 5 percent carbohydrate and 0.2 percent acid-hydrolyzed casein was made. This study showed that dextrin and sucrose gave the highest yields. When these carbohydrates were added to soybean oil meal, no material change occurred. Analysis of the pigments showed 95 percent was *trans*-β-carotene. The remaining fraction contained seven pigments. Study of the time course of the fermentation showed that the pH did not fall below 5.9 and that maximum carotene production occurred at 5 or 6 days. When the + and — strains were mated 3 days prior to inoculation, the increase in β-carotene was not significant. The mycelium in these fermentations showed distorted growth with large irregular swellings with the pigment uniformly dispersed. In some, a red crystalline precipitate that resembled carotene was seen in the mycelium.

Since vegetable oils enhanced production of β-carotene in *B. trispora*, additional work was done on the effect of fats and oils on the fermentation (*31,33*). Each fat was added at the rate of 40 ml. per liter of media. Of the 19 oils tested, all but one greatly stimulated growth and β-carotene production. Growth, as measured by dry weight, was greatest with peanut oil, but the most β-carotene was formed with white grease. Oils and fats that contain large amounts of oleic and linoleic acids, such as cottonseed and soybean oils or white and brown grease, produce the largest β-carotene yields. When white grease was used, dry weight increased with increase in amounts of grease to 8 percent, but maximum β-carotene per gram of dried solids was found at 4 percent. Fatty acids were not as effective in stimulating β-carotene formation as neutral oils and fats. Analysis of dried mycelium from these experiments did not indicate any shift in the carotene pigments. Thus, *trans*-β-carotene constituted about 95 percent.

The third ingredient that stimulated β-carotene formation was β-ionone. Carotene synthesis increased with greater concentrations of β-ionone, but the dry weight of the fermentations became progressively less. To obtain maximum stimulation, the β-ionone had to be added on the second day. If it were added at the start of the fermentation, a 1-day lag in growth initiation occurred. The effect was no different when α-ionone was used.

The use of a detergent (Triton X–100) had two effects: (1) it emulsified the fat, making it more available to the mold, and (2) it brought about a dispersed growth of mycelia, a condition necessary for high yields of carotene. Other surface detergents were tried, but the best results were obtained with Tritons 45 through 100.

Patents covering the process of making β-carotene by combining + and − strains of the Choanephoraceae have recently been issued (*1,34,76*).

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