as in the first harvest of alfalfa. Tunis rock, −325-mesh size, shows an approximate 20% increase over the −100-mesh size in the residual test. The data from the fourth harvest of alfalfa, however, show the two size classes to be practically comparable in uptake for the Tunis rock. Various mesh sizes of Curaçao rock at the first harvest of alfalfa and the residual test are more nearly in accord than is the fourth harvest of alfalfa with the residual test. All data relative to South Carolina rock seems to be comparable for both alfalfa harvests and the residual test. Probably the most pronounced change has taken place between the various mesh fractions of Tennessee brown rock in the residual test. Here increases favoring the −325-mesh size have increased to as much as 45% over the −100-mesh size fraction.

A Value Measurements. A residual test was made using an adaptation of the *A* value technique as a direct measure of residual value (*3*). These results (Figure 5) indicate that the residual agronomic value of the various mesh sizes of the phosphate rocks is similar to their initial agronomic value at the various alfalfa harvests. The phosphorus uptake data from the pots containing radioactive phosphorus can also be utilized to measure relative residual value of the various particle sizes in the more conventional manner. The results of this calculation are presented in Figure 6.

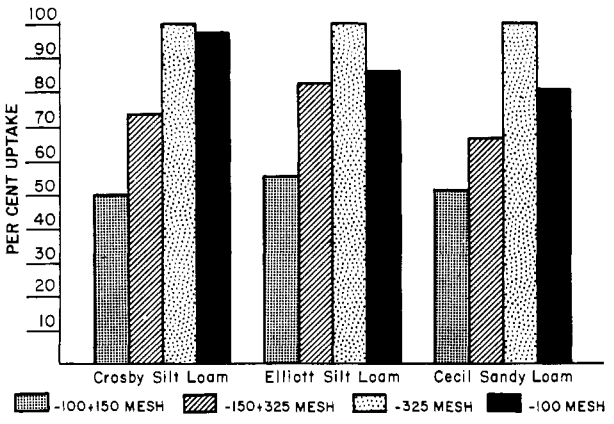

Figure 7. Residual effect of phosphate rock particle size on phosphorus uptake by buckwheat as determined by the *A* value technique

The two methods of evaluating particle size importance are nearly comparable. If anything, the *A* value seems to accentuate the differences between the various sizes of like materials.

The use of the *A* value to determine soil interaction effects on phosphorus source and particle size is shown in Figure 7. A very definite pattern of results is shown by this data. None of the soils show a variation of more than approximately 15% for any one given particle size. There is excellent agreement between the three soils in regard to phosphorus uptake by the various particle sizes.

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FEED SUPPLEMENTS PRODUCTION

Microbiological Production of Beta-Carotene in Shaken Flasks

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When appropriate plus and minus strains of various members of the Choanephoraceae were grown together in a grain-based medium in shaken flasks, carotene production was increased four- to fivefold over that obtained with unmated strains. Production of carotene by mated cultures was further enhanced by the addition to the medium of vegetable oils, detergent, and β-ionone. Chromatographic analysis showed that 75% of the pigments produced was all-*trans*-β-carotene.

CARTENOID-CONTAINING MATERIALS are incorporated into feeds for poultry and livestock to provide a source of vitamin A and to impart a desirable color to the skin, fat, and other tissues of the animals. From the standpoint of feed efficiency, a high-potency carotene supplement that is relatively low in crude fiber would be advantageous for use in formulating mixed feeds. The well-known synthetic powers of certain groups of microorganisms prompted a search for those capable of synthesizing carotene in practical amounts.

Barnett, Lilly, and Krause (*2*), Plempel (*5*), and Hesseltine and Anderson (*3*) demonstrated that mating of appropriate heterothallic strains of various members of the order Mucorales increased production of carotene within the mycelium. Further investigations were undertaken at this laboratory to ascertain the effects of certain adjuncts on the yield of carotene produced and to determine the problems involved in developing a practical fermentation process using the mating technique.

Table I. Effect of Medium Adjuncts on Carotene Production by *Blakeslea trispora*[a]

Organism	Basal Control	Adjuncts						
		Oil[b]	β-Ionone[c]	Detergent[d]	Oil + β-ionone	Oil + detergent	β-Ionone + detergent	Oil + detergent + β-ionone
NRRL 2456 (+)	420	560	300	40	390	1550	24	820
NRRL 2457 (−)	470	400	320	390	420	1080	75	1490
NRRL 2456 } × NRRL 2457 }	1980	4000	1760	640	11000	6500	280	12960

[a] Yield reported as γ of carotene per 100 ml. of fermentation medium.
[b] 4% vegetable oils (2% cottonseed plus 2% soybean).
[c] 0.1% β-ionone added after 2 days of incubation.
[d] 0.12% detergent (Triton X-100).

Fermentation Methods

To initiate production of inoculum, pieces of agar containing mycelium were transferred from parent cultures to test tube slants. Plus and minus strains were cultured separately until ready for use in the fermentation. The culture medium was as follows:

	Grams/Liter
Glucose	2.0
Asparagine	1.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
$FeCl_3$, $ZnSO_4$, $MnSO_4$	Trace
Thiamine.HCl	1.0 mg./l.
Agar	20
pH adjusted to 6.2 with NaOH	

The slants were incubated 5 to 6 days at 28° C. The entire vegetative growth was then scraped off each slant and transferred to 500-ml. Erlenmeyer flasks containing 100 ml. of basal medium of the following composition:

	Grams/Liter
Corn (acid hydrolyzed)	75.0
Casein (acid hydrolyzed)	2.0
Corn steep liquor	5.0
KH_2PO_4	0.5
Thiamine.HCl	1.0 mg./l.
pH adjusted to 6.2 with NaOH	

Ground corn was hydrolyzed by autoclaving a 15% slurry in 0.2*N* sulfuric acid at 121° C. for 90 minutes.

The flask cultures were incubated at 28° C. for 48 hours on a rotary shaker at 200 r.p.m. Both the plus and minus strains usually grew as large solid clumps which were macerated in a sterilized Waring Blendor cup for 5 seconds. Ten milliliters of the homogenates (5 ml. of each mating type) were then used to inoculate 100 ml. of the fermentation medium. This medium had the same composition as the basal medium described and, in addition, contained the following adjuncts as noted: 4% vegetable oil (2% cottonseed plus 2% soybean), 0.1% β-ionone, and 0.12% of a nonionic detergent. The β-ionone was sterilized by Seitz-filtration and then added to the flasks aseptically after 2 days' incubation.

Following an incubation period of 6 days, the flasks were steamed 10 to 15 minutes to prevent enzymatic decomposition of the carotene; the solids were removed by filtration and dried at 50° to 55° C. in a vacuum oven.

Table II. Carotene Production by Intraspecifically and Interspecifically Mated Cultures[a]

Organism	Dry Weight, G./100 Ml.	Carotene, γ/G. Dry Wt.	Total Yield γ/100 Ml.
INTRASPECIFICALLY MATED			
C. cucurbitarum NRRL A-6097 (+)	3.62	45	163
C. cucurbitarum NRRL A-6098 (−)	3.05	45	136
6097 × 6098	2.75	122	336
C. conjuncta NRRL 2560 (+)	1.46	67	98
C. conjuncta NRRL 2561 (−)	2.55	25	64
2560 × 2561	2.50	185	463
C. circinans NRRL A-6680 (+)	2.79	103	288
C. circinans NRRL A-6777 (−)	2.52	90	227
6680 × 6777	2.10	240	504
C. circinans NRRL 2546 (−)	2.58	19	49
C. circinans NRRL 2548 (+)	3.24	29	94
2546 × 2548	2.81	115	322
INTERSPECIFICALLY MATED			
C. circinans NRRL 2547 (+)	1.84	32	59
B. trispora NRRL 2457 (−)	3.89	138	537
2547 × 2457	3.36	640	2150
C. cucurbitarum NRRL A-6098 (−)	3.70	56	207
B. trispora NRRL 2456 (+)	3.70	150	555
A-6098 × 2456	3.49	260	906
C. conjuncta NRRL 2562 (−)	2.48	107	266
B. trispora NRRL 2456 (+)	3.70	150	555
2562 × 2456	3.84	368	1412
C. conjuncta NRRL 2561 (−)	2.05	56	115
C. circinans NRRL 2546 (+)	2.52	23	58
2561 × 2546	2.64	44	116

[a] These organisms (*4*) were obtained from the culture collection of the Northern Utilization Research and Development Division.

Analytical Methods

Carotene was removed from the dried material by solvent extraction with petroleum ether (boiling point, 33° to 57° C.) and the intensity of color determined at 450 mμ with a Coleman spectrophotometer. The concentration of carotene present in the sample was then determined by comparison with a standard calibration curve prepared from pure all-*trans*-β-carotene dissolved in petroleum ether.

The per cent moisture, ash, crude fat, crude fiber, and total nitrogen in the dried solids were determined by standard methods (*1*).

The dried solids were placed on top of a large magnesium oxide–Celite (1 to 1) column, 95 by 5 cm., and washed with petroleum ether until fatty material no longer appeared in the effluent. The column was then developed with 4% acetone in petroleum ether. The single large orange-yellow band was carved out and the pigment eluted with 10% acetone in petroleum ether. The carry-over of some fatty impurities in this first step necessitated two additional passages through the column. The final eluate was evaporated to dryness at room tem-

perature under reduced pressure and the resulting crystals subjected to repeated recrystallization from acetone-benzene and, finally, from petroleum ether–benzene. Microcarbon and microhydrogen analyses, as well as melting point determinations, were performed on the final crystalline product. Further evidence on the purity of the final product was obtained by comparing the product absorption curve, determined with an automatic recording spectrophotometer, with values given in the literature.

Results and Discussion

On the basis of preliminary screening, two strains of *Blakeslea trispora*, NRRL 2456 (+ mating type) and NRRL 2457 (− mating type) were selected for further investigation. Results obtained from both mated and unmated cultures are shown in Table I. Growing the two mating strains together in the basal medium resulted in an approximate four- to fivefold increase in the total yield of carotene over that produced by either culture alone. The addition of 4% vegetable oils to the basal medium afforded an additional twofold increase in carotene production by the mated cultures. Adding 0.12% of a nonionic detergent (Triton X-100, Rohm & Haas Co., Philadelphia, Pa.) together with the vegetable oils resulted in a further increase in carotene production by both the mated and control cultures. The function of the detergent appears to be emulsification of the oil, thereby making it more readily available to the molds.

β-Ionone, a component of the β-carotene molecule, exhibited slight toxicity when added alone to the basal medium but afforded a fivefold stimulation of carotene production by the mated cultures when added in conjunction with the vegetable oils. The addition of all three adjuvants—i.e., vegetable oils, detergent, and β-ionone—to the basal medium resulted in the best total yield given by a mated culture—a six- to sevenfold increase over yields obtained with the basal medium alone. Other adjuncts which were tested (2,4-dinitrophenol, lipoic acid, chloroform, yeast extract, liver extract, and potato extract) had no influence on carotene production.

The promising results obtained by mating strains NRRL 2456 and NRRL 2457 encouraged application of the mating principle to other members of the family Choanephoraceae. Mating was accomplished both intraspecifically—i.e., between strains representing the same species—and interspecifically—i.e., pairing of opposite mating types of different species. The results of these experiments utilizing the basal medium in which all three adjuncts were incorporated are presented in Table II. Recent work by Poitras (*6*) indicates that the slight morphological differences between *Blakeslea* and *Choanephora* may not be of generic significance. Thus the data presented in Table II (under Interspecifically Mated) may not represent the results of true cross-generic mating but only that of interspecific mating. Mating in most cases resulted in a marked increase in carotene production over that given by the unmated cultures. However, under the experimental conditions utilized, none of the paired strains gave yields of carotene greater than that produced by the paired strains NRRL 2456 and NRRL 2457.

Identification of β-Carotene. The carotenoid pigments produced by the mated strains NRRL 2456 and NRRL 2457 were separated on the described columns. The major component, constituting about 75% of the total pigment produced, was eluted and crystallized. These crystals dissolved in petroleum ether gave an absorption curve and extinction identical with that of pure all-*trans*-β-carotene. Results of microanalyses of the crystals were: 10.7% hydrogen (calculated, 10.5%) and 88.9% carbon (calculated, 89.5%). The melting point of the crystals was 185° to 186° C. (corrected). Literature values for the melting point of all-*trans*-β-carotene range from 181° to 187° C. Admixture of the crystals with a pure commercial preparation of all-*trans*-β-carotene did not depress the melting point. In addition, column chromatography of the mixture on magnesium oxide–Celite and calcium hydroxide columns revealed only a single homogeneous band. On the basis of this evidence, the major pigment produced by the mated cultures was thought to be all-*trans*-β-carotene.

Feed Stuff Analysis. Analyses of the dried solids from typical fermentations with the mated strains NRRL 2456 and NRRL 2457 gave the following data: moisture, 5.0 to 6.2%; ash, 3.0 to 3.2%; fat, 52.2 to 53.0%; fiber, 5.2 to 5.5%; and total nitrogen, 5.15 to 5.35%. Standard microbiological assays of the dried solids for the essential amino acids, lysine and methionine, gave 2.5% and 0.4%, respectively. Biological assays utilizing rats indicated that the β-carotene within the dried solids was available as a precursor of vitamin A.

Microscopic Observations. When opposite mating types of the same species were grown together on potato-dextrose-agar medium, a narrow band of zygospores (the spore resulting from a sexual interaction) was observed to form at the zone where the two mating types met. The mycelial growth in this zone became yellow; the carotene appeared to be concentrated mostly within the suspensors supporting the zygospores. However, when these strains were paired in fermentation medium, no formation of zygospores was observed but pigment formation appeared to occur throughout the mycelial growth in the flasks. When opposite mating types of unlike species were paired on agar plates, zygospore formation did not occur although occasional progametangia were observed to form at the line of contact. The mycelium in this region became pigmented.

These observations indicate that mating per se with the resulting formation of zygospores may not be necessary for the increased degree of carotene production noted when opposite mating types are grown together. Similar observations were recorded by Barnett, Lilly, and Krause (*2*) and by Hesseltine and Benjamin (*4*) utilizing different experimental methods.

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