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CAROTENOGENESIS IN *PHYCOMYCES*

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Abstract—Time course studies of carotenoid production and of mycelial growth in liquid cultures of *Phycomyces blakesleeanus* wild type [NRRL 1555 (−)], red mutants C9, C10 and C13 and the heterokaryon C2 * C9 are reported The ratios of the concentrations of lycopene, γ-carotene and β-carotene in the red mutant C13 and in the heterokaryon C2 * C9 during the growth periods were measured In these strains the concentration of lycopene is close to its final value after 2 days of growth, at a time at which β-carotene is just beginning to be produced It is suggested that the β-carotene produced late is possibly synthesized via β-zeacarotene

INTRODUCTION

THE WILD type strain of *Phycomyces* [NRRL 1555 (−)] accumulates appreciable amounts of β-carotene in its mycelium, in the sporangiophores, and in the sporangium. These structures are therefore yellow during the early stages of development. Later, other pigments tend to blacken the organism Some mutant strains, which are red, accumulate lycopene, others produce small amounts of colored carotenoids and are white. De la Guardia *et al.*[1] studied carotenogenesis in heterokaryons of the white strain, C2, and the red strain, C9, containing varying proportions, p, of C2 nuclei, and $1-p$ of C9 nuclei. They observed that in the resulting heterokaryons lycopene, γ-carotene and β-carotene were accumulated in the ratios of $(1-p)$, $p(1-p)$, and p^2, respectively. They explained these results by postulating the existence of a carotenogenic enzyme aggregate in which the individual enzymes, leading to the eventual production of β-carotene, function sequentially as in an assembly line. In the heterokaryon, each strain will supply enzymes which are defective in the other; they assumed that the defective ones, while inactive, are still capable of taking the place of their normal counterpart in the aggregate. Accordingly, it should be possible for the heterokaryon to form enzyme aggregates containing nondefective enzymes only in addition to those containing various patterns of normal and defective enzymes. The relative number of each kind of aggregate, and thus the amount of the corresponding carotenoid produced, should then depend solely on the relative numbers of nuclei of each strain present in the heterokaryon.

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[1] DE LA GUARDIA, M., ARAGÓN, C M G., MURILLO, F J and CERDÁ-OLMEDO, E (1971) *Proc Nat Acad Sci* **68**, 2012

We assume that the model of De la Guardia *et al* predicts constant proportions of the carotenoids, lycopene, γ-carotene and β-carotene, in either the homokaryon or heterokaryon, during growth To test this idea, time course experiments of mycelial growth and carotenoid production in agitated liquid media for the wild type strain, the red mutants C9, C10 and C13 and the heterokaryon C2 * C9 were carried out The results obtained indicated that the enzyme aggregate hypothesis does not provide a complete description of carotenogenesis in *Phycomyces* and they disagree with the results reported by De la Guardia *et al* [1]

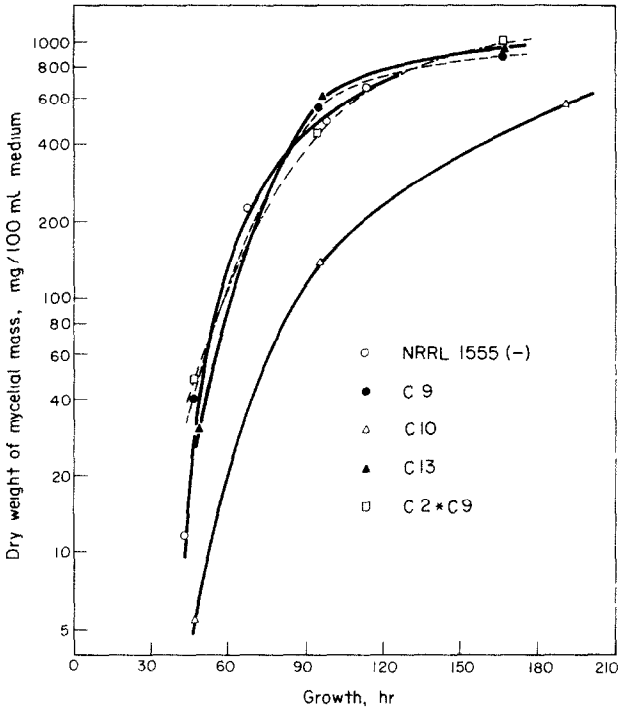

FIG 1 MYCELIAL GROWTH IN THE *Phycomyces* WILD TYPE (−) STRAIN NRRL 1555 (−) RED MUTANTS C9, C10 AND C13, AND HETEROKARYON C2 * C9

RESULTS AND DISCUSSIONS

For each of the *Phycomyces* strains tested, there was an exponential increase in the mycelial mass, doubling every 5 or 6 hr at early stages of development, followed by gradual slowing at 90 hr after inoculation, and approaching a saturation state (Fig. 1). Carotenoid samplings were taken at 3 points such that one occurred in the exponential growth region, one at the onset of saturation, and one well into the saturation region

Table 1 shows that in the wild-type strain the concentration of β-carotene reaches a high value after 4 days' growth, and that there was very little lycopene at any stage of growth This agrees with earlier findings of others.[3] Table 2 shows that at 2 days' growth the mutant C13 had a very high lycopene concentration (330 μg/g dry wt). The concentration of lycopene declined slowly as the culture grew, although the total amount of lycopene

[2] CERDÁ-OLMEDO, E and REAL, P (1970) *Mutat Res* **9**, 369
[3] GOODWIN, T W (1952) *J Biochem* **50**, 550

continues to rise. In contrast, concentrations of β- and γ-carotene were very low at 2 days and rose sharply between the second and fourth day after which they were constant. A similar pattern was observed for the C2 * C9 heterokaryon (Table 3).

TABLE 1 CAROTENOGENESIS AND GROWTH IN THE WILD TYPE (−) STRAIN OF *Phycomyces blakesleeanus* [NRRL 1555 (−)]

Age of culture (hr) . Carotenoids	43 75 (μg/g dry wt)	68 (μg/g dry wt)	98 25 (μg/g dry wt)	114 (μg/g dry wt)
Phytofluene	—	4 9	8 0	16 6
ζ-Carotene	—	—	—	—
Neurosporene	—	1 4	1 3	4 5
Lycopene	1 6	—	0 7	1·5
γ-Carotene	—	—	—	3 2
β-Carotene	10 0	58 0	430 0	880 0
β-Zeacarotene	—	3 4	17 0	29 0
Total carotenoids	11 6	67 7	457 0	934·8
Total dry mycelial mass (mg)/100 ml medium	12 0	229 0	480 0	677 0
Dry wt of sample (g)	0 09	1 74	2 78	1 34

—Means less than 0 2 μg/sample

The hypothesis formulated by De la Guardia *et al.*[1] predicted that at a fixed p value the proportions of β-, γ-carotene and lycopene should remain fixed at the ratios of $p^2:p(1-p):(1-p)$, where p represents the proportion of white C2 nuclei (8:21:71 for $p = 0{\cdot}29$, as measured for this heterokaryon). In Table 3, the ratios between the three carotenoids for the 4- and 7-day samples were similar to each other (55:14:31, 62:13:25), but widely different from the predicted value. Only the 2-day sample had a ratio (15:6:79) close to the predicted one. These results indicated that if the model of De la Guardia *et al.* applied at all it could be applied only at the earliest stage of development.

Since our measurements did not agree with those reported by De la Guardia *et al.*,[1] they were repeated, using both liquid medium and solid medium (as used by De la Guardia). In both cases the results of Table 3 were reproduced. We then suspected that our

TABLE 2 CAROTENOGENESIS AND GROWTH IN THE RED *Phycomyces* MUTANT C13

Age of culture (hr)	48 5		96		169	
Carotenoids	(μg/g dry wt)	(% of total)	(μg/g dry wt)	(% of total)	(μg/g dry wt)	(% of total)
Phytofluene	52	12	36	4 0	59	6·0
ζ-Carotene	12	3	19	2 0	7	0 7
Neurosporene	9	2	13	1 5	19	2 0
Lycopene	330	77	260	27 7	224	23 6
γ-Carotene	18	4	70	7 5	80	8 4
β-Carotene	9	2	538	57	556	58 5
β-Zeacarotene	—	—	3	0 3	8	0 8
Total carotenoids	430		939		953	
Total dry mycelial mass (mg)/100 ml medium	32		610		944	
Dry wt of sample (g)	0 4		2 4		2 8	

—Means less than 0 2 μg/sample

strain C9 might be different from the one used in Sevilla. Professor Cerdá-Olmedo kindly supplied us with the heterokaryon used in the experiments by De la Guardia A repetition using this strain again gave results in agreement with Table 3 and in sharp disagreement with those reported by De la Guardia, except for the earliest growth stage.

Table 3 Carotenogenesis and growth in the heterokaryon C 2 * C9, ($p = 0.29$)

Age of culture (hr)	48		96		168	
Carotenoids	(μg/g dry wt)	(% of total)	(μg/g dry wt)	(% of total)	(μg g dry wt)	(% of total)
Phytofluene	13	4	24	4	65	7
ζ-Carotene	9	3	9	1 5	29	3
Neurosporene	7	2	6	1	9	1
Lycopene	221	72	173	28 5	211	22
γ-Carotene	16	5	80	13	110	12
β-Carotene	43	14	315	52	521	55
β-Zeacarotene	- -	-	—	—	--	-
Total carotenoids	309		607		945	
Total dry mycelial mass (mg)/100 ml medium	51		477		982	
Dry wt of sample (g)	0 74		2 81		3 85	

—Means less than 0 2 μg/sample

In every strain which accumulates lycopene, the lycopene concentration is close to its final level after only 2 days of growth, i.e at a time at which β-carotene is just beginning to be produced in the wild type, in C13, and in the heterokaryon (Tables 1–5). Clearly, the machinery for lycopene synthesis is ready at the early growth stage, whereas the one for the cyclic carotenoids appears later.

Table 4 Carotenogenesis and growth in the red mutant C9

Age of culture (hr)	48	96	168
Carotenoids	(μg/g dry wt)	(μg/g dry wt)	(μg/g dry wt)
Phytofluene	—	10	10
ζ-Carotene	4	12	11
Neurosporene	15 6	16	7
Lycopene	680	1300	1630
γ-Carotene	1 4	12	7
β-Carotene	6 9	9	--
β-Zeacarotene	1 3	-	-
Total carotenoids	709 2	1359	1665
Total dry mycelial mass (mg/100 ml medium)	42	573	916
Dry wt of sample (g)	0 59	2 2	2 65

—Means less than 0 2 μg/sample

We might then expect the relative amounts of the carotenoids present to agree with the prediction of De la Guardia *et al.* in the later samples As mentioned, our results do not bear this out; on the contrary, we find that the enzyme aggregate hypothesis may be correct during the exponential stage of growth, a second mechanism for β-carotene production might come into being during the later stages

Both lycopene and neurosporene have been proposed as the substrates for the first cyclization reaction of the carotenoids.[4,5] The accumulation of β-zeacarotene by *Phycomyces* in the presence of the inhibitor diphenylamine favors the concept of neurosporene as the first substrate for cyclization in *Phycomyces*.[6] The extra accumulation of β- and γ-carotene at the later stages and the fairly constant lycopene level throughout the growth indicate a second pathway leading to β-carotene synthesis, bypassing lycopene, via β-zeacarotene and γ-carotene; and that this second pathway becomes dominant over the first one in later growth stages in the *Phycomyces* system. The results also may indicate that the defective cyclases formed by the strain C9 do not compete for the enzyme aggregate with the normal cyclases provided by strain C2; or due to the excess production of the normal cyclases from strain C2, thus, most of the enzyme aggregates, present at the later stages, would contain normal cyclases only, causing the massive accumulation of β-carotene at later growth stages.

TABLE 5 CAROTENOGENESIS AND GROWTH IN THE RED MUTANT C10

Age of culture (hr)	48	96	192
Carotenoids	(μg/g dry wt)	(μg/g dry wt)	(μg/g dry wt)
Phytofluene	—	—	37
ζ-Carotene	—	—	19
Neurosporene	—	15	25
Lycopene	692	750	1190
γ-Carotene	—	—	—
β-Carotene	—	—	—
β-Zeacarotene	—	—	—
Total carotenoids	692	765	1271
Total dry mycelial mass (mg)/100 ml medium	5 6	139 2	587
Dry wt of sample (g)	0 06	0 39	1 25

—Means less than 0 2 μg/sample

EXPERIMENTAL

Homokaryotic cultures were obtained by inoculating groups of *ca* 100 heat-shocked vegetative spores each into 100 ml liquid glucose-asparagine-yeast (GAY) medium[2] in 250-ml flasks The cultures were grown at 21°C under white light (250 μW/cm²) on a gyrotary shaker (120 rpm) To increase the sensitivity of the analyses, 25 flasks of culture were used for each strain, for the earliest sampling point of mycelial growth (2 day), 10–15 flasks of culture were harvested and the mycelia pooled

The heterokaryon C2* C9 was made according to the procedure of Ootaki,[7] spores thus obtained were plated out on solid agar containing GAY and acid at pH 3 3 On the acid medium the mycelia grow into relatively small colonies A stage I sporangiophore from a well-isolated orange-colored colony was removed and submerged into a solid GAY plate The sporangiophore then formed a mycelium, which eventually filled the surface of the plate Before the new sporangiophores formed on this plate, squares (0 2 × 0 2 cm) of mycelium were removed and used as inocula, one for each flask. The cultures then were subjected to the same treatment as the homokaryons The remaining mycelium was allowed to form sporangiophores whose spores were collected for subsequent determination of the nuclear ratio *p* Since the mycelial inocula used in the heterokaryotic cultures were derived from a single heterokaryotic stage I sporangiophore, the cultures had identical ratios of white and red nuclei and thus could be characterized by a single value of the nuclear ratio, *p*

[4] GOODWIN, T W (1965) *Chemistry and Biochemistry of Plant Pigments*, p 143, Academic Press, New York
[5] PORTER, J W and ANDERSON, D G (1967) *Ann Rev Plant Physiol* **18**, 197
[6] DAVIES, B H, VILLOUTREIX, J, WILLIAMS, R J H and GOODWIN, T W (1963) *J Biochem* **89**, 96
[7] OOTAKI, T (1973) *Mol Gen Genet* **121**, 49
[8] HEISENBERG, M and CERDÁ-OLMEDO, E (1968) *Mol Gen Genet* **102**, 187

The nuclear ratio p was determined by the "little tube assay" procedure.[1, 7, 8] 400 tubes were inoculated with an average of one heterokaryotic spore each. The fractions of red (C9), white (C2) and yellow (heterokaryotic) mycelia were determined and subjected to analysis involving Poisson statistics. Our analysis indicated that $p = 0.29$ for the heterokaryon used in this experiment (i.e. 29% of the nuclei came from C2, contributing a normal cyclase).

The procedures used in the determination of carotenoid content are as follows. The mycelia were sampled at different stages of growth, thoroughly extracted with acetone and transferred into petrol (30–60°) (PE). The lipid thus obtained was chromatographed on MgO–Hyflo-Supercel (1:1, w/w) column, and the various fractions were eluted with increasing amounts of acetone in PE. The carotenoid pigments were identified by their UV and visible spectra and by their adsorption behavior on the column relative to known compounds. The concentrations of these pigments were calculated according to the method described by Davies.[9]

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[9] DAVIES, B. H. (1965). *Chemistry and Biochemistry of Plant Pigments*. (GOODWIN, T. W., ed.), Academic Press, New York.