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Activation of β-Carotene Synthesis in *Blakeslea trispora* by Certain Terpenes

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Various pine oil fractions predominantly composed of terpenes stimulated β-carotene synthesis without affecting growth of *Blakeslea trispora*.

Among the individual terpenes tested, the bicyclic terpenes α- and β-pinene, (+)-camphene and, to a lesser degree, Δ^3-carene stimulated the carotene synthesis, whereas the monocyclic terpenes (+)- and (−)-limonene were without effect.

The results indicate that the terpenes stimulating carotene formation may act at some specific site(s) during the biosynthesis of β-carotene.

The biosynthesis of β-carotene is activated by β-ionone as first observed by MacKinney *et al.*[1] in cultures of *Phycomyces blakesleeanus*. The original hypothesis that β-ionone thereby acts as a precursor of β-carotene could not be confirmed in later studies.[2] It is now well established that the biosynthesis of the carotenes proceeds from mevalonic acid-5-pyrophosphate to a C_{40} chain precursor through a stepwise addition of "active isoprene" units (for a recent review *cf.* Ref. 3). It has been suggested from studies with *Phycomyces blakesleeanus* that the stimulating action of β-ionone on carotene synthesis may involve a reversal of a feedback control mechanism on the route from mevalonic acid-5-phosphate to γ,γ-dimethylallyl pyrophosphate.[2] Another member of the Phycomycetes, *Blakeslea trispora*, a heterothallic member of the order *Mucorales*, has received considerable attention during recent years because of the extensive synthesis of β-carotene in mated cultures of this organism (for review *cf.* Ref. 4). The process has been extensively studied by Anderson, Ciegler and their co-workers [5,6] who have also found a stimulatory effect of β-ionone and of certain inexpensive by-products from the citrus industry.[7,8] Our own studies on this subject were originally oriented towards utilization of industrial waste products, especially Swedish ones, for activation of the carotenogenesis. Various pine oil fractions, usually obtained as by-products in the sulphate (kraft) pulp industries, were considered to be

of interest in this connection. These materials are rich in terpenoid compounds, which, because of their structural relationship to β-ionone, could be expected to affect the carotene synthesis. On the basis of these studies, we investigated the effects of individual terpenes known to occur in those pine oil fractions which gave stimulatory effects. Growth and carotene synthesis in *Blakeslea trispora* are subject to considerable variations between different fermentation series, even under carefully standardized experimental conditions. These variations seem to depend on the state of the inoculum with respect to factors which are beyond experimental control at present. We considered it essential, therefore, to repeat every fermentation series on many occasions, and to express the yields as a percentage of those obtained using different reference media fermented at the same time. The resulting values were subjected to statistical analysis.

EXPERIMENTAL PROCEDURE

Organisms, growth media and conditions of fermentation. Mated cultures of *B. trispora* (NRRL 2456 (+) and 2457 (−) were used in all experiments. The strains were obtained through the courtesy of Dr. Hesseltine of the Northern Regional Research Laboratory, Peoria, Ill., USA. The strains were maintained on potato dextrose agar at room temperature with transfer to fresh medium every 8−10 days, essentially as described by Ciegler *et al.*[9] They were then homogenized and mixed for 7 sec in a sterilized Ato-Mix (MSE), in the proportion of 67 % of strain (−) to 33 % of strain (+). The mixture was used as inoculum at a 10 % level.

The composition of the fermentation medium was essentially similar to that described by Ciegler *et al.*,[9] but was modified to give a medium of the following composition per litre: acid-hydrolyzed corn meal 23 g, acid-hydrolyzed soybean meal 47 g, raw cottonseed oil (Karlshamns Oljefabriker, Sweden) 50 ml, Nonyl-phenol (Sunlight) 1.2 ml, thiamine-HCl 2 mg, NaOH to pH 6.5, deodorized paraffin for oil lamps (sold by Shell under the name of "Shellsol K") 50 ml. The hydrocarbon components were sterilized separately by filtration and added after 2 days of fermentation. The acid hydrolysis of the mixed grains was carried out in 0.1 M H_2SO_4 for 1½ h at 121°C, the pH of the hydrolysate being adjusted to approximately 6.5 before it was combined with the other components of the medium.

The first part of the investigation was concerned with nine different fractions of pine oil obtained from two plants (Nos. 1−9). Fractions 1−6 contained various fatty acids, resin acids, and aldehydes, whereas fractions 7−9 were mainly composed of terpenes. Fraction 7 was a crude mixture of terpenes. Fraction 8, which was a more refined mixture than fraction 7, contained 51−58 % α-pinene, 25−33 % Δ^3-carene, 3−7 % β-pinene, 2−4 % limonene, 2−4 % terpinolene, and 1.5−3 % camphor. Fraction 9 was essentially similar to fraction 8. It contained 50−56 % α-pinene and 36−42 % Δ^4- and Δ^3-carenes, the remainder consisting of monocyclic terpenes.

In the second part of the investigation, the effects of most of the respective terpenes present in fraction 8 were studied individually. The compounds thus tested were: α-pinene, β-pinene, Δ^3-carene, (+)-limonene, (−)-limonene, and (+)-camphene. They were all obtained from Fluka AG.

The pine oil fractions were added at several levels, the individual terpenes at a level of 0.4 %. The additions of both were made together with the addition of the hydrocarbon component of the medium after two days of fermentation. Each fermentation series contained one or more reference media containing 0.1 % β-ionone. Several series contained in addition medium without any activator, and one series also contained medium with 0.05 % β-ionone. All fermentations were carried out in quadruplicate.

Harvest of the mycelium and determination of β-carotene. The quadruplicate portions of the fermented media were pooled, the mycelium filtered off on filter paper discs under suction, washed with distilled water and freeze-dried, usually for 40−46 h. The dry

cakes were weighed and ground in a mill equipped with a sieve. Two 150 mg portions were extracted for 12 h with 75 ml light petroleum in 200 ml Erlenmeyer flasks and the residues washed with 50 ml light petroleum. Aliquots of the extracts were chromatographed on 1×10 cm columns of Al_2O_3 and water-free sodium sulphate (2:1). Elution was carried out with light petroleum containing 2 % acetone. The eluate corresponding to the β-carotene band was collected in a volumetric flask. The β-carotene content was determined from optical density measurements at 452 nm in a Hitachi Perkin-Elmer spectrophotometer, Model 14, using $E_{1\,\%}^{1\,cm} = 2560$. As far as possible, the carotene containing material was protected from light and from excessive contact with air during all the operations involved in the analysis.

RESULTS AND DISCUSSION

The effects of the various pine oil fractions on β-carotene synthesis as compared to the effect of β-ionone are shown in Fig. 1. Each value is expressed as percent of the value obtained in a reference medium, fermented in the absence of an activator, and refers to mg β-carotene/g mycelium solids. This latter yield varied between 1.24 and 5.42 mg/g, with an average of 3.1 mg/g as calculated from 9 separate fermentations. Fig. 1 shows that the pine oil fractions 1—6 inhibited the β-carotene synthesis to a varying degree. However, a stimulating effect was obtained with the fractions 7—9, *i.e.* those mainly composed of terpenes. The corresponding effects on mycelial growth were insignificant or, at higher concentrations of fractions 1—5, inhibitory. Growth usually amounted to 38.6—53.7 g mycelial solids/litre medium with an average of 47.8 g/l as calculated from 9 separate fermentations. The effect

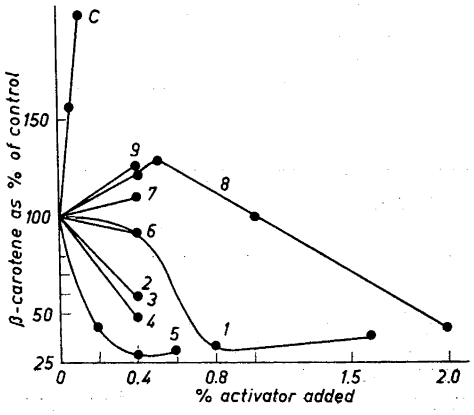

Fig. 1. The effect of various pine oil fractions on β-carotene synthesis in mated cultures of *Blakeslea trispora*. Fractions 1—6 contain mainly fatty acids, resin acids, and aldehydes. Fractions 7—9 contain mainly terpenes. Curve C refers to fermentation with β-ionone.

of fraction 8, the composition of which was best defined, was studied at several levels. It can be seen in Fig. 1 that maximum stimulation of carotene synthesis was obtained with a 0.5 % addition, whereas higher levels were inhibitory.

Table 1 shows the effects of the individual terpenes comprising fraction 8, as well as the effect of β-ionone. Again, the effects are expressed as a percentage of those observed with reference media fermented simultaneously

without any specific activator of carotenogenesis, and refer to mg β-carotene/g mycelial solids, averaged as above. In this case too, the growth was practically unaffected by the respective additions (*cf.* Table 1). Apart from β-ionone

Table 1. Activation of β-carotene synthesis in mated cultures of *Blakeslea trispora* by certain terpenes.

Activator [a]	No. of expts.	Mycelial growth [b] Mean	β-Carotene [a] Mean	Range
		%	%	%
None	6	100	100	—
α-Pinene	6	98	142	112–173
β-Pinene	5	96	128	96–168
(+)-Camphene	6	97	132	72–197
Δ^3-Carene	6	101	116	99–135
(+)-Limonene	6	100	98	78–123
(−)-Limonene	6	100	96	85–118
β-Ionone	5	98	190	132–249

[a] Terpenes added at 0.4 % level: β-ionone at 0.1 % level.
[b] These percentages have been calculated as follows:

$$\frac{\text{g dry mycelium per litre medium fermented in the presence of activator}}{\text{g dry mycelium per litre medium fermented without activator}} \times 100.$$

[c] These percentages have been calculated as follows:

$$\frac{\text{mg β-carotene per g mycelium solids in the presence of activator}}{\text{mg β-carotene per g mycelium solids without activator}} \times 100.$$

the synthesis of β-carotene was stimulated most by α-pinene, less so by (+)-camphene and β-pinene and considerably less by Δ^3-carene. Both (+)- and (−)-limonene were practically without effect or possibly slightly inhibitory. The respective significance probabilities of the observed effects were: for α-pinene 99.9 %; for β-ionone 99.0 %; for β-pinene 98.0 %, for (+)-camphene and Δ^3-carene 90.0 % in both cases.

The effect obtained with a 0.4 % level of α-pinene was 75 % of that obtained with the 0.1 % level of β-ionone, a concentration found to be optimal in fermentations for β-carotene production.[6]

A generally stimulating effect of several terpenes and terpene derivatives on β-carotene synthesis in *Blakeslea trispora* has also been reported by Ciegler, Nelson and Hall.[7] According to these workers (*d*)-limonene stimulated the synthesis too, which is at variance with our results. A direct comparison of the results is difficult, however, because of possible differences between the American and European terpene preparations and also because the above report [7] does not contain any information concerning the range of variations in the observed effects.

It may be of interest to consider in this connection the molecular structure of the terpenes tested in the present study. It seems that the bicyclic terpenes with their more rigid structure, *e.g.* α- and β-pinene as well as (+)-camphene, do have a distinct activating effect, whereas the two monocyclic limonenes with their flexible conformation do not. Δ^3-carene occupies an intermediate position in both these respects.

The fact that the activating effect of the terpenes studied seems to depend on their particular conformation points towards a specific site of action rather than a generally stimulating effect.

It is interesting to recall in this connection the group of stimulatory substances isolated from the mated cultures of *Blakeslea trispora* by Prieto *et al.*[10,11] and also by Sebek and Jäger.[12] These compounds, named *trisporic acids*, were suggested to act as de-repressors at some site of carotene synthesis, not defined as yet.[13] They have a terpenoid type of structure.

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REFERENCES

1. MacKinney, G., Nakayama, T., Buss, C. D. and Chichester, C. O. *J. Am. Chem. Soc.* **74** (1952) 3456.
2. Reyes, Ph., Chichester, C. O. and Nakayama, T. O. M. *Biochim. Biophys. Acta* **90** (1964) 578.
3. Porter, J. W. and Anderson, D. C. *Ann. Rev. Plant Physiol.* **18** (1967) 197.
4. Ciegler, A. *Advan. Appl. Microbiol.* **7** (1965) 19.
5. Anderson, R. F., Arnold, M., Nelson, G. E. N. and Ciegler, A. *J. Agr. Food Chem.* **6** (1958) 543.
6. Ciegler, A., Arnold, M. and Anderson, R. F. *Appl. Microbiol.* **7** (1959) 98.
7. Ciegler, A., Nelson, G. E. N. and Hall, H. H. *Appl. Microbiol.* **11** (1963) 128.
8. Pazola, Z., Ciegler, A. and Hall, H. H. *Nature* **210** (1966) 1367.
9. Ciegler, A., Nelson, G. E. N. and Hall, H. H. *Appl. Microbiol.* **10** (1962) 132.
10. Prieto, A., Spalla, C., Bianchi, M. and Biffi, G. *Chem. Ind. (London)* **13** (1964) 551.
11. Cagliotti, L., Cainelli, G., Camerino, B., Mondelli, R., Prieto, A. Quilico, T., Salvalori, T. and Selva, A. *Chim. Ind. (Milan)* **46** (1964) 1.
12. Sebek, O. and Jager, H. *Abstr.* **148***th Meet. Am. Chem. Soc.* (1964) 90.
13. Thomas, D. M., Harris, R. C., Kirk, J. T. O. and Goodwin, T. W. *Phytochemistry* **6** (1967) 361.

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