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Optimization of β-Carotene Production from Synthetic Medium by *Blakeslea trispora*

A Mathematical Modeling

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Abstract

The effect of inoculum, pH, carbon and nitrogen source, natural oils, fatty acids, antioxidant, and precursors on β-carotene production by *Blakeslea trispora* in shake-flask culture was investigated. The highest concentration of β-carotene was obtained in the medium (pH 7.0) inoculated with one loop of each culture. Sucrose, glycerol, cornmeal, soy protein acid hydrolysate, and distiller's solubles did not improve the production of β-carotene. By contrast, glucose, corn steep liquor, antioxidant, olive oil, soybean oil, cottonseed oil, oleic and linoleic acids, and kerosene significantly increased the β-carotene production. A central composite design was employed to determine the maximum β-carotene production at optimum values for the process variables (linoleic acid, kerosene, and antioxidant). The fit of the model was found to be good. Linoleic acid, kerosene, and antioxidant had a strong linear effect on β-carotene production. The concentration of β-carotene was significantly affected by linoleic acid–kerosene and linoleic acid–antioxidant interactions as well as by the negative quadratic effects of these variables. The interaction between kerosene and antioxidant had no significant linear effect. The maximum β-carotene concentration (2.88 g/L) was obtained at concentrations of 17.15 g/L of linoleic acid, 39.25 g/L of kerosene, and 9.04 g/L of antioxidant.

Index Entries: β-Carotene; synthetic medium; *Blakeslea trispora*; shake flask; mathematical modeling.

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Introduction

Carotenoids are highly unsaturated isoprene derivatives. Naturally occurring carotenoids are tetraterpenoids consisting of eight isoprene residues. β-Carotene is an important compound because of its role as a precursor of vitamin A in food and feed products. In addition, it is used as an antioxidant to reduce cellular or tissue damage and as a coloring agent for food products, such as margarine, soft drinks, and baked goods *(1,2)*. β-Carotene is produced primarily by fungi, yeasts, some species of bacteria, algae, and lichens. The greatest yields have been obtained with the mixture of + and – strains of *Blakeslea trispora (3)*.

The production of β-carotene in a chemically defined medium by *Phycomyces blakesleeanus* and *Rhodotorula glutinis* has been described *(4,5)*. A number of researchers have investigated different aspects of β-carotene production from sucrose, glycerol, cellobiose, sugarcane juice, citrus molasses, and cheese whey *(6–12)*. Ciegler et al. *(13,14)* and Kim et al. *(1)* studied the effect of various grains, lipids, and related substances and nonionic surfactants on the production of β-carotene by different strains of *B. trispora*. In all previous works, the maximum concentration of β-carotene reported was very low.

In the present study, the effect of the composition of medium on β-carotene production by *B. trispora* was explored in an attempt to increase the β-carotene-producing capacity of the culture. In addition, the effect of various process parameters, such as inoculum size, ratio of inoculum, and pH, on kinetic parameters of β-carotene fermentation was examined. A mathematical design was used to determine the optimum levels of linoleic acid, kerosene, and antioxidant in order to obtain the maximum β-carotene production. A semiautomatic image analysis system was also used to determine the morphology of the microorganism.

Materials and Methods

Microorganisms and Culture Conditions

The two strains of *B. trispora* used were *B. trispora* ATCC 14271, mating type (+), and *B. trispora* ATCC 14272, mating type (–). Both strains were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and maintained at 4°C on potato dextrose agar slants. Cells for inoculation of the growth medium or the production medium were obtained from cultures grown on potato dextrose agar slants at 26°C for 72 h. One loop of each culture was transferred to 500-mL conical flasks containing 100 mL of growth medium (pH 7.0) with the following composition: 30.0 g/L of glucose, 5.0 g/L of corn steep liquor (CSL), 2.0 g/L of casein acid hydrolysate, 1.0 g/L of yeast extract, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, and 5.0 mg/L of thiamine · HCl. The flasks were incubated at 26°C for 48 h in a rotary shaker incubator (Orbit-Environ shaker; Lab-Line, Melrose

Park, IL) at 200 rpm. These cultures were used to inoculate the basal medium.

Fermentation Conditions

The fermentation was carried out in 500-mL conical flasks containing 100 mL of basal medium (pH 7.0) with the following composition: 50.0 g/L of glucose, 80.0 g/L of CSL, 2.0 g/L of casein acid hydrolysate, 1.0 g/L of yeast extract, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, and 5.0 mg/L of thiamine · HCl. The flasks were inoculated with one loop of each culture and incubated at 26°C in the rotary shaker incubator at 200 rpm.

Effects of Inoculum

A set of conical flasks containing 100 mL of basal medium (pH 7.0) was inoculated with one loop of the strain ATCC 14271 or ATCC 14272; one loop of each culture; 5% (v/v) of premated inocula (the two mating strains were grown together to produce premated inocula); and 2, 5, or 10% (v/v) of each culture grown separately. The flasks were incubated as already described.

Effects of Initial pH

A series of conical flasks containing 100 mL of basal medium was adjusted at different initial pHs (6.0, 7.0, 8.0, 9.0, 10.0, 11.0), inoculated with one loop of each culture, and incubated at 26°C for 8 d.

Effects of Carbon Source

A set of conical flasks containing 100 mL of basal medium consisting of all the nutrients (except glucose) was supplemented with 30, 50, and 70 g/L of glucose, sucrose, or glycerol. The flasks were inoculated and incubated as already described.

Effects of Nitrogen Source

The basal medium consisting of all the nutrients (except CSL) was supplied with 50, 80, and 100 g/L of CSL (2.0, 3.2, and 4.0 g/L of total nitrogen), soy protein acid hydrolysate (6.3, 10.1, and 12.6 g/L of total nitrogen), distiller's solubles (2.1, 3.4, and 4.2 g/L of total nitrogen), and cornmeal (0.8, 1.3, and 1.6 g/L of total nitrogen), or 5, 10, and 15 g/L of NH_4NO_3 or $(NH_4)_2SO_4$. The substrate was inoculated with one loop of each culture and incubated at 26°C for 8 d.

Effects of Vegetable Oils and Fatty Acids

A series of conical flasks containing 100 mL of basal medium was supplemented with 10, 30, and 50 g/L of olive oil, cottonseed oil, and soybean oil, or 10, 20, 30, 40, and 50 g/L of oleic or linoleic acid. The flasks were inoculated and incubated as already described.

Effects of Precursors

Precursors are chemicals added to production medium and are directly incorporated into the desired product. A set of shake-flask experiments was performed at different concentrations of precursors to examine their effect on β-carotene production. The flasks containing 100 mL of basal medium were supplied with different concentrations of β-ionone, kerosene, isoniazid, cyclohexane, cyclohexanone, succinimide, or a mixture of these chemicals. The flasks were inoculated with one loop of each culture and incubated at 26°C for 10 d. Precursors were added on the second day of fermentation *(10)*.

Analytical Techniques

At appropriate time intervals, fermentation flasks were removed and the contents analyzed. β-Carotene concentration was determined according to Roukas and Mantzouridou *(15)*. The pigment was extracted from the cells with ethanol, and the intensity of color was measured at 450 nm with a Zeiss spectrophotometer. In all experiments, only all-*trans*-β-carotene was determined by UV detection (UV 120A; Shimadzu). Biomass dry wt was determined by centrifuging the broth at 10,000*g* for 20 min, washing the sediment with distilled water (twice), and drying at 105°C, overnight. Residual sugars in the supernatant were determined, as glucose, according to Dubois et al. *(16)*.

The morphology of the *B. trispora* during the fermentation was characterized using a semiautomatic image analysis system consisting of a phase contrast microscope (Nicon E 200), a charge-coupled device (CCD) camera (JVC), a PC with a frame-grabber (LEADEC), and image analysis software (Matrox Inspector 32). The CCD camera captured images of 768 × 568 × 24 pixels, with a grayness level from 0 (black) to 255 (white). Samples for morphologic characterization were taken at the maximum concentration of β-carotene. The morphologic parameters measured were the area of zygospores and vacuolated regions (percentage of total area of mycelium), and the length of mycelium included the equivalent diameter of zygospores and the mean width of vacuoles (Table 1) *(17)*. Primary measurements on individual objects in images were perimeter, area, and length. The raw data were used to calculate circularity, equivalent diameter for zygospores (d_z), and diameter (width) (d_m) for vacuoles. The shape of zygospores was close to spherical (circularity of <1.3). The shape of the vacuoles was approximating short cylinders (1.3 ≤ circularity < 2.0). For each sample, the process was repeated at least 20 times using new positions on the same and on different filaments, and the morphologic parameters were expressed as the mean values of each sample.

The reported data are the average values ± SD of three separate experiments.

Variability was also expressed by coefficient of variation (CV) values ($CV = SD/\bar{x} \cdot 100$).

Table 1
List of Image Analysis Parameters

Parameter	Source formula	Units	Use
Perimeter (P)	Pixel counting, around the boundary of detected image	μm	Mycelium (P_m), zygospores (P_z), and vacuoles (P_v)
Area (A)	Pixel counting, total number of detected pixels	μm × μm	Mycelium (A_m), zygospores (A_z), and vacuoles (A_v)
Circularity (C)	$C = P^2/4\pi A$	Dimensionless	Zygospores and vacuoles
Equivalent diameter (d_z)	$d_z = (4A/\pi)^{1/2}$	μm	Zygospores
Mean width (d) for cylindrical objects	$d = [P - (P^2 - 16A)^{1/2}]/4$	μm	Vacuoles (d_v)

Table 2
Levels of Factors Used in Experimental Design

		Level				
Factor	Name	$-a$	−1	0	+1	$+a$
x_1	Linoleic acid (g/L)	13.18	20.00	30.00	40.00	46.82
x_2	Kerosene (g/L)	13.18	20.00	30.00	40.00	46.82
x_3	Antioxidant (g/L)	6.59	10.00	15.00	20.00	23.40

Experimental Design and Statistical Analysis

The statistical analysis of the data was performed using the Minitab package. Details of response surface methodology can be found elsewhere *(18)*. The levels of factors used in the experimental design are listed in Table 2. The data of the factors were chosen after a series of preliminary experiments. In this design, there were five experimental levels: $-a$, −1, 0, +1, $+a$, in which $a = 2^{n/4}$, n = number of variables, and 0 corresponds to the central point. The actual level of each factor was calculated using the following equation *(17)*:

$$\text{Coded value} = \frac{\text{Actual level} - (\text{High level} + \text{Low level}/2)}{(\text{High level} - \text{Low level}/2)}$$

Twenty experiments were conducted using a central composite statistical design for the study of three factors each at five levels (Table 3). The most commonly used empirical model, polynomial response surface, was fitted to measure the response variable, β-carotene concentration (g/L).

Table 3
Experimental Design

Run	Linoleic acid (g/L)	Kerosene (g/L)	Antioxidant (g/L)	β-Carotene (g/L)
1	20.00	40.00	10.00	2.87
2	30.00	13.18	15.00	0.81
3	46.82	30.00	15.00	1.10
4	20.00	20.00	20.00	0.61
5	30.00	30.00	15.00	2.29
6	40.00	20.00	20.00	0.68
7	30.00	46.82	15.00	2.04
8	40.00	40.00	20.00	1.34
9	30.00	30.00	15.00	2.50
10	30.00	30.00	15.00	2.50
11	20.00	20.00	10.00	1.93
12	13.18	30.00	15.00	1.96
13	40.00	40.00	10.00	1.70
14	30.00	30.00	15.00	2.29
15	30.00	30.00	15.00	2.50
16	30.00	30.00	15.00	2.29
17	20.00	40.00	20.00	1.54
18	30.00	30.00	6.59	2.10
19	30.00	30.00	23.40	0.71
20	40.00	20.00	10.00	1.20

The second-order response function for three quantitative factors is given by the following equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (1)$$

in which x_1, x_2, and x_3 are the levels of the factors according to Table 2; and β_0, β_1, …, β_{23} are coefficient estimates with β_0 having the role of a scaling constant.

Results and Discussion

Effects of Inoculum

The effect of inoculum on β-carotene concentration is shown in Fig. 1. The maximum concentration of β-carotene (170 mg/L) was obtained after 8 d of fermentation, when the basal medium was inoculated with one loop of each culture. In media inoculated with 2, 5, or 10% of each culture grown separately, the β-carotene concentration was 80, 100, and 90 mg/L, respectively. When the media were inoculated only with the strain ATCC 14271 or ATCC 14272, the concentrations of the pigment were 68 and 56 mg/L, respectively. Finally, the amount of β-carotene was very low (10 mg/L) when the substrate was inoculated with premated inoculum. This may be

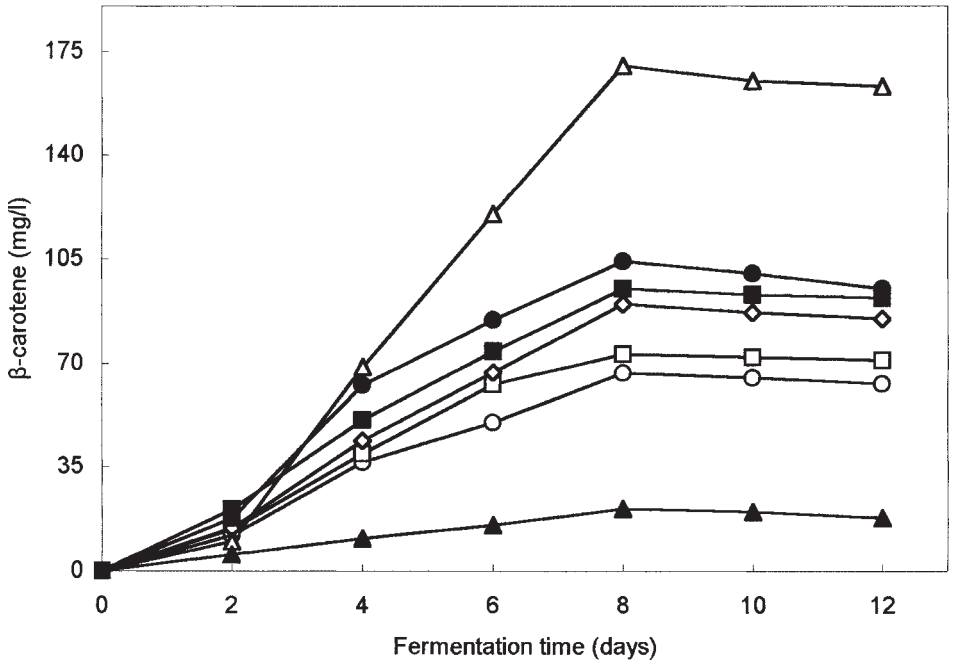

Fig. 1. Effect of inoculum on β-carotene production from synthetic medium by *B. trispora* in shake-flask culture. The medium was inoculated with one loop of the strain ATCC 14271 (—○—), one loop of the strain ATCC 14272 (—□—), one loop of each culture (—△—), 2.0% (v/v) of each culture (—◇—), 5.0% (v/v) of each culture (—●—), 10.0 % (v/v) of each culture (—■—), and premated culture (—▲—). Data are means of triplicate experiments; CVs for all measured parameters did not exceed 5.0% in all cases. Basal medium consisted of the following: 50.0 g/L of glucose, 80.0 g/L of CSL, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, and 5 mg/L of thiamine·HCl (fermentation time of 8 d).

explained by the fact that the microorganism lost its activity when it was precultured in growth medium before the inoculation of the production medium. Microscopic examination using the image analysis showed that the medium inoculated with one loop of each culture appeared to have a higher number of zygospores than those inoculated with the cultures grown separately. These results clearly show that the medium inoculated directly with the cells of the microorganism gave the highest concentration of β-carotene. These results are in contrast to those of other researchers who studied the production of β-carotene in synthetic medium by different strains of *B. trispora*. Those researchers used strains of *B. trispora* grown separately as inocula and then added to the production medium at a ratio of 1:1. Based on the foregoing observations, all the media were inoculated directly with one loop of each strain of *B. trispora* for all subsequent studies.

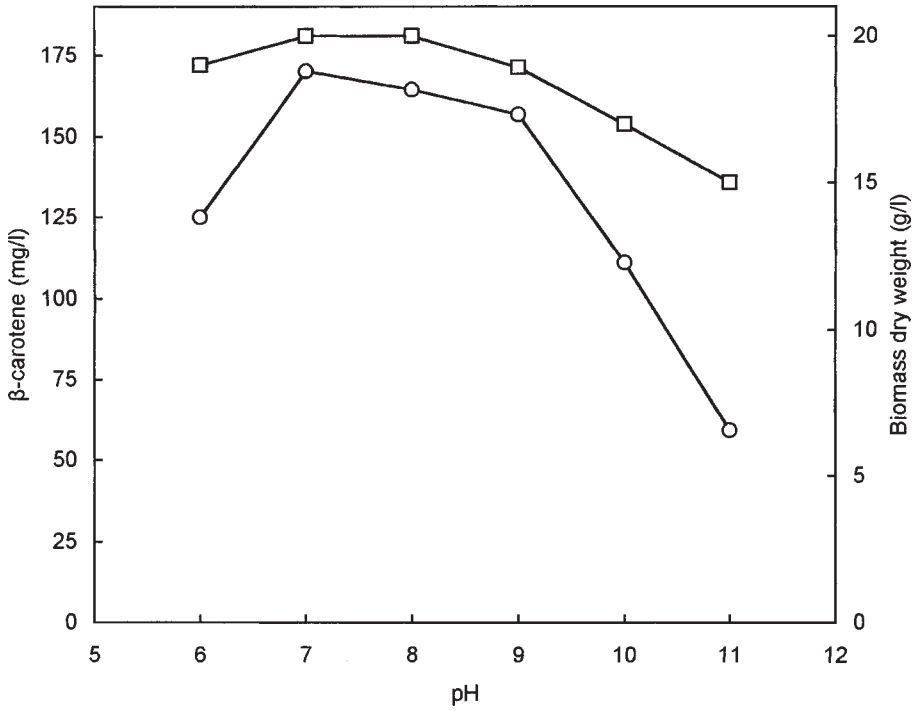

Fig. 2. Effect of initial pH on biomass dry wt and β-carotene concentration from synthetic medium by *B. trispora* in shake-flask culture. (—○—) β-carotene concentration; (—□—) biomass dry wt. Data are means of triplicate experiments; CV values for all measured parameters did not exceed 4.7 % in all cases. Basal medium consisted of the following: 50.0 g/L of glucose, 80.0 g/L of CSL, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, and 5 mg/L of thiamine·HCl (fermentation time of 8 d).

Effects of Initial pH

As shown in Fig. 2, the β-carotene concentration increased with the increase in initial pH from 6.0 to 7.0 and then decreased. The biomass dry wt slightly increased with the increase in pH from 6.0 to 7.0, remained practically constant between pH 7.0 and 8.0, but decreased beyond that value. The highest concentration of β-carotene (170.0 mg/L) and biomass dry wt (18.0 g/L) was obtained in culture grown at an initial pH of 7.0. Martelli et al. *(7)* and Kim et al. *(19)* found that an initial pH of 5.0 and 10.0–11.0 is optimal for β-carotene production by *Rhodotorula lactosa* and *B. trispora* ATCC 14271, respectively. It would appear that the optimal initial pH for β-carotene biosynthesis depends on several fermentation parameters. These include the chemical composition of the substrate; the strain of the microorganism; the fermentation system; and generally, the conditions under which the fermentation takes place.

Table 4
Effects of Carbon Source on Fermentation Parameters
During β-Carotene Production by *B. trispora* in Shake-Flask Culture[a]

Glucose (g/L)	Sucrose (g/L)	Glycerol (g/L)	β-Carotene concentration (mg/L)	Biomass dry wt (g/L)	Residual sugars as glucose (g/L)
0.0	0.0	0.0	0.0	No growth	0.0
30.0	0.0	0.0	5.9 ± 0.29	1.1 ± 0.05	21.5 ± 0.86
50.0	0.0	0.0	63.2 ± 3.16	9.4 ± 0.47	6.7 ± 0.25
70.0	0.0	0.0	50.6 ± 2.53	14.0 ± 0.70	40.3 ± 1.61
0.0	30.0	0.0	0.0	1.2 ± 0.06	30.0 ± 1.50
0.0	50.0	0.0	0.0	1.3 ± 0.06	50.0 ± 2.50
0.0	70.0	0.0	0.0	2.2 ± 0.11	70.0 ± 2.80
0.0	0.0	30.0	0.0	0.6 ± 0.03	0.0
0.0	0.0	50.0	0.0	0.7 ± 0.03	0.0
0.0	0.0	70.0	0.0	4.2 ± 0.21	0.0

[a]Basal medium consisted of the following: 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 8 d).

Effects of Carbon Source

The effect of carbon source on β-carotene production by *B. trispora* is shown in Table 4. The concentration of β-carotene increased with the increase in glucose concentration up to 50 g/L but decreased beyond this value. On the other hand, the biomass dry wt increased with the increase in glucose concentration from 30 to 70 g/L. This means that there is not a parallel relationship between biomass and product concentration. Increasing the glucose concentration above 50 g/L resulted in a decrease in β-carotene production. The decreased concentration encountered with the highest concentration treatment was probably owing to osmotic effects. It has been reported that above a critical substrate concentration, the decreased water activity and the onset of plasmolysis combine to cause a decrease in the rates of fermentation and product concentration *(20)*. The addition in the medium of sucrose and glycerol at concentrations of 30, 50, and 70 g/L did not produce β-carotene. These results showed that the addition of glucose improved the production of β-carotene. For this reason, further experiments were performed with the addition of glucose in the medium at a concentration of 50 g/L.

Effects of Nitrogen Source

In an attempt to increase the β-carotene-producing capacity of the culture, several nitrogen sources such as CSL, cornmeal, soy protein acid hydrolysate, distiller's solubles, NH_4NO_3, and $(NH_4)_2SO_4$ were explored. The results in Table 5 show that these nitrogen compounds influenced the

Table 5
Effects of Nitrogen Source on Fermentation Parameters During β-Carotene Production by *B. trispora* in Shake-Flask Culture

Nitrogen source (g/L)	Equal nitrogen basis (g/L)	β-Carotene concentration (mg/L)	Biomass dry wt (g/L)	Residual sugars as glucose (g/L)
Basal medium[a]	—	63.2 ± 3.16	9.4 ± 0.47	6.7 ± 0.25
CSL				
50.0	2.0	100.5 ± 4.00	25.6 ± 0.77	4.5 ± 0.14
80.0	3.2	170.0 ± 6.80	27.8 ± 0.83	3.6 ± 0.14
100.0	4.0	108.4 ± 4.34	28.5 ± 0.86	4.1 ± 0.12
Cornmeal				
50.0	0.8	56.0 ± 2.24	6.2 ± 0.19	10.8 ± 0.27
80.0	1.3	92.6 ± 3.71	8.8 ± 2.32	6.4 ± 0.16
100.0	1.6	33.4 ± 1.34	5.3 ± 0.16	10.5 ± 0.42
Soy protein acid hydrolysate				
50.0	6.3	90.0 ± 3.60	28.0 ± 0.84	3.5 ± 0.07
80.0	10.1	70.5 ± 2.82	30.0 ± 0.90	3.0 ± 0.06
100.0	12.6	65.2 ± 2.61	29.5 ± 0.89	2.7 ± 0.05
Distiller's solubles				
50.0	2.1	93.5 ± 3.74	32.5 ± 0.98	10.5 ± 0.32
80.0	3.4	81.0 ± 3.24	30.1 ± 0.90	10.7 ± 0.32
100.0	4.2	72.6 ± 2.91	30.0 ± 0.75	11.0 ± 0.33
NH_4NO_3				
5.0	1.8	23.5 ± 0.94	8.7 ± 0.22	7.4 ± 0.15
10.0	3.5	20.7 ± 0.83	11.6 ± 0.29	10.5 ± 0.52
15.0	5.3	13.8 ± 0.55	14.0 ± 0.35	10.5 ± 0.52
$(NH_4)_2SO_4$				
5.0	1.1	56.0 ± 2.24	11.5 ± 0.29	2.9 ± 0.06
10.0	2.1	45.2 ± 1.81	7.9 ± 0.19	3.0 ± 0.06
15.0	3.2	28.0 ± 1.12	6.4 ± 0.16	3.2 ± 0.08

[a]Basal medium consisted of the following: 50.0 g/L of glucose, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 8 d).

production of β-carotene and growth of microorganism to different degrees. All the nitrogen sources (except NH_4NO_3 and $[NH_4]_2SO_4$) significantly increased the biosynthesis of β-carotene. The β-carotene concentration increased with the increase in CSL from 50 to 80 g/L but decreased above this value. The maximum β-carotene concentration (170 mg/L) was obtained in culture grown at a concentration of 80 g/L of CSL. In cultures grown in cornmeal, soy protein acid hydrolysate, distiller's solubles, NH_4NO_3, and $(NH_4)_2SO_4$, the maximum concentration of β-carotene was 92.6, 90.0, 93.5, 23.5, and 56.0 mg/L, respectively. Ciegler et al. *(13)* studied the effect of various grains such as oats, wheat, barley, corn, rice, and rye on β-carotene production and found that the maximum pigment concen-

Table 6
Effects of Natural Oils on Fermentation Parameters
During β-Carotene Production by *B. trispora* in Shake-Flask Culture

Natural oils (% [w/v])	β-Carotene concentration (mg/L)	Biomass dry wt (g/L)	Residual sugars as glucose (g/L)
Basal medium[a]	170.0 ± 6.80	27.8 ± 0.83	3.6 ± 0.14
Olive oil			
1.0	858.6 ± 25.76	32.3 ± 0.81	5.3 ± 0.11
3.0	796.2 ± 23.89	45.5 ± 1.14	4.2 ± 0.08
5.0	750.7 ± 22.52	46.2 ± 1.16	4.1 ± 0.08
Cottonseed oil			
1.0	757.9 ± 22.74	40.5 ± 1.00	4.5 ± 0.09
3.0	871.7 ± 26.15	48.2 ± 1.21	3.2 ± 0.06
5.0	786.4 ± 23.59	48.9 ± 1.22	3.0 ± 0.06
Soybean oil			
1.0	690.6 ± 17.27	41.5 ± 1.25	4.3 ± 0.09
3.0	765.6 ± 22.97	47.4 ± 1.42	3.3 ± 0.07
5.0	680.2 ± 20.41	48.3 ± 1.45	3.0 ± 0.06

[a]Basal medium consisted of the following: 50.0 g/L of glucose, 80.0 g/L of CSL, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, 2.5 g/L of antioxidant (butylated hydroxytoluene), and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 8 d).

tration varied between 36.4 and 177.4 mg/L. Lampila et al. *(11)* found that the maximum β-carotene concentration (2.4 mg/L) was obtained when *B. trispora* was grown in cheese whey in shake-flask culture. Ciegler et al. *(10)* reported that a high concentration of β-carotene (1200 mg/L) was obtained when *B. trispora* was grown in a chemically defined medium containing 50 g/L of citrus peel. These results clearly show that CSL is an appropriate medium for the production of β-carotene by *B. trispora*. The replacement of CSL with other nitrogenous compounds resulted in a lesser increase in β-carotene concentration (Table 5), although there was considerable variation in growth of microorganism. In the culture grown in medium containing CSL, the biomass dry wt ranged between 25.6 and 28.5 g/L, while it increased slightly in the presence of soy protein acid hydrolysate and distiller's solubles. On the other hand, in cultures grown in cornmeal, NH_4NO_3, and $(NH_4)_2SO_4$, the biomass concentration remained rather low (5.3–14.0 g/L). Generally, the results showed that CSL, a low-cost byproduct of the starch industry, gave the maximum concentration of β-carotene compared to other nitrogen compounds.

Effects of Natural Oils

A variety of natural oils (olive oil, cottonseed oil, soybean oil) was incorporated into the medium to determine their effect on β-carotene production. Representative data are shown in Table 6. In all cases, the addition

Table 7
Effects of Fatty Acids on Fermentation Parameters
During β-Carotene Production by *B. trispora* in Shake-Flask Culture

Fatty acids (% [w/v])	β-Carotene concentration (mg/L)	Biomass dry wt (g/L)	Residual sugars as glucose (g/L)
Basal medium[a]	170.0 ± 6.80	27.8 ± 0.83	3.6 ± 0.14
Oleic acid			
1.0	175.0 ± 7.00	24.3 ± 0.73	4.5 ± 0.11
2.0	182.5 ± 7.30	34.7 ± 1.10	5.2 ± 0.13
3.0	391.6 ± 15.66	44.9 ± 1.35	5.4 ± 0.16
4.0	848.3 ± 33.93	46.0 ± 1.38	4.7 ± 0.12
5.0	594.1 ± 23.76	43.0 ± 1.29	5.1 ± 0.13
Linoleic acid			
1.0	500.0 ± 20.00	28.4 ± 0.85	5.4 ± 0.14
2.0	1322.0 ± 52.88	37.8 ± 1.13	4.2 ± 0.11
3.0	1064.1 ± 42.56	45.3 ± 1.36	4.5 ± 0.11
4.0	750.0 ± 30.00	51.7 ± 1.55	4.6 ± 0.12
5.0	550.0 ± 22.00	46.8 ± 1.41	6.2 ± 0.16

[a]Basal medium consisted of the following: 50.0 g/L of glucose, 80.0 g/L of CSL, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, 2.5 g/L of butylated hydroxytoluene, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 8 d).

of these oils significantly increased the production of β-carotene. Moreover, the addition of olive oil, cottonseed oil, and soybean oil in a concentration up to 1, 3, and 3%, respectively, gave the maximum concentration of the pigment (858.6, 871.7, and 765.6 mg/L, respectively), while the addition of greater amounts of these oils caused a decrease in the product's concentration (Table 6). In all culture systems, the biomass dry wt increased with the increase in the concentration of natural oils. The maximum biomass concentration (46.2–48.9 g/L) was obtained in cultures grown in media containing 5% (w/v) olive oil, cottonseed oil, or soybean oil.

The addition of a mixture of natural oils at different concentrations (1 to 2% [w/v]) to the medium resulted in a slight increase in β-carotene concentration (data not shown) compared with the medium supplied only with olive oil, cottonseed oil, or soybean oil (Table 6). The highest concentration of β-carotene (924.4 mg/L) was obtained in culture grown in medium supplemented with 1% (w/v) olive oil and 2% (w/v) cottonseed oil.

Effects of Fatty Acids

The effect of fatty acids (oleic and linoleic acid) on β-carotene production by *B. trispora* is shown in Table 7. The concentration of β-carotene and the biomass dry wt increased significantly with the increase in oleic acid

concentration from 1 to 4% (w/v) and then decreased. The maximum β-carotene concentration (848.3 mg/L) and biomass dry wt (46.0 g/L) were obtained in culture grown at a concentration of oleic acid of 4% (w/v). On the other hand, when the linoleic acid concentration increased from 1 to 2% (w/v), the pigment level increased from 500.0 to 1322.0 mg/L (Table 7). With a further increase in linoleic acid concentration to 5% (w/v), the level of β-carotene decreased to 550.0 mg/L. By contrast, the biomass constantly increased with linoleic acid concentration up to 4% (w/v) and then decreased. The results in Table 7 indicate that the optimal oleic and linoleic acid concentration for β-carotene production by *B. trispora* was 4 and 2% (w/v), respectively. As shown in Tables 6 and 7, the concentration of β-carotene was almost the same when the media were supplemented with olive oil or oleic acid (858.6 vs 848.3 mg/L). This means that the olive oil contains all the necessary substances for the growth of microorganism and biosynthesis of β-carotene. On the other hand, the medium supplied with linoleic acid gave a very high concentration of the pigment compared with the media containing cottonseed oil and soybean oil (1322.0 vs 871.7 mg/L and 765.6 mg/L, respectively) (Tables 6 and 7). These results show that linoleic acid is more effective in stimulating carotene synthesis than oleic acid. This may be explained by the fact that linoleic acid contains two double bonds instead of one of oleic acid.

Effects of Precursors

The effects of precursors on fermentation parameters during β-carotene production by *B. trispora* in shake-flask culture are shown in Table 8. All the precursors tested (except kerosene) decreased the concentration of β-carotene, while the biomass dry wt was maintained at appropriate levels (21.3–37.8 g/L). This means that they inhibit the activity of enzymes, responsible for the biosynthesis of the product, and not the growth of microorganism. The addition of kerosene into the medium significantly increased the concentration of the pigment. These results agree with those of Atkinson and Mavituna *(3)*, who reported that kerosene increased the production of β-carotene, but are in contrast with those of Ciegler et al. *(2,14)*, who found that the addition of β-ionone into the medium increased the concentration of β-carotene. Moreover, these researchers reported that the olive oil and cottonseed oil, which contain a significant amount of oleic and linoleic acid, respectively, stimulated the biosynthesis of the pigment. The maximum concentration of β-carotene (2300.0 mg/L) was obtained in cultures grown in media supplemented with 4% (w/v) kerosene. In this case, the maximum biomass concentration was 34.0 g/L.

The mixture of precursors at different concentrations (β-ionone, isoniazid, cyclohexane, cyclohexanone 0.1% , and 1 to 2% [w/v] kerosene) did not increase the concentration of β-carotene compared to basal medium (data not shown).

Table 8
Effects of Precursors on Fermentation Parameters
During β-Carotene Production by *B. trispora* in Shake-Flask Culture

Precursor (% [w/v])	β-Carotene concentration (mg/L)	Biomass dry wt (g/L)	Residual sugars as glucose (g/L)
Basal medium[a]	1322.0 ± 52.88	37.8 ± 1.13	4.2 ± 0.11
β-Ionone			
0.1	271.6 ± 10.86	27.9 ± 0.84	5.7 ± 0.14
0.3	130.7 ± 5.23	21.3 ± 0.64	4.6 ± 1.12
Kerosene			
1.0	1529.5 ± 61.18	36.2 ± 1.10	4.6 ± 1.12
2.0	1725.0 ± 69.00	35.1 ± 1.05	5.2 ± 0.13
3.0	1960.0 ± 78.40	34.5 ± 1.04	6.3 ± 0.16
4.0	2300.0 ± 92.00	34.0 ± 1.02	6.1 ± 0.15
5.0	1250.0 ± 50.00	35.5 ± 1.07	5.5 ± 0.14
Isoniazid			
0.1	1180.0 ± 47.20	28.5 ± 0.86	4.2 ± 0.11
0.3	650.0 ± 26.00	32.4 ± 0.97	4.5 ± 0.11
0.5	116.5 ± 4.66	28.6 ± 0.86	6.7 ± 0.17
0.7	62.9 ± 2.52	29.5 ± 0.89	5.8 ± 0.15
1.0	53.5 ± 2.14	29.9 ± 0.90	7.2 ± 0.18
Cyclohexane			
0.1	473.6 ± 18.94	37.8 ± 1.13	4.3 ± 0.11
0.3	208.2 ± 8.33	37.7 ± 1.13	4.3 ± 0.11
Cyclohexanone			
0.1	715.0 ± 28.60	32.3 ± 0.97	4.1 ± 0.10
0.3	265.5 ± 10.62	27.8 ± 0.83	5.5 ± 0.14
Succinimide			
0.5	864.4 ± 34.58	32.3 ± 0.97	4.0 ± 0.10

[a]Basal medium consisted of the following: 50.0 g/L of glucose, 80.0 g/L of CSL, 20.0 g/L of linoleic acid, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, 2.5 g/L of butylated hydroxytoluene, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 10 d).

Effects of Medium Composition on Culture Morphology

Five image analysis criteria (Table 9) were investigated for describing the morphology of *B. trispora* during β-carotene production from synthetic medium in shake-flask culture. These were compared against β-carotene concentration. Microscopic examination showed that the length of mycelium and the size and number of zygospores changed significantly with the change in the medium's composition. In culture grown in basal medium consisting of 50 g/L of glucose and all the nutrients (except CSL), a small number of zygospores with small diameter were observed. In this case, the length of the mycelium was also small (Fig. 3). When the basal medium was supplied with 80 g/L of CSL, there was a significant increase in all the

Table 9
Effects of Medium Composition on Morphologic Parameters During β-Carotene Production by *B. trispora* in Shake-Flask Culture

Medium[a]	Length of mycelium (μm)	Equivalent diameter of zygospores (μm)	Mean width for vacuoles (μm)	Area of zygospores (% of total area of mycelium)	Area of vacuoles (% of total area of mycelium)	β-Carotene concentration (mg/L)
Basal medium + 5% glucose	5.62	13.88	—	4.71	—	63.2 ± 3.16
Basal medium + 5% glucose + 8% CSL	14.07	15.70	—	12.80	—	170.0 ± 6.80
Basal medium + 5% glucose + 8% CSL + 2% linoleic acid + 0.25% antioxidant	19.00	18.90	8.05	31.24	8.51	1322.0 ± 52.88
Basal medium + 5% glucose + 8% CSL + 3% kerosene + 0.25% antioxidant	14.50	18.00	5.18	12.04	14.15	550.0 ± 22.00
Basal medium + 5% glucose + 8% CSL + 2% linoleic acid + 4% kerosene + 1.0% antioxidant	19.50	22.39	—	54.24	—	2870.0 ± 114.80

[a]Basal medium consisted of the following: 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 8 d).

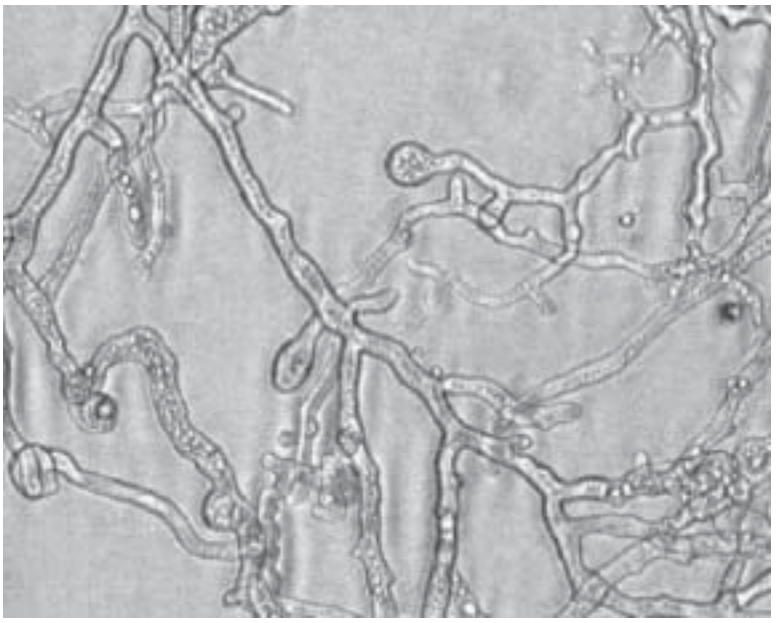

Fig. 3. Morphology of *B. trispora* during β-carotene production from synthetic medium in shake-flask culture. Basal medium consisted of the following: 50.0 g/L of glucose, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 8 d).

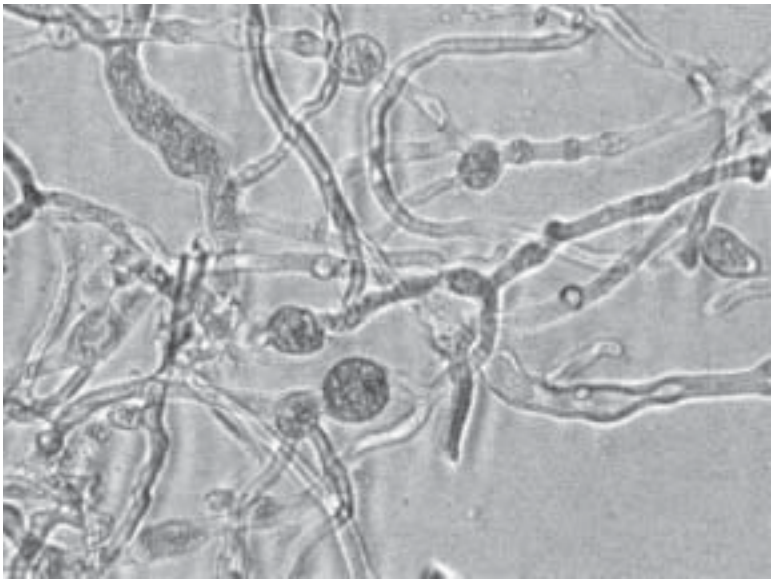

Fig. 4. Morphology of *B. trispora* during β-carotene production from synthetic medium in shake-flask culture. Basal medium consisted of the following: 50.0 g/L of glucose, 80.0 g/L of CSL, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 8 d).

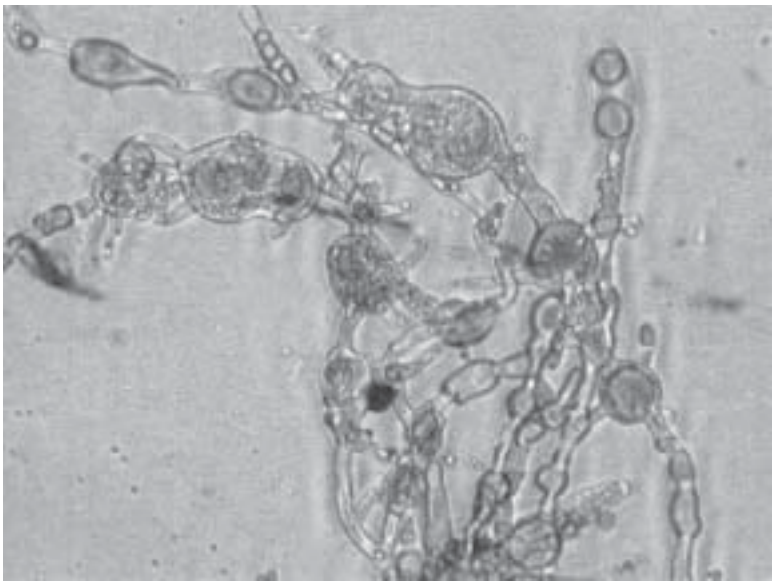

Fig. 5. Morphology of *B. trispora* during β-carotene production from synthetic medium in shake-flask culture. Basal medium consisted of the following: 50.0 g/L of glucose, 80.0 g/L of CSL, 20.0 g/L of linoleic acid, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, 2.5 g/L of butylated hydroxytoluene, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 8 d).

morphologic variables (Fig. 4). This was owing to the fact that CSL contains several necessary substances (amino acids, vitamins, and trace elements) important for the growth of the microorganism and β-carotene production. The addition of 20 g/L of linoleic acid increased the number of zygospores with long diameter and high proportion of the total area of mycelium. Moreover, small vacuoles appeared but their area (percentage of total area of mycelium) was not large (Fig. 5). On the other hand, when kerosene (30 g/L) was added to the medium, vacuolization increased, and a large number of dispersed spores were observed. Although the mean width for the vacuoles was small, there was an increase in their number and their proportion of the overall area of mycelium. Moreover, there was a decrease in the length of mycelium and the area of zygospores (percentage of total area of mycelium). The size of the zygospores slightly decreased (Fig. 6).

In all the culture systems, sexual reproduction occurred when colonies of opposite mating types produced aerial branches (zygophores), which grew toward each other and produced progametangia at their tips. Septation in the progametangia led to the production of terminal gametangia, which fused to form thick-walled resting spores, the zygospores. In culture grown in medium containing kerosene or linoleic acid, meiosis occurred in most or a few zygospores, respectively, and they germinated to produce sporangiophores or a hyphae. The sporangia were released as

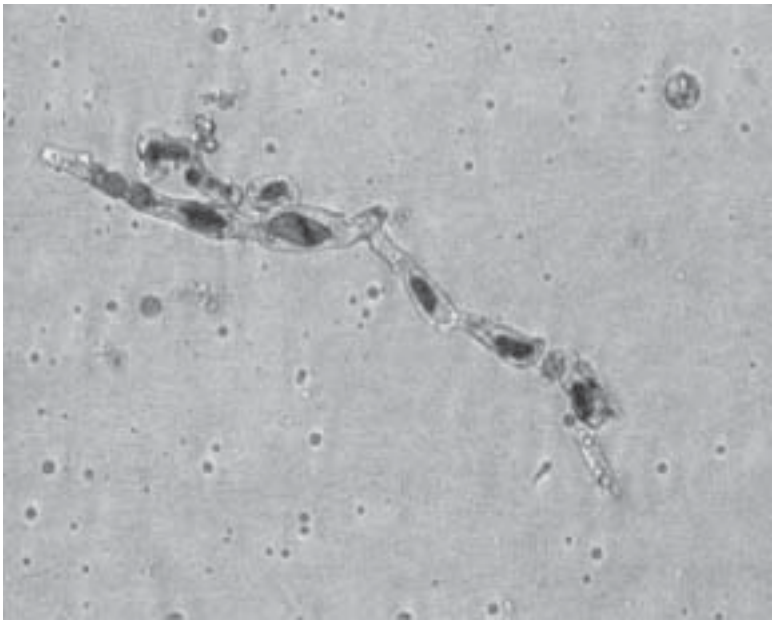

Fig. 6. Morphology of *B. trispora* during β-carotene production from synthetic medium in shake-flask culture. Basal medium consisted of the following: 50.0 g/L of glucose, 80.0 g/L of CSL, 30.0 g/L of kerosene, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, 2.5 g/L of butylated hydroxytoluene, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 10 d).

single cells and functioned as dispersal spores and cylindrically shaped vacuoles also appeared. Vacuoles were important storage sites for β-carotene.

As shown in Table 9, the maximum concentration of β-carotene (2.87 g/L) was observed in culture grown in basal medium supplemented with 80 g/L of CSL, 20 g/L of linoleic acid, 40 g/L of kerosene, and 10 g/L of antioxidant. In this case, a maximum area and a large size of zygospores were observed. Moreover, the length of the mycelium was very long (Fig. 7).

The zygospores are responsible for the biosynthesis of the product, while the hyphae do not play a role in the production of β-carotene. In addition, there is a parallel relationship between the area of zygospores and β-carotene concentration. Generally, *B. trispora* is a polymorphic microfungus that produces β-carotene with a life cycle involving hyphae, zygophores, and zygospores.

Optimization of β*-Carotene Production*

The aforementioned results showed that the media supplemented with linoleic acid, kerosene, and antioxidant gave the highest concentration of β-carotene. For this reason, a central composite design was used to determine the optimum level of these parameters leading to a maximum

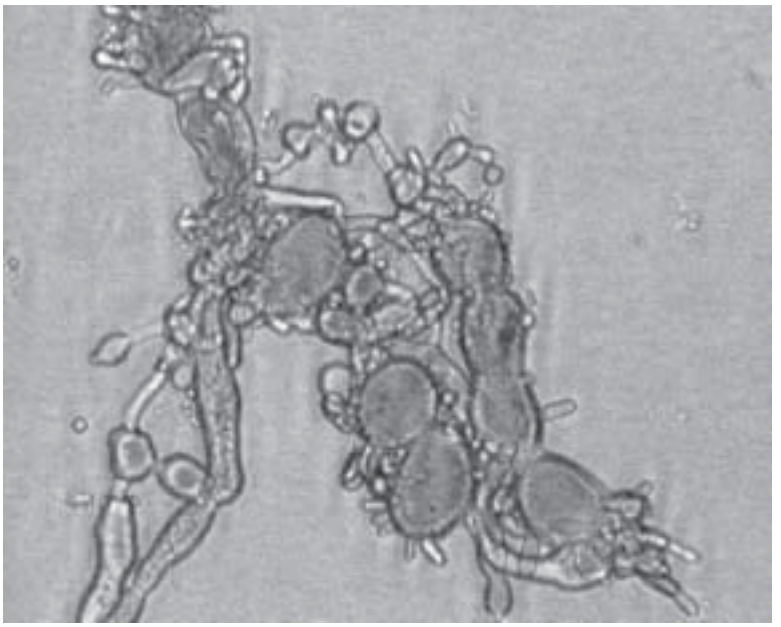

Fig. 7. Morphology of *B. trispora* during β-carotene production from synthetic medium in shake-flask culture. Basal medium consisted of the following: 50.0 g/L of glucose, 80.0 g/L of CSL, 20.0 g/L of linoleic acid, 40.0 g/L of kerosene, 1.0 g/L of yeast extract, 2.0 g/L of casein acid hydrolysate, 2.0 g/L of L-asparagine, 1.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of Tween-80, 10.0 g/L of Span 20, 10.0 g/L of butylated hydroxytoluene, and 5 mg/L of thiamine·HCl (pH 7.0, fermentation time of 10 d).

β-carotene production. The effects of the three variables, each at five levels, and their interactions on β-carotene concentration were determined. Analysis of variance (ANOVA) for β-carotene production is presented in Table 10. The analysis gives the value of the model and determines whether a more complex model could provide a better fit. If the *F*-test for the model is significant at the 5% level ($p < 0.05$), then the model can adequately account for the variation observed. If the *F*-test for lack of fit is significant, then a more complicated model is needed. As shown in Table 10, R^2 was 0.99 and the *F*-test for the regression was significant at a level of 5% ($p < 0.05$). Moreover, the lack of fit was not significant at the 5% level ($p > 0.05$). Consequently, the model chosen can satisfactorily explain the effects of the three factors (linoleic acid, kerosene, antioxidant) on β-carotene production by *B. trispora*. The following model was fitted for β-carotene concentration:

$$Y = -3.804 + 0.107x_1 + 0.253x_2 + 0.168x_3 - 0.003x_1^2 - 0.003x_2^2 - 0.013x_3^2 - 0.001x_1x_2 + 0.004x_1x_3 \quad (2)$$

in which x_1, x_2, and x_3 are the actual levels of factors shown in Table 2. The full second-order model, Eq. 1, was simplified by omitting the terms (x_2x_3) that were not statistically significant (Table 11). As shown in Table 11,

Table 10
ANOVA for β-Carotene Concentration (R^2 = 0.99)

Source	*D F*	Seq SS	Adj SS	Adj MS	*F*	*p*
Regression	9	9.35169	9.35169	1.03908	106.64	0.000
Linear	3	5.30890	1.35544	0.45181	46.37	0.000
Square	3	3.58534	3.58534	1.19511	122.66	0.000
Interaction	3	0.45744	0.45744	0.15248	15.65	0.000
Residual error	10	0.09743	0.09743	0.00974		
Lack of fit	5	0.03128	0.03128	0.00626	0.47	0.785
Pure error	5	0.06615	0.06615	0.01323		
Total	19	9.44912				

DF, degrees of freedom; Seq SS, sequential sum of squares; Adj SS, adjusted sum of squares; Adj MS, adjusted mean square.

Table 11
Estimated Regression Coefficients for β-Carotene Concentration

Term	Coefficient	SE coefficient	*T*	*p*
Constant	–3.804	0.695598	–5.469	0.000
Linoleic acid	0.107	0.021671	4.943	0.000
Kerosene	0.253	0.021671	11.671	0.000
Antioxidant	0.168	0.043360	3.882	0.003
Linoleic acid * linoleic acid	–0.003	0.000260	–11.048	0.000
Kerosene * kerosene	–0.003	0.000260	–12.476	0.000
Antioxidant * antioxidant	–0.013	0.001041	–12.756	0.000
Linoleic acid * kerosene	–0.001	0.000349	–2.543	0.029
Linoleic acid * antioxidant	0.004	0.000698	6.340	0.000
Kerosene * antioxidant	0.000	0.000698	0.537	0.603

linoleic acid, kerosene, and antioxidant had a strong positive linear effect on the response ($p << 0.05$). There were also significant negative quadratic effects of these substances. Additionally, significant interactions were noted among linoleic acid and kerosene, as well as linoleic acid and antioxidant. By contrast, the interactions between kerosene and antioxidant appeared to have no significant effect on β-carotene production ($p >> 0.05$).

The contour plots of β-carotene presented in Figs. 8–10 were produced for each pair of factors, whereas the third factor was kept constant at its middle level. Figure 8 shows that the concentration of β-carotene increased with the increase in concentration of linoleic acid and kerosene, reaching a maximum at 25.0 and 35.0 g/L of linoleic acid and kerosene, respectively. In addition, for the same concentration of kerosene, the concentration of β-carotene changed with the concentration of linoleic acid. As shown in Fig. 9, the concentration of β-carotene increased with the increase in concentration of antioxidant up to 11.0 g/L, reaching a maximum at a concentration of linoleic acid of 22.0 g/L. When the concentration of antioxidant remained constant, the concentration of the pigment decreased with the

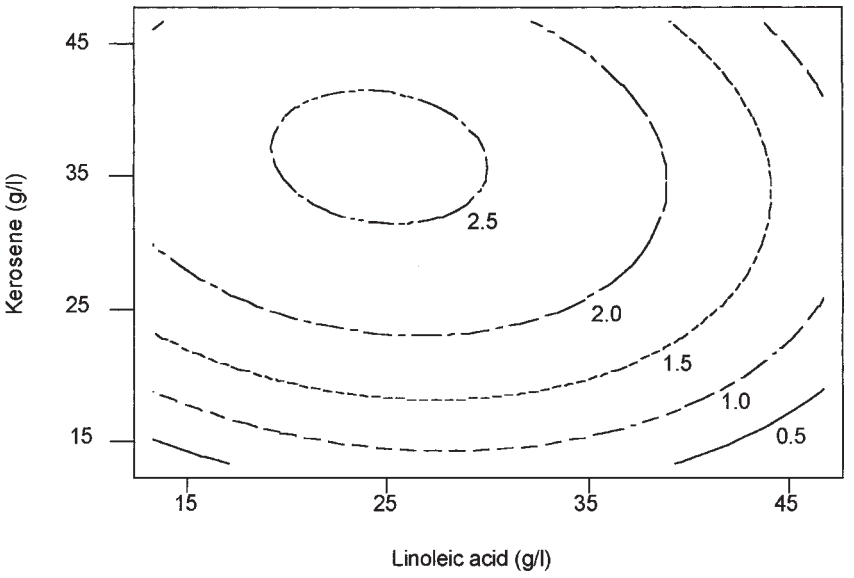

Fig. 8. Contour plot for β-carotene concentration at varying concentrations of linoleic acid and kerosene at a constant (15.0 g/L) antioxidant concentration.

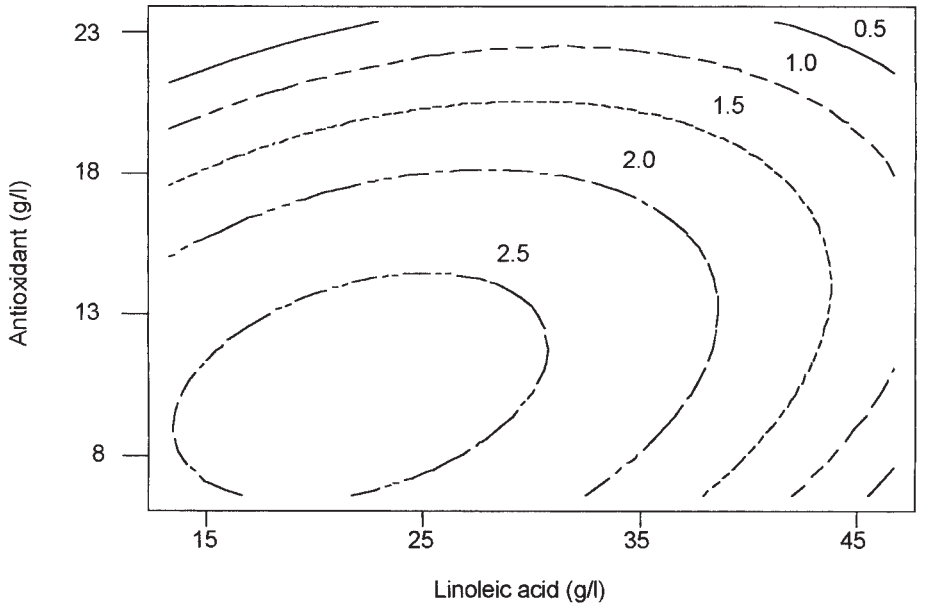

Fig. 9. Contour plot for β-carotene concentration at varying concentrations of linoleic acid and antioxidant at a constant (30.0 g/L) kerosene concentration.

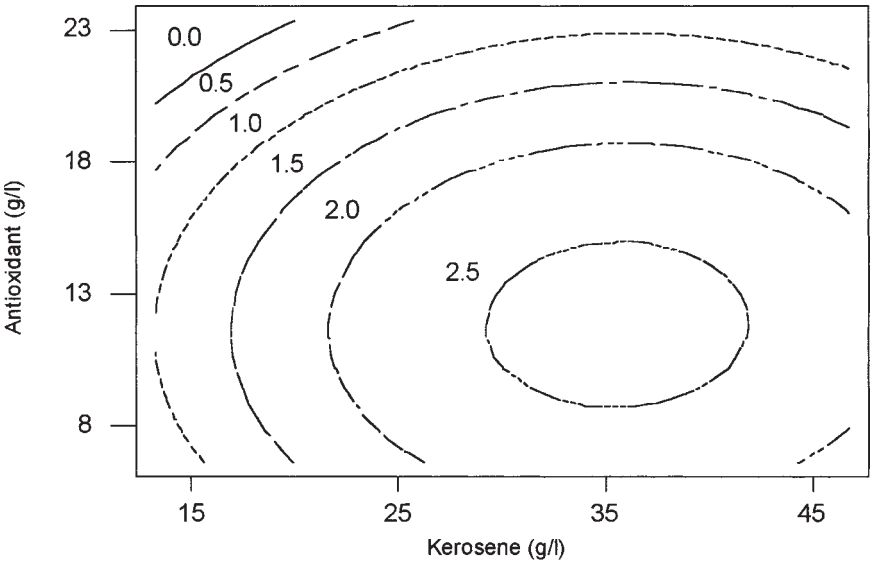

Fig. 10. Contour plot for β-carotene concentration at varying concentrations of kerosene and antioxidant at a constant (30.0 g/L) linoleic acid concentration.

increase in linoleic acid. Finally, at the middle level of linoleic acid, the concentration of β-carotene increased with the increase in concentration of antioxidant and kerosene. The maximum concentration of β-carotene was obtained at concentrations of 11.0 g/L of antioxidant and 35.0 g/L of kerosene (Fig. 10). Moreover, for the same concentration of antioxidant, the concentration of β-carotene decreased with the increase in concentration of kerosene above 35.0 g/L. To determine the maximum concentration of β-carotene corresponding to the optimum levels of linoleic acid, kerosene, and antioxidant, a second-order polynomial model was used to calculate the values of these variables. The fitting of the experimental data to Eq. 2 allowed the determination of the concentrations of linoleic acid (x_1 = 17.15 g/L), kerosene (x_2 = 39.25 g/L), and antioxidant (x_3 = 9.04 g/L) giving a maximum concentration of β-carotene (2.88 g/L) and, therefore, the optimization of β-carotene production by *B. trispora*.

The concentrations of β-carotene reported until now from different media and strains of *B. trispora*, *R. lactosa*, and *R. glutinis* are very low (2.8, 28.0, 37.5, 100.0, 110.0, 400.0 and 410.0 mg/L) (12, 7, 9, 18, 8, 14, 11). Only three research works reported a high concentration of β-carotene (1000.0, 1200.0, and 2000.0 mg/L) (10, 2, 1). Our results show that our experiments gave the highest concentration of β-carotene reported until now (2880.0 mg/L) using as medium CSL supplemented with linoleic acid, kerosene, and an antioxidant.

Conclusion

The results showed some important aspects of β-carotene production by *B. trispora*. The concentration of β-carotene increased when the medium was inoculated with one loop of each culture. Zygospores are the morphologic forms, which are responsible for the biosynthesis of β-carotene. Maximum concentration of β-carotene was obtained at pH 7.0. CSL was an attractive medium for the production of β-carotene. The addition to the medium of natural oils, fatty acids, antioxidant, and kerosene improved the production of the pigment. Linoleic acid, kerosene, and antioxidant were of paramount importance in β-carotene production.

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