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Production of β-Carotene From Beet Molasses by *Blakeslea trispora* in Stirred-Tank and Bubble Column Reactors

Development of a Mathematical Modeling

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Abstract

The effect of aeration rate and agitation speed on β-carotene production from molasses by *Blakeslea trispora* in a stirred-tank fermentor and optimization of the production of the pigment in a bubble column reactor were investigated. In addition, a central composite design was employed to determine the maximum β-carotene concentration at optimum values for the process variables (aeration rate, sugar concentration, linoleic acid, kerosene). By image analysis of the morphology of the fungus, a quantitative characterization of the hyphae and zygospores formed was obtained. The hyphae were differentiated to intact hyphae, vacuolated hyphae, evacuated cells and degenerated hyphae. An increased proportion of zygospores was correlated to high β-carotene production. In the stirred-tank fermentor, the highest concentration of the carotenoid pigment (92.0 mg/L) was obtained at an aeration rate of 1.5 vvm and agitation speed of 60 rpm. In the bubble column reactor, the aeration rate and concentration of sugars, linoleic acid, kerosene, and antioxidant significantly affected the production of β-carotene. In all cases, the fit of the model was found to be good. Aeration rate, sugar concentration, linoleic acid, and kerosene had a strong positive linear effect on β-carotene concentration. Moreover, the concentration of the pigment was significantly influenced by the negative quadratic effects of the given variables and by their positive or negative interactions. Maximum β-carotene concentration (360.2 mg/L) was obtained in culture grown in molasses solution containing 5% (w/v) sugar supplemented with linoleic acid (37.59 g/L), kerosene (39.11 g/L), and antioxidant (1.0 g/L).

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Index Entries: β-carotene; beet molasses; *Blakeslea trispora*; stirred-tank fermentor; bubble column reactor; response surface methodology.

Introduction

β-Carotene is a highly unsaturated isoprene derivative, used as an antioxidant and as a coloring agent for food products. It is a precursor of vitamin A, and it is effective in treating cancer and cardiovascular disease by reducing reactive oxygen species *(1)*. β-Carotene is produced primarily by *Blakeslea trispora*, a heterothallic Zygomycota with two mating types (termed *plus* and *minus*). These two opposite mating types produce zygophores, which grow toward each other and produce progametangia on their tips. Septation in the progametangia leads to the production of terminal gametangia, which fuse to form the zygospores, which are responsible for the biosynthesis of the pigment *(2)*.

The production of β-carotene from glucose, sucrose, glycerol, cellobiose, lipids and related substances, oats, wheat, barley, corn, rice, rye, nonionic surfactants, grape must, citrus byproducts, and cheese whey by different strains of fungi and yeasts has been described *(3–20)*. Molasses is a convenient raw material for fermentation applications since it contains a high concentration of sucrose, is cheap and abundant, and requires only a little handling before fermentation. It contains water, approx 50% (w/w) sugars (sucrose, glucose, fructose, raffinose), nitrogen compounds, organic acids, amino acids, heavy metals, and so on. Very little published information is available about the utilization of molasses as a carbohydrate raw material to produce β-carotene by *Rhodotorula glutinis (21)*. In our previous works *(2,22)*, the production of β-carotene from synthetic mediums by *B. trispora* was examined. All the studies just cited reported on the production of β-carotene in shake-flask culture and a stirred-tank fermentor. There is no study of the production of β-carotene from molasses in a bubble column reactor.

A bubble column reactor is an elongated nonmechanically stirred fermentor with a high aspect ratio (i.e., height/diameter) through which there is a unidirectional flow of gases. It is particularly attractive for applications in biotechnology in which a strong mixing action must be avoided. Bubble column reactors offer many advantages such as easier control of the fermentation process, simplicity of construction and scale-up, low operating costs, high mass transfer efficiency, low shear stress, and easy maintenance *(23,24)*.

B. trispora is an aerobic microorganism and therefore requires the provision of oxygen. The effect of agitation and aeration on β-carotene production is extremely important for the successful progress of the fermentation. Agitation is important not only for adequate mixing and mass transfer between the different phases present in the culture, but also for maintaining homogeneous chemical and physical conditions in the culture by continuous mixing. Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product/byproduct, and oxygen *(2)*.

The aim of the present investigation was to study the production of β-carotene from molasses by *B. trispora* in a stirred-tank fermentor as well as in a bubble column reactor. Moreover, two mathematical models were proposed to determine the optimum levels of initial sugar concentration and aeration rate as well as the optimum concentrations of linoleic acid and kerosene in order to obtain maximum β-carotene production. In addition, a semiautomatic image analysis system was used to determine the morphology of the microorganism and the relationship between the morphology of the fungus and β-carotene concentration.

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 production medium were obtained from cultures grown on potato dextrose agar dishes at 26°C for 72 h.

Hydrolysis of Molasses

Beet molasses was obtained from the Greek Sugar Industry (Platy, Thessaloniki). *B. trispora* does not possess constitutive enzymes for the hydrolysis of sucrose. To overcome this problem, enzymatic hydrolysis of sucrose with invertase was accomplished to hydrolyze sucrose contained in molasses. The molasses solution containing 5% (w/v) sucrose was adjusted to pH 4.5 with concentration. HCl and treated with invertase (I-9253; Sigma, St. Louis, MO) at a ratio of 0.5% (w/v) at 55°C for 2 h in a water bath. At the end of hydrolysis, the pH of the medium was adjusted to 7.0 with 10 *N* NaOH and the substrate sterilized at 121°C for 20 min. Samples of molasses prepared in this way (basal medium) were used for the production of β-carotene by *B. trispora*.

Fermentation Conditions

Fermentation was carried out in a stirred-tank fermentor and in a bubble column reactor. The stirred-tank fermentor (Bellco Glass, Vineland, NJ) was a 12-L reactor with a working volume of 6 L. The fermentor containing 6 L of basal medium supplemented with 0.5% (w/v) Span 20 and 0.1% (w/v) Tween-80 was sterilized at 121°C for 30 min. The medium was inoculated with 60 loops of scrapings from each vegetative culture in Petri dishes. To study the effect of agitation speed and aeration rate on β-carotene production, different experiments were carried out at impeller speeds of 60 and 120 rpm at aeration rates of 0.75, 1.5, and 2.5 vvm. The bubble column reactor was a 2-L glass bioreactor (height of 65 cm, diameter of 6.3 cm) with a working volume of 1.2 L. The reactor was sterilized at 121°C

for 15 min. After cooling, 1.2 L of basal medium was added to the bioreactor. The medium was inoculated with 15 loops of scrapings from each vegetative culture in Petri dishes. Dry air was supplied from the bottom of the column with an air pump at rates of 1, 2, 3, and 4 vvm. The bioreactor was incubated at 26°C in a controlled-temperature chamber.

A set of experiments in the bubble column reactor was performed with basal medium supplemented with different concentrations of linoleic acid (L-1626; Sigma), kerosene (32,946-0; Aldrich), and antioxidant (butylated hydroxytoluene) (B-1378; Sigma) to examine their effect on β-carotene production.

Analytical Techniques

At appropriate time intervals, fermentation broth was removed from the bioreactor and the contents were analyzed. β-Carotene concentration was determined according to Roukas and Mantzouridou *(25)*. The pigment was removed from the cells of the microorganism with ethanol, and the intensity of color was measured at 450 nm with a Zeiss spectrophotometer. Biomass dry weight 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 were measured as glucose in the supernatant according to Dubois et al. *(26)*. The pH of the fermentation broth was measured using a Jenway 370 pH meter equipped with a glass electrode. The reported data are the average values ± SD of three separate experiments.

The morphology of the *B. trispora* was characterized using a semi-automatic 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). The morphologic parameters were measured as described by Paul et al. *(27)*. For each sample, the process was repeated at least 100 times using new positions on the same and on different filaments, and the morphologic parameters were expressed as the mean values of each sample.

Experimental Design and Statistical Analyses

The statistical analyses of the data were performed using the Minitab package. Details of response surface methodology can be found elsewhere *(28)*. The levels of factors used in the experimental design are provided in Tables 1 and 2. The data of the factors were chosen after a series of preliminary experiments. In the case of aeration rate and sucrose concentration, there were three experimental levels (–1, 0, +1), while in the case of linoleic acid and kerosene there were five levels (–a, –1, 0, +1, +a) in which $a = 2^{n/4}$ where n is the number of variables, and 0 corresponds to the central point. The actual level of each factor was calculated using the following equation *(28)*:

Table 1
Levels of Factors Used in the Experimental Design
for Aeration Rate and Initial Sugar Concentration

		Level		
Factor	Name	–1	0	+1
X_1	Aeration rate (vvm)	2.0	3.0	4.0
X_2	Sugar concentration (g/L)	30.0	50.0	70.0

Table 2
Levels of Factors Used in the Experimental Design
for Linoleic Acid and Kerosene

		Level				
Factor	Name	–a	–1	0	+1	+a
X_1	Linoleic acid (g/L)	25.86	30.00	40.00	50.00	54.14
X_2	Kerosene (g/L)	25.86	30.00	40.00	50.00	54.14

$$\text{Coded value} = \frac{\text{actual level} - (\text{high level} + \text{low level})/2}{(\text{high level} - \text{low level})/2}$$

Thirteen experiments were carried out using a face central composite statistical design for the study of two factors each at three or five levels (Table 3). The response variables (β-carotene concentration [mg/L] and biomass dry weight [g/L]) were measured using the polynomial response surface model. The second-order response function for two quantitative factors is given by Eq. 1:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

in which X_1 and X_2 represent the levels of the factors according to Tables 1 and 2, and β_0, β_1, …, β_{12} represent coefficient estimates with β_0 having the role of a scaling constant.

Results and Discussion

Effect of Aeration Rate and Agitation Speed on β-Carotene, Biomass, and Residual Sugar Concentration in Stirred-Tank Fermentor

As shown in Fig. 1A–C, when the agitation speed remained constant (60 rpm), the concentration of β-carotene increased with the increase in the aeration rate from 0.75 to 1.5 vvm and then decreased. Aeration could be beneficial to the growth and performance of microorganism cells by improving the mass transfer characteristics with respect to substrate, products/byproducts, and oxygen. Aeration results in better mixing of the

Table 3
Experimental Design for Aeration Rate and Initial Sugar Concentration and Linoleic Acid and Kerosene

Aeration rate and initial sugar concentration					Linoleic acid and kerosene				
Run	Aeration rate (vvm)	Sugar concentration (g/L)	β-Carotene (mg/L)	Biomass dry wt (g/L)	Run	Linoleic acid (g/L)	Kerosene (g/L)	β-Carotene (mg/L)	Biomass dry wt (g/L)
1	3.0	50.0	162.59	13.70	1	40.00	40.00	361.20	35.40
2	3.0	50.0	165.00	13.35	2	40.00	40.00	359.00	34.80
3	3.0	50.0	162.59	13.70	3	30.00	30.00	172.00	31.70
4	3.0	30.0	120.51	9.30	4	30.00	50.00	154.89	24.80
5	4.0	50.0	110.08	13.10	5	54.14	40.00	31.45	25.00
6	4.0	30.0	49.63	8.60	6	25.86	40.00	68.12	23.20
7	4.0	70.0	80.50	9.10	7	40.00	54.14	235.50	31.50
8	2.0	70.0	50.26	10.50	8	40.00	25.86	267.90	36.40
9	2.0	50.0	115.50	12.85	9	50.00	30.00	148.85	29.80
10	3.0	70.0	113.10	10.60	10	40.00	40.00	361.20	35.40
11	3.0	50.0	165.00	13.35	11	40.00	40.00	359.00	34.80
12	3.0	50.0	162.59	13.70	12	40.00	40.00	361.20	35.40
13	2.0	30.0	89.50	7.70	13	50.00	50.00	125.50	29.50

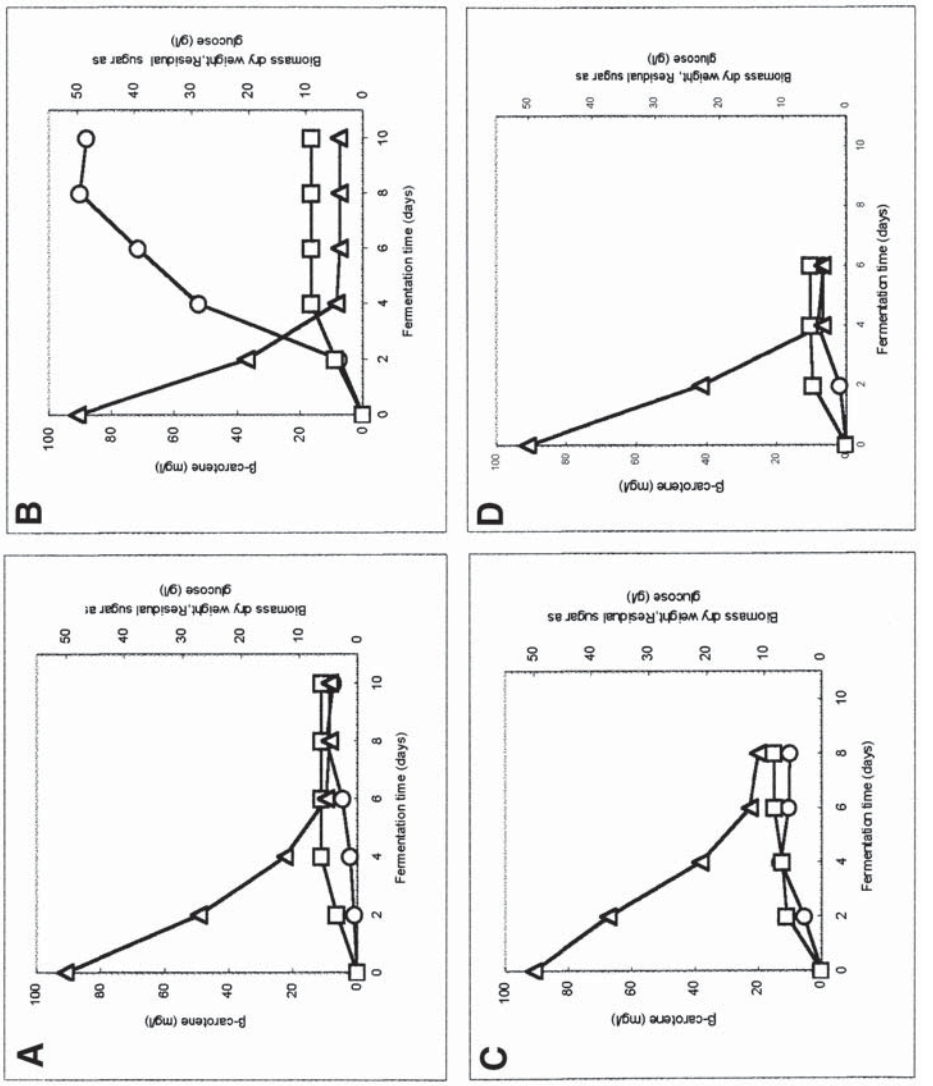

Fig. 1. Effect of aeration rate and agitation speed on β-carotene production from molasses by *B. trispora* in stirred-tank fermentor. **(A)** 0.75 vvm, 60 rpm; **(B)** 1.5 vvm, 60 rpm; **(C)** 2.5 vvm, 60 rpm; **(D)** 1.5 vvm, 120 rpm. (—○—) β-carotene; (—□—) biomass dry weight; (—△—) residual sugars as glucose.

fermentation broth, thus helping maintain a concentration gradient between the interior and the exterior of the cells. This concentration gradient works in both directions; through better diffusion it helps maintain a satisfactory supply of sugars and other nutrients to the cells, while it facilitates the removal of gases and other byproducts of catabolism from the microenvironment of the cells. Air supply favors oxygen supply to the cells, and this is especially important for high biomass concentration. Moreover, as shown in Table 4, the morphology of the microorganism was changed at different aeration rates. The proportion of the vacuolated hyphae, evacuated cells, and degenerated hyphae was increased when the aeration rate was increased from 1.5 to 2.5 vvm, whereas the percentage of the zygospores in the total biomass dry weight was decreased at the same conditions.

In a previous work in our laboratory *(2)*, we studied the production of β-carotene from a synthetic medium by *B. trispora* and reported that the maximum concentration of β-carotene was correlated with the maximum area and the great number of zygospores formed. On the other hand, when the area of zygospores (percentage of total area of mycelium) was very small, the concentration of the pigment was very low. This means that the zygospores were responsible for the biosynthesis of β-carotene. When the agitation speed was increased from 60 to 120 rpm, the concentration of β-carotene was decreased significantly (Fig. 1B,D). This may be explained by the fact that high agitation speed causes shear damage of the cells and oxidation of the pigment *(2,29)*. The maximum concentration of the pigment (92.0 mg/L) and biomass dry weight (17.0 g/L) were obtained in the cultures grown at an aeration rate of 1.5 vvm and agitation speed of 60 rpm after 8 d of incubation, and then decreased. The decline in the concentration of β-carotene was owing to the oxidation of the pigment in the relevant isomers and epoxides by the oxidative enzymes of the fungus *(2)*. As shown in Fig. 1, when the aeration rate increased from 1.5 to 2.5 vvm and the agitation speed from 60 to 120 rpm, the biomass dry weight reached the maximum concentration on the d 2 of incubation and then remained practically constant. In the other cases, the biomass reached the highest concentration on d 4 of fermentation. This means that the better aeration and agitation, the earlier the maximal biomass occurred.

On the other hand, high values of the given fermentation parameters significantly decreased the concentration of the pigment. Thus, a large amount of sugars was converted to biomass instead of β-carotene. The concentration of residual sugars decreased during the fermentation, coinciding with an increase in biomass and β-carotene production. The concentration of residual sugars fell rapidly during the first 2–4 d of the process, after which it decreased slowly. This was owing to a rapid increase in the biomass observed at the same time. Almost complete sugar depletion was observed when the maximum concentration of the pigment was obtained. In the case of the maximum concentration of β-carotene (Fig. 1B), the total amount of sugar utilization was 92%. When the concentration of the sugars

Table 4
Hyphal Morphology of *B. trispora* on Maximum Concentration of β-Carotene in Stirred-Tank Fermentor and in Bubble Column Reactor Using Molasses Solution Containing Different Concentrations of Sugars and Additives[a]

Concentration (w/v)	Fermentation system[b]	Aeration rate (vvm)	Agitation speed (rpm)	β-Carotene	Intact hyphae	Vacuolated hyphae	Evacuated cells	Degenerated hyphae	Zygospores
5% Sugars	STF	1.5	60	1.00	63.10	5.40	6.70	8.30	15.50
5% Sugars	STF	2.5	60	0.10	29.41	15.20	19.03	30.57	5.69
10% Sugars	STF	1.5	60	0.15	55.52	8.51	10.88	16.42	8.52
3% Sugars	BCR	3.0	—	0.90	58.51	7.64	8.52	10.24	14.19
5% Sugars	BCR	3.0	—	1.21	52.17	2.56	3.28	5.50	35.28
5% Sugars	BCR	4.0	—	0.84	55.97	8.32	9.50	11.65	13.72
7% Sugars	BCR	3.0	—	1.03	55.44	3.89	4.42	6.44	28.78
5% Sugars + 1.0% Span 20 + 0.1% Tween-80	BCR	3.0	—	1.07	55.96	3.15	4.15	6.03	29.64
5% Sugars + 1.0% linoleic acid + 1.0% kerosene + 0.1% antioxidant	BCR	3.0	—	1.02	54.25	4.53	4.60	7.12	28.48
5% Sugars + 1.0% linoleic acid + 1.0% kerosene + 0.25% antioxidant	BCR	3.0	—	0.08	22.11	17.12	21.74	33.52	5.43
5% Sugars + 4% linoleic acid + 4.0% kerosene + 0.1% antioxidant	BCR	3.0	—	1.40	35.44	1.48	1.55	4.38	55.75

[a]Percentage of the different hyphal compartments and intracellular β-carotene in the total biomass dry weight.
[b]STF, stirred-tank fermentor; BCR, bubble column reactor.

was increased from 50 to 100 g/L, the concentration of the pigment was decreased (data not shown). This was owing to the significant decrease in the percentage of zygospores in the biomass (Table 4). In addition, preliminary experiments in our laboratory (data not shown) showed that the addition to the molasses solution of a mixture of natural oils (olive oil, soybean oil, and cottonseed oil at a concentration of 1% each) and antioxidant (0.1%), or linoleic acid (4%), kerosene (4%), and antioxidant (0.1%) decreased significantly the concentration of the product. These results showed that the supply of the molasses solution with nutrients did not improve the production of β-carotene in a stirred-tank fermentor.

Effect of Aeration Rate on β-Carotene, Biomass, and Residual Sugar Concentration in Bubble Column Reactor

One important factor that affects the production of β-carotene in a bubble column reactor is the aeration rate. We conducted an experiment to determine the optimum level of the aeration rate that would result in the highest concentration of β-carotene. As shown in Fig. 2, the concentration of β-carotene increased significantly when the aeration rate was increased from 1 to 3 vvm. A further increase in aeration rate at values >3 vvm resulted in a decrease in the amount of the pigment produced. On the other hand, the biomass dry weight increased with the increase in aeration rate from 1 to 3 vvm and then remained practically constant. The maximum β-carotene concentration (162.5 ± 6.5 mg/L) and the maximum biomass dry weight (13.7 ± 0.5 g/L) were obtained in cultures grown at an aeration rate of 3 vvm after 6 and 4 d of fermentation, respectively. These results show that the production of β-carotene was carried out in two phases: in the first phase, in which the biomass of the microorganism increased and the pigment was formed; and in the second phase, in which the biomass remained constant and only the production of the β-carotene was carried out. In all cultures, the pH of the fermentation broth decreased slightly during the first 2 d of the fermentation from 7.0 to 6.0 and then increased slowly up to 7.8 at the end of the fermentation (data not shown). This was probably owing to the deamination of amino acids of molasses by the fungus and the production of ammonia, which increased the pH of the fermentation broth. In all culture systems, the lowest concentration of sugars was observed on the maximum concentration of the pigment (Fig. 2). In this case, the total amount of sugar utilization was 90–92% in all cultures grown at different aeration rates.

Effect of Mixture of Linoleic Acid, Kerosene, and Antioxidant on β-Carotene Concentration in Bubble Column Reactor

In a relevant work from our laboratory *(22)*, we found that the addition of linoleic acid, kerosene, and antioxidant to the synthetic medium improved the production of β-carotene in shake-flask cultures. To examine the influence of the mixture of these substances on β-carotene production,

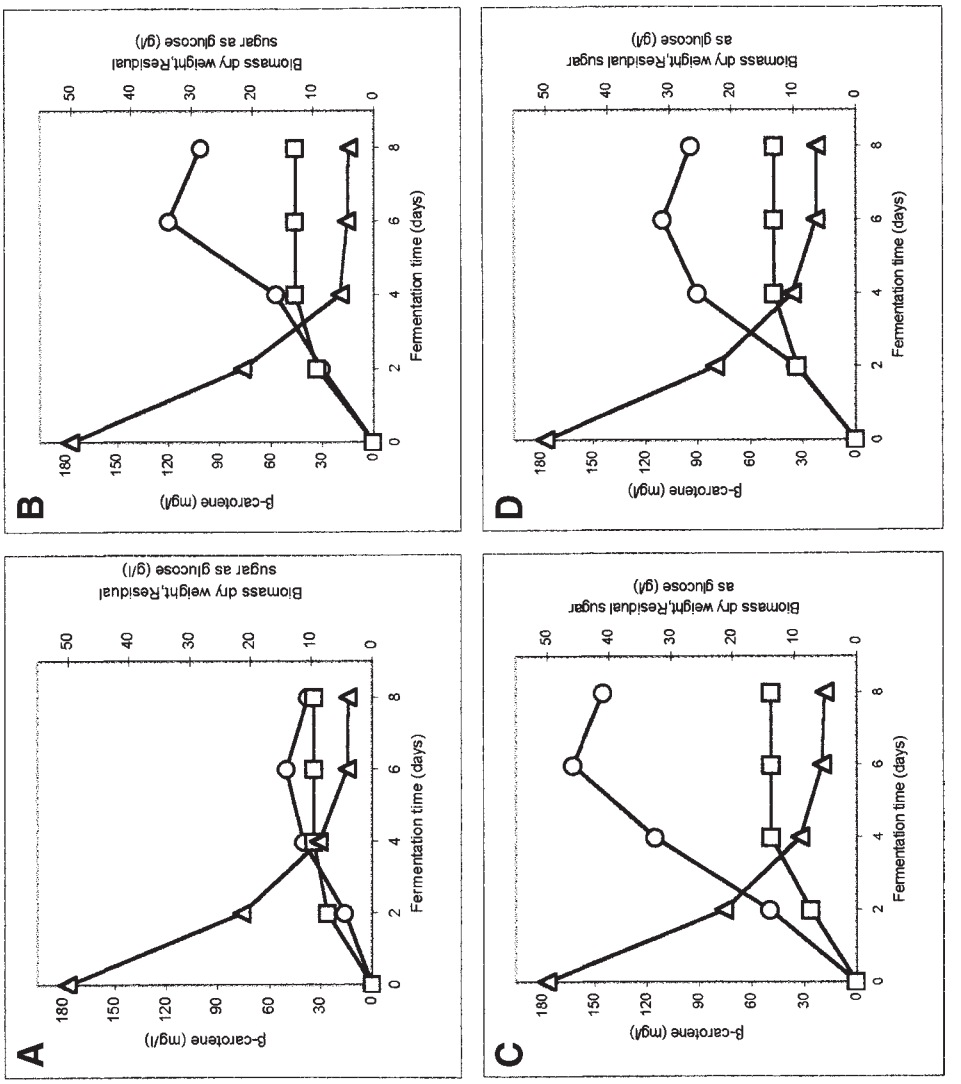

Fig. 2. Effect of aeration rate on β-carotene production from molasses by *B. trispora* in bubble column reactor. **(A)** 1 vvm; **(B)** 2 vvm; **(C)** 3 vvm; **(D)** 4 vvm. (—○—) β- carotene; (—□—) biomass dry weight; (—△—) residual sugars as glucose.

the molasses medium was supplemented with a mixture of these substances at different concentrations. The results showed that the addition of 4% linoleic acid, 4% kerosene, and 0.1% antioxidant, or 3% linoleic acid, 3% kerosene, and 0.1% antioxidant to the basal medium increased the production of the pigment. The highest concentration of β-carotene (358.42 ± 16.13 mg/L) was obtained in culture grown in medium supplemented with 4% linoleic acid, 4% kerosene, and 0.1% antioxidant. This was owing to the high percentage of the zygospores in the biomass dry weight (Table 4). When the basal medium was supplemented with high concentrations of linoleic acid and antioxidant, no growth of microorganism occurred. In addition, when the basal medium was supplemented only with linoleic acid and kerosene, the concentration of β-carotene was low, whereas when it was supplied with these substances and 0.1% antioxidant the concentration of the pigment increased significantly (data not shown). The combination of these substances therefore had a synergistic effect on β-carotene production. This may be explained by the fact that the antioxidant protected the oxidation of linoleic acid during fermentation. Moreover, kerosene might have had a profound effect on the metabolism of sugars by *B. trispora*. The mechanism by which kerosene stimulates β-carotene production from sugars is not clear. Kerosene may affect the permeabilization of the cell membrane, allowing sugars and other nutrients to be diffused into the cell and secondary metabolites to be excreted from the cell to the fermentation broth. The cell then responds by increasing β-carotene production, in an attempt to maintain an adequate intracellular level of the metabolite. Generally, the preceding results show that supplementation of molasses with linoleic acid, kerosene, and antioxidant significantly affects β-carotene production by *B. trispora* in a bubble column reactor.

Effect of Composition of Medium on Culture Morphology

The morphology of *B. trispora* during β-carotene production from molasses in a stirred-tank fermentor and in a bubble column reactor was investigated as described in Table 4. Microscopic examination showed that in all culture systems, the composition of the medium significantly affected the morphology of the fungus concerning the proportion of the vacuolated hyphae, evacuated cells, degenerated hyphae, and zygospores. In the stirred-tank fermentor, when the aeration rate increased from 1.5 to 2.5 vvm and the agitation speed was 60 rpm, the percentages of the vacuolated hyphae, evacuated cells, and degenerated hyphae were increased whereas the zygospores and the pigment in the biomass dry weight were decreased (Table 4). Similar results were observed in cultures grown in the same medium in the bubble column reactor by increasing the aeration rate from 3 to 4 vvm. The high aeration rate probably resulted in a drastic increase in the percentage of the evacuated cells and degenerated hyphae in the mycelium. For this reason, in both fermentation systems at high aeration rates the percentage of the pigment in the biomass was low. As shown in Table 4, when the sugar concentration was increased from 3 to

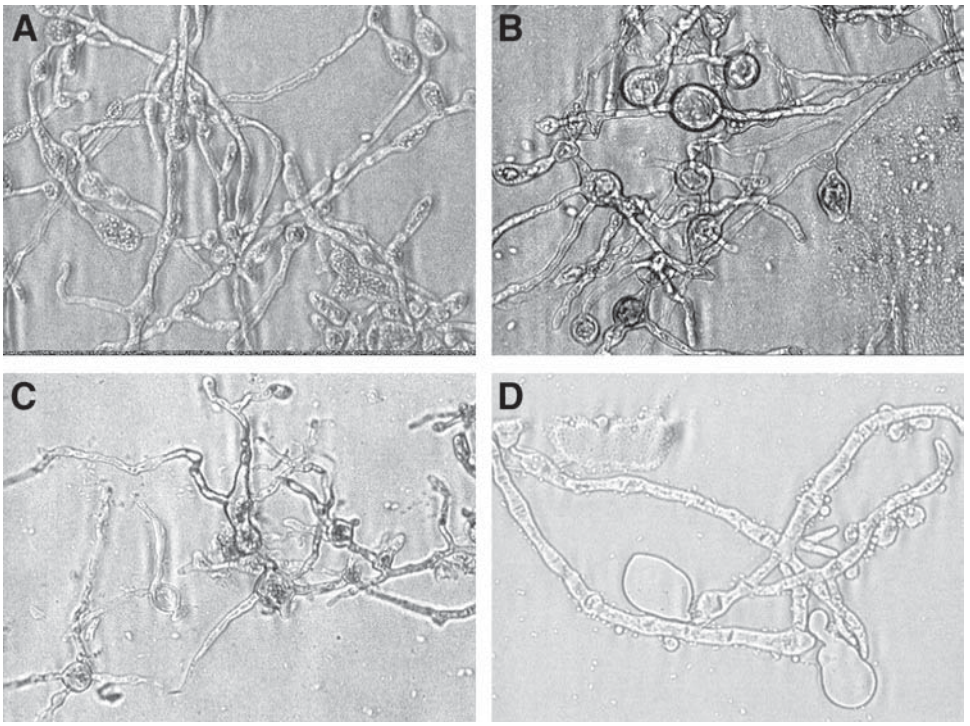

Fig. 3. Phase contrast images of morphologic characteristics of *B. trispora* on the maximum concentration of β-carotene in stirred-tank fermentor and in bubble column reactor. **(A)** Stirred-tank reactor: 5% sugars, 60 rpm, 1.5 vvm; **(B)** bubble column reactor: 5% sugars, 3 vvm; **(C,D)** autolysis of *B. trispora* in stirred-tank fermentor and in bubble column reactor, respectively.

5%, the percentage of the zygospores increased whereas the percentages of the vacuolated hyphae and evacuated cells were decreased. A further increase in the sugar concentration from 5 to 7% resulted in a decrease in the zygospores, whereas the proportion of the vacuolated hyphae, evacuated cells, and degenerated hyphae was increased. These results show that low levels of the carbon source in combination with high aeration rates increased the formation of the vacuolated hyphae, evacuated cells, and degenerated hyphae, whereas at the same conditions, a negative effect on the formation of zygospores and the biosynthesis of the pigment was observed. The highest percentage of the zygospores in the biomass dry weight and the maximum concentration of β-carotene were observed at a sugar concentration of 5% and an aeration rate of 3 vvm in the bubble column reactor.

After the maximum concentration of pigment, in both fermentation systems autolysis of the cells was observed (Fig. 3A–D). The cell wall of *B. trispora* consists of a fibrillar structure built on chitin and chitosan and a complex gel-like matrix composed of polyglucuronic acid, glucuronomannoproteins, and polyphosphate *(30)*. In the stirred-tank fermentor,

fragmentation of vacuolated regions of the mycelium as indicated in Fig. 3C was attributed to enzymatic activity and shear forces *(31)*. Although vacuoles are important storage sites for β-carotene, the great percentage of vacuolated hyphae was not correlated with a high concentration of the pigment, as indicated in Table 4. The extrinsic factors, which have been demonstrated to bring about the process of autolysis in fungi, are carbon and nitrogen starvation, increased temperature, and the presence of toxic compounds or metabolites. Regardless of the extrinsic factors, which bring about autolysis, there is evidence that the process may take place in a number of stages involving an initial disruption of normal cellular metabolism. This leads to progressive loss of membrane function, and failure of compartmentalization within the cell, allowing release/activation of the enzymes stored in compartments such as the vacuole *(32–35)*.

Mathematical Modeling for β-Carotene Concentration and Biomass Dry Weight

Our results showed that the aeration rate and sugar concentration influenced the production of the pigment in a bubble column reactor. Furthermore, it was observed that the molasses solution containing 5% sugars and 0.1% antioxidant supplemented with linoleic acid and kerosene 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 production of the pigment. Two operating variables (aeration rate and sugar concentration, and linoleic acid and kerosene) each at three and five levels, respectively, and their interaction on the β-carotene concentration and the biomass dry weight have been studied. For each response variable, an analysis of variance (ANOVA) was produced (Tables 5 and 6). This analysis gives the value of the model and determines whether a more complex model would have a better fit. If the *F*-test for lack of fit is significant, then a more complicated model is needed. As shown in Tables 5 and 6, R^2 was 0.999 for β-carotene concentration and ranged from 0.994 to 0.998 for biomass dry weight. The *F*-test for the regression was significant at a level of 5% ($p < 0.05$) in both responses. In addition, the lack of fit was not significant at the 5% level ($p > 0.05$). These results show that the model chosen can satisfactorily explain the effects of the two factors (sugar concentration and aeration rate, and linoleic acid and kerosene) on β-carotene concentration and biomass dry weight during the production of the pigment from molasses solution by *B. trispora* in a bubble column reactor. The models fitting these response variables were as follows:

$$P_1 = -429.6 + 254.1X_1 + 8.8X_2 - 50.1X_1^2 - 0.1X_2^2 + 0.9X_1X_2 \quad (2)$$

$$B_1 = -23.19 + 6.04X_1 + 1.07X_2 - 0.77X_1^2 - 0.01X_2^2 - 0.03X_1X_2 \quad (3)$$

$$P_2 = -2926.0 + 123.62X_1 + 43.08X_2 - 1.55X_1^2 - 0.54X_2^2 - 0.02X_1X_2 \quad (4)$$

$$B_2 = -32.81 + 3.85X_1 - 0.34X_2 - 0.06X_1^2 - 0.01X_2^2 - 0.02X_1X_2 \quad (5)$$

Table 5
ANOVA for β-Carotene and Biomass Dry Weight vs Aeration Rate and Sugar Concentration

Source	*D F*	*F*	*p*
β-Carotene concentration (R^2 = 0.999)			
Regression	5	3E + 03	0.000
Linear	2	2E + 03	0.000
Square	2	6E + 03	0.000
Interaction	1	718.52	0.000
Residual error	7		
Lack of fit	3	0.96	0.494
Pure error	4		
Total	12		
Biomass dry wt (R^2 = 0.994)			
Regression	5	225.72	0.000
Linear	2	443.16	0.000
Square	2	519.06	0.000
Interaction	1	24.62	0.002
Residual error	7		
Lack of fit	3	2.08	0.246
Pure error	4		
Total	12		

Table 6
ANOVA for β-Carotene and Biomass Dry Weight vs Linoleic Acid and Kerosene

Source	*D F*	*F*	*p*
β-Carotene concentration (R^2 = 0.999)			
Regression	5	3E + 04	0.000
Linear	2	4E + 04	0.000
Square	2	6E + 04	0.000
Interaction	1	6.93	0.034
Residual error	7		
Lack of fit	3	0.92	0.506
Pure error	4		
Total	12		
Biomass dry wt (R^2 = 0.998)			
Regression	5	771.24	0.000
Linear	2	1E + 03	0.000
Square	2	2E + 03	0.000
Interaction	1	165.55	0.000
Residual error	7		
Lack of fit	3	0.09	0.963
Pure error	4		
Total	12		

in which P_1, B_1 and P_2, B_2 are the concentrations of β-carotene and biomass dry weight according to sugar concentration and aeration rate, and linoleic acid and kerosene, respectively. X_1 and X_2 are the actual levels of factors shown in Table 1. As shown from the Eqs. 2 and 4, aeration rate, sugar concentration, linoleic acid, and kerosene had a strong positive linear effect on β-carotene concentration. The same results (except kerosene) were observed on the biomass dry weight; kerosene had a strong negative linear effect on biomass (Eqs. 3 and 5). Moreover, there was a significant negative quadratic effect of these factors on β-carotene concentration and biomass dry weight (Eqs. 2–5). Additionally, significant positive interactions were noted among aeration and sugar concentration on β-carotene concentration, as well as linoleic acid and kerosene on biomass dry weight (Eqs. 2 and 5). Finally, significant negative interaction effects were observed between aeration rate and sugar concentration and linoleic acid and kerosene on biomass and concentration of the pigment, respectively (Eqs. 3 and 4). This indicates that β-carotene and biomass concentration increased with the increase in aeration rate and sugar concentration or linoleic acid and kerosene; they reached a maximum and then decreased at high values of the given factors.

To determine the maximum concentration of the pigment and the biomass dry weight corresponding to the optimum levels of sugar concentration and aeration rate or linoleic acid and kerosene, a second-order polynomial model was used to calculate the values of these variables. Fitting of the experimental data to Eqs. 2–5 allowed determination of sugar concentration and aeration rate or concentration of linoleic acid and kerosene giving a maximum concentration of the pigment or biomass dry weight and, therefore, the optimization of β-carotene production by *B. trispora*. Thus, Eqs. 2 and 4 gave the maximum concentration of β-carotene (212.87 and 360.20 mg/L) corresponding to the optimum levels of aeration rate (3.0 vvm) and sugar concentration (57.92 g/L) or linoleic acid (37.59 g/L) and kerosene (39.11 g/L), respectively. On the other hand, Eqs. 3 and 5 determined the maximum concentration of biomass dry weight (12.01 g/L) at an aeration rate of 2.9 vvm and a sugar concentration of 49.24 or 31.68 g/L at a concentration of 35.02 g/L of linoleic acid and 18.02 g/L of kerosene.

Lampila et al. *(12,13)* reported maximum β-carotene concentrations of 2.8–4.0 mg/L when various strains of *B. trispora* were grown in cheese whey in shake-flask culture. Buzzini *(5)* reported that a high concentration of β-carotene (7.0 mg/L) was obtained when *R. glutinis* DBVPG 3853 was grown in concentrated rectified grape must using shake-flask culture. Martelli et al. *(14)* and Bhosale and Gadre *(21)* found that a maximum concentration of carotenoid pigment (37.5 and 130.0 mg/L, respectively) was obtained when *R. glutinis* was grown in sugarcane juice and cane molasses, respectively. There are some possible reasons for these differences, including the strain of the organism used; the chemical composition of the substrate; the fermentation system; and, generally, the conditions under which the fermentation takes place. The cited results show that the

amounts of β-carotene produced from different agroindustrial byproducts were very low (2.8–130.0 mg/L) compared with our results, which gave 360.2 mg/L of the pigment using as fermentation medium molasses solution supplemented with linoleic acid, kerosene, and an antioxidant. This means that beet molasses is an attractive medium for the production of β-carotene by *B. trispora*.

Conclusion

The results showed some important aspects of β-carotene production from molasses by *B. trispora*. The bubble column reactor was a better fermentation system than the stirred-tank fermentor. In both fermentation systems, vacuolated hyphae, evacuated cells, degenerated hyphae, and zygospores were formed during the fermentation. The zygospores were responsible for the biosynthesis of β-carotene. In the bubble column reactor, the concentration of the pigment was significantly influenced by the aeration rate; concentration of sugars; and addition of linoleic acid, kerosene, and antioxidant in molasses solution.

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