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An Improved Method for Extraction of β-Carotene from *Blakeslea trispora*

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Abstract

An improved method for the extraction of β-carotene from *Blakeslea trispora* is described. The fermentation broth was steamed at 121°C for 15 min, and the liquid was centrifuged at 5000*g* for 20 min. β-Carotene was removed from the biomass by extraction with absolute ethanol at a ratio of 1:100 at 30°C for 2 h in a rotary shaker incubator at 300 rpm. The carotenoid pigment was completely removed from the cells after three repeated extractions. The removal of β-carotene from *B. trispora* was higher during the first stage (75%) whereas in the other stages it was very slow.

Index Entries: β-Carotene; extraction; *Blakeslea trispora*.

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 and has also been used 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 and yeasts and by some species of bacteria, algae, and lichens. The greatest yields have been obtained with a mixture of + and – strains of *Blakeslea trispora (3)*.

For the extraction of β-carotene from the cells of microorganisms, several methods have been reported *(4–10)*. These methods rely on the centrifugation of fermentation broth, drying the biomass under vacuum at 50°C overnight, followed by the extraction of β-carotene with different

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solvents such as petroleum ether, acetone, hexane, and hexane:methanol. These methods do not describe in detail the extraction conditions of carotenoid pigments from the cells. We tested all these methods and found that they were not sufficiently definitive. There were considerable differences with respect to the pretreatment of fermentation broth, time, and temperature of extraction; the number of extractions; and the biomass to solvent ratio. Also, the methods tended to be time-consuming, requiring 18 h for drying of the biomass.

The aim of this investigation was to improve the extraction method of β-carotene from *B. trispora* using new solvents, several treatments of biomass before extraction, and various experimental conditions during the process.

Materials and Methods

Microorganisms and Culture Conditions

The two strains of *B. trispora* used throughout this investigation were *B. trispora* ATCC 14271, mating type (+), and *B. trispora* ATCC 14272, mating type (–). Both strains were obtained from the American Type Culture Collection (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 slants at 26°C for 72 h.

Fermentation Conditions

Fermentation was carried out in 250-mL conical flasks containing 50 mL of production medium (pH 6.5) of the following composition: 70.0 g/L of glucose, 50.0 g/L of starch, 5.0 g/L of corn steep liquor, 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 for 6 d in a rotary shaker incubator (Lab-Line, Melrose Park, IL) at 200 rpm.

Extraction Procedures

At the end of the fermentation, the flasks were removed and the fermentation broth was treated for the extraction of β-carotene. Fifty milliliters of the liquid was centrifuged at 5000*g* for 20 min, the sediment was washed with distilled water, and the liquid was centrifuged as described above. This process was repeated until the supernatant was colorless. The precipitated biomass was dried at 50°C under vacuum to constant weight. The biomass was pulverized with a pestle, and the particles were used for the extraction of β-carotene by the following procedures.

Method A: Extraction at Constant Temperature and Different Extraction Times

The biomass was treated with different solvents such as ethanol, methanol, acetone, petroleum ether, hexane, and a mixture of hexane: methanol (1:1) at a ratio of 100 mL of solvent/g of biomass dry wt. The

extraction was carried out in a rotary shaker incubator at 30°C and at different extraction times (30, 60, 90, 120, and 150 min) at 300 rpm.

Method B: Extraction at Constant Time and Different Extraction Temperatures

The carotenoid pigment was extracted from the cells at different extraction temperatures (30, 35, 40, 45, and 50°C) for 2 h. The solvents were the same as described in Method A, but instead of petroleum ether acetone and petroleum ether were used. In this case, the biomass was treated with acetone at the aforementioned different temperatures for 2 h and the extract was centrifuged at 5000*g* for 20 min. The acetone extract was decanted into a separator funnel, mixed with an equal amount of petroleum ether, and the mixture was shaken gently. On addition of 20 mL of distilled water, a separation of the two phases occurred, and 5 g of sodium sulfate was added to the petroleum ether layer. The mixture was maintained at 4°C for 1 h, and the absorption spectra were obtained at 450 nm, using a Zeiss PMQII spectrophotometer.

Method C: Removal of β-Carotene from Cells by Repeated Extractions

The carotenoid pigment was removed from the cells by extraction with absolute ethanol. The extraction of β-carotene was carried out in a rotary shaker incubator at 300 rpm for 2 h and at temperatures that were the optimum for each solvent as reported in Fig. 2. After 2 h of extraction, the liquid was centrifuged at 5000*g* for 20 min, the supernantant was removed, and the sediment was extracted with fresh solvent. The extractions were repeated four times.

Pretreatment of Fermentation Broth for Extraction of β-Carotene

The fermentation broth was treated using different methods to improve the removal of β-carotene from the cells.

Method A

Fifty milliliters of fermentation broth was heated at 100°C for 15, 30, and 60 min or steamed at 121°C for 15 min. The liquid was then treated as described in Extraction Procedures.

Method B

Fifty milliliters of the broth was steamed at 121°C for 15 min and the liquid centrifuged at 5000*g* for 20 min. The sediment was washed with distilled water until the supernatant was colorless, and the β-carotene was directly removed from the cells by extraction with ethanol. In Methods A and B, the extraction was carried out in a rotary shaker incubator at 30°C for 2 h at 300 rpm using ethanol as the solvent at a ratio of biomass dry wt to solvent of 1:100. The carotenoid pigment was completely removed from the cells after three extractions. The liquid was centrifuged at 5000*g* for 20 min, and the supernatant was used for the determination of β-carotene. The intensity of color was measured at 450 nm with a Zeiss spectrophotometer. The concentration of carotene present in the sample was then determined

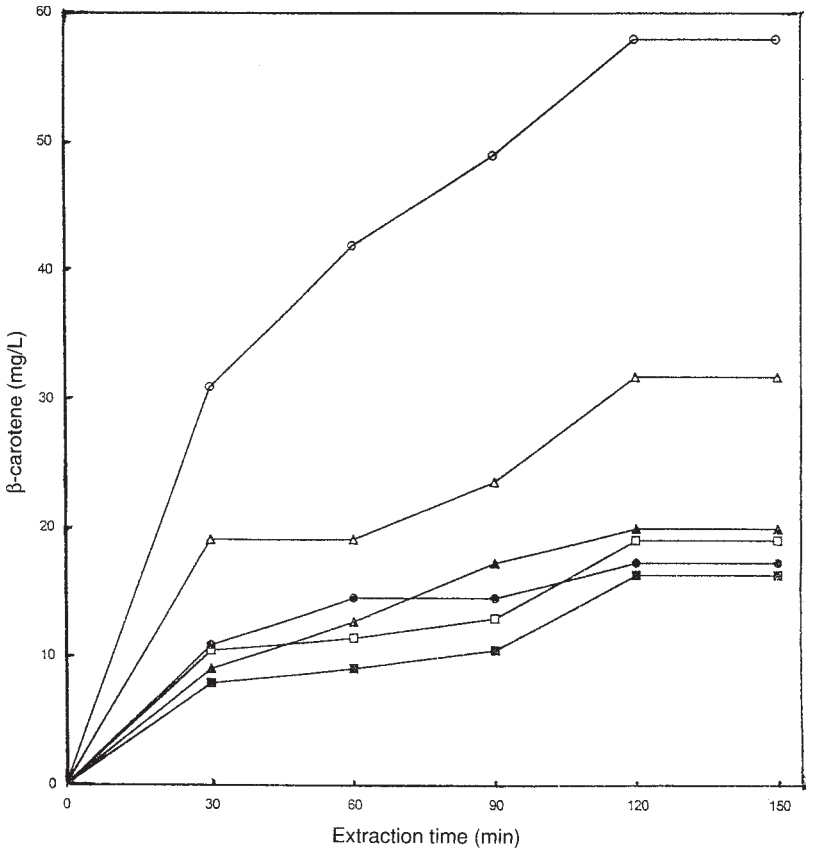

Fig. 1. Effect of extraction time on β-carotene removal from *B. trispora* with different solvents. (—○—), Ethanol; (—□—), methanol; (—△—), acetone; (—●—), petroleum ether; (—■—), hexane; (—▲—), hexane:methanol (1:1). Extraction temperature was 30°C. Each point is the mean of three repetitions. CV values for all measured parameters did not exceed 2.5% in all cases.

by comparison with a standard calibration curve prepared from pure all-*trans*-β-carotene (C-9750; Sigma).

The amount of β-carotene extracted from the cells of the microorganism was expressed as milligrams of β-carotene/liter of substrate. In all experiments, only all-*trans*-β-carotene was determined by ultraviolet detection (UV 120A; Shimadzu). The data are the average values of three separate experiments. Variability was also expressed by coefficient of variation (CV) values.

Results and Discussion

Effect of Time on β*-Carotene Extraction from* B. trispora

Figure 1 shows the influence of extraction time on β-carotene removal from *B. trispora* with different solvents. β-Carotene extraction increased by

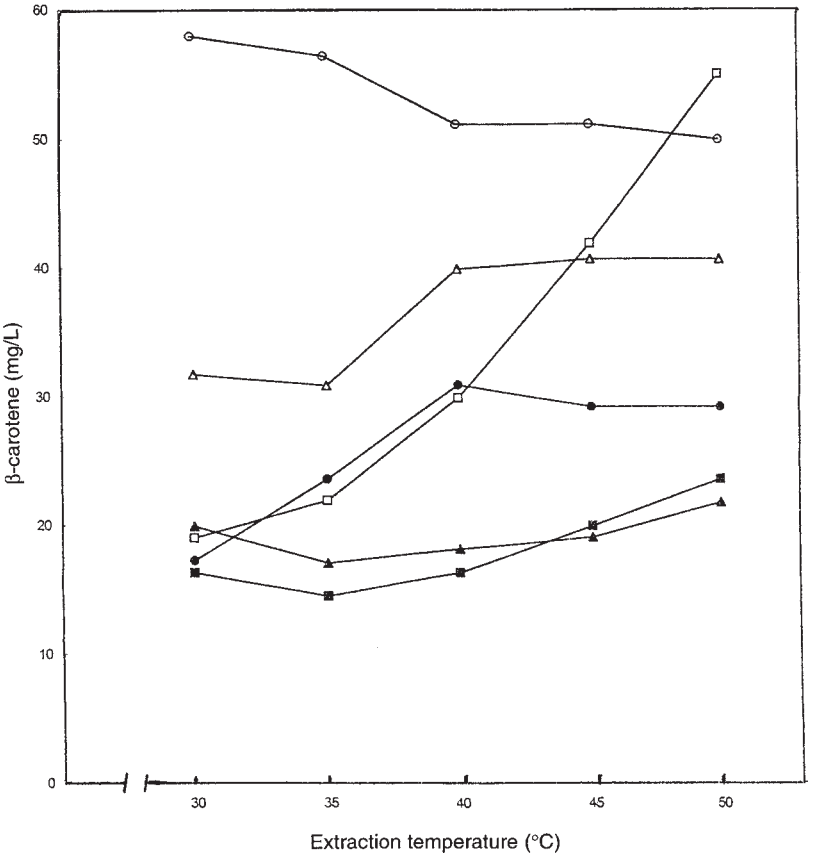

Fig. 2. Effect of temperature on β-carotene extraction from the cells of *B. trispora* with different solvents. Extraction time was 2 h. Each point is the mean of three repetitions. (—○—), Ethanol; (—□—), methanol; (—△—), acetone; (—●—), extraction with acetone followed by petroleum ether; (—■—), hexane; (—▲—), hexane:methanol (1:1). CV values for all measured parameters did not exceed 3.0% in all cases.

increasing the extraction time up to 2 h, and thereafter remained constant at longer time. The maximum amount of β-carotene removal from the cells was obtained by extraction with ethanol. In the other solvents such as acetone, methanol:hexane (1:1), methanol, petroleum ether, and hexane, the amount of β-carotene extracted from the cells was lower by 45.0, 65.5, 67.0, 71.5, and 72.0%, respectively. The results showed that ethanol (a solvent that has not been used until now) was the better solvent for the removal of β-carotene from the cells. Two other variables that can be taken into consideration in an extraction process are biomass to solvent ratio and concentration of solvent. We studied ethanol at concentrations of 100, 60, and 40% with a biomass dry wt to solvent ratio of 1:100, 1:50, and 1:30. The results showed that a concentration of 100% ethanol and a biomass to solvent ratio of 1:100 gave the maximum amount of β-carotene extracted from the cells (data not shown).

Effect of Temperature on β*-Carotene Extraction from* B. trispora

Figure 2 depicts the effect of temperature on the extraction of β-carotene from *B. trispora*. The results showed that solvents had a positive or negative effect on β-carotene extraction from the cells of microorganism. Methanol increased the extraction of β-carotene from the cells with an increase in temperature from 30 to 50°C, whereas ethanol decreased the removal of carotenoid pigment from the cells with the same increase in temperature. On the other hand, hexane and the mixture of hexane and methanol decreased the extraction of β-carotene with an increase in temperature from 30 to 35°C, and then the extraction of pigment was increased as the temperature increased up to 50°C. The amount of β-carotene extracted with acetone followed by petroleum ether increased from 30 to 40°C and decreased at higher temperatures. Finally, a decrease in β-carotene extracted by acetone was observed from 30 to 35°C, increased between 35 and 40°C, and then remained practically constant as the temperature was increased from 40 to 50°C. At temperatures higher than 50°C, a relatively high evaporation of solvents was observed. These results showed that both ethanol and methanol were appropriate solvents for the extraction of β-carotene from *B. trispora*. Moreover, the maximum amount of β-carotene removed from the cells was obtained by extraction with ethanol at 30°C for 2 h.

Removal of β*-Carotene Using Repeated Extractions of Biomass*

Repeated extractions of biomass with ethanol, methanol, acetone, petroleum ether, hexane, and hexane:methanol (1:1) were performed to assess the efficacy of these solvents in the removal of β-carotene. The results given in Fig. 3 show that the amount of β-carotene was completely removed from the cells of microorganism after three repeated extractions of biomass. The extraction rate was higher during the first stage when 75, 79.5, 68, 77.5, 81.5., and 90% of the corresponding total amount of β-carotene was removed with ethanol, methanol, acetone, hexane, hexane:methanol (1:1), and petroleum ether, respectively, whereas in the other stages it was very slow.

Pretreatment of Fermentation Broth to Increase β*-Carotene Extraction from* B. trispora

In a further attempt to improve the β-carotene extraction from *B. trispora*, the fermentation broth was treated with different methods, as reported in Table 1. The results showed that pretreatment of fermentation broth before the extraction of β-carotene had a positive effect on the removal of β-carotene from the cells of the microorganism. The amount of β-carotene extracted from untreated fermentation broth was low in comparison to treated broth (except the case in which the liquid was heated at 100°C for 60 min). The amount of β-carotene removed from the cells increased with an increase in heating time of liquid from 15 to 30°C and then decreased as

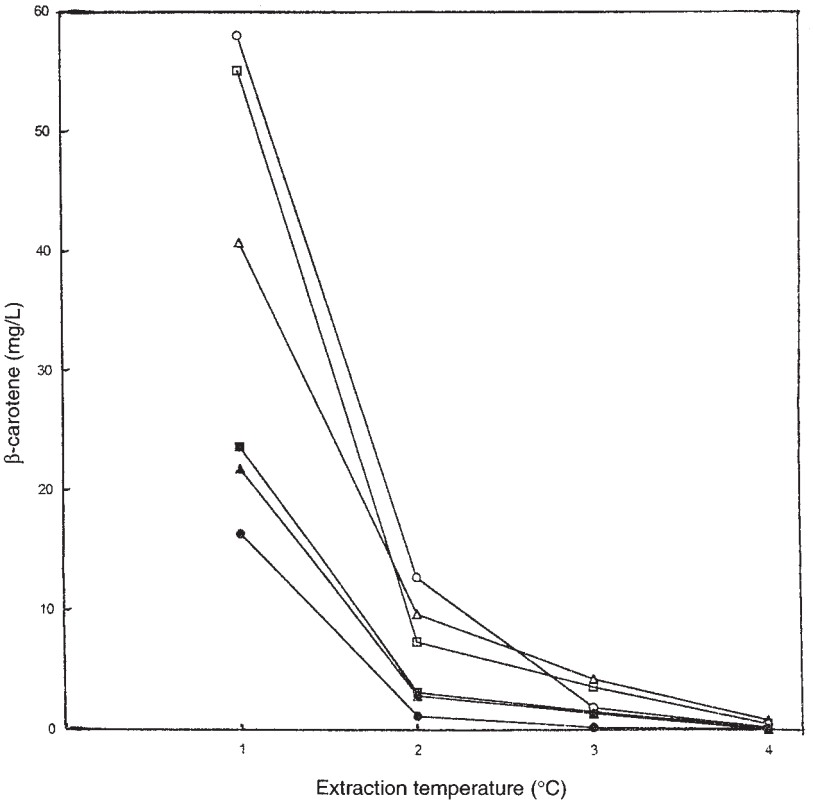

Fig. 3. Removal of β-carotene from *B. trispora* with different solvents using repeated extractions. The procedure was carried out for 2 h and at the temperature that was the optimum for each solvent. Each point is the mean of three repetitions. CV values for all measured parameters did not exceed 2.7% in all cases. Symbols are as shown in Fig. 1.

the heating time was increased up to 60 min. This may be owing to the oxidation of carotenoid pigment when the fermentation broth was heated for a long time. Increasing the heating temperature of fermentation broth from 100 to 121°C resulted in an increase in β-carotene extraction from *B. trispora*. The highest amount of carotenoid pigment extracted from the cells was obtained using a method in which the fermentation broth was steamed at 121°C for 15 min and the β-carotene was removed directly from the cells by extraction with ethanol without drying the biomass. The superiority of this treatment over other methods may be owing to the following reasons: the oxidation of carotenoid pigment during drying of the biomass was omitted; the activity of enzymes that causes oxidation of β-carotene was eliminated; and cell permeabilization during steaming was obtained, allowing extraction of β-carotene from the cells. Generally, the results showed that the pretreatment of fermentation broth significantly improved the extraction of β-carotene from *B. trispora*.

Table 1
Pretreatment of Fermentation Broth
to Increase β-Carotene Extraction Produced by *B. trispora*[a]

	Procedure	β-Carotene (mg/L)
1.	Centrifugation of fermentation broth at 5000*g* for 20 min, drying of sediment, and extraction by ethanol	76.0
2.	Heating of the fermentation broth at 100°C for 15 min and then treatment as in procedure 1	82.5
3.	Heating of the fermentation broth at 100°C for 30 min and then treatment as in procedure 1	109.0
4.	Heating of the fermentation broth at 100°C for 60 min and then treatment as in procedure 1	62.5
5.	Steaming of the fermentation broth at 121°C for 15 min and then treatment as in procedure 1	122.0
6.	Steaming of the fermentation broth at 121°C for 15 min, centrifugation of the fermentation broth at 5000*g* for 20 min, and extraction of β-carotene with ethanol directly from the sediment	160.0

[a]Each value is the mean of three repetitions and expresses the total amount of β-carotene extracted from the cells after three repeated extractions. CV values for all measured parameters did not exceed 2.5% in all cases.

Conclusion

The procedure described herein allows the recovery of β-carotene from *B. trispora* by heating the fermentation broth and extracting the carotenoid pigment with ethanol. The method differs from those previously described by the use of steaming of fermentation broth and direct removal of β-carotene from the cells. Thus, the problem associated with oxidation of β-carotene during the stage of drying of the biomass was omitted. The present method, compared to those proposed by other investigators, was found to be accurate, rapid, and very reproducible. Moreover, the amount of β-carotene extracted from *B. trispora* was higher than that removed by other methods.

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