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Performance of Crude Olive Pomace Oil and Soybean Oil during Carotenoid Production by *Blakeslea trispora* in Submerged Fermentation

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Crude olive pomace oil (COPO) and crude soybean oil (CSO), two low-cost carbon sources, were examined as cosubstrates of glucose for carotenoid production by *Blakeslea trispora*. Results were compared to those obtained in glucose as a sole carbon source (medium 1) and glucose plus the respective end-line refined oil counterparts. Microbial growth in the presence of oils resulted in an increase in total carotenoid production. The performance of crude oils was better than that of the respective refined forms. Carotenoid production depended on both type and added oil amount. An increase in added oil amount did not necessarily favor carotenoid accumulation. The addition of 10 g oil/L of substrate stimulated carotenoid synthesis, mainly that of β-carotene, more than 14 (COPO) and 40 times (CSO) in comparison to that observed in medium 1. The maximum total carotenoid content (as mg β-carotene per g of biomass dry weight) was 75 (COPO) and 235 mg (CSO), respectively. Growth, substrate assimilation, and lipid accumulation−degradation also depended on the presence of oil in the substrate.

KEYWORDS: Crude olive pomace oil; crude soybean oil; microbial carotenoids; *Blakeslea trispora*; submerged fermentation

INTRODUCTION

Current industrialization in agricultural practice has led to overproduction of solid and liquid residual matter. Among food industry residues/byproducts, crude olive pomace oil (COPO), derived from one of the major oil cakes in Mediterranean countries, and crude soybean oil (CSO), a byproduct of soybean processing, are of great economic potential (*1*, *2*). The chemical composition of both oils differs in the degree of triacylglycerol unsaturation and the content and presence of minor components. Thus, olive pomace oil is rich in oleic acid whereas soybean oil is rich in both linoleic and linolenic acids. Differences in the composition and levels of phytosterols, phospholipids, tocopherols, pigments, and trace elements are well-established in the literature (*1*).

On the basis of the upgrading concept, crude oils are usually transformed into value-added products in dependency of the adequate technology either as raw materials for secondary processes (e.g., refinement, soap production) or, currently, as ingredients of novel products. Being economically competitive, crude vegetable oils offer excellent possibilities to be used as low-cost carbon sources for the production of value-added products by biotechnological processes. Yet, these byproducts have to fulfill certain requirements as shown in **Figure 1** (*2*). The utilization of vegetable oils as growth substrates has recently attracted attention since reports indicate enhancement of the production of commercially interesting products (e.g., lipases, lipid-soluble materials, biopolyesters, riboflavin, single-cell proteins, and citric acid) (*3*, *4*). Moreover, it has been claimed that oils protect fragile microorganisms, possess natural antifoam properties, and are cheaper than other carbon sources (i.e., carbohydrates) (*5*).

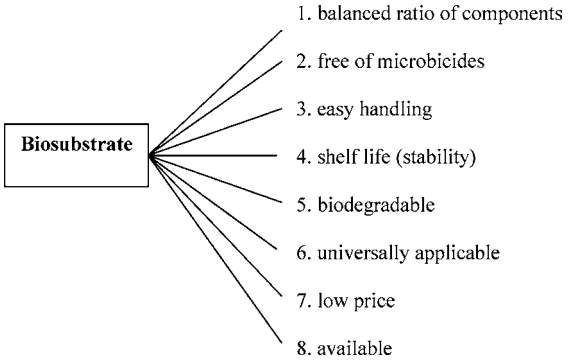

Figure 1. Natural properties of bioconvertible substrates in biotechnology.

Carotenoids, the most common, naturally occurring terpenoid pigments, are of great interest in many scientific fields because of their wide distribution, diverse functions, and interesting

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properties. Several carotenoids such as β-carotene, astaxanthin, lutein, canthaxanthin, and lycopene are important industrially as nutrient supplements, food colorants, and feed additives. Scientific interest in dietary carotenoids has increased in recent years due to claims for beneficial effects on human health, such as a reduced risk of cancer and enhancement of immune system function (*6*), attributed to their antioxidative potential. Carotenoids used in industry are mostly manufactured using chemical synthesis or are natural extracts or concentrates. The increasing interest in microbial carotenoid sources is related to consumer preferences for natural additives and improvements in knowledge related to fungal biotechnology though considerations about the cost effectiveness of such a process (*7*). Among the types of microbes tested for the production of carotenoids, *Blakeslea trispora* and *Dunaliella* species seem to be the most promising ones for commercial exploitation (*8*). In particular, production by *B. trispora* has in recent years gained considerable interest due to the capacity of the fungus to convert numerous raw materials or even industrial waste products into value-added end products (*9*, *10*). There are a few examples of the use of hydrophobic substances in fermentation processes for the production of carotenoids by the fungus *B. trispora*. Among them, some research has been carried out on the use of oils rich in unsaturated C18 fatty acids, for example, olive, cottonseed, and soybean oils (*11*, *12*). To date, the biochemical pathways involved in the regulation of carotenoid production by *B. trispora* from vegetable oils and fats have not been studied in depth. Moreover, crude vegetable oils have not been used for microbial carotenoid synthesis, although they cost significantly less than other carbon sources.

The present study aims at investigating the dynamics of carotenoid production by *B. trispora* growing in glucose in the presence or absence of crude vegetable oils, to optimize the production of these microbial metabolites from low-cost carbon sources used as substrates. Experiments were also conducted using culture media with the respective end-line refined oil counterparts as reference materials. Biochemical and kinetic interpretations concerning growth, substrate assimilation, lipid accumulation−degradation, and carotenoid production were considered and discussed.

MATERIALS AND METHODS

Oil Substrates. COPO and the respective refined oil (ROPO) were donated by Minerva S. A. Edible Oils (Shimatari, Viotia, Greece). CSO and the respective refined oil (RSO) were a gift of Soya Hellas S. A. (Athens, Greece).

Microorganisms. The microorganisms used in this work were *B. trispora* ATCC 14271, mating type (+), and *B. trispora* ATCC 14272, mating type (−). Both strains were donated by the German chemical industry BASF Aktiengesellschaft (Ludwigshafen, Germany). The strains were stored in cryotubes at −80 °C.

Reagents and Solvents. Potato dextrose agar (PDA) was from Fluka (Hannover, Germany). D-Glucose monohydrate, casein acid hydrolysate, yeast extract L-asparagine, KH_2PO_4, $MgSO_4 \times 7H_2O$, and thiamine−HCl used for preparation of the culture medium and emulsifiers Span 20 and Tween 80 were from Scharlau Chemie S. A. (Barcelona, Spain). Corn steep liquor (CSL) was supplied by Amylum Hellas (Thessaloniki, Greece).

High-performance liquid chromatography (HPLC)-grade solvents were used without further purification. HPLC-grade acetone, acetonitrile, and methanol were from Panreac (Barcelona, Spain). 2-Propanol (Chromasolv), *n*-hexane, chloroform, methanol, and acetone of analytical reagent-grade were from Riedel-de Haën (Seelze, Germany). β-Carotene (for biochemistry, purity 97%) was purchased from Merck (Darmstadt, Germany).

Apparatus. A Zeiss PMQII spectrophotometer (Oberkochen, Germany) was used in the determination of total carotenoid, residual sugar, and nitrogen content. Reversed phase (RP)-HPLC for the analysis of carotenoids was performed isocratically using a solvent delivery system consisting of two Marathon IV Series HPLC pumps (Rigas Labs, Thessaloniki, Greece), a Rheodyne 7125 injection valve with a 20 μL fixed loop (Rheodyne, Cotati, CA), and a Linear UVIS-206 diode array multiple-wavelength detector (Linear Instruments, Fermont, CA). The data from the detector were processed with the chromatographic software EZChrom (Sci Software, Inc., San Ramon, CA). An autoclavable Mettler Toledo 4100 pH probe (Ohio) was used to measure the pH of the culture medium. Inoculated flasks were incubated on a rotary shaker incubator (Lab-line, Melrose Park, IL).

Culture Conditions. The strains were grown on PDA at 26 °C for 3 days and used for the inoculation in the culture medium.

Fermentation Conditions. The batch fermentation was carried out in 250 mL conical flasks at a filling volume of 50 mL of the culture medium (medium 1). The quantities used as g/L of culture medium were 50.0 (glucose), 80.0 (CSL), 2.0 (casein acid hydrolysate), 1.0 (yeast extract), 2.0 (L-asparagine), 1.5 (KH_2PO_4), 0.5 ($MgSO_4 \times 7H_2O$), 10.0 (Span 20), 1.0 (Tween 80), and 0.005 (thiamine−HCl). The initial pH of the culture medium was adjusted to 7.5. Sets of experiments were performed with medium 1 supplemented with crude or refined forms of olive pomace oil or soybean oil. The oils were added at 10.0 and 30.0 g/L of culture medium. The above experiments were conducted at a shaking speed of 250 rpm and at a shaking diameter of 2.5 cm. The flasks were inoculated with a spore suspension of each microorganism containing 5.0×10^6 spores/mL and incubated at 26 °C.

Analytical Techniques. At specific time intervals, the fermentation broth was removed from the flasks and subjected to the following determinations.

Determination of Cell Growth and Residual Oil. An aliquot of the culture liquid (~5 mL) was filtered under reduced pressure through a Whatman 1 filter paper, and cells were washed with *n*-hexane. To verify the efficiency of the method, specifically that no losses of cell components occurred during washing with *n*-hexane, cells produced in glucose were washed with distilled water or as previously described (*n*-hexane). In both cases, the biomass concentration was determined from dry matter (80 °C/24h).

The aqueous filtrate used for the oil content determination was washed twice with *n*-hexane. *n*-Hexane extracts were collected into a glass flask. Lipids from the aqueous layer were extracted with chloroform and then combined with the *n*-hexane extract. The combined lipid extract was dried over anhydrous sodium sulfate. Solvents were then removed using a vacuum (40 °C) and a nitrogen stream prior to taking the lipid weight.

Carotenoid Extraction and Determination of Total Carotenoid Content. The carotenoids were removed from the cells after cell rupture by freezing and thawing, using liquid nitrogen, and then by manual grinding in the presence of quartz sand until complete cell breakage occurred. The preparation was examined under a phase contrast microscope (Nikon E 200, United States) to ensure complete destruction. Magnification × 100 was used for image analysis (Matrox Inspector version 3.0, Matrox Electronic Systems Ltd., Canada). Carotenoids were extracted with acetone (30 min at 30 °C) to achieve decolorization of the suspension. The acetone/treated fermentation broth ratio was 1:1, v/v. The colored organic layer was then centrifuged at 10000*g* for 10 min to remove cells, passed through a water-free Na_2SO_4 layer, and rotary-evaporated to dryness. Crude extract was dissolved in *n*-hexane. The total carotenoid content estimated as β-carotene was quantified spectrophotometrically at 450 nm by using a five-point standard calibration curve. Control of standard β-carotene solution concentrations was carried out through spectrometry using the appropriate extinction coefficient value for the solvent found in the literature (2590 at 450 nm) (*13*).

RP-HPLC Analysis of Cellular Carotenoids at the Time of Maximum Carotenoid Accumulation. Acetonitrile/methanol/2-propanol (90:6:4, v/v/v) was used as an eluent (*14*). Separation was achieved on a Nucleosil C18, 5 μm (250 mm × 4 mm i.d.) column (Macherey-Nagel, Düren, Germany) at a 1.5 mL/min flow rate. The injection volume was 5 μL. The column was carefully conditioned between two injections with the elution mixture for 15 min. Crude extracts and β-carotene standard solution were first dissolved in a small quantity of tetra-

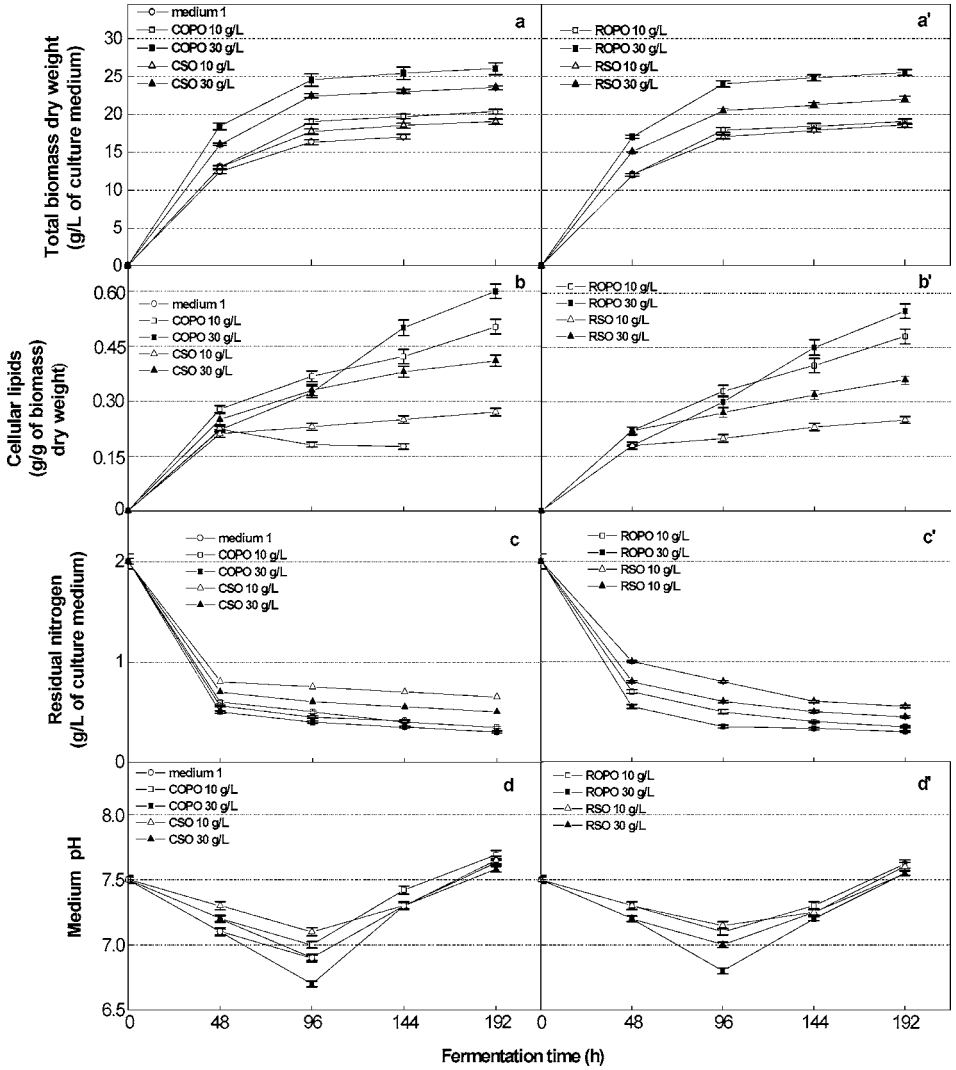

Figure 2. Kinetics of total biomass dry weight, cellular lipids, residual nitrogen, and medium pH during *B. trispora* growth in medium 1 in the presence/absence of different initial levels of COPO, CSO, ROPO, or RSO. Error bars represent the SD of the mean.

hydrofuran and then in methanol. All analytical samples were filtered through a 0.45 μm membrane filter (Schleicher & Schuell, Dassel, Germany) just before HPLC analysis. Care was taken to prevent exposure of samples and standard solution to direct light throughout the analytical procedure. Peak identification and purity were based on retention time and peak spiking with a β-carotene standard solution. The percent β-carotene was calculated on the basis of the sum of all peaks recorded at 453 nm. Analysis of samples was carried out in duplicate (CV% = 6.2, $n = 5$ for a 10 ppm standard β-carotene solution).

Determination of Cellular Lipids. A cellular lipid magnetically stirred extraction with a chloroform−methanol (2:1, v/v) mixture was performed three times (each session lasted 1 h) using the Folch method (*15*). Cell rupture was achieved as described above. The extract was dried over anhydrous sodium sulfate. Solvents were then removed using a vacuum (40 °C) and a nitrogen stream.

Determination of Residual Nitrogen and Sugar Content. Residual sugars and nitrogen in the supernatant were determined as described by Mantzouridou et al. (*12*). Nitrogen consumption was determined spectrophotometrically (*16*).

Statistics. Tables and figures contain data that are mean values of three independent experiments. Error bars represent the standard deviation (SD) of the mean value.

RESULTS AND DISCUSSION

The results of the experiments conducted using culture media containing mixtures of glucose (at 50.0 g/L of culture medium) and COPO or CSO (at 10.0 or 30.0 g of oil/L of culture medium) are presented in **Tables 1**−**3** and in **Figures 2** and **3**. Culture media having glucose as the sole substrate (at 50.0 g/L of culture medium) (medium 1) and glucose plus 10.0 or 30.0 g of oil/L of culture medium of the ROPO or RSO were used as control systems. The two latter control systems were used to compare the performance of crude oils on primary and secondary metabolism with that of the respective refined oils.

Microbial Growth and Cellular Lipid Production. In all culture media, good microbial growth was observed while noticeable quantities of intracellular lipids were produced. The curves of total biomass (lipid-free material plus cellular lipids) and of cellular lipid accumulation vs time in the different culture media are presented in **Figure 2a**,**a′**,**b**,**b′**. In all cases, the total biomass value increased dramatically in the first 96 h and then it increased slightly until the end of the fermentation process, although significant quantities of the available extracellular nitrogen had been consumed within the first 2 days ($t = 48$ h) (**Figure 2c**,**c′**). Lipid-free material together with cellular lipids was synthesized, indicating that both glucose and oil substrates were absorbed for lipogenesis as well as for anabolic activities other than accumulation of lipids. Biomass formation together with the synthesis of the metabolite has already been reported in the literature for the cases of lipid-, citric acid-, or polysaccharide-producing microorganisms, after nitrogen depletion

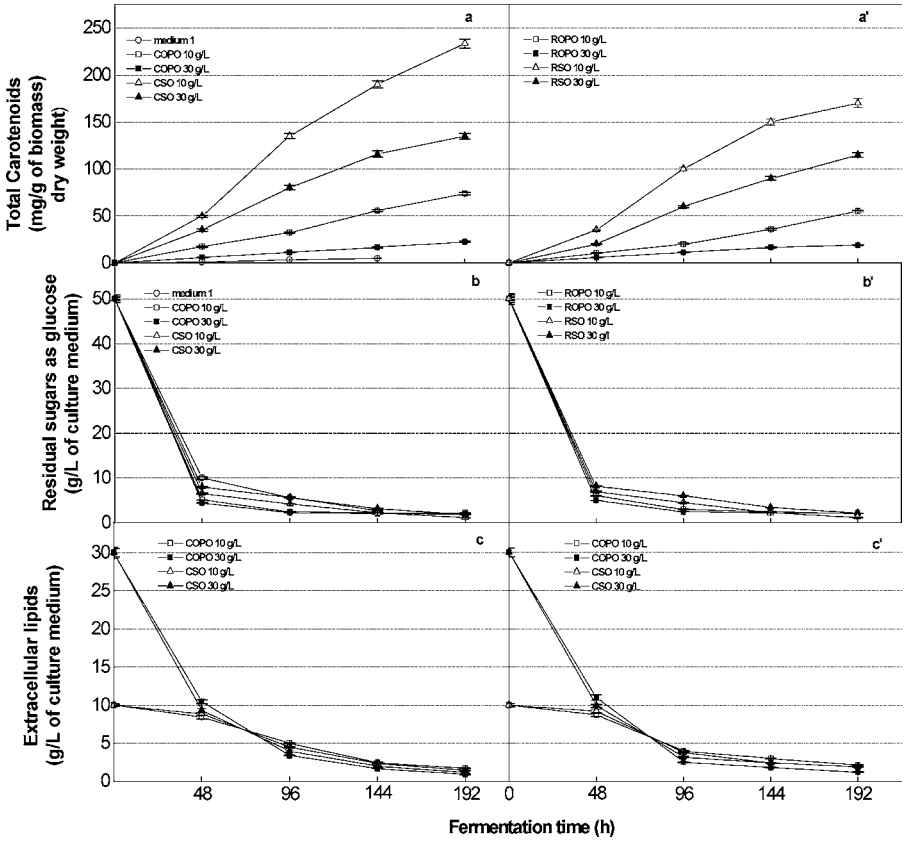

Figure 3. Kinetics of total carotenoids, residual sugars as glucose, and extracellular lipids during *B. trispora* growth in medium 1 in the presence/absence of different initial levels of COPO, CSO, ROPO, or RSO. Error bars represent the SD of the mean.

from the medium, presumably through the anaplerotic pathway of glyoxylate bypass (*17*−*19*). The enzyme key regulating the reactions conducted through the glyoxylic bypass pathway is the one of iso-citrate lyase, the activity of which increases considerably on cells cultivated on *C*2 compounds or on substrates leading to the generation of *C*2 units (e.g., *n*-alkanes, fats) (*4*, *18*).

In **Figure 2b**,**b′**, quantitative changes of cellular lipids during the different fermentation processes are given. Lipid accumulation in the cells of *B. trispora* varied significantly and was critically influenced by the presence of oil substrate in the culture medium. In medium 1, it was observed that during the first growth step (0−48 h), the kinetics of cellular lipid formation was similar to that of total biomass evolution (**Figure 2b**). In this stage, remarkable quantities of lipids were produced (ca. 20% w/w of dry matter) (**Figure 2b**) despite the presence of significant nitrogen amounts into the growth medium. In *B. trispora*, hence, lipid accumulation from glucose appears to be a growth-coupled process, occurring in the presence of assimilable nitrogen into the medium in agreement with the data reported by Reshamwala and Modi (*20*). In contrast with the above finding, microorganisms capable of producing lipids from glucose or similarly metabolized compounds accumulate lipids after nitrogen depletion from the growth medium (*21*, *22*). Additionally, between 48 and 192 h, a degradation of the cellular lipids occurred. This indicates that the biosynthetic abilities of the microbial population cannot be supported at this stage solely by the extracellular carbon source; hence, the carbon pool needs to be supplemented by biodegradation of the lipid reserves. However, in the media with mixed substrates (glucose plus COPO or CSO), reserve lipids were not mobilized at the late growth phase (e.g., 192 h after inoculation), despite the relatively low concentration of extracellular carbon sources (**Figures 2b**,**b′** and **3c**,**c′**,**d**,**d′**). Reserve lipid breakdown is a phenomenon routinely observed for lipid-accumulating microorganisms, such as molds (*23*, *24*), yeasts (*4*), or bacteria (*25*). When growth has been conducted on sugars, reserve lipid degradation should be realized through the glyoxylate bypass pathway, due to previous inactivation of the Krebs cycle enzymes (e.g., NAD^+-isocitrate dehydrogenase) at nitrogen-limited conditions (*24*). Lipid mobilization has also been successfully simulated with the aid of numeric models (*23*). Moreover, it was observed that from 48 to 96 h, the total biomass value increased by more than 45.0% and a significant acidification of the culture medium occurred (**Figure 2d**). It can be deduced that carbon is directed to primary metabolism yielding fat-free biomass and organic acids in line with observations made by Papanikolaou et al. (*26*) for cell growth and formation of lipids in *Yarrowia lipolytica* using lipid substrates with different fatty acid profiles.

In low COPO or CSO medium, the final total biomass presented almost equivalent values to that obtained during growth in medium 1 (**Tables 1** and **2**). The addition of higher oil level in the medium allowed an increase of total biomass value. In all cases, the incorporated oil substrate was assimilated for growth as well as cellular lipid synthesis.

As can be noted from **Table 1**, in cases where COPO and glucose were used as simultaneous carbon substrates, a high quantity of cellular lipids was evidenced. Interestingly, the addition of high COPO level in the medium, which allowed an increase of total biomass, resulted in a higher production of microbial lipids as compared with the culture presenting low COPO levels (0.6 against 0.5 g of lipids per g of biomass dry weight, which corresponds to 15.6 g against 10.0 g of cellular lipids per L of culture medium). This trend was repeated in the case of CSO. Thus, in a culture in which a low level of the latter was employed as the cosubstrate, the quantities of cellular

Table 1. Values of the Fermentation Parameters Measured at the Time of Maximum Carotenoid Production by *B. trispora* Grown in Medium 1 in the Presence/Absence of Different Initial Levels of COPO or ROPO

culture medium	g/L of culture medium: added oil	g/L of culture medium: total biomass dry weight[c]	total carotenoids[c] (mg/g of biomass dry weight)	cellular lipids[c] (g/g of biomass dry weight)	g/L of culture medium: extracellular lipids[c]	g/L of culture medium: residual sugars[c]	g/L of culture medium: residual nitrogen[c]	medium pH[c]
medium 1[a]		17.0 ± 0.31	5.0 ± 0.10	0.18 ± 0.007		2.80 ± 0.03	0.40 ± 0.01	7.54 ± 0.03
+ COPO[b]	10.0	20.0 ± 0.30	74.0 ± 1.51	0.50 ± 0.020	1.70 ± 0.050	2.00 ± 0.02	0.35 ± 0.01	7.69 ± 0.03
+ COPO[b]	30.0	26.0 ± 0.40	23.0 ± 0.65	0.60 ± 0.02	0.90 ± 0.025	1.95 ± 0.02	0.30 ± 0.01	7.63 ± 0.02
+ ROPO[b]	10.0	19.0 ± 0.35	55.0 ± 0.94	0.48 ± 0.020	2.10 ± 0.055	2.10 ± 0.03	0.35 ± 0.01	7.62 ± 0.03
+ ROPO[b]	30.0	25.5 ± 0.40	19.0 ± 0.49	0.55 ± 0.020	1.20 ± 0.034	2.00 ± 0.03	0.30 ± 0.01	7.55 ± 0.02

[a] Fermentation time, 6 days. [b] Fermentation time, 8 days. [c] Mean values of three different experiments ± SD.

Table 2. Values of the Fermentation Parameters Measured at the Time of Maximum Carotenoid Production by *B. trispora* Grown in Medium 1 in the Presence/Absence of Different Initial Levels of CSO or RSO

culture medium	g/L of culture medium: added oil	g/L of culture medium: total biomass dry weight[c]	total carotenoids[c] (mg/g of biomass dry weight)	cellular lipids[c] (g/g of biomass dry weight)	g/L of culture medium: extracellular lipids[c]	g/L of culture medium: residual sugars[c]	g/L of culture medium: residual nitrogen[c]	medium pH[c]
medium 1[a]		17.0 ± 0.31	5.0 ± 0.10	0.18 ± 0.003		2.80 ± 0.03	0.40 ± 0.01	7.30 ± 0.03
+ CSO[b]	10.0	19.0 ± 0.34	235.0 ± 4.67	0.27 ± 0.010	1.40 ± 0.036	1.10 ± 0.01	0.65 ± 0.01	7.65 ± 0.03
+ CSO[b]	30.0	23.5 ± 0.28	135.0 ± 2.88	0.41 ± 0.015	1.10 ± 0.028	1.90 ± 0.02	0.50 ± 0.01	7.58 ± 0.02
+ RSO[b]	10.0	18.5 ± 0.29	170.0 ± 4.65	0.25 ± 0.010	1.90 ± 0.036	1.20 ± 0.02	0.55 ± 0.01	7.60 ± 0.03
+ RSO[b]	30.0	22.0 ± 0.37	115.0 ± 2.42	0.36 ± 0.012	1.65 ± 0.044	2.15 ± 0.02	0.45 ± 0.01	7.55 ± 0.02

[a] Fermentation time, 6 days. [b] Fermentation time, 8 days. [c] Mean values of three different experiments ± SD.

lipids were slightly higher when compared with that obtained during growth in medium 1. A higher CSO level enhanced cellular lipid accumulation although less than the respective level of COPO (**Tables 1** and **2**). In accordance with the finding reported in the present study, lipid accumulation from fatty materials is largely dependent on the initial concentration of the latter, and the more the concentration of fat increases, the more lipid accumulation is favored (*4*, *27*). Vegetable fat conversion into cell biomass with variations in cellular lipid content also reported for other microorganisms (*28*) suggests mechanisms that regulate the partition of carbon flow between cellular lipid and nonlipid material synthesis.

Carotenoid Production. As can be seen in **Figure 3a**,**a′**, in all cases, carotenoid synthesis was slow within 48 h after inoculation, whereas a sharp increasing trend of carotenoids was evidenced after the second day of fermentation and, essentially, in the last stage of cell growth, where residual nitrogen content in the medium was low (**Figure 2c**,**c′**). It is well-established that a low nitrogen concentration induces a nitrogen/carbon imbalance that should drive most of the assimilated carbon to secondary metabolism pathways yielding N-lacking intermediates (*29*). Carotenoid production is the outcome of the increase of carbon flux through N-lacking secondary routes. Indeed, a low nitrogen content in the presence of excess organic carbon substrates has been proved effective in enhancing carotenoid production in different carotenogenic strains (*29*, *30*).

Despite the exo-cellular nitrogen limitation, the carotenoid yield in medium 1 was low (**Figure 3a**). This might occur as a result of the low glucose content in the medium at the late fermentation stage (**Figure 3b**) indicating that nonlimiting carbon conditions are important for carotenoid biosynthesis. Thus, it becomes attractive to utilize two major carbon sources: one for the initial growth phase and the second for the production phase. The selection of appropriate carbon source can have a dramatic effect on the production of secondary metabolites as well as raw material costs (*31*). The second carbon source should be a slowly metabolized nutrient such as a low-cost crude vegetable oil.

The performance of COPO as a secondary carbon source upon carotenoid production is shown in **Figure 3a** and in **Table 1**. In the medium supplemented with a low COPO level, a significant increase of carotenoid accumulation in the cells of *B. trispora* was observed with respect to medium 1. The maximum carotenoid concentration achieved was 74 mg/g of biomass dry weight (or 1480 mg/L of culture medium). In rich COPO medium, total carotenoids were produced in considerable amounts (25 mg/g of biomass dry weight), although significantly lower than that obtained during growth in low COPO medium. It can be concluded that the effect of COPO on carotenoid biosynthesis is concentration-dependent. Carotenoid accumulation in the presence of high oil level seems to be influenced by the fatty acid profile since one or more fatty acids may be inhibitory when found in excess. As the surplus of cellular fatty acids represses nicotinamide adenine dinucleotide phosphate (NADPH) production through glucose 6-phosphate inhibition (*31*, *32*), carotenoid biosynthesis could be restrained. Nevertheless, other more specific regulatory control mechanisms are expected to contribute including feedback regulation of synthetases or oxygen availability.

Over the range of levels tested, a low CSO level has been shown to increase dramatically the total carotenoid accumulation in the cells of *B. trispora*, as compared to medium 1 in the presence or absence of COPO (**Figure 3a** and **Table 2**). The higher concentration of carotenoids achieved during the CSO−glucose coutilization experiments was 235 mg/g of biomass dry weight. This value is one of the highest reported in the literature (*33*). As in the case of COPO, a further increase of the CSO level caused a decrease in carotenoid production. However, the quantity of cellular lipids continued to increase. It is noticeable that the overproduction of total carotenoids did not coincide with a sharp increase of cellular lipid accumulation (**Table 2**). The stronger positive effect of CSO, as compared to COPO, on carotenoid yield, accompanied by a lower lipid accumulation, indicates that the enhancement of carotenoid biosynthesis and lipid production obtained by the addition of oils cannot be attributed solely to the high caloric value of these substances.

Table 3. β-Carotene Content (% of Total Carotenoids) at the Time of Maximum Carotenoid Accumulation in *B. trispora* Grown in Medium 1 in the Presence/Absence of Different Initial Levels of COPO or CSO

culture medium	added oil (g/L of culture medium)	β-carotene (% of total carotenoids[a])
medium 1[b]		28.0
+ COPO[c]	10.0	62.0
+ COPO[c]	30.0	44.0
+ CSO[c]	10.0	75.0
+ CSO[c]	30.0	58.0

[a] The total carotenoid content at the time of maximum carotenoid accumulation in medium 1 in the presence/absence of different initial levels of COPO or CSO is estimated in **Tables 1** and **2**. [b] Fermentation time, 6 days. [c] Fermentation time, 8 days.

Because carotenoid overproduction seems to be attended by the development of intracellular triacylglycerols with specialized structure (*34*), further research is in progress for the investigation of the relationship between the biosynthesis of lipids and carotenoids as well as the role of the reserve lipid structure/activity relationship on carotenoid accumulation. Moreover, the relatively higher carotenoid yields in crude oils, as compared to those obtained in the respective refined forms (**Figure 3a**,**a′**), lead to the idea that minor constituents present in crude oils at higher levels to those found in the end products, such as phospholipids and natural tocopherols, could contribute to a higher carotenoid production (*35*, *36*).

To gain insight on the composition of carotenoid mixture produced in the presence of crude oils, RP-HPLC was carried out for the lipid extracts at the time of maximum carotenoid accumulation. In an extract of fungal cells grown in medium 1, β-carotene constituted only 28% of the total carotenoid content (**Table 3**). In low COPO medium, there was an increase in β-carotene proportion by more than 2-fold (62% of the total carotenoid content). A higher induction in β-carotene synthesis, as compared to the two previous ones, was found in low CSO-enriched medium. In this case, β-carotene constituted 75% of total carotenoids. The addition of higher COPO or CSO levels did not favor so much β-carotene biosynthesis (44 and 58% of the total carotenoid content, respectively) (**Table 3**). This finding needs further investigation to be better justified.

Carbon Substrate Utilization. The presence or absence of oil substrate into the culture medium had no effect upon the consumption of glucose, while in the case of its coutilization with glucose, the extracellular lipid uptake rate varied significantly and was critically influenced by initial oil amount in the culture medium (**Figure 3b**,**b′**,**c**,**c′**). In low added oil system, for conditions of low oil/low biomass that simulate the beginning of the fermentation process (0−48 h), a small amount of lipids was taken by the microorganism, whereas for the high added oil system the respective quantity consumed at the beginning of the fermentation was notably higher. The latter was possibly due to a better dispersion of the oil in the culture medium. The small quantities of biomass dispersed in the medium at the beginning of the fermentation indicate that segregation controls the oil and biomass dispersion. Coutilization of fats and hydrophilic components by other microorganisms (e.g., *Torulopsis bombicola* and oleaginous Mucorales) has also been recorded regardless of the configuration of the fermentation process (*37*, *38*).

For conditions simulating the end of the fermentation (high biomass), a high consumption rate of oil substrate was evident in all cases. The high amounts of biomass present might be the factor that enhanced oil degradation as a result of increased dispersion in the medium (*4*). Despite the changes in the rheology of the broth, it was observed that homogeneity was higher as fermentation proceeded. This phenomenon was associated with the increase in biomass/oil ratio and the role of biomass as an oil carrier.

In conclusion, COPO and CSO are types of readily sterilizable, inexpensive, concentrated carbon sources that appear to be promising substrates for carotenoid production by *B. trispora* in submerged fermentation. Despite the inhibition of carotenoid biosynthesis at higher COPO or CSO quantities, it is important to note that the addition of 10 g oil/L of culture medium stimulated carotenoid synthesis, mainly that of β-carotene, more than 14 (COPO) and 40 times (CSO) in comparison to that observed in medium 1. In favorable growth conditions, nitrogen present in the medium is exhausted in a few days, allowing high cell density. This supports massive accumulation of carotenoids and oil dispersion in the aqueous phase, as well as carotenoid/lipid accumulation to be triggered under nonlimiting carbon conditions. COPO, although less effective than CSO, may be of some economical importance for oil-producing countries such as Spain, Italy, and Greece, which urgently seek multiple uses for oil mill wastes.

ABBREVIATIONS USED

ATP, adenosine triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; acetyl-coA, acetyl-coenzyme A; TCA, tricarboxylic acid cycle.

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