JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Industrial Glycerol as a Supplementary Carbon Source in the Production of β-Carotene by *Blakeslea trispora*

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The dynamics of industrial types of glycerol as a supplementary carbon source to glucose for β-carotene production by *Blakeslea trispora* was investigated in batch cultures. The growth kinetics, cellular lipid accumulation−degradation, substrate assimilation, and β-carotene production were clearly dependent on the level of addition of pure glycerol. The highest β-carotene production (15.0 mg/g of dry biomass) was obtained at an initial glycerol concentration of 60.0 g/L. Substitution of pure glycerol by the nonpurified soap byproduct did not inhibit cell growth. Conversely, partial purification of the biodiesel byproduct by removing methanol and fatty acids was unavoidable for cell growth. Both types of industrial glycerol stimulated β-carotene synthesis more than 10 (soap byproduct) and 8 times (biodiesel byproduct) compared to control medium. The maximum β-carotene contents were 10 and 8 mg/g of dry biomass, respectively, and its relative content in the carotenoid fraction was 86–88%.

**KEYWORDS: Industrial glycerol; glucose; β-carotene production; biodiesel; *Blakeslea trispora***

## INTRODUCTION

The production of β-carotene by *Blakeslea 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 (e.g., citrus byproducts, various grains, cellobiose, beet molasses, and cheese whey) into valuable end products (*1–5*). On the basis of the upgrading concept we recently introduced crude olive pomace and soybean oils as inexpensive cosubstrates to glucose in the production of β-carotene by this fungus (*6*). Extending our effort to other byproducts from the agricultural industry in our country, the present study explores the potential of two types of industrial glycerol as a supplement to the glucose substrate.

Glycerol, the major byproduct of soap and detergent manufacturing, is currently also generated at appreciable quantities in the production of biofuels (*7*). The interest in the latter at a large commercial scale is definitely within the priorities of the European Union for energy self-reliance (*8*). Glycerol is expected to be available at a low cost, and its competence as a fermentation feedstock in the production of high-value goods seems to be promising (*9*). Glycerol has been used as a carbon source in an array of bacterial-assisted fermentations for the production of compounds of industrial interest such as 1,3-propanediol, succinic acid, dihydroxyacetone, and polyhydroxyalkanoates (*10*). Moreover, its utilization as a low-cost carbon source is of special interest in the production of γ-linolenic acid by Mucorales (*11*), *Rhizopus oryzae* lipase by recombinant *Pichia pastoris* (*12*), and citric acid and α-amylase by wild-type and recombinant strains of *Yarrowia lipolytica*, respectively (*13, 14*).

In a previous investigation, Dandekar and Modi (*15*) studied the growth of *B. trispora* and β-carotene production on glycerol instead of glucose, using a typical batch culture. Although the substitution of glucose by glycerol resulted in a 1.6 times higher yield of β-carotene (1.0 mg/g of dry biomass), the latter cannot be considered to be competitive to others reported in the literature for practical applications. In our work, using a similar fermentation process, we investigated the dynamics of glycerol as a supplementary carbon source to glucose on growth characteristics of *B. trispora*.

Batch culture control conditions used in the study had been repeatedly checked in previous works (*6, 16*) to ensure sufficient cell growth and β-carotene production (0.5–1.0 mg/g of dry biomass). The major fermentation outcome on glucose as substrate is efficient cell growth parallel with lipid production, wherein almost total glucose consumption occurs within the early growth phase period. Nonetheless, β-carotene production shows an increasing trend associated with degradation of the cellular lipids within the late growth phase. The latter indicates that the biosynthetic abilities of the fungus cannot be supported solely at this stage by glucose (*17*). Such a burden may be overcome by the utilization of supplementary carbon sources, one for the initial growth phase and the second for the production one. Considering that carotenoid production is the outcome of the increase of carbon flux through N-lacking

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10.1021/jf703667d CCC: $40.75 
Published on Web 03/28/2008

secondary routes (*18, 19*), selection of an appropriate supplementary carbon source may have a dramatic effect on its yield. Thus, we were interested in whether microbial metabolism could be directed by using glycerol and glucose as cosubstrates.

The kinetic behavior of *B. trispora* (cell growth, substrate assimilation, product formation) was studied on glucose in the presence of pure glycerol at different levels (10–180 g/L of culture medium). Biochemical and kinetic elucidation regarding extracellular pH, total acidity (expressed as citric acid, g/L of culture medium), and dissolved oxygen (DO) level in the culture medium under selected conditions was also considered to support the discussion. In addition, experiments were conducted with two types of industrial glycerol to investigate the effect of impurities on cell growth and carotenoid production. Industrial glycerol was used at the level that triggered maximum β-carotene accumulation using pure glycerol.

## MATERIALS AND METHODS

**Glycerol Samples.** The types of glycerol employed were (a) pure glycerol (Panreac, Barcelona, Spain) (purity = 99% w/w), (b) industrial glycerol solution from a soap manufacturing unit (Papoutsanis AS, Athens, Greece) (glycerol content = 40% w/w), and (c) industrial glycerol solution from a biodiesel production unit (Hellenic Biodiesels, Kilkis, Greece) (glycerol content = 15% w/w). From information provided by the two industries, impurities in the soap byproduct were mainly composed of non-glycerol organic materials (5% w/w), sodium salts (10% w/w), and water (40% w/w), whereas those in the biodiesel byproduct were soap (20.0% w/w), methanol (25% w/w), and water (30.0% w/w).

**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.

**Standards, Reagents, and Solvents.** *all-trans*-β-Carotene standard (for biochemistry, purity = 97%) was purchased from Merck (Darmstadt, Germany). *all-trans*-Lycopene (Redivivo 10% FS, DSM, Nutritional Products Ltd., Basel, Switzerland) was a gift of BASF Aktiengesellschaft (Ludwigshafen, Germany) to our laboratory. Potato dextrose agar (PDA) was from Fluka (Hannover, Germany).

D-Glucose monohydrate, casein acid hydrolysate, yeast extract, L-asparagine, $KH_2PO_4$, $MgSO_4 \cdot 7H_2O$, and thiamin−HCl used for the 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). Acetic acid (Panreac), ammonium acetate (Riedel-de Haën, Seelze, Germany), acetylacetone (Fluka), and sodium *m*-periodate (Riedel-de Haën) were used for glycerol determination. All reagents were of analytical grade.

High-performance liquid chromatography (HPLC) grade solvents were used without further purification. HPLC grade acetone, acetonitrile, and tetrahydrofuran were from Panreac. Chloroform, methanol, and ethanol of analytical reagent grade were from Riedel-de Haën.

**Apparatus.** A Hitachi U-2000 spectrophotometer (Tokyo, Japan) was used in the determination of glycerol, glucose, 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 Laboratories, Thessaloniki, Greece), a Rheodyne 7125 injection valve with a 20 μL fixed loop (Rheodyne, Cotati, CA), and a Linear UV−vis 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). Absorbance measurements were recorded on a U-2000 spectrophotometer (Hitachi, Tokyo, Japan). Measurements such as pH and DO level of the culture medium were carried out using appropriate probes. Inoculated flasks were incubated in a rotary shaker (model MkX, Stoke Poges, U.K.).

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

**Fermentation Conditions.** Fermentation experiments were carried out in triplicate. The batch fermentation was conducted in 250 mL conical flasks at a filling volume of 50 mL of the culture medium (control medium) optimized elsewhere (*16*). The quantities used as grams per liter 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 ($MgSO_4 \cdot 7H_2O$), and 0.005 (thiamin−HCl). Dispersed growth of *B. trispora* was obtained by the addition of Span 20 (10.0 g/L) and Tween 80 (1.0 g/L). The initial pH of the culture medium was adjusted to 7.0. Experiments were performed with control medium supplemented with pure glycerol at 10.0, 30.0, 60.0, 90.0, or 180.0 g/L of culture medium or with industrial glycerol solutions diluted with the control medium to 60.0 g/L glycerol. The shaking speed was 250 rpm at a shaking diameter of 2.5 cm. The flasks were inoculated with a spore suspension of each microorganism containing $5.0 \times 10^6$ spores/mL and incubated at 26 °C for 288 h (in the case of pure glycerol or the soap byproduct) and 336 h (in the rest of the experiments). Constant working volume was achieved throughout the experiment by periodic addition of sterilized water. Aliquots (3 × 3 mL) were sampled under sterilized conditions for further analysis with the exception of the experiment using biodiesel byproduct, for which the aliquot obtained was less due to the lengthy fermentation period (3 × 1 mL).

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

*Determination of Total Dry Biomass, Total Acidity, and Residual Carbon and Nitrogen Content.* An aliquot of the culture medium was filtered under reduced pressure through a Whatman no. 1 filter paper, and cells were washed with distilled water and dried at 80 °C until constant weight (about 24 h). Total acidity of the filtered aliquots was titrated with 0.01 N NaOH solution and expressed as grams of citric acid per liter of culture medium. Residual sugars and nitrogen consumption were determined in the filtered aliquots spectrophotometrically (*6*). All measurements were obtained in triplicate.

*Determination of Glycerol.* The method used was an adaptation of a procedure developed for biodiesel (*20*) as follows: After removal of biomass by centrifugation, glycerol was directly determined in the hydrophilic culture media, properly diluted with a water/ethanol mixture (50:50, v/v) using periodate anions to oxidize glycerol to formaldehyde. The latter then reacts with acetylacetone in the presence of ammonium acetate (Hantzsch reaction) to give a quantifiable derivative that absorbs strongly at 410 nm. The linear range of determination was between 3.75 and 30 mg/L (RSD < 4%, $n = 5$) and recovery was satisfactory (93 and 92% recovery, respectively, for samples containing glycerol at 10 and 180 g/L, $n = 3$).

*DO Level Estimation.* DO level was measured off-line as described in a recent paper (*21*). DO measurements were performed in separate shake flasks so as not to interrupt determination of other fermentation parameters. All measurements were obtained in triplicate.

*Extraction of Cellular Lipids.* Lipids 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. Cellular lipid extraction with chloroform/methanol (2:1, v/v) mixture was performed three times (each session lasted 1 h at room temperature) using the Folch method (*22*). The extract was centrifuged at 10000 *g* for 10 min to remove cell and dried over anhydrous $Na_2SO_4$, and finally the solvent was removed using a vacuum (40 °C) under a nitrogen stream. All treatments were accomplished away from light exposure. Repeatability of extraction was satisfactory (CV% = 5.0, $n = 3$).

*RP-HPLC Analysis of Carotenoids.* Analysis of carotenoids by RP-HPLC was performed using the method previously developed by Mantzouridou and Tsimidou (*23*). Peak identification was achieved by comparison of retention time with that of authentic standards and confirmed by spiking and comparison of spectral data. Owing to the lack of availability of standard γ-carotene, its identification was solely based on elution order and spectral data in the visible region with regard to published information. Quantification of β-carotene was carried out at 453 nm. Percent carotenoid content was calculated on the basis of

the sum of the three peaks recorded at 453 nm. Analysis of samples was carried out in duplicate (CV% = 6.2, $n = 5$, for a 10 ppm standard solution).

*RP-HPLC Analysis of Triacylglycerols (TAGs).* Analysis of TAGs by RP-HPLC was performed as described in a previous work (*24*). Characterization of TAG groups was based on the equivalent carbon number (ECN) principle (ECN = CN-2DB, where CN = carbon number and DB = double-bond number in the TAG molecule). Percent of ECN TAG groups was calculated on the basis of the total area of all TAG groups from ECN 38 to ECN 50. Analysis of samples was carried out in duplicate (CV% = 9.2 and 9.6, respectively, for ECN 46 and 48 TAG of a 5 wt %/vol olive oil solution, $n = 5$).

**Statistics.** Tables and figures contain data that are mean values of at least nine measurements. Error bars represent the standard deviation (SD) of the mean value.

## RESULTS AND DISCUSSION

**Pure Glycerol as a Supplementary Substrate to Glucose: Effect on Microbial Growth and Carotenoid Production.** *B. trispora* growth in batch cultures on glucose or on other hydrophilic carbon sources (e.g., cellobiose, beet molasses, whey) is usually monitored with respect to biomass formation, substrate assimilation, and $\beta$-carotene production (*1–5*). When lipophilic materials are used to supplement glucose, lipid accumulation (g/g of dry biomass) is also followed (*25*). In our work, all of the above parameters and, additionally, pH, total acidity (expressed as citric acid, g/L of culture medium), and DO levels were monitored using appropriate methods.

Panels **a** and **b** of **Figure 1** show the kinetics of growth and cellular lipid accumulation−degradation during *B. trispora* cultivation on the different amounts of glycerol. The experimental results indicated remarkable growth in all culture media (**Figure 1a**). Growth dependence on the level of glycerol addition was clear. For example, the maximum value of total biomass (lipid-free material plus cellular lipids) ranged from 20.0 to 60.0 g/L on 10.0 and 180.0 g of glycerol/L of culture medium, respectively. In the cultures supplemented with glycerol up to 90.0 g/L the total biomass level was higher than that in the control medium during all growth phases. In these cases, total biomass value increased dramatically in the first 60 h (early growth phase). Beyond 60 h two types of total biomass kinetics were observed regarding the culture conditions: (a) total biomass remained constant in media containing glucose as a sole carbon source or glucose enriched with glycerol up to 30 g/L of culture medium, and (b) total biomass increased to some extent in media containing 60 and 90 g of glycerol/L of culture medium. Moreover, in the culture containing 180.0 g of glycerol/L of culture medium total biomass was lower than that in the control medium until about 36 h, indicating an adaptation period for growth under high glycerol conditions (*26*). After 36 h, growth was stimulated and the total biomass increased significantly compared to that of the control medium until the end of the process.

In all culture media, synthesis of cellular lipids was stimulated only when the extracellular nitrogen content reached the critical value of 0.2 g/L of culture medium (occurring at 36 and 60 h after inoculation in cultures growing on glycerol up to 90 and at 180 g/L of culture medium, respectively) (**Figure 1b**,**c**). At the late stage of the early growth phase (between 36 and 60 and 60–108 h after inoculation in cultures growing on glycerol up to 90 and at 180 g/L of culture medium, respectively), cellular lipids were synthesized together with lipid-free material, despite the low nitrogen content in the culture media (**Figure 1a**,**b**). Actually, the synthesis of cellular lipids was clearly dependent on the level of glycerol, and the greater the amount of glycerol

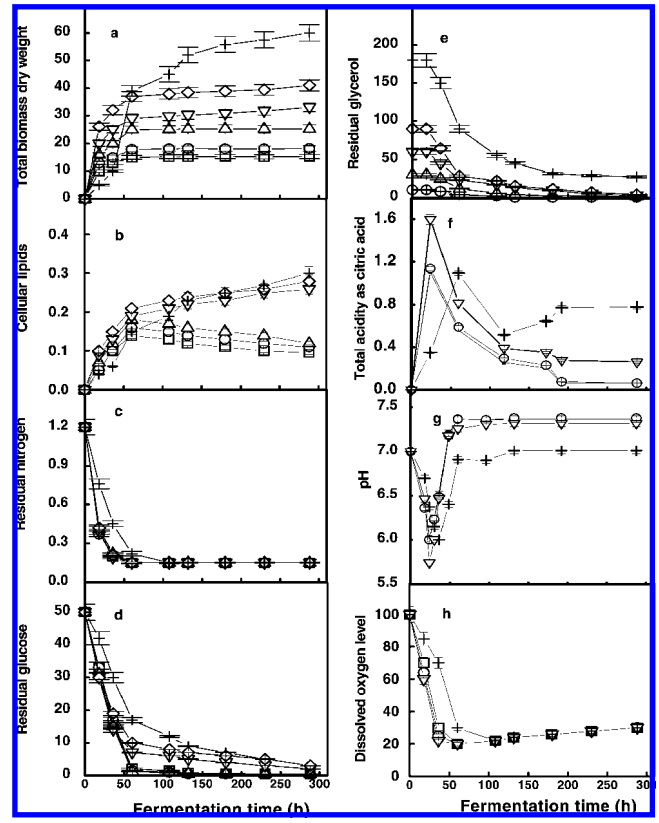


**Figure 1.** Kinetics of (**a**) total biomass dry weight (g/L of culture medium), (**b**) cellular lipids (g/g of biomass dry weight), (**c**) residual nitrogen (g/L of culture medium), (**d**) residual glucose (g/L of culture medium), (**e**) residual glycerol (g/L of culture medium), (**f**) total acidity expressed as citric acid (g/L of culture medium), (**g**) pH, and (**h**) dissolved oxygen (DO) level (% of saturation) during *B. trispora* growth in control medium in the absence/presence of different initial levels of glycerol. (□) Control medium containing glucose as a sole carbon source; control medium plus glycerol: 10 (○), 30 (△), 60 (▽), 90 (◇), 180 (+) (g/L of culture medium). Error bars represent the SD of the mean of at least nine measurements (three independent experiments × three measurements for each parameter).

added, the more lipid accumulation was favored. Our view is that increased production of acetyl-CoA, the building unit for the biosynthesis of fatty acids and from fatty acids to other lipids, resulting from the catabolism of high amounts of glycerol via glycolysis may be the key to increased cellular lipid content. Still, a significantly lower cellular lipid content was observed (0.15–0.31 g/g of total dry biomass) compared with that occurring from glucose supplemented with vegetable oils (0.40–0.60 g/g of total dry biomass) (*6*). The above findings are in agreement with those reported for the oleaginous yeast *Yarrowia lipolytica* when grown on mixtures of glucose and glycerol and on industrial fats in shake flasks (*14, 17*).

According to Tereshina and collaborators (*27*), the main fraction of total lipids in mated cultures of *B. trispora* cells is expected to be in the form of TAGs. Cellular TAGs represent the energy storage lipids and also the site into which lipophilic carotenoids are deposited. In media containing glucose as a sole carbon source or glucose plus glycerol at 10 or 30 g/L of culture medium, utilization of storage lipids was triggered during the stationary growth phase (i.e., 60–288 h), when most of the extracellular carbon was consumed (**Figure 1b**,**d**,**e**). Storage lipid breakdown is a phenomenon observed for other oleaginous *Zygomycetes* following the transition to carbon-deficient growth conditions (*21, 28, 29*). It indicates that the biosynthetic abilities of the microbial population cannot be supported at this stage

solely by the extracellular carbon source and that the carbon pool needs to be supplemented by biodegradation of the storage lipids. Because total biomass remained always constant during the storage lipid utilization period, it can be concluded that synthesis of lipid-free material occurred. This is in agreement with results obtained for other members of *Zygomycetes* growing on glucose (*21, 28*). On the other hand, in the media with higher glycerol contents (i.e., 60, 90, and 180 g/L of culture medium), no degradation of storage lipids was observed due to culture conditions concerning mainly the carbon source availability (**Figure 1b**,**d**,**e**).

The growth of *B. trispora* on mixtures of glucose and pure glycerol resulted in a sequential consumption of hydrophilic carbon components; glucose was primarily assimilated, and when around 40.0% of initial glucose content was consumed, glycerol metabolism occurred (**Figure 1c**,**d**). This phenomenon can be explained by assuming higher specificity of *B. trispora* on glucose with regard to that on glycerol. This assumption is strengthened by the findings of Mantzouridou et al. (*16*), according to which the performance of microorganism was poorer on glycerol compared with that on glucose, each one used as the sole carbon source. This is in line with outcomes of the study of Dandekar and Modi (*15*), in which cultures of plus and minus strains of *B. trispora* were first grown separately on glucose-supplemented medium to obtain sufficient growth and subsequently inoculated into glycerol-supplemented medium for β-carotene production. However, our results are in contrast with those reported for the cases of *Y. lipolytica* and *Clostridium butyricum* grown on mixtures of glucose and glycerol, in which the substrate uptake rate was significantly higher for glycerol compared with that of glucose (*14, 30*). The different patterns suggest the strain-dependent specificity on these substrates.

In the cultures supplemented with glycerol up to 90.0 g/L, >80.0% consumption of the available glucose and nitrogen was observed during the early growth phase (0–60 h) (**Figure 1c**), whereas the quantities of glycerol available during different stages of fermentation depended on its initial level (**Figure 1d**). Specifically, at glycerol addition levels up to 30 g/L of culture medium, glycerol limitation occurred after 108 h of incubation. At 60 and 90 g/L of culture medium, glycerol limitation occurred after 230 h of incubation, indicating that part of the assimilated glycerol was absorbed at the late stage of cell growth. This observation strengthens our previous suggestion that in the latter cases reserve lipid breakdown was not observed during the late growth phase due to sufficient carbon availability for biosynthetic purposes. In all of the above cases, residual glycerol values were lower than 5.0 g/L at the end of fermentation, which is indicative of almost complete utilization of glycerol (**Figure 1d**). In cultures with a higher glycerol concentration (180.0 g/L), the lag phase was followed by the late appearance of glucose and nitrogen limitation (108 h). In this case, at the end of the fermentation, ∼16% of the initial glycerol amount was left unconsumed (**Figure 1d**). This finding suggests that the current medium formulation supports only ∼85% of the initial glycerol amount.

In all cases, the yield of the supplementary amount of total biomass produced per quantity of glycerol consumed was not proportional to the initial level of glycerol and the amount of glycerol consumed. Specifically, at low glycerol addition level (10 g/L of culture medium) total biomass yield per glycerol consumed was around 0.5 g/g, decreasing to 0.30 g/g at higher glycerol addition levels (30−180 g/L of culture medium). Thus, carbon flow could have been either channeled to the synthesis of extracellular organic acids or contributed to maintenance

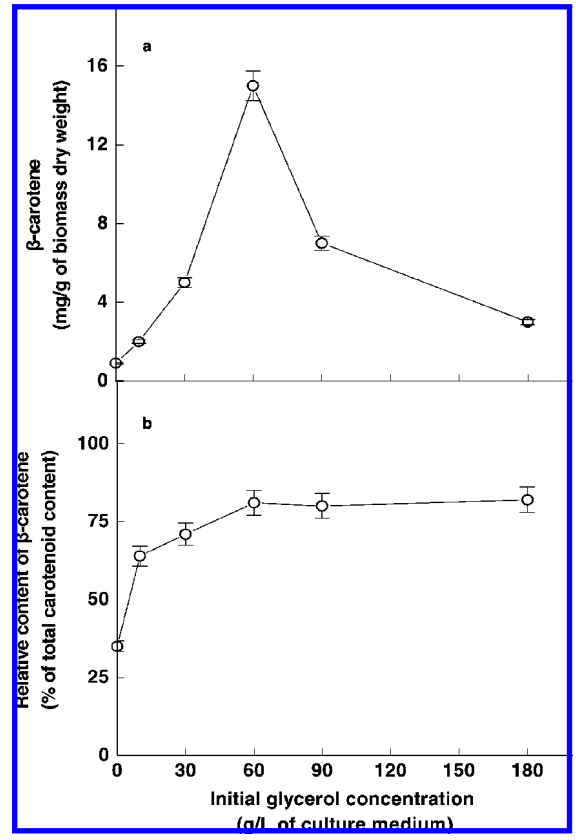


**Figure 2.** β-Carotene production by *B. trispora* grown in control medium in the absence/presence of different initial levels of pure glycerol. β-Carotene is expressed as mg/g of biomass dry weight (**a**) and as a percentage with reference to the total carotenoids produced (**b**). Error bars represent the SD of the mean of at least six measurements (three independent experiments × two measurements for each parameter).

energy requirements or to secondary routes. To respond to all of these assumptions, pH and total acidity expressed as citric acid, the predominant organic acid exuded from *B. trispora* (*2*), as well as DO level, were monitored in the culture medium under selected conditions (i.e., glycerol addition level of 10, 60, and 180 g/L of culture medium). The curves shown in **Figure 1f** indicate that in the cultures supplemented with glycerol at 10.0 and 60.0 g/L, small amounts of citric acid (1.1 and 1.6 g /L of culture medium) were produced during the first 24 h of the bioprocess. The production of citric acid was followed by a slight decrease in pH of the culture media (pH values between 5.75 and 6.0), after which it increased to pH value of 7.3 until the end of the fermentation process (**Figure 1g**). In these cases, between the late growth phase (60 and 288 h), some consumption of citric acid was observed. This trend was also evidenced in the case of glycerol addition level of 180 g/L, but with a delay of 36 h. Moreover, in all cultures the level of DO fell rapidly during the early growth phase and reached values near 20% of saturation (**Figure 1h**). This can be attributed to the strong respiratory activity accompanied by biomass formation. During the late growth phase teh DO level increased to almost 40% of saturation, indicating the cessation of respiration in all cases (*31*). On the basis of the above findings, the increased maintenance energy requirements, as well as enhancement of secondary metabolite production and more specifically carotenoid production, could explain the low carbon recovery.

**Figure 2** shows experimental results of β-carotene amount at the end of the fermentation process as a function of initial glycerol concentration in the culture medium. In all cases,

β-carotene was accumulated in mold cells in higher amounts as compared to when glucose was used as the sole carbon source. Over the range of glycerol levels tested, the highest β-carotene production (15.0 mg/g of dry biomass) was achieved in cells grown on an initial glycerol concentration of 60.0 g/L (**Figure 2a**). Under the above fermentation conditions the formation of lycopene and γ-carotene, the immediate precursors of β-carotene, was significantly restricted (<19% of total carotenoids). However, when the glycerol concentration was increased to 90.0 and 180.0 g/L, β-carotene production decreased to 7.0 and 3.0 mg/g of dry biomass, respectively, although the composition of carotenoid mixture remained almost unchanged (β-carotene constituted 80.0% of total carotenoids) (**Figure 2a**,**b**). In view of the fact that in *B. trispora* a catabolite repression mechanism has been related to secondary metabolism (*15*), it can be suggested that β-carotene synthesis is under catabolite repression in the presence of an initial glycerol level above 60.0 g/L.

Given that fatty acid and carotenoid synthesis share several common features (i.e., acetyl-CoA, NADPH), interpretation of cellular TAG composition under culture conditions that triggered the maximum β-carotene accumulation (i.e., glycerol addition at 60 g/L of culture medium) as a function of the fermentation time (e.g., 60 and 288 h after inoculation) was based on comparison with that of cells grown in control medium (glucose used as the sole carbon source) (data provided as Supporting Information). Cellular TAG composition during growth on a mixture of glucose and glycerol (60.0 g/L) showed an increase of the more unsaturated TAG groups of ECN 38, 40, and 42 at the expense of those of ECN 46 and 48 species during fermentation. However, this phenomenon was much stronger during growth on glucose. This indicates that, in contrast to cell growth on the mixture of glucose and glycerol, the microorganism grown on glucose as a sole carbon source exhibited significant desaturase activity during the late growth stages. These results are in agreement with changes observed in cellular lipid profile with regard to the age of other oleaginous microorganisms grown on glucose or on mixtures of glucose and glycerol (*14, 32*). In all cases, the intermediate degradation products diacyl- and monoacylglycerols were present at elevated levels at the end of the bioprocess compared with those at the beginning.

It can be argued that glycerol in the presence of glucose critically influences cellular metabolism and accumulation of β-carotene. Our view is that the key to efficient acetyl-CoA and NADPH production required for increased β-carotene content may be the energy excess by the catabolism of glycerol via glycolysis that directed glucose toward the pentose phosphate pathway, as also suggested in the case of *Y. lypolytica* growing on glycerol/glucose-based media for citric acid production (*14*). Moreover, enhancement of the synthesis of trisporic acid, a metabolic intermediate that activates carotenogenesis, by glycerol cannot be excluded (*15*). Maximum product concentration obtained in our batch experiments with pure glycerol (15 mg/g of dry biomass) was lower than those obtained using certain vegetable oils in our previous works (30–90 mg/g of dry biomass) (*6, 16*), because the latter are slowly metabolized nutrients and are mainly used for the production phase. Even so, β-carotene production achieved in this study is indeed noteworthy, in particular when compared with yields reported from batch cultures growing on other inexpensive hydrophilic carbon substrates (3–8 mg/g of dry biomass) (*1–5*).

From the results obtained so far, pure glycerol was found to be a suitable secondary carbon source for β-carotene production by *B. trispora*. On such grounds it was challenging to investigate the potential of crude types of glycerol as a feedstock of this fermentation process.

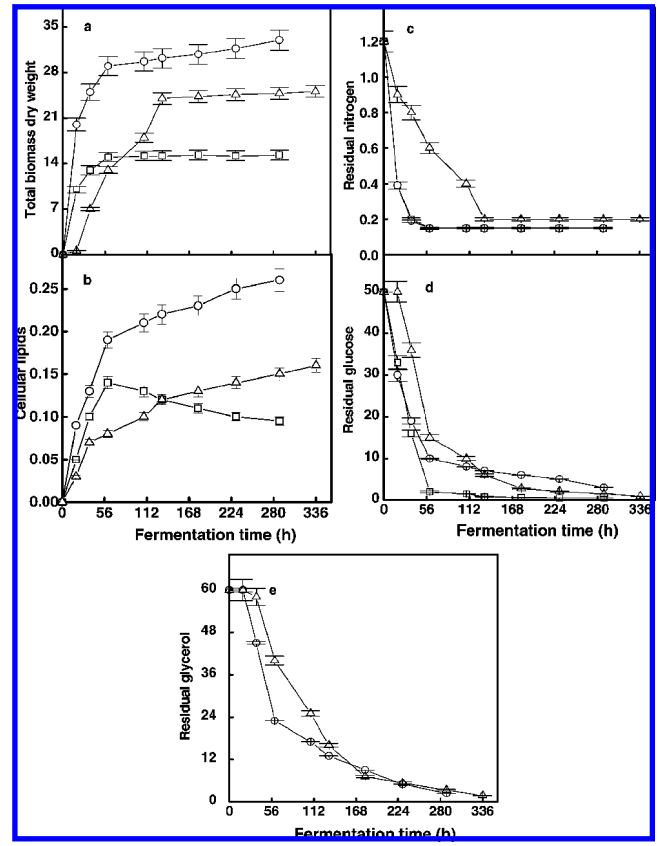


**Figure 3.** Kinetics of (**a**) total biomass dry weight (g/L of culture medium), (**b**) cellular lipids (g/g of biomass dry weight), (**c**) residual nitrogen (g/L of culture medium), (**d**) residual glucose (g/L of culture medium), and (**e**) residual glycerol (g/L of culture medium) during *B. trispora* growth in control medium plus industrial glycerol from soap manufacture at 60.0 g/L of culture medium (△) compared with those on glucose in the absence (○) and in the presence (□) of pure glycerol at the same level of addition. Error bars represent the SD of the mean of at least nine measurements (three independent experiments × three measurements for each parameter).

**Potential of Industrial Glycerol in the Production of β-Carotene.** *Effect of Industrial Glycerol from a Soap Manufacturing Process.* Nonpurified industrial glycerol solution from a soap manufacturing unit did not inhibit cell growth. The kinetics of *B. trispora* on this byproduct (**Figure 3**) showed a prolonged lag phase (18 h) accompanied by nonsignificant substrate uptake. Then an efficient cell growth with simultaneous cellular lipid accumulation and utilization of glucose and nitrogen substrate took place. Only when residual glucose became low did glycerol consumption occur (**Figure 3d**,**e**). Moreover, in the late growth stage, glycerol assimilation was uninterrupted. The final biomass value (25 g/L of culture medium) and cellular lipid quantity (0.16 g/g of dry biomass) were lower than the respective ones obtained in culture with pure glycerol (33 g/L and 0.26 g/g, respectively) but higher than those in control medium (15 g/L and 0.095 g/g, respectively) (**Figure 3a**,**b**). As shown in **Figure 3c**−**e**, lag phase and growth inhibition were followed by much lower utilization rates of carbon and nitrogen sources in the culture medium with industrial glycerol than in the medium with pure glycerol within the period of 120 h. This resulted in delayed nitrogen depletion and early growth phase termination (132 h) and, consequently, extension of the total fermentation process to 336 h.

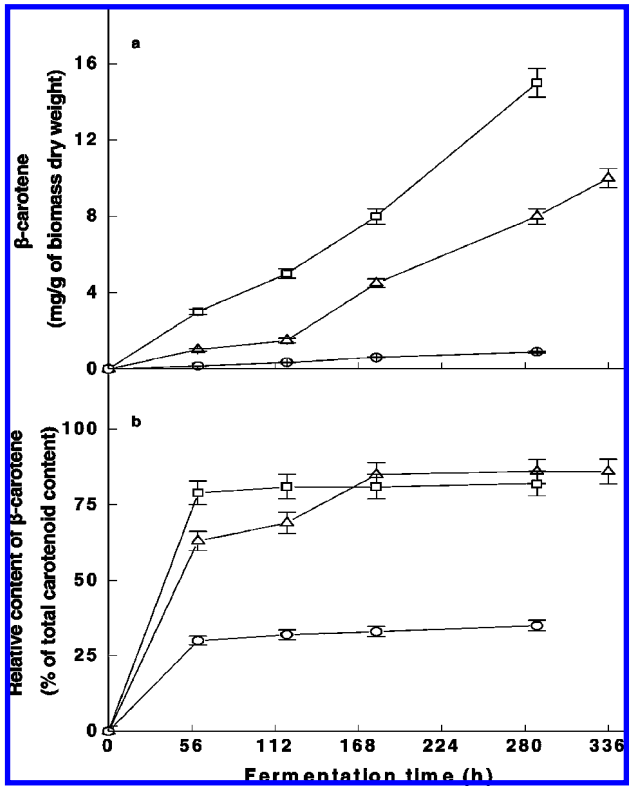


**Figure 4.** Kinetics of β-carotene production by *B. trispora* grown in control medium plus industrial glycerol from soap manufacture at 60.0 g/L of culture medium (△) compared with that on glucose in the absence (○) and in the presence (□) of pure glycerol at the same level of addition. β-Carotene is expressed as mg/g of biomass dry weight (**a**) and as a percentage with reference to the total carotenoids produced (**b**). Error bars represent the SD of the mean of at least six measurements (three independent experiments × two measurements for each parameter).

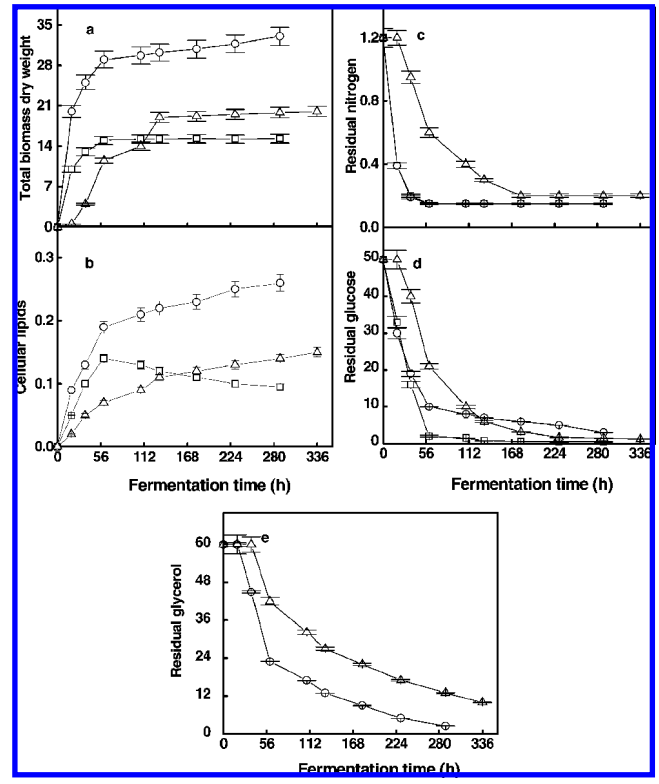


**Figure 5.** Kinetics of (**a**) total biomass dry weight (g/L of culture medium), (**b**) cellular lipids (g/g of biomass dry weight), (**c**) residual nitrogen (g/L of culture medium), (**d**) residual glucose (g/L of culture medium), and (**e**) residual glycerol (g/L of culture medium) during *B. trispora* growth in control medium plus industrial glycerol from biodiesel production at 60.0 g/L of culture medium (△) compared with those on glucose in the absence (○) and in the presence (□) of pure glycerol at the same level of addition. Error bars represent the SD of the mean of at least nine measurements (three independent experiments × three measurements for each parameter).

Because sodium chloride is used to facilitate separation of soap from glycerol byproduct, the latter is mixed with high quantities of this salt. Given that 10% (w/w) sodium chloride was present in industrial glycerol used in this study (verified by atomic emission measurements), about 1.5% (w/v) sodium chloride should be present in culture medium supplemented with such type of glycerol (60.0 g/L). Thus, salt stress could be one of the causal factors for the lag phase observed.

The kinetic behavior of *B. trispora* grown on industrial glycerol from the point of view of β-carotene accumulation is shown in **Figure 4**. As expected, β-carotene production was correlated with the late growth phase (*6*). β-Carotene production was slow within the first 120 h of the fermentation process, whereas a sharp increasing trend of β-carotene was evidenced in the last stage of cell growth (132–336 h), when residual nitrogen content in the medium was low. Although β-carotene was the main carotenoid during all stages of the fermentation process, its relative content within the early growth phase was less than that observed within the last stage of cell growth. This may be due to the low amount of industrial glycerol consumed in the early growth phase (**Figure 3e**). As a result, the amount of glucose directed toward the pentose phosphate pathway is low, so that the available NADPH is also low.

It should be noted that β-carotene production was higher than the respective in control medium (**Figure 4a**,**b**). At the end of the fermentation process β-carotene accumulated at 10 mg/g of dry biomass with maximum volumetric productivity of 20.8 mg/L/d and constituted 88.0% of total carotenoid content; these values were almost 11-, 20-, and 2.5-fold higher than those observed in control medium. On the other hand, β-carotene production and maximum volumetric productivity were lower than those observed using pure glycerol (15.0 mg/g and 38.8 mg/L/day, respectively), although the relative compositions of β-carotene in both cultures were similar within the last stage of cell growth (88 and 82%, respectively) (**Figure 4a**,**b**). It can be assumed that this negative response was due to the inhibitory effect of sodium salt on carotenogenesis, as reported earlier (*33*).

Glycerol produced as a byproduct of soap manufacture contributes significantly to the present world production of glycerol. However, the advent of the biodiesel industry has strengthened the link between glycerol generation and its economic exploitation. We next focused on the dynamics of biodiesel-originated glycerol as a fermentation feedstock.

*Effect of Industrial Glycerol from a Biodiesel Production Unit.* On the basis of information for the residual matter on crude glycerol sample, we calculated that the culture medium enriched with such type of glycerol (60 g/L) should contain ∼10% methanol. Hence, in this study, partial purification of crude glycerol by removing methanol and fatty acids was unavoidable. The mixture was first treated with hydrochloric acid to liberate free acids from the contaminating soaps. Removal of the lipid phase was achieved using a separation funnel after centrifugation. Methanol was then recovered by vacuum evaporation. At that point the glycerol concentration was 40% (w/w), whereas the amount of methanol left was <2%, w/w (measured as weight difference).

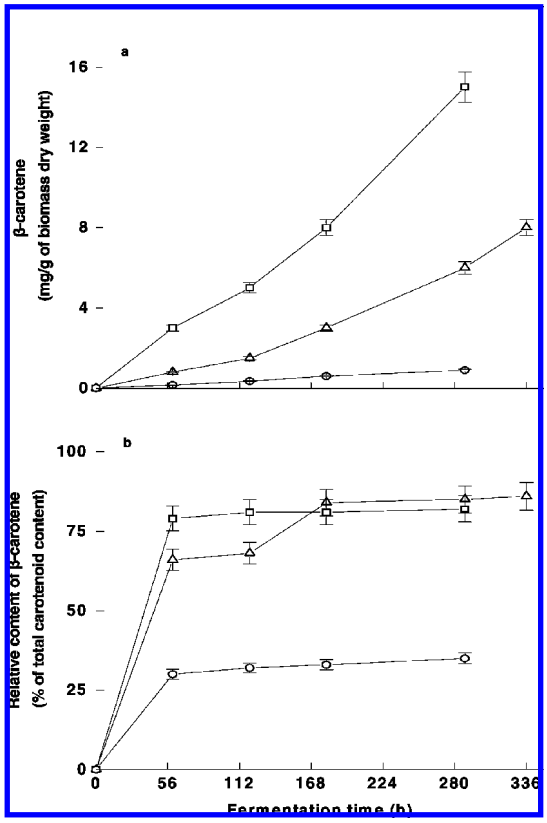


**Figure 6.** Kinetics of $\beta$-carotene production by *B. trispora* grown in control medium plus industrial glycerol from biodiesel production at 60.0 g/L of culture medium (△) compared with that on glucose in the absence (○) and in the presence (□) of pure glycerol at the same level of addition. $\beta$-Carotene is expressed as mg/g of biomass dry weight (**a**) and as a percentage with reference to the total carotenoids produced (**b**). Error bars represent the SD of the mean of at least six measurements (three independent experiments × two measurements for each parameter).

The growth characteristics of the culture grown on the pretreated industrial glycerol were compared to those obtained on pure glycerol (60 g/L) or in control medium. As can be observed (**Figure 5**), the use of the industrial glycerol provoked a clear delay of growth stimulation. At the end of the fermentation process, lower amounts of total biomass (20 g/L of culture medium) and cellular lipids (0.15 g/g of dry biomass) were detected when compared with those on pure glycerol (31 g/L and 0.26 g/g, respectively). On the other hand, these values were higher than those obtained during growth in control medium (15 g/L and 0.095 g/g, respectively). The growth characteristics of the culture with glycerol from the biodiesel production unit presented similarities with those of the culture with glycerol from the soap manufacturing unit in the feed (see **Figure 3**). It may be considered, hence, that the lag phase is partially due to a high inorganic salt content in the industrial feedstocks.

As shown in **Figure 6a**, the late growth phase (132–336 h) favored carotenoid production. Significant $\beta$-carotene yield (8.0 mg/g of dry biomass) was achieved at the end of the fermentation process. In addition, the accumulation of precursors lycopene and $\gamma$-carotene was avoided, which resulted in an increased production of the target carotenoid (86% of total carotenoid content) (**Figure 6b**). These values were much higher than that obtained using only glucose (0.9 mg/g of dry biomass and 35% of total carotenoid content). However, there was some decrease in $\beta$-carotene production and maximum volumetric productivity compared with those on pure glycerol (8.0 mg/g and 11.4 mg/L/day versus 15.0 mg/g and 38.8 mg/L/day, respectively), although the carotenoid patterns in both cultures were similar. It seems that the impurities present in industrial glycerol, such as sodium chloride, partially inhibit carotenogenesis in *B. trispora* cells.

Our findings suggest that industrial glycerol from soap manufacture or biodiesel production has a potential as a supplement to glucose substrate. Research with other types of industrial glycerol (e.g., physical refining process) may also have a similar effect. *B. trispora* produced satisfactory amounts of $\beta$-carotene at the expense of the formation of intermediate compounds. It is necessary to increase the productivity of the fermentation process, thereby decreasing the running costs. Optimization of the use of glycerol should also employ trials under feed-batch or continuous operations. Such efforts are under consideration in our laboratory.

## ABBREVIATIONS USED

DO, dissolved oxygen; PDA, potato dextrose agar; CSL, corn steep liquor; HPLC, high-performance liquid chromatography; RP, reversed phase; TAGs, triacylglycerols; ECN, equivalent carbon number; SD, standard deviation; NADPH, nicotinamide adenine dinucleotide phosphate; acetyl-coA, acetyl-coenzyme A.

## ACKNOWLEDGMENT

We are indebted to Dr. M. Sofoniou (Analytical Chemistry Laboratory, School of Chemistry, Aristotle University of Thessaloniki) for his assistance in verification of the sodium content of industrial glycerol samples through atomic emission spectroscopy and Professor Tr. Roukas (Department of Food Science and Technology, School of Agriculture, Aristotle University of Thessaloniki) for permission to use an oxygen probe.

**Supporting Information Available:** RP-HPLC profile of different ECN TAG species in *B. trispora* cells grown on glucose as the sole carbon source (Figure 1A,B, 60 and 288 h after inoculation, respectively) and on glucose plus pure glycerol at 60 g/L of culture medium (Figure 1C,D, 60 and 288 h after inoculation, respectively) using refractive index detection (chromatographic conditions as in Materials and Methods). This material is available free of charge via the Internet at http://pubs.acs.org.

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**Received for review December 17, 2007. Revised manuscript received February 1, 2008. Accepted February 12, 2008.**

JF703667D