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Lycopene Production Using *Blakeslea trispora* in the Presence of 2-Methyl Imidazole: Yield, Selectivity, and Safety Aspects

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The potential role of 2-methyl imidazole in improving lycopene production by *Blakeslea trispora* with regards to yield, selectivity, and safety aspects was investigated in batch culture. Optimization of the bioprocess conditions in terms of (a) (+) and (−) strain ratio in the inoculum, (b) initial crude soybean oil (CSO) addition level, and (c) the amount of 2-methyl imidazole was based on response surface methodology to achieve maximum lycopene production. The dependence of growth kinetics, lycopene yield, and selectivity of the bioprocess on the above factors was clear. 2-Methyl imidazole at 50 mg/L was found equally active in terms of lycopene cyclase inhibition with that at 200 or 100 mg/L; in all cases, lycopene accounted for 94% of the total carotenoids. The highest yield was observed at a 50 mg/L level of addition (24 mg/g of biomass dry weight,) in a substrate supplemented with CSO (48 g/L of culture medium) and inoculated with 1(+)/7(−) strain ratio.

KEYWORDS: Lycopene; 2-methyl imidazole; *Blakeslea trispora*; mating type; safety aspects; lycopene cyclase inhibitor

INTRODUCTION

The filamentous fungus *Blakeslea trispora* takes a prominent place among microbial producers of β-carotene, and more recently, lycopene due to its legal status (*1, 2*) and the ability to synthesize excess amounts of certain substances that promote high carotenoid yield (*3*). Commercially viable processes for the production of carotenoids from *B. trispora* have become an important alternative to synthetic manufacturing or extraction from natural sources considering: (a) the relatively high price of natural extracts, (b) the drawback of the non-natural character of synthetic carotenoids, and (c) the increasing preference of consumers for natural products. Production of β-carotene by *B. trispora* on a large scale involves well-developed technologies optimized for yield and purity of the final product. In the last few decades, several cultural and environmental factors have been studied extensively for their inducing effect on β-carotene production (*3, 4*). Still, the bioprocess of lycopene production at industrial scale is at early stages whereas basic research is rather limited and nonsystematic, especially when compared to the progress made for β-carotene production by this fungus. In view of these, further studies are motivated for the optimization and selectivity of the bioprocess routes.

In *B. trispora*, β-carotene, and not lycopene, is synthesized as the main carotenoid under the conventional cultivation conditions that involve the joint cultivation of mycelia of opposite mating type (i.e., plus (+) and minus (−)). To obtain lycopene, suppression of lycopene cyclase activity by chemical or genetic means is required in order to prevent the formation of β-carotene and promote the accumulation of lycopene. Literature review revealed that the use of special chemical compounds such as substituted amines and nitrogenous heterocyclic bases is an efficient approach for the regulation of lycopene formation. A great number of inhibitors have been tried over the last forty years (*5–8*). Imidazole appears to be the chemical inhibitor used in the formulation currently available as a novel food in the EU (*2*). Indeed, its use in the range 200−800 mg/L of culture medium in a recent semi-industrial scale trial (*9*) was successful in terms of yield and selectivity.

The main limitations of the process of lycopene production by *B. trispora* include the low yield of the final product and the relative toxicity of chemical stimulators. These parameters are pointed out in our recent review (*10*) concerning structure−activity relationships for compounds stimulating lycopene biosynthesis. The basic character of the inhibitors used so far and conformation seem to be more critical than lipophilicity. Basicity appears to ensure selectivity rather than total carotenoid yield. Still, increased basicity does not always coincide with increased stimulatory effect. Moreover, though some compounds present suitable basicity (pK_a values within the range 5−7), because of deviation from planarity they are not effective. Apart from the structural features necessary for inducer activity, the toxic character of the examined stimulants should not be overlooked when a chemical compound is selected for trial. This view is strengthened by current legislative demands on food safety precautions. In the present work, taking into account the

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relative effectiveness and toxicity of various inhibitors applied so far, we introduced 2-methyl imidazole instead of imidazole to reduce risks of this source of hazard for the bioprocess (*11*).

The total carotenoid yield as well as the lycopene production is directly dependent on sexual activity of *B. trispora*. The former is enhanced under conditions favoring trisporic acids synthesis. These compounds are sexual hormones that are extensively produced when (+) and (−) mating types are cultivated together (*3*). Since β-carotene is a precursor of trisporic acids, lycopene synthesis and cessation of β-carotene formation in the presence of a lycopene cyclase inhibitor are accompanied by the interruption of the sexual reproduction process (*12*). Therefore, inhibition of the end-carotene production would amount to a decrease in the production of total carotenoids and a new growth pattern of combined (+) and (−) cells of *B. trispora* heterothallic strains. For this reason, its examination was included in the present study.

The low lycopene yield in the *B. trispora* system does not conform to the requirements for commercialization of a biotechnological process. Our previous knowledge on effective β-carotene production using crude soybean oil (CSO) as a cosubstrate with glucose (*13, 14*) led us to the decision of using it in the case of lycopene production, too. This decision was supported by the fact that, in the presence of CSO, enhanced carotenoid content could be related to increasing levels of acetyl-CoA, NADPH, and ATP directed toward the carotenogenic pathway in the fungal cells. Moreover, in the major studies on lycopene production so far, vegetable oils (cottonseed oil, sunflower oil) were used in the culture media (*5, 7–9*), though not systematically.

Our work has been designed to manipulate external or cultural stimulants in terms of (a) the (+) and (−) strain ratio in the inoculum, (b) the initial CSO addition level, and (c) the amount of 2-methyl imidazole to achieve maximum lycopene production. Optimization of the bioprocess conditions was based on experiments arranged according to a central composite statistical design. A high-performance liquid chromatography (HPLC) procedure was employed to monitor the lycopene yield and selectivity of the bioprocess. Biochemical and kinetic elucidation regarding biomass formation, extracellular pH evolution, substrate assimilation, and lipid accumulation-degradation were also considered and discussed. To our knowledge, this is the first attempt to optimize the bioprocess for lycopene production by *B. trispora* in the presence of 2-methyl imidazole.

MATERIALS AND METHODS

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 TM 10% FS, DSM, Nutritional Products Ltd., Basel, Switzerland) was a gift of BASF Aktiengesellschaf (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 thiamine-HCl used for preparation of the culture medium and emulsifiers Span 20 and Tween 80 were from Scharlau Chemie S. A. (Barcelona, Spain). Crude soybean oil (CSO) was a gift of Soya Hellas S. A. (Athens, Greece). Corn steep liquor (CSL) was supplied by Amylum Hellas (Thessaloniki, Greece). 2-Methyl imidazole was from Sigma-Aldrich (Steinheim, Germany). All reagents were of analytical grade.

Table 1. Levels of Factors in Actual and Coded Values Used in the Experimental Design

factor	name	level				
		coded value				
		−a	−1	0	+1	+a
		actual value				
X_1	CSO level of addition (g/L of culture medium)	17	30	60	90	120
X_2	strain ratio (+)/(−)	0.040	0.070	0.135	0.200	0.230

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

Apparatus. A Hitachi U-2000 spectrophotometer (Tokyo, Japan) was used in the determination of the 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 UVVIS-206 diode array multiple-wavelength detector (Linear Instruments, Fremont, CA). The data from the detector were processed with the chromatographic software EZChrom (Sci Software, Inc., San Ramon, CA). A Consort C532 pH probe (Turnhout, Belgium) was used to measure the pH of the culture medium. Inoculated flasks were incubated in a rotary shaker (Model MkX, Stoke Poges, UK).

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. 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 (thiamine-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 shaking speed was 250 rpm. The flasks were inoculated with a spore suspension of each microorganism containing 5.0×10^6 spores/mL and incubated at 26 °C.

Initial Experiments. 2-Methyl imidazole was fed at 200 mg/L into the culture medium in the absence (medium 1) or in the presence of CSO at 10.0 g/L (medium 2). The fermentation conditions were the same as described above.

To determine the separate growth pattern of (+) and (−) strains of *B. trispora* on 2-methyl imidazole (200 mg/L), experiments were conducted using medium 2 inoculated with 5.0×10^6 spores/mL of (+) or (−) spore suspension. The fermentation conditions were the same as described above.

Experimental Design. Thirteen experiments were set according to an unblocked full factorial central composite statistical design (*15*) for the study of two factors, namely CSO level of addition (X_1) (g/L of culture medium) and strain ratio (+)/(−) (X_2), each at five experimental levels. The levels of the Xj factor are coded as follows: −a, −1, 0, +1, +a, where $a = 2^{n/4}$ (n = number of variables) and −1, +1, and 0 correspond to the low, high, and midlevel of Xj (**Table 1**). The actual levels of each factor were calculated using the following equation:

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

The 13 runs were set by the use of the software Minitab Release 13.20 (Minitab, Inc., State College PA) (**Table 2**). The design had five of the factorial points at the center of the design replicated for the estimation of error.

The most commonly used empirical models, polynomial response surfaces, were fitted to three response variables, namely lycopene yield (*LY*) (mg/g of biomass dry weight), productivity (*P*) (mg/(L d)), and biomass (*B*) (g/L). Statistical analysis of the experimental data was

Table 2. Experimental Design for Five-Level-Two-Factor Central Composite Design and the Comparison between Observed and Predicted Responses for Lycopene Yield (*LY*), Productivity (*P*), and Biomass (*B*)

			LY (mg/g of biomass dry weight)		*P* (mg/(L h))		*B* (g/L of culture medium)	
run	CSO level of addition (g/L of culture medium)	strain ratio (+)/(−)	actual	predicted	actual	predicted	actual	predicted
1	60.000	0.040	3.500	3.699	14.000	14.336	25.500	25.886
2	30.000	0.200	3.300	3.344	6.500	6.544	17.000	17.426
3	17.574	0.135	4.100	4.165	9.500	9.810	13.900	13.736
4	60.000	0.135	7.000	6.820	17.500	17.540	16.000	16.220
5	60.000	0.135	6.700	6.820	18.000	17.540	16.300	16.220
6	60.000	0.135	6.400	6.820	17.200	17.540	16.200	16.220
7	30.000	0.070	4.800	4.634	14.500	14.033	18.100	17.928
8	60.000	0.135	7.000	6.820	17.500	17.540	16.000	16.220
9	60.000	0.230	3.600	3.501	10.000	9.614	25.000	24.539
10	60.000	0.135	7.000	6.820	17.500	17.540	16.600	16.220
11	90.000	0.070	2.900	2.756	14.000	14.006	31.000	30.649
12	90.000	0.200	3.700	3.766	14.300	14.817	29.000	29.247
13	102.426	0.135	3.100	3.135	16.000	15.640	31.000	31.089

performed by response surface methodology (RSM) using the software Minitab Release 13.20 (Minitab, Inc., PA). Details of response surface methodology can be found elsewhere (*15*). Initially, the second-order polynomial model was fitted to each response giving an equation of the form:

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

where Y is the predicted *LY*, *P*, and *B* response, X_1 and X_2 represented the levels of the coded factors according to **Table 1**, and β_0, β_1,..., β_{12} represented the estimated coefficients with β_0 having the role of a scaling constant. The quality of the fit of the model was evaluated by the coefficients of determination (R^2), the significance of each parameter through the F-test (calculated p value), and the lack of fit of the model. Coefficients with a p value lower than 0.05 were considered significant. Where possible, the model was simplified by omission of terms, which were not statistically significant. Optimization of the fitted polynomials for *LY* and *P* was performed using the same software. The combination of factor optimal values resulting in optimal responses was verified by conducting a simulation experiment in triplicate. Results were compared with model predictions.

Effect of 2-Methyl Imidazole Concentration on Carotenoid Biosynthesis. A set of experiments was performed under response surface optimum conditions using 2-methyl imidazole at concentrations from 10 to 200 mg/L of culture medium. The fermentation conditions were the same as described above.

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

Determination of Total Biomass Dry Weight. An aliquot of the culture liquid 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 grown on glucose as a sole carbon source were washed with distilled water or as previously described (*n*-hexane). In both cases, the biomass was determined from dry matter (80 °C/24 h). 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 3 times (each session lasted 1 h at room temperature) using the Folch method (*16*). The extract containing carotenoids and other neutral lipids was then centrifuged at 10 000*g* for 10 min to remove cells, 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. The repeatability of extraction was satisfactory (CV% = 5.0, n = 3). Crude extracts were kept at −18 °C until further analysis.

RP-HPLC Analysis of Carotenoids. Crude extracts were first dissolved in a small quantity of tetrahydrofuran and then in acetone. All samples were filtered through a 0.45 μm membrane filter (Schleicher Schnell, Dassel, Germany) just before HPLC analysis. The analysis of the carotenoids by RP-HPLC was performed using the method previously developed by Mantzouridou and Tsimidou (*17*). 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 regards to published information. Quantification of lycopene was carried out at 472 nm. Percent carotenoid content was calculated on the basis of the sum of the three peaks recorded at 472 nm. Analysis of samples was carried out in duplicate (CV% = 6.2, n = 5 for a 10 ppm standard solution).

Determination of Residual Oil, Nitrogen, and Sugar Content. 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. Residual sugars and nitrogen in the supernatant were determined as described by Mantzouridou et al. (*13*). All measurements were obtained in triplicate.

RESULTS AND DISCUSSION

Initial Experiments. The evolution of the fermentation parameters for lycopene production by mated cultures of *B. trispora* using 2-methyl imidazole at a predetermined addition level (200 mg/L) is presented in **Figure 1**. The addition level of the inhibitor was that which had been found effective under certain microbial growth conditions in the past (*5*). Typical fermentation media for lycopene production in the presence of lycopene cyclase (LC) inhibitor (e.g., imidazole) are either hydrophilic (e.g., glucose) (*18*) or mixtures of hydrophilic (e.g., corn starch, corn condensed distiller's solubles) and hydrophobic substances (e.g., vegetable oils) (*5, 9*). Since 2-methyl imidazole has been used only in vegetable enriched medium (*5*), in our initial experiments, we comparatively tested the efficiency of the culture media containing glucose (50 g/L of culture medium; medium 1) and glucose plus crude soybean oil (CSO) (10 g/L of culture medium; medium 2) on lycopene production. The effectiveness of the above environmental conditions for sufficient cell growth, carotenoid yield, and β-carotene production has been presented in the past (*13, 14*).

The curves of total biomass (lipid-free material plus cellular lipids) and of cellular lipid accumulation versus time in the different culture media are presented in **Figure 1a**,**b**. Noticeably, the growth pattern of the fungus was similar to that reported in our previous work for carotenoid production in the absence of the inhibitor (*13*). In all cases, the total biomass value increased

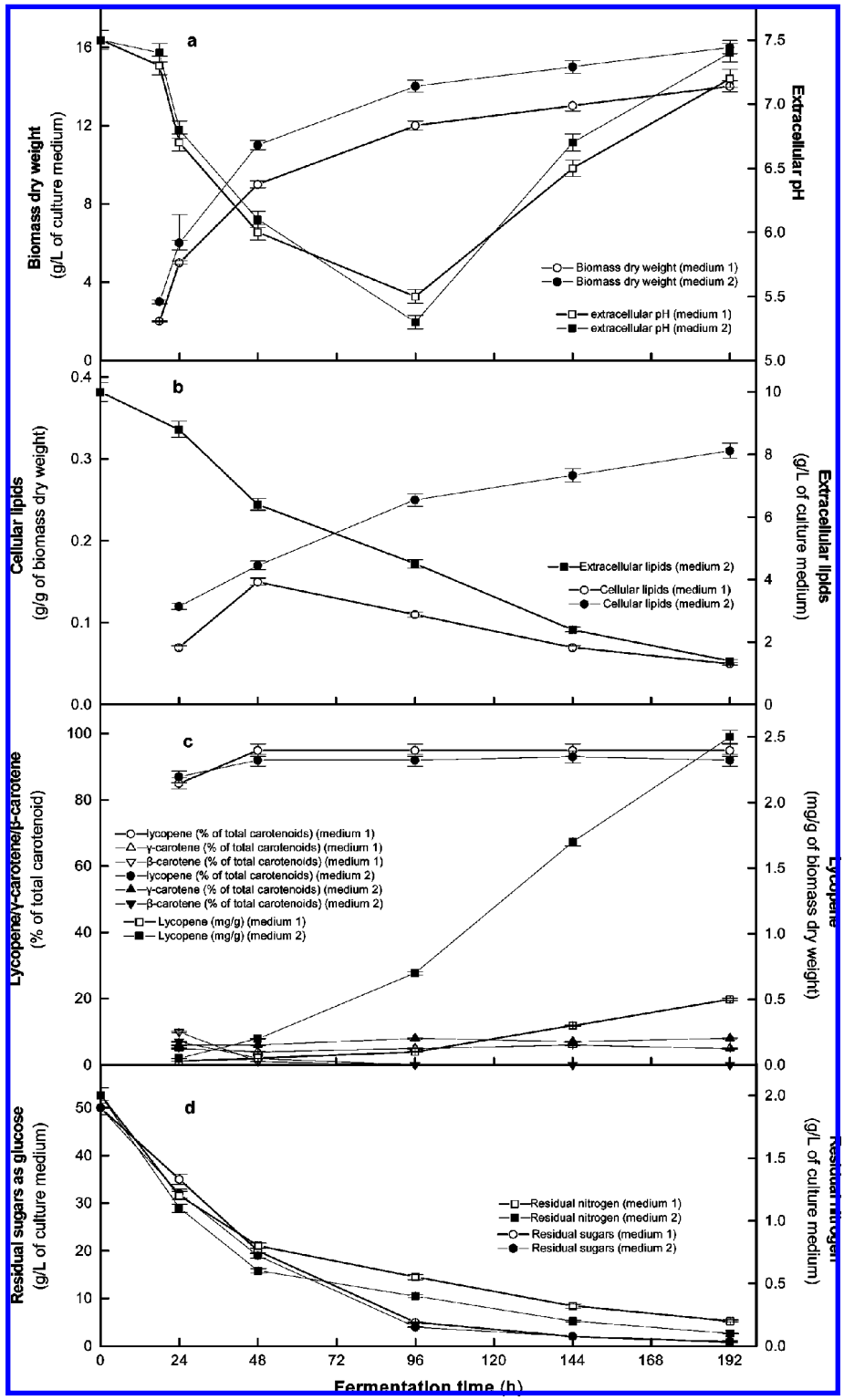

Figure 1. Kinetics of total biomass dry weight (**a**), medium pH (**a**), cellular lipids (**b**), extracellular lipids (**b**), carotenoid content (**c**), residual sugars (**d**), and residual nitrogen (**d**) during *B. trispora* growth in media 1 and 2 using 2-methyl imidazole at a predetermined addition level (200 mg/L) (See the Materials and Methods section for descriptions of media composition). Error bars represent the SD of the mean.

dramatically in the first 96 h (early growth phase) coinciding with significant acidification of the culture medium, and then it increased slightly until the end of the fermentation process (**Figure 1a**). In medium 1, the kinetics of cellular lipid formation within the first 48 h was similar to that of total biomass evolution (**Figure 1b**). Additionally, between 48 and 192 h, a degradation of the cellular lipids occurred. However, in medium 2, reserve lipids were not mobilized at the late growth phase (96−192 h after inoculation) (**Figure 1b**). In this medium, the final total biomass and the quantity of cellular lipids were higher when compared with those obtained during growth in medium 1. It seems that the incorporated oil substrate was assimilated for growth as well as cellular lipid synthesis.

As can be seen in **Figure 1c**, in both media, addition of 2-methyl imidazole at 200 mg/L of culture medium led to an almost complete inhibition of lycopene cyclization and allowed high selectivity of the bioprocess. Specifically, in all fermentation stages, lycopene was the main component of the total

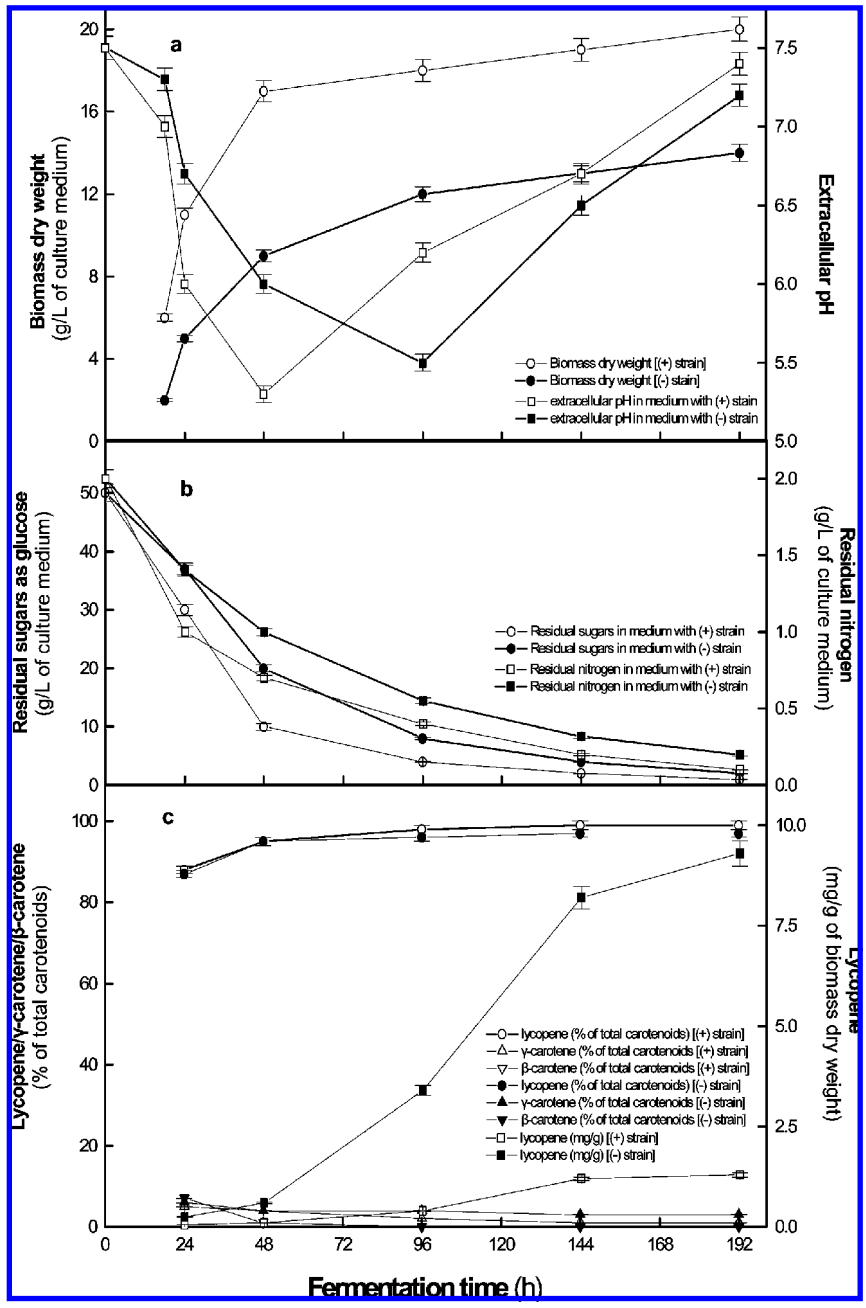

Figure 2. Kinetics of total biomass dry weight (**a**), medium pH (**a**), residual sugars (**b**), residual nitrogen (**b**), and carotenoid content (**c**) during separate growth of (+) and (−) strains of *B. trispora* in medium 2 using 2-methyl imidazole at a predetermined addition level (200 mg/L) (See the Materials and Methods section for descriptions of media composition). Error bars represent the SD of the mean.

carotenoid content (e.g., 85−87% after 24 h, and 92−95% from that time till the end of the fermentation process). Production of γ-carotene accounted only for 5−8% of the total carotenoids whereas no β-carotene was found after 24 h. Lycopene synthesis was slow for 48 h after inoculation, but a sharp increase was evidenced later on, especially during the late growth phase. In the latter, residual glucose and nitrogen content were low, and most of the oil of medium 2 was taken by the fungal cells (**Figure 1b**,**d**). Lycopene yield in medium 1 was significantly low (0.5 mg/g of biomass dry weight or 5.5 mg/L of culture medium) (**Figure 1c**). Addition of CSO as a supplementary carbon source to glucose caused an 8-fold increase in lycopene accumulation (**Figure 1c**). In this case, the maximum lycopene yield achieved was 2.4 mg/g of biomass dry weight (or 45 mg/L of culture medium). However, carotenogenesis was significantly restricted in the presence of LC inhibitor when compared with that observed in its absence (*13*).

The presence or absence of oil substrate into the culture medium had no effect upon the consumption of glucose (**Figure 1d**). In medium 2, at stages 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 (**Figure 1b**,**d**). At later stages characterized by high biomass accumulation, a high consumption rate of oil substrate was evident. Based on the above findings, the next experiments for the optimization of lycopene production in the presence of 2-methyl imidazole were conducted using glucose and CSO as cosubstrates.

Taking into account that *B. trispora* cells grow under stress in the presence of LC inhibitor (*12*), the next objective was to clarify the growth pattern of each mating type cultured separately

Table 3. Model Equations for Prediction of the Optimum Response Values

model	response	polynomial equation[a]	
		coded value of factors	actual value of factors
1	Lycopene yield (*LY*) (mg/g of biomass dry weight)	$LY = 6.820 - 0.364X_1 - 0.070X_2 - 1.585X_1^2 - 1.610X_2^2 + 0.575X_1X_2$ (2)	$LY = -3.2 + 0.16X_1 + 84.12X_2 - 0.002X_1^2 - 381.1X_2^2 + 0.29X_1X_2$ (3)
2	Productivity (*P*) (mg/(L h))	$P = 17.540 + 2.062X_1 - 1.670X_2 - 2.408X_1^2 - 2.782X_2^2 + 2.075X_1X_2$ (4)	$P = 3.87 + 0.25X_1 + 88.3X_2 - 0.003X_1^2 - 658.6X_2^2 + 1.1X_1X_2$ (5)
3	Biomass (*B*) (g/L)	$B = 16.22 + 6.135X_1 - 0.476X_2 + 3.096X_1^2 + 4.496X_2^2$ (6)	$B = 35.78 - 0.19X_1 - 287.73X_2 + 0.003X_1^2 + 1064.2X_2^2$ (7)

[a] X_1 and X_2 are the coded (eqs 2, 4, 6) or actual (eqs 3, 5, 7) values of factors presented in **Table 1**.

in media fed with 2-methyl imidazole (200 mg/L of culture medium). The evolution of the fermentation parameters during the different growth stages of each mating type are shown in **Figure 2**. As it is observed, the growth characteristics for the (+) and (−) strains were different. The results showed that: (a) (+) strain had a higher growth rate resulting in 1.4-fold higher values of final biomass compared with those of the (−) one (**Figure 2a**); (b) extracellular pH decreased more rapidly in medium with (+) cells than in that with (−) ones, due to the higher growth rate of the former (**Figure 2a**); and (c) glucose and nitrogen uptake rates were higher in medium with (+) cells than that with (−) ones (**Figure 2b**). Clearly, although the bioprocess for lycopene production was highly selective in both (+) and (−) cells, maximum lycopene accumulation in the latter was 6- and 3-fold higher than that of the (+) and mated cells, respectively (**Figure 2c**). Thus, optimization of the (+)/(−) strain ratio seemed necessary to avoid wash-out of the (−) strain during their cocultivation that would result in a decrease in carotenoid production.

Optimization Process for Lycopene Production Using the Response Surface Methodology. A central composite statistical design was used to select the experimental conditions under which the effects of the (+)/(−) strain ratio (X_1) and the initial CSO addition level (X_2) on lycopene yield (*LY*) (mg/g of biomass dry weight), productivity (*P*) (mg/(L d)), and biomass formation (*B*) were investigated. The model allowed assessment of factor interactions so that the response surface models fitted enabled optimization of the bioprocess conditions. Verification of the model was then tested experimentally.

Model Fitting for LY, P, and B. Experimental results for *LY*, *P*, and *B* were analyzed by ANOVA (for data, see the Supporting Information) to test the validity of each model on the basis of F-test values (see the Experimental Design section). The models, in terms of coded and actual factor values (**Table 1**), fitted for each one of the response variables are shown in **Table 3** (eqs 2–7). The experimental *LY* and *P* data were fitted to a full second-order polynomial model, since most of the factors have *p* values below 0.05 (for data, see the Supporting Information). X_2 appears to have no significant linear effect on *LY*; however, X_2 was included in eq 2, as it gave interactions with X_1 that were significant at the 5% level. Only in the case of the *B* response, the full second-order model was reduced by omitting the insignificant term X_1X_2 ($p \gg 0.05$) (**Table 3**) that describes the interaction effect of X_1 and X_2 on biomass formation. The ANOVA showed that there is no significant lack of fit ($p \gg 0.05$) of the models 1−3. Moreover, the coefficient of determination (R^2) was found to be within 0.988 and 0.998 indicating that more than 98% of the variability in the responses could be explained by the models. Consequently, the above equations can give an adequate description of the experimental data and are suitable for use in the optimization of the *LY*, *P*, and *B* responses.defau

Main Effects of Factors and Interactions on the LY, P, and B Responses. Eqs 2, 4, and 6 describe the significant linear effects of the factors (X_1, X_2) tested on *LY*, *P*, and *B*. It is apparent from the equations that X_1 has the greatest effect. Specifically, X_1 has a negative effect on *LY* and a positive one on *P* and *B*. As mentioned above, X_2 has no significant linear effect on *LY* ($p < 0.05$), whereas this factor showed a strong negative one on *P*. *B* was slightly affected. There were also significant negative quadratic effects of X_1 and X_2 on *LY* and *P*, indicating that these responses reach a maximum when X_1 and X_2 approach their midlevel (**Table 1**). In the *B* response, quadratic effects of both factors were positive. Significant

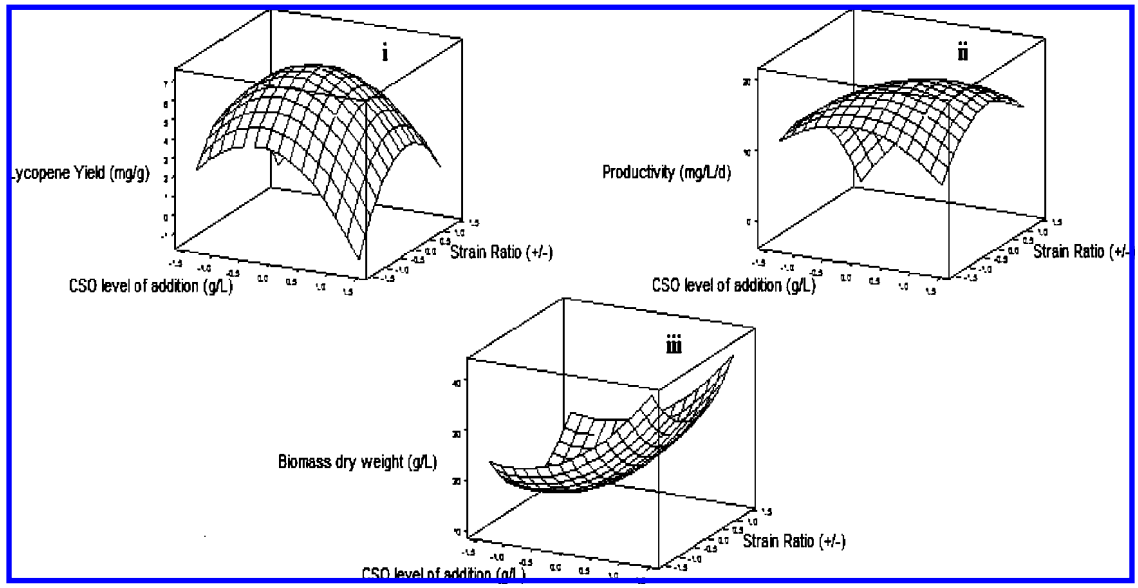

Figure 3. Surface plot for lycopene yield (*LY*) (**i**), productivity (*P*) (**ii**), and biomass (*B*) (**iii**) at varying levels of CSO level of addition and strain ratio (+)/(−).

Table 4. Optimum Values of CSO Level of Addition and (+)/(−) Strain Ratio and Maximum Predicted and Experimental Values of Lycopene Yield (*LY*) and Productivity (*P*)

factor	optimum actual value	predicted response value	mean expt response value[a]
		lycopene yield (mg/g of biomass dry weight)	
CSO level of addition (g/L of culture medium)	48	12.6	12.9 ± 0.6
strain ratio (+)/(−)	1/7		
		productivity(mg/(L d))	
CSO level of addition (g/L of culture medium)	60	17	19.5 ± 1.5
strain ratio (+)/(−)	1/7		

[a] Mean value of three independent experiments.

positive interaction was noted for X_1 and X_2 on *LY* and *P*, while there was no significant interaction effect on *B*.

Then, the fitted polynomial equations (eqs 2, 4, 6) were expressed as response surface plots (**Figure 3i**−**iii**) to visualize the relationship between the response of *LY*, *P*, and *B* and the experimental levels of each factor (X_1, X_2) and to deduce the optimum conditions. **Figure 3i** shows increasing *LY* as a function of CSO level and (+)/(−) ratio, with a maximum near the middle level of both factors (**Table 1**). In the same figure, the negative quadratic effect of both factors can be seen by the characteristic curvature of the response surface, especially at values of factors higher than their respective middle level. It is well established that carotenoid production is the outcome of the increase of carbon flux through N-lacking secondary routes. Indeed, low nitrogen content in the presence of excess organic carbon substrates has been proven effective in enhancing carotenoid production in different carotenogenic strains (*19, 20*). However, our results suggest that the effect of CSO on lycopene biosynthesis is concentration-dependent. **Figure 3i** reflects that this effect is more pronounced at low (+)/(−) ratio. As the surplus of cellular fatty acids represses nicotinamide adenine dinucleotide phosphate (NADPH) production through glucose 6-phosphate inhibition (*21, 22*), lycopene biosynthesis could be restrained in the presence of high oil level. Nevertheless, other more specific regulatory control mechanisms are expected to contribute, including feedback regulation of synthetases or oxygen availability (*13*). For the same CSO level, a trend toward higher *LY* appeared when using (+)/(−) ratios near 1(+)/7(−), whereas a significant decrease in lycopene yield was observed at ratios lower than 1 (+)/14(−) or higher than 1(+)/5(−). This effect suggests that extreme proportions of both strains may favor the growth of one strain and not the other, blocking carotenoid stimulation by mating (*9*).

In the *P* response surface plot (**Figure 3ii**), a trend toward higher *P* values is noted by increasing the factor values to middle level. By comparing **Figure 3i** and **ii**, the maximum value of *P* corresponds to higher values of CSO level than the respective ones for the *LY* maximum value. As shown in our initial experiments, in a low added oil system, especially at the beginning of the bioprocess where the biomass/oil ratio is low, a small amount of lipids is taken by the microorganism. Still, for a higher added oil level, the respective oil quantity consumed at the beginning of the fermentation is expected to be higher, due to a better dispersion of the oil in the culture medium, resulting in faster oil incorporation into the cells. This supports intracellular lipid synthesis and massive lycopene accumulation to be triggered under nonlimiting carbon conditions. **Figure 3ii** reveals also the negative quadratic effect of each factor on *P*. This means that, as in the case of *LY*, further increase of X_1 or X_2 values above their middle level cause a decrease in *P*. The negative quadratic effect of X_1 on *P* is more pronounced while keeping X_2 at the low level and vice versa, indicating a synergistic effect between the factors.

Figure 3iii shows the response surface of the factors X_1 and X_2 with respect to biomass formation. For the same X_2 value, the lowest value of *B* corresponds to the minimum of the X_1 and the highest value to its maximum. This means that higher CSO levels in the culture medium will increase the amount of biomass formed. A similar observation was also noted in β-carotene production by *B. trispora* using CSO as a cosubstrate with glucose (*13*). In the corresponding *B* response surface plot, a minimum point can be seen at the middle level of the X_2 values. By comparing the slopes of the surfaces shown in **Figure 3i** and **iii**, it is clear that there is a reversion in this region of the X_2 values. Thus, it appears that the use of (+)/(−) ratios near 1(+)/7(−) controls cell growth and favors secondary metabolism and more specifically carotenogenesis.

Optimum Conditions for LY and P. The X_1 and X_2 values determined to be optimum for *LY* and *P* responses by RSM

Table 5. Effect of Addition of 2-Methyl Imidazole Level on Biomass Formation and Carotenoid Pattern in *B. trispora*[a]

Inhibitor level (mg/L of culture medium)	carotenoid pattern (% of total carotenoids)			Lycopene(mg/g of biomass dry weight)	Biomass dry weight (g/L of culture medium)
	lycopene	γ-carotene	β-carotene		
200	93 ± 1	7 ± 1	–	12.5 ± 0.6	12 ± 0.4
100	95 ± 2	5 ± 2	–	14.0 ± 0.5	12 ± 0.3
50	94 ± 1	6 ± 1	–	24.0 ± 1.0	14 ± 0.4
10	85 ± 2	15 ± 2	–	8.5 ± 0.3	15 ± 0.5

[a] Data represent the mean values of three independent experiments.

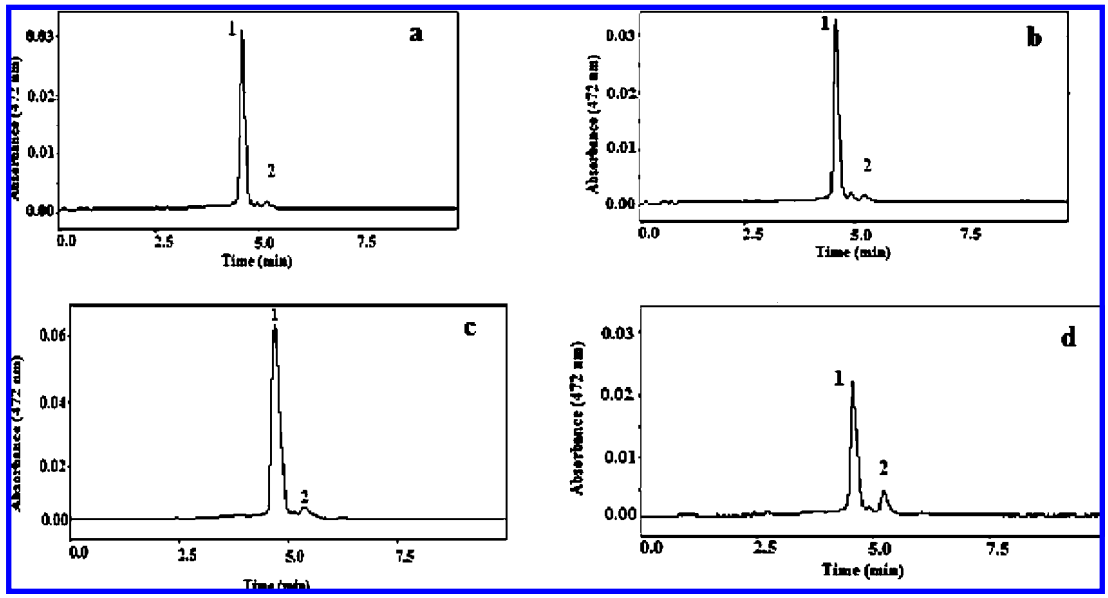

Figure 4. RP-HPLC carotenoid profile in *B. trispora* cells grown in culture medium fed with 2-methyl imidazole at 200 mg/L (**a**), 100 mg/L (**b**), 50 mg/L (**c**), and 10 mg/L(**d**) (1, lycopene; 2, γ-carotene) (chromatographic conditions as in Materials and Methods section).

optimization approach are presented in **Table 4**. The CSO level of addition and the (+)/(−) ratio combination of (48, 1/7) and (60, 1/7 ratio) can be recommended as optimum for *LY* and *P*, respectively. The above sets of conditions were substituted into eq 3 and 5, respectively, to predict maximum *LY* and *P* values; the optimum values calculated were 12.6 mg/g of biomass dry weight and 17 mg/(L d) for *LY* and *P*, respectively (**Table 4**).

Verification of Results. The suitability of the model equations for predicting the optimum response values was tested experimentally using the predicted optimum conditions. The experimental values were found to be in agreement with the predicted ones (**Table 4**). Under the predicted optimum conditions, growth of the mated culture was satisfactory (14−16 g/L of culture medium), and also, lycopene production was highly selective (lycopene accounted for 93% of total carotenoids). Moreover, maximum lycopene yield and productivity obtained experimentally using the optimized conditions were 12.9 ± 0.6 mg/g of biomass dry weight and 19.5 ± 1.5 mg/(L d). These values fit well with the respective predicted ones.

Effect of 2-Methyl Imidazole Level of Addition on Lycopene Production. Due to current legislative demands on food safety precautions, it is expected that the toxic character of the chemical added to the fermentation broth to inhibit the formation of γ- and β-carotene from lycopene has to be investigated by interested parties (i.e., food industry, regulatory authorities, consumers). For example, Feofilova and collaborators in their recently published data (*12*) on the use of 6-methyl-2-aminopyridine (MAP) that almost completely inhibited LC pointed out that this azine is nontoxic. Moreover, purity specifications set as maximum residue limits of imidazole not more than 1 mg/kg lycopene (*2*) in order to ensure that this product licensed as novel food ingredient is safe. Therefore, in this part of our work, we paid particular attention to practices that may reduce risk from this potential hazard. In this view, 2-methyl imidazole was fed into the fermentation broth at a descending order of addition level (200, 100, 50, 10 mg/L of culture medium). Carotenoid pattern in cells cultured under the above fermentation conditions are shown in **Table 5** and **Figure 4a−d**.

Inhibitor addition at 200, 100, and 50 mg/L was found equally effective and selective in terms of lycopene formation (**Figure 4a−c**). Moreover, at 50 mg/L of inhibitor, a 2-fold increase in yield was observed with regards to higher ones. The lowest level of addition used (i.e., 10 mg/L) was not effective in terms of both yield and selectivity (**Figure 4d**). It is of interest that in the presence of 2-methyl imidazole, the highest yield of lycopene was observed at a level of inhibitor that was significantly lower than those reported in previous studies for other chemicals (*5, 8, 9, 23*). In other words, the process of carotene cyclization was found to be more sensitive to 2-methyl imidazole than to other heterocyclic nitrogenous compounds used so far. For example, imidazole has been found effective at levels of addition exceeding 500 mg/L, though contradictory are the data for selectivity (*5, 9, 23*). Obviously, introduction of an electron donating group ($-CH_3$) affected positively the inhibitory activity of the tested compound. It is known that an electron donor substituent increases the electron density at the aromatic ring and, consequently, reduces the positive charge at the reaction center (nitrogen atom). This tends to stabilize the imidazole ion and increases the basicity of the molecule. In our recent review on structure−activity relationships for compounds stimulating lycopene biosynthesis in *B. trispora* cells (*10*), we stressed the

fact that between compounds of the same lipophilic character in terms of log *P* value, stabilization of the carbocation seemed to be important. This may explain the satisfactory performance of 2-methyl imidazole on LC inhibition.

The optimized bioprocess in the presence of 2-methyl imidazole at 50 mg/L of culture medium yielded to 2.5% of lycopene (94% of total carotenoids) in fungal biomass. The lycopene yield is 360 mg/L of culture medium. Taking into account that tomatoes contain 0.05−0.1% of lycopene in their dry biomass (*24*), the prospective of 2-methyl imidazole on lycopene production in future trials on both laboratory and industrial scale should be considered a promising area of research.

ABBREVIATIONS USED

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

Supporting Information Available: Analysis of variance of lycopene yield (*LY*), productivity (*P*), and biomass (*B*) for response surface quadratic model and estimated regression coefficients and significance (*p*) values for lycopene yield (*LY*), productivity (*P*), and biomass (*B*) after analysis using coded values of factors. This material is available free of charge via the Internet at http://pubs.acs.org.

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