Appl Microbiol Biotechnol (2004) 66: 153–159
DOI 10.1007/s00253-004-1669-4

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Biotechnological lycopene production by mated fermentation of *Blakeslea trispora*

Received: 18 March 2004 / Revised: 5 May 2004 / Accepted: 15 May 2004 / Published online: 10 July 2004

Abstract A semi-industrial process (800-l fermentor) for lycopene production by mated fermentation of *Blakeslea trispora* plus (+) and minus (−) strains has been developed. The culture medium was designed at the flask scale, using a program based on a genetic algorithm; and a fermentation process by means of this medium was developed. Fermentation involves separate vegetative phases for (+) and (−) strains and inoculation of the production medium with a mix of both together. Feeding with imidazole or pyridine, molecules known to inhibit lycopene cyclase enzymatic activity, enhanced lycopene accumulation. Different raw materials and physical parameters, including dissolved oxygen, stirring speed, air flow rate, temperature, and pH, were checked in the fermentor to get maximum lycopene production. Typical data for the fermentation process are presented and discussed. This technology can be easily scaled-up to an industrial application for the production of this carotenoid nowadays widely in demand.

Introduction

Carotenoids are an important group of natural pigments with specific applications as colorants, feed supplements, and nutraceuticals, and for medical, cosmetic, and biotechnological purposes. Despite the availability of a variety of natural and synthetic carotenoids, only a few have been exploited commercially, including β-carotene, lycopene, astaxanthin, canthaxanthin, lutein, and capxanthin (Liaaen-Jensen and Andrewes 1972; Nelis and DeLeenheer 1991; Johnson and Schroeder 1996; Bhosale 2004). Although more than 600 different carotenoids have been described from carotenogenic microorganisms (Pszczola 2002), only β-carotene and astaxanthin are commercially produced by microbial fermentation. These compounds have various biological functions, such as species-specific coloration, photo-protection, light-harvesting, antioxidant, and hormone precursor (Vershinin 1999; Lee and Schmidt-Dannert 2002). Dietary carotenoids have beneficial effects on the onset of many diseases, such as arteriosclerosis, cataracts, age-related macular degeneration, multiple sclerosis, cardiovascular diseases, and some kinds of cancer (Bhosale 2004); and probably for that reason the demand and market for carotenoids have grown drastically (Ulrich 2000). Lycopene also inhibits the harmful effect of ferric nitrilotriacetate on DNA in rats and prevents liver necrosis (Matos et al. 2001).

Commercial lycopene production relies on chemical synthesis and extraction from natural sources, e.g. tomatoes (Zelkha et al. 1997). However, due to the increasing importance of this molecule and to the mixture of carotenoids produced in plants and algae (Britton 1998), alternative ways of producing higher and more specific amounts have been sought. *Streptomyces chrestomyceticus* (Bianchi et al. 1969), *Blakeslea trispora* (Ninet et al. 1968; Lampila et al. 1985; Mehta et al. 2003), *Phycomyces blakesleeanus* (Murillo et al. 1978), and an engineered *Flavobacterium* sp. (Hohmann et al. 2000) have been described as lycopene-producing microorganisms, but always at the bench scale and, in most cases, with a weak specific yield. Scale-up to 20-l fermentation has been described for β-carotene production (Ciegler et al. 1963). Lycopene $C_{40}H_{56}$; molecular weight 526.8) is a red-colored intermediate of the β-carotene biosynthetic pathway. This pathway has been studied in fungi such as *P. blakesleeanus* (Arrach et al. 2001), *Mucor circinelloides* (Velayos et al. 2000), and *B. trispora* (Mehta and Cerdá-Olmedo 1995; Fig. 1); and a similar biosynthetic pathway is known in carotenogenic organisms (Lee and Schmidt-Dannert 2002). Either *B. trispora* strains without lycopene cyclase (Mehta and Cerdá-Olmedo 1995) or specific

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chemical inhibition of this enzymatic activity during the fermentation (either feeding tertiary amines, aminomethylpyridines or using a heterocyclic nitrogen basis, such as imidazole, pyridine, morpholine, quinolone, methylheptenone, etc.; Ninet et al. 1969; Bhosale 2004) are required for lycopene production. *B. trispora* produces a high level of lycopene (> 5% dry matter), with a yield greater than 95% of the total accumulated carotenoids (Ninet et al. 1968), a much higher level than those obtained by direct extraction from natural sources. Furthermore, the process can be enhanced by strain improvement programs and optimization of culture medium composition. For medium optimization in shake-flask experiments, the "one-at-a-time" method and statistical approaches, such as response surface methodology or genetic algorithms, are usually used. The "one-at-a-time" method assumes that no interactions occur among the different medium components, but in most medium optimizations this assumption is not true and gives only sub-optimal medium composition. However, genetic algorithms are non-model-based optimization methods with the ability to search a large parameter space in a highly directed way (Weuster-Botz and Wandrey 1995).

The objective of this study was the development of an optimized method for lycopene production by fermentation of *B. trispora* at the 800-l scale. The main parameters governing the fermentation process are presented and discussed. The industrial scale-up of this technology will provide a cost-effective process for the commercial exploitation of lycopene.

Materials and methods

Microorganisms and culture conditions

The sexual mating types *B. trispora* F-816 (+) and *B. trispora* F-744 (−) were obtained from the Russian National Collection of Industrial Microorganisms and maintained either in potato dextrose agar (Difco Laboratories) slants at room temperature for short-term maintenance, or at −180°C as glycerol stock solutions (40% v/v) for long-term maintenance. Spores from (+) and (−) strains were inoculated in separate vegetative flasks with 100 ml of VM culture medium (23 g/l soybean meal, 47 g/l corn meal, 0.5 g/l KH_2PO_4, 2 mg/l thiamine hydrochloride; pH 6.3) and incubated at 25°C for 48 h. For flask fermentation, a mix of both vegetative cultures was used to inoculate 50 ml of FM culture medium (44 g/l soybean meal, 19 g/l corn meal, 1.5 g/l KH_2PO_4, 2 mg/l thiamine

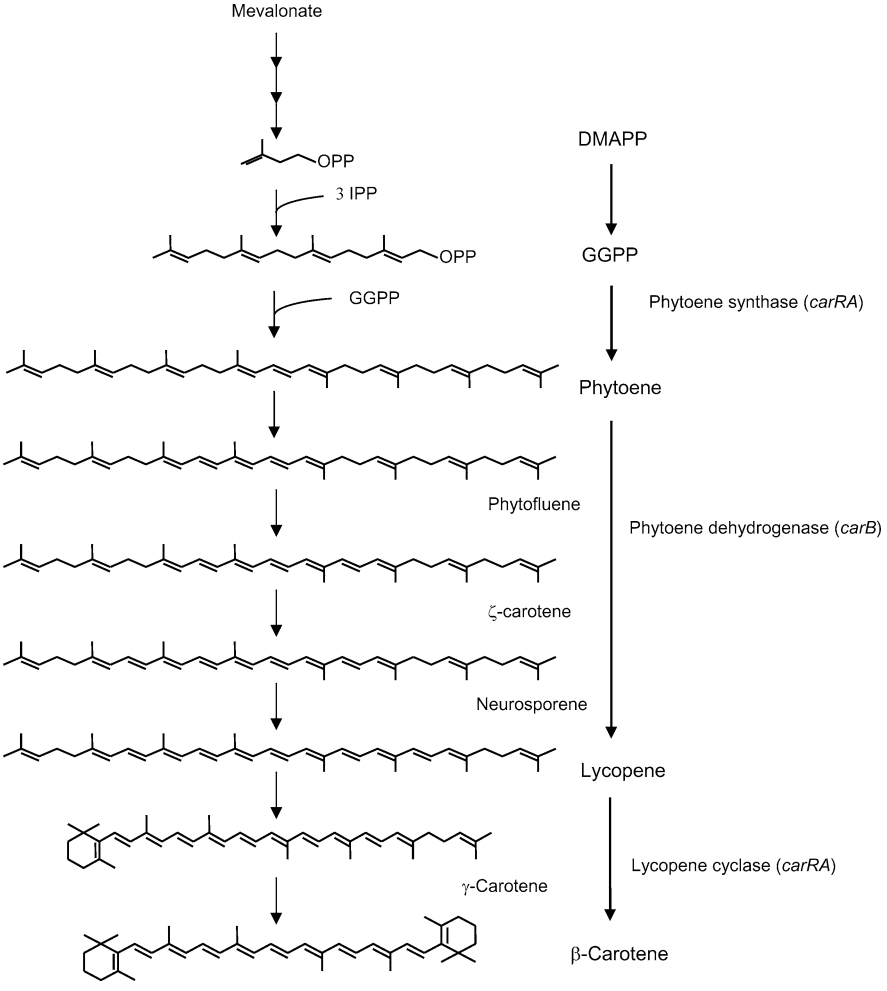

Fig. 1 Biosynthetic pathway leading to β-carotene in *B. trispora*. One molecule of dimethylallyl pyrophosphate (*DMAPP*) and three molecules of isopentenyl pyrophosphate (*IPP*) are coupled to form geranylgeranyl pyrophosphate (*GGPP*) and, subsequently, two molecules of GGPP are joined by phytoene synthase, encoded by the A domain of the *carRA* gene, to form phytoene. Afterwards, phytoene dehydrogenase, encoded by the *carB* gene, introduces four double bonds in the molecule of phytoene to yield lycopene. Finally, lycopene cyclase, encoded by the R domain of the *carRA* gene, sequentially converts one of the ψ acyclic ends of lycopene as a β-ring to form γ-carotene and subsequently the other to generate β-carotene

hydrochloride, 0.1 g/l isoniazide, 100 ml/l soybean oil; pH 7.5) and fermentation was carried out at 25°C for 120 h. For 800-l fermentation, vegetative flasks were transferred independently to separated 200-l pre-culture fermentors (first seed stage) with VM and were grown independently for 48 h under controlled conditions of temperature (25°C), airflow rate (0.66–1.50 vol./min), agitation rate (150 rpm), and backpressure (1 kg/cm^2). Afterwards, the packed cell volume (PCV; 2,000 *g* for 10 min at room temperature), pH, viscosity (Brookfield DV-II+ viscometer, Stoughton, Mass.), glucose, and phosphate were checked; and a mix of both vegetative cultures was used to inoculate the production stage. Fermentation was carried out for 120 h in FM (fed with polypropylene glycol as antifoam), using controlled conditions of temperature (25°C), airflow rate (1.00–1.25 vol./min), agitation rate (150–250 rpm), and back-pressure (0.5 kg/cm^2). PCV, pH, viscosity, CO_2, lycopene, and other carotenoids were checked during the process. Feedings were made via mass-flow meters or diaphragm dosing pumps. A chemical inhibitor of lycopene cyclase activity (pyridine or imidazole) was fed to improve lycopene production.

Carotenoid analysis

Cell homogenization for off-line analysis of intracellular carotenoids was performed with a laboratory vibration mill for 15 min, using glass beads (diameter 0.5 mm), 1–2 ml of cell sample, and 4 ml of methanol/dichloromethane (50:50). Carotenoids were extracted with methanol/dichloromethane (50:50) and finally re-dissolved in acetone. HPLC analyses were performed with the Hypersil ODS column (5 μm, 100×4.6 mm), using acetonitrile/methanol/chloroform (47:47:6) with a flow rate of 1 ml/min as the mobile phase.

Genetic algorithm

A suitable culture medium for lycopene production was designed, using the GALOP program based on a genetic algorithm (Weuster-Botz and Wandrey 1995) to set up a population of experiments (first generation) and, after evaluation, to produce a new population of experiments (second generation), and so on. The process continued until a suitable result was achieved.

Results and discussion

Medium design by a genetic algorithm at the flask scale

Five generations (Table 1, Fig. 2) were done, using soybean meal (44 g/l) and corn meal (19 g/l) as predetermined raw materials, and checking different concentrations of compounds known to improve lycopene production, including triton X-100, kerosene, isoniazide, KH_2PO_4, thiamine hydrochloride, soybean oil, and β-ionone (Lampila et al. 1985). After the first generation, a strong negative relationship between triton X-100 and lycopene formation was determined, mainly due to growth inhibition (Fig. 2). Therefore, the triton X-100 concentration was decreased for the second generation. Results of the second generation showed that the isoniazide concentration was too high; and this was decreased for the third generation. The third and fourth generations notably improved lycopene production. Finally, the results of the fifth generation did not improve on the best result of the fourth generation; and culture medium 11 was chosen as the most suitable for further fermentations (see FM medium in "Materials and methods").

Mated fermentation of *B. trispora* in 800-l fermentor for lycopene production

Separate vegetative stages of the sexual mating types F-816 (+) and F-744 (−) showed a faster growth rate for (+) as deduced from the evolution of the culture parameters (Fig. 3): (1) dextrin and glucose were minimal after 40 h for (+) and after 60 h for (−), (2) PCV of (+) reached higher values than that of (−), (3) pH and ammonium nitrogen began to rise earlier in (+) due to the use of nitrogen sources from the culture medium (soybean meal, corn meal) for growth.

The typical development of the fermentation stage is displayed in Fig. 4. Fungal growth was initially sustained

Table 1 Medium design by a genetic algorithm at the flask scale. Soybean meal (44 g/l) and corn meal (19 g/l) were included as predetermined raw materials in the culture medium

Raw material	Stock solution	Range of variation (μl stock solution)				
		First generation	Second generation	Third generation	Fourth generation	Fifth generation
Triton X-100	100 g/l	72–3,384	7–338	7–352	7–331	7–360
Kerosene	100%	120–6,000	120–5,880	120–4,560	120–960	120–2,400
Isoniazide	100 g/l	45–2,250	45–1,935	4–117	4–117	4–90
KH_2PO_4	100 g/l	150–1,470	150–1,470	150–1,440	60–1,440	120–900
Thiamine hydrochloride	10 g/l	6–294	6–228	6–228	6–228	6–156
Soybean oil	100%	600–5,760	1,440–4,920	720–5,160	2,400–5,160	2,400–5,280
β-Ionone	100%	48–528	60–504	12–456	48–432	24–576

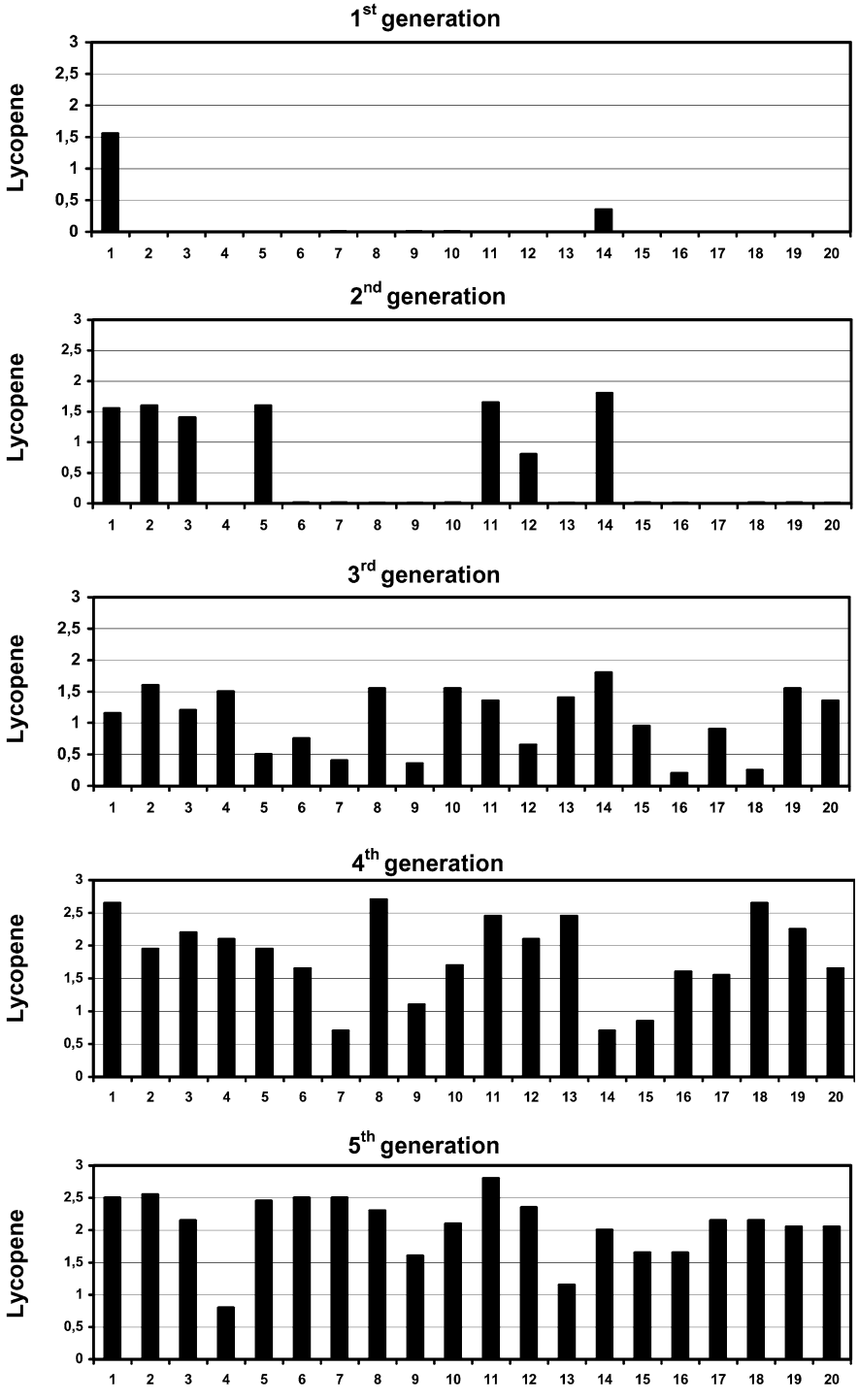

Fig. 2 Development of a fermentation medium for lycopene production using genetic algorithm. A total of 20 medium compositions were analyzed every generation according to the ranges showed in Table 1. Results are the average of duplicate fermentations (40 fermentations every generation) and, although lycopene production was analyzed after 96 h and 120 h (80 results every generation), only results from 120 h (arbitrary units) are shown. Culture medium 11 from the fifth generation was selected for further fermentations

by dextrin and glucose (coming from starch metabolism) and was then replaced by soybean meal and soybean oil, according to the carbon dioxide and respiratory quotient evolution rates (data not shown). The rise of phosphate and ammonium nitrogen after 120 h pointed to mycelium lysis. Mycelial biomass (measured as PCV) increased progressively until 72 h and then began to decrease. There was no defined set point for pH. Dissolved oxygen in the broth went down quickly until 20 h, due to the high rate of metabolism. It was maintained at 30–40% until 72 h and then increased progressively to 85% by the end of the batch cycle. Lycopene production was mostly associated with the second half of the fermentation cycle, when fungal metabolism was lower, glucose was exhausted, and most of the soybean oil had been incorporated into the mycelium. The specific lycopene production rate decreased after 140 h. Therefore, the biosynthesis and accumulation of lycopene seem to display a specific pattern in the secondary metabolism of *B. trispora*.

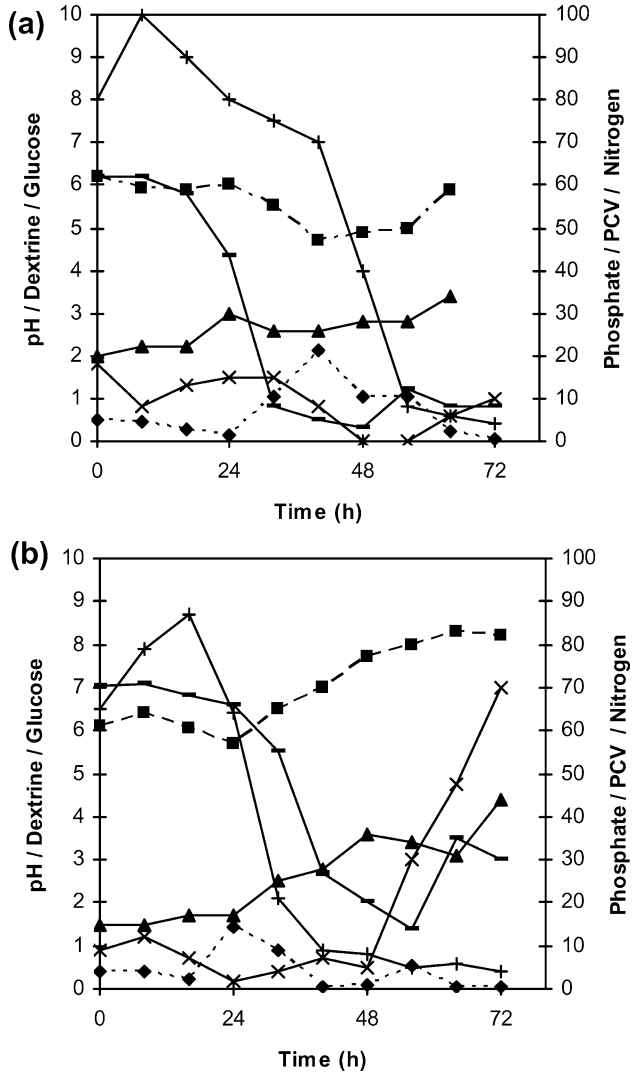

Fig. 3 **a,b** Standard development of *B. trispora* pre-cultures for 800-l fermentation. **a** F-744 (−) strain, **b** F-816 (+) strain. Parameters: pH (*filled squares*), glucose (g/l; *filled diamonds*), dextrin (g/l; *plusses*), PCV (%; *filled triangles*), ammonium nitrogen (1/10, mg/l; *crosses*), and phosphate (1/10, mg/l; *solid lines*)

The presence of pyridine or imidazole (Table 2), compounds known to inhibit the lycopene cyclase activity, reduced the level of β-carotene and γ-carotene, confirming that the β-carotene biosynthetic pathway in *B. trispora* is similar to that described in *P. blakesleeanus*, *M. circinelloides*, algae, and higher plants.

Effect of the vegetative phases on lycopene production

The effect of the ratio of (+) and (−) strains in the mated culture, the proportion of the seed, and the time-span of the vegetative phases were checked in order to get the best lycopene production. Taking into account the poor growth rate of the (−) strain, vegetative cultures were mixed in ratios from 1(+)/3(−) to 1(+)/20(−) before the fermentor was seeded. The maximum production of lycopene was achieved using ratios from 1(+)/10(−) to 1(+)/15(−), but

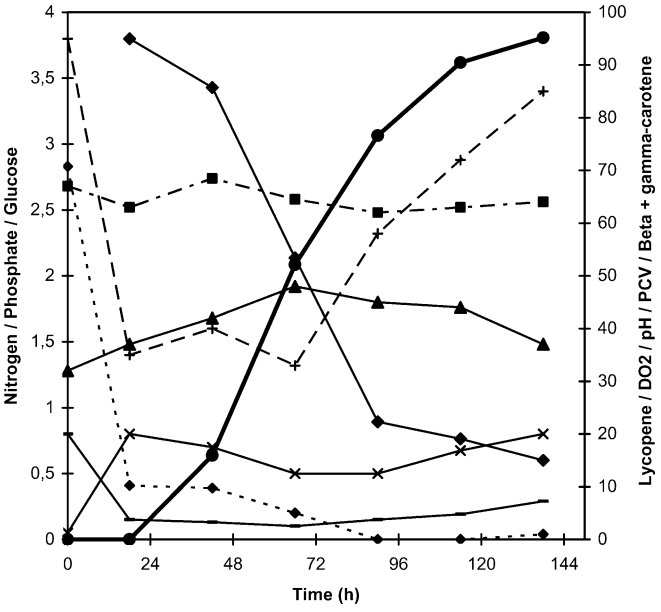

Fig. 4 Time-course of a standard 800-l fermentation of *B. trispora* for lycopene production. Lycopene is expressed as a percentage with reference to the standard production (100%). β-Carotene and γ-carotene are expressed as a percentage with reference to total carotenoids produced (lycopene, β-carotene, γ-carotene). Parameters: lycopene (*filled circles*), pH (*filled squares*), glucose (g/l; *filled diamonds in dotted line*), PCV (%; *filled triangles*), ammonium nitrogen (1/10, mg/l; *crosses*), phosphate (1/10, mg/l; *solid line*), dissolved oxygen (%; *DO2*, *plusses in dotted line*), and β-carotene and γ-carotene (*filled diamonds*)

Table 2 Lycopene production by feeding imidazole at 800-l scale. Average results from two independent cultures are shown. Similar results were obtained using pyridine. Lycopene is expressed as a percentage with reference to the standard production (100%). β-Carotene + γ-carotene are expressed as a percentage with reference to the total carotenoids produced (lycopene, β-carotene, γ-carotene). PCV (biomass volume) is expressed as a percentage with reference to the broth volume

Concentration (g/l)	Lycopene (%)	β-Carotene + γ-carotene (%)	PCV (%)
0.0	6	93	48
0.2	84	31	52
0.3	68	55	45
0.4	90	18	47
0.5	87	21	45
0.6	100	15	41
0.8	113	11	39

the accumulation of β-carotene and γ-carotene reached its highest concentration when using 1(+)/15(−). The use of extreme proportions of both strains, i.e. higher than 1 (+)/15(−) or lower than 1(+)/5(−), led to a significant decrease in lycopene, β-carotene, and γ-carotene production, probably due to a complete wash-out of one of the strains, avoiding carotenoid stimulation by mated culture. The highest lycopene production was obtained using a 20% seed proportion (with reference to the culture medium volume) and a 1(+)/10(−) ratio. Seed proportions higher than 20% led to an uncontrolled rate of growth

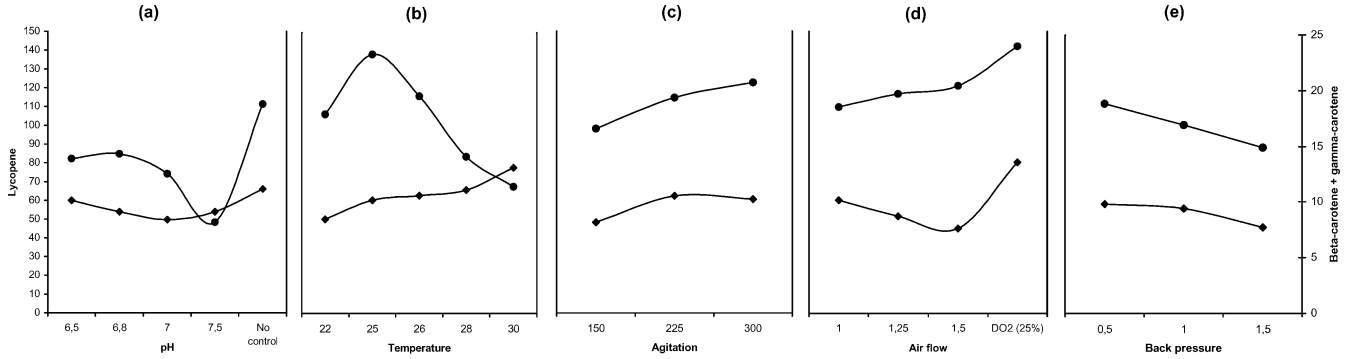

Fig. 5 **a–e** Effect of physical and chemical parameters of the 800-l fermentation process on lycopene production. Lycopene (*filled circles*) is expressed as a percentage with reference to the standard production (100%). β-Carotene and γ-carotene (*filled diamonds*) are expressed as a percentage with reference to total carotenoids produced (lycopene, β-carotene, γ-carotene). Parameters: **a** pH, **b** temperature, **c** agitation rate (rpm), **d** air flow rate [vol./min; *DO2 (25%)* 25% oxygen-enriched air], **e** backpressure (bar; 1 bar = 100 kPa)

(high viscosity) and consequently to poor oxygenation, deficient mass transfer, and lower lycopene production. Likewise, the time-span (from 24 h to 60 h) of pre-culture fermentors (first seed stage) was checked, showing an optimum for lycopene production around 48 h. Biosynthesis of β-carotene and γ-carotene was greatly enhanced using vegetative cultures after 60 h.

Physical and chemical fermentation parameters

After determining the optimal seed conditions for the production stage, the influence of the main fermentation parameters was studied. With reference to pH, the best lycopene production was obtained when this parameter was not controlled (Fig. 5a). The pH before sterilization of the medium was adjusted close to neutrality (pH 7.5), but pH control during the fermentation near this value showed poor results. pH evolution ranged from 6.3 to 6.6 (Fig. 4). Good results obtained without pH control are probably related to nutrient availability, the suitable development of both strains in the culture, and the inhibitory effect of pyridine or imidazole on lycopene cyclase activity. An incubation temperature of 25°C was optimal for lycopene production (Fig. 5b). Higher temperatures (28–30°C) led to poor lycopene production and increased accumulation of β-carotene and γ-carotene.

The main physical parameters affecting dissolved oxygen in the fermentor (agitation rate, air flow rate, backpressure) had an effect on lycopene production. Lycopene biosynthesis occurred when the fungal metabolism decreased and the dissolved oxygen level was not a limiting parameter (Fig. 4). Using low agitation rates (around 150 rpm), lycopene production was lower due to the failure of the fermentor to maintain the required level of dissolved oxygen in the culture broth (Fig. 5c); and the viscosity of the culture was higher, pointing to a great filamentous development of the biomass. When the agitation rate increased to 300 rpm, hyphae were shorter and lycopene production improved, but above 300 rpm carotenoid production and biomass decreased, due to shear stress. Lycopene biosynthesis was also improved by increasing the air flow rate (Fig. 5d), showing a positive effect of high dissolved oxygen and low carbon dioxide concentrations on lycopene production. The enrichment of oxygen concentration (up to 25%) in the air stream increased the accumulation of lycopene, but also β-carotene and γ-carotene. Finally, lycopene production decreased when backpressure was intensified (Fig. 5e), probably linked to the rise in dissolved carbon dioxide.

Inhibitory molecules of lycopene cyclase

Imidazole or pyridine, molecules known to inhibit lycopene cyclase enzymatic activity and promote lycopene accumulation (Lampila et al. 1985; Bhosale 2004), were fed into the fermentation broth at concentrations from 0.2 g/l to 0.8 g/l (Table 2). Using low inhibitor concentrations (0.2–0.3 g/l), the biosynthetic pathway was not completely inhibited at the lycopene cyclase step, which allowed a high accumulation of β-carotene and γ-carotene. A notable drop in β-carotene and γ-carotene and a great improvement in lycopene production were achieved by increasing inhibitor concentrations to 0.8 g/l. Concentrations of inhibitor higher than 0.8 g/l affected biomass growth and drastically reduced lycopene production.

Fermentation raw materials

Alternative raw materials and the feeding of effectors or nutrients during the fermentation cycle were assayed to improve mass transfer and mixing, by means of enhancing dissolved oxygen and reducing viscosity. Specifically, the addition of lecithin or citrus meal allowed lycopene production improvements (26% and 58%, respectively) with a drop in β-carotene and γ-carotene accumulation and a higher biomass (Table 3). The positive effect of citrus derivatives was also described in β-carotene production (Lampila et al. 1985) and could be related to activating molecules or to specific pentoses coming from the catabolism of citrus meal complex sugars. Soybean meal was in turn substituted by corn gluten, cottonseed meal and soybean grits, without success. Lycopene

Table 3 Effect of raw materials on lycopene production at the 800-l scale. Average results from two independent cultures are shown. Lycopene, β-carotene, γ-carotene and PCV are expressed as in Table 2

Raw material change			Production (%)		
Test nutrient	Concentration (g/l)	Replaced nutrient	Lycopene	β-Carotene + γ-carotene	PCV
–	–	–	100	11	39
Lecithin	3	–	113	11	41
Lecithin	5	–	116	10	46
Lecithin	7	–	119	9	42
Lecithin	10	–	123	7	45
Lecithin	20	–	126	7	47
Citrus meal	12	–	158	7	43
Corn gluten	44	Soybean meal	68	11	45
Cottonseed meal	44	Soybean meal	119	13	44
Soybean grits	44	Soybean meal	94	6	37
Sunflower oil	100	Soybean oil	116	6	39
Rape seed oil	100	Soybean oil	110	8	38
Glucose	10	Soybean oil (20 g/l)	95	12	41
Glucose	10	Soybean oil (40 g/l)	94	12	37
Glucose	20	Soybean oil (20 g/l)	74	18	39
Feed soybean oil	20	Soybean oil (20 g/l)	90	6	38
Feed soybean oil	40	Soybean oil (40 g/l)	77	5	37

production was improved (19%) using cottonseed meal, but this also increased β-carotene and γ-carotene and broth viscosity. With rape seed oil or sunflower oil instead of soybean oil, lycopene production was improved (10–16%), with a decrease in β-carotene and γ-carotene. Any attempt to replace the initial soybean oil, either by glucose or by delayed feedings of the same oil, always led to a drop in lycopene production. This behavior points to the need for a preliminary uptake of oil before the biosynthesis and storage of lycopene take place.

As a conclusion, a biotechnological process for the production of lycopene by mated culture of *B. trispora* at a semi-industrial scale has been developed. This technology can be scaled-up as a profitable industrial process for the production of this carotenoid nowadays widely in demand.

Acknowledgments Part of this work was supported by the grants FIT-010000-2001-132, FIT-010000-2002-121, and FIT-010000-2003-122 from Spanish Ministry of Science and Technology. The authors are indebted to Eva Suárez and the personnel of the Área de Biotecnología and Planta Piloto de Fermentación for their excellent technical assistance.

References

Arrach N, Fernández-Martín R, Cerdá-Olmedo E, Avalos J (2001) A single gene for lycopene cyclase, phytoene synthase and regulation of carotene biosynthesis in *Phycomyces*. Proc Natl Acad Sci USA 98:1687–1692

Bianchi ML, Francheschi G, Marnati MP, Spalla C (1969) Microbiological process for the production of lycopene. US patent 3,467,579

Bhosale P (2004) Environmental and cultural stimulants in the production of carotenoids from microorganisms. Appl Microbiol Biotechnol 63:351–361

Britton G (1998) Overview of carotenoid biosynthesis. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids: biosynthesis and metabolism. Birkhäuser, Basel, pp 13–147

Ciegler A, Lagoda AA, Sohns VE, Hall HH, Jackson RW (1963) Beta-carotene production in 20-l fermentors. Biotechnol Bioeng 5:109–121

Hohmann HP, Pasamontes L, Tessier M, Loon A van (2000) Fermentative carotenoid production. US patent 6,124,113

Johnson E, Schroeder W (1996) Microbial carotenoids. Adv Biochem Eng Biotechnol 53:119–178

Lampila LE, Wallen SE, Bullerman LB (1985) A review of factors affecting biosynthesis of carotenoids by the order Mucorales. Mycopathologia 90:65–80

Lee PC, Schmidt-Dannert C (2002) Metabolic engineering towards biotechnological production of carotenoids in microorganisms. Appl Microbiol Biotechnol 60:1–11

Liaaen-Jensen S, Andrewes AG (1972) Microbial carotenoids. Annu Rev Microbiol 26:225–248

Matos HR, Capelozzi VL, Gomes OF, Mascio PD, Medeiros MH (2001) Lycopene inhibits DNA damage and liver necrosis in rats treated with ferric nitrilotriacetate. Arch Biochem Biophys 396:171–177

Mehta BJ, Cerdá-Olmedo E (1995) Mutants of carotene production in *Blakeslea trispora*. Appl Microbiol Biotechnol 42:836–838

Mehta BJ, Obraztsova IN, Cerdá-Olmedo E (2003) Mutants and intersexual heterokaryons of *Blakeslea trispora* for production of beta-carotene and lycopene. Appl Environ Microbiol 69:4043–4048

Murillo FJ, Calderon IL, López-Díaz I, Cerdá-Olmedo E (1978) Carotene-superproducing strains of *Phycomyces*. Appl Environ Microbiol 36:639–642

Nelis HJ, DeLeenheer AP (1991) Microbial sources of carotenoid pigments used in food and feeds. J Appl Bacteriol 70:181–191

Ninet L, Renaut J, Tissier R (1968) Production of lycopene. US patent 3,369,974

Ninet L, Renaut J, Tissier R (1969) Activation of the biosynthesis of carotenoids by *Blakeslea trispora*. Biotechnol Bioeng 11:1195–1210

Pszczola DE (2002) Timely ingredient developments via fermentation. Food Technol 56:52–65

Ulrich M (2000) Business report: the global market for carotenoids. Business Communication, Norwalk

Velayos A, Eslava AP, Iturriaga EA (2000) A bifunctional enzyme with lycopene cyclase and phytoene synthase activities is encoded by the *carRP* gene of *Mucor circinelloides*. Eur J Biochem 267:5509–5519

Vershinin A (1999) Biological functions of carotenoids—diversity and evolution. Biofactors 10:99–104

Weuster-Botz D, Wandrey C (1995) Medium optimization by genetic algorithm for continuous production of formate dehydrogenase. Process Biochem 30:563–571

Zelkha M, Ben-Yehuda M, Hartal D, Raveh Y, Garti N (1997) Industrial processing of tomatoes and lycopene extraction. WO patent 97/48287