Improved β-Carotene and Lycopene Production by *Blakeslea trispora* with Ultrasonic Treatment in Submerged Fermentation

Hong-Bo Wang, Rong-Gang Xu, Long-Jiang Yu, Jun Luo, Li-Wei Zhang, Xiao-Yan Huang, Wen-An Zou, Qian Zhao, and Ming-Bo Lu*

Institute of Resource Biology and Biotechnology, Department of Biotechnology, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, 430074, China. Fax: 86-27-87 79 22 65. E-mail: mblu@mail.hust.edu.cn

* Author for correspondence and reprint requests

Z. Naturforsch. **69c**, 237 – 244 (2014) / DOI: 10.5560/ZNC.2013-0122
Received July 17, 2013 / February 19, 2014 / published online June 5, 2014

Response surface methodology (RSM) based on Box-Behnken design (BBD) was employed to investigate the effect of ultrasonic treatment on β-carotene production by *Blakeslea trispora*. The optimized strategy involved exposing three-day-old mycelial cultures to ultrasonic treatment at a fixed frequency of 20 kHz, power of 491 W, treatment time of 3 min, working time of 3 s, and rest time of 5.8 s, repeated four times at a 24-h interval. Mycelium growth was not significantly promoted under ultrasonic stimulation; however, the glucose metabolism increased by about 10%, the average size of the aggregates significantly decreased, and the uptake rate of imidazole into cells was increased about 2.5-fold. After a 6-d culture, the technique produced 173 mg/L of β-carotene and 82 mg/L of lycopene, which represented an increase of nearly 40.7% and 52.7%, respectively, over the yields obtained in cultures without ultrasonic treatment.

Key words: Blakeslea trispora, Carotenoid, Ultrasonic Treatment

Introduction

The biological function of β-carotene and lycopene in human wellness has drawn significant attention because of their commercialization in functional food, medicine, and cosmetic industries. Compared with other microorganisms, *Blakeslea trispora* exhibits a higher potential for β-carotene and lycopene production (Mantzouridou and Tsimidou, 2008). Different methods for improving the carotenoid production by *B. trispora* have drawn considerable research interest. In most studies, ingredients were added into the culture medium to promote carotenoid synthesis. The use of natural vegetable oils as co-substrate enhanced the fungal growth, subsequently increasing the carotenoid production (Mantzouridou *et al.*, 2006; Papaioannou and Liakopoulou-Kyriakides, 2010). Trisporoids and their analogues enhanced the expression of key metabolic enzymes and improved the carotenoid production (Sun *et al.*, 2012). Isopentenyl compounds and metabolic precursors (geraniol, isopentenyl alcohol, and mevalonic acid) were found to improve the lycopene production by *B. trispora* (Shi *et al.*, 2012). The addition of exogenous butylated hydroxytoluene enhanced the oxidative stress in *B. trispora* and increased the carotene production (Nanou and Roukas, 2010). *n*-Hexane and *n*-dodecane were added into the medium as oxygen vector to improve the lycopene and β-carotene production by *B. trispora* (Xu *et al.*, 2007). However, physical methods such as microwave and ultrasonic stimulation for carotenoid production by *B. trispora* have not been reported. High-frequency ultrasound (beyond 20 kHz) is widely employed for damaging cells and breaking cell walls to release intracellular products at the laboratory scale. Nevertheless, suitably applied ultrasound can enhance the productivity of bioprocesses involving live cells and bioactive enzymes (Sulaiman *et al.*, 2011). The effects of ultrasonic treatment on productivity enhancement have been reported for filamentous fungi (Sainz Herrán *et al.*, 2008). Ultrasonic treatment appears to provoke a highly complex process; thus, a new method for improving the β-carotene and lycopene production by *B. trispora* is proposed. The effects of ultrasonic

treatment on mycelium growth, glucose metabolism, β-carotene and lycopene production, mycelium morphology, and the uptake of imidazole into cells are discussed.

Materials and Methods

Chemicals and reagents

All chemicals, reagents, and solvents were of analytical or high-performance liquid chromatography (HPLC) grade (Aladdin, Shanghai, China).

Microorganisms

The microorganisms were *B. trispora* ATCC 14060 mating type (+) and *B. trispora* ATCC 14059 mating type (−). Both strains were purchased from the China Center for Type Culture Collection (Wuhan, China).

Culture conditions

The strains were grown on potato dextrose agar medium at 28 °C for 3 d, and the spores were used for inoculation of the culture medium.

Fermentation conditions

About 10^6 spores of *B. trispora* [1:4 mixture, by spore number, of ATCC 14060 (+) and ATCC 14059 (−)] were inoculated into 300-mL Erlenmeyer flasks containing 100 mL sterile medium for β-carotene and lycopene production (Choudhari and Singhal, 2008). Fermentation was conducted at 28 °C on a rotary shaker (200 rpm) for 7 d. The composition of the fermentation medium was as follows: 2 g/L Span® 20 emulsifier, 100 g/L glucose, 12 g/L yeast extract, 1.5 g/L Na_2HPO_4, 3 g/L KH_2PO_4, and 0.5 g/L $MgSO_4$. A solution of imidazole with a final concentration of 2 g/L in the culture medium was added for lycopene production after 72 h of fermentation.

Lycopene and β-carotene extraction

The mycelium was vacuum freeze-dried for 48 h and ruptured with liquid nitrogen by manual grinding until complete cell disruption. The samples were then subjected three times to stirring-assisted extraction with petroleum ether at room temperature.

Extraction of intracellular imidazole

The dry mycelium was ruptured by freezing using liquid nitrogen and then by manual grinding until complete cell disruption. Ultrasonic extraction of imidazole with distilled water was performed three times, for 1 h each, at room temperature.

Analytical methods

Lycopene and β-carotene analysis

β-Carotene and lycopene were separated using an Agilent (Santa Clara, CA, USA) HPLC system equipped with an Ascentis (Supelco, Bellefonte, PA, USA) RP column (RP-amide, 15 cm × 2.1 mm × 5 μm). The operating conditions were as follows: elution solvent, acetonitrile; flow rate, 0.4 mL/min; injection volume, 10 μL. Carotenoid absorption was measured at 472 nm.

Glucose analysis

Glucose was quantitated by the 3,5-dinitrosalicylic acid method for reducing sugars (Miller, 1959).

Imidazole analysis

The HPLC analytical procedure for 4-methylimidazole was modified for the analysis of imidazole (Klejdus *et al.*, 2003). The operating conditions were as follows: separation column (Agilent), RP column (Zorbax SB-C18, 4.6 mm × 250 mm × 5 μm); elution solvent, 95% 0.05 M K_3PO_4, 0.025 M sodium heptanesulfonate buffer, and 5% methanol, pH 3.0; flow rate, 1 mL/min; injection volume, 10 μL; detection wavelength, 220 nm. Intracellular imidazole was continuously determined for 3 h after the addition of imidazole to the culture.

Image analysis of mycelium morphology

The method reported by Nanou *et al.* (2011) was used for the image analysis of the mycelium morphology. For the determination of the aggregate size, 0.1 g of 6-d-old wet biomass was mixed with 15 mL of sterile distilled water, and the diluted sample was observed microscopically (Eclipse TS100; Nikon, Tokyo, Japan). Ten samples of 0.1 g wet biomass each were analysed per ultrasonic and non-ultrasonic culture, respectively, and the results were expressed as the percentage of aggregated mycelia.

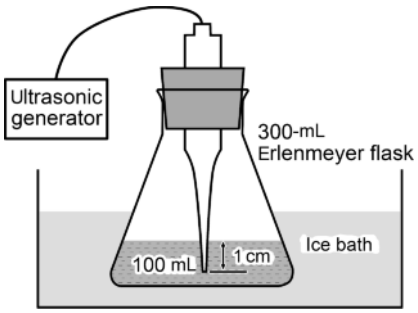

Fig. 1. Ultrasonic treatment device and ultrasonic operation. The ice bath was used only during sonication.

Experimental setup for ultrasonic treatment

An ultrasonic cell crusher (Scientz-IID; Ningbo Scientz Biotechnology, Ningbo, China) was used to sonicate the *B. trispora* culture flasks. The ultrasonic unit released a maximum power output of 950 W and a fixed frequency of 20 kHz. After fermentation of *B. trispora* in a shaking culture, the ultrasonic generator probe was placed in the flasks. All samples were divided into six groups, including three control groups of non-ultrasonic and three groups of ultrasonic treatment, respectively. From day 3 on, all mycelium cultures underwent ultrasonic stimulation at a constant frequency of 20 kHz once every 24 h, repeated four times. The ultrasonic treatment device and operation are shown in Fig. 1.

Optimization of ultrasonic parameters by the response surface methodology (RSM)

The operating time of ultrasonication was set to 3 s. The ultrasonic parameters consisted of power, treatment time, and rest time. RSM was employed to elucidate the effects of these important parameters on the β-carotene production by *B. trispora* using the Box-Behnken design (BBD). Table I presents the design matrix. The β-carotene yield was regarded as the response. The experiments were conducted in triplicate to predict measurement variability. The relationship of the independent variables and the response were investigated by the following second-order polynomial equation:

$$Y = X_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i<j} a_{ij} X_i X_j \,, \qquad (1)$$

where Y is the predicted response (β-carotene yield), X_0, a_i, a_{ii}, a_{ij} are constant coefficients, and X_i and X_j ($i = 1, 2, 3$; $j = 1, 2, 3$; $i \neq j$) represent the independent variables (power, treatment time, and rest time). The quality of fit of the second-order model equation was determined by the coefficient of determination, R^2, and

Table I. Experimental design for β-carotene production using *B. trispora* by ultrasonic treatment.

Run	Power X_1 [W]	Treatment time X_2 [min]	Rest time X_3 [s]	Response (β-carotene yield) [mg/L]	
				Observed	Predicted
1	400	4	6	170.4 ± 4.2	164.8
2	400	6	4	135.5 ± 5.3	128.6
3	200	4	4	125.4 ± 5.6	124.7
4	200	6	6	132.1 ± 5.0	139.8
5	400	4	6	155.9 ± 5.9	164.8
6	400	4	6	159.3 ± 6.0	164.8
7	600	4	8	150.5 ± 7.4	151.2
8	600	6	6	112.9 ± 6.4	119.7
9	400	6	8	165.9 ± 5.5	158.4
10	200	2	6	105.8 ± 5.4	99.0
11	400	2	4	146.6 ± 10.2	154.2
12	400	4	6	167.5 ± 7.4	164.8
13	200	4	8	125.7 ± 4.3	125.6
14	600	2	6	169.8 ± 7.3	162.1
15	400	2	8	127.5 ± 7.6	134.4
16	600	4	4	142.0 ± 4.4	142.1
17	400	4	6	170.9 ± 7.7	164.8

The observed values are means ± SD.

its statistical significance was calculated by an F-test. Three-dimensional (3D) response surface analysis was conducted by assigning a middle value to one independent variable and varying the values of the two other independent variables. The computer software used was Design Expert version 8.0. 5.0.

Results and Discussion

Optimization of β-carotene production

To estimate the combined effect of the three different ultrasonic parameters (independent variables) on the β-carotene yield, a BBD of 17 experiments was performed. Table I shows the experimental design and the results of the 17 experiments. By substituting factor levels into the regression equation (2), the maximum predictable response for the β-carotene production was calculated and experimentally verified. The results were analysed using ANOVA, and the resulting parameters calculated based on the β-carotene yield as responses are presented in Table I. Substituting the co-

Table II. ANOVA for the regression equation (2).

Source	Sum of squares	Degree of freedom	Mean square	F value	P value
Model	6519.09	9	724.34	8.36	0.0053
X_1	928.80	1	928.80	10.72	0.0136
X_2	1.36	1	1.36	0.016	0.9038
X_3	50.50	1	50.50	0.58	0.4702
X_1^2	1912.52	1	1912.52	22.07	0.0022
X_2^2	749.01	1	749.01	8.64	0.0217
X_3^2	242.40	1	242.40	2.80	0.1383
X_1X_2	1730.56	1	1730.56	19.97	0.0029
X_1X_3	16.81	1	16.81	0.19	0.6729
X_2X_3	612.56	1	612.56	7.07	0.0325
Residual	606.60	7	86.66		
Cor total	7125.69	16			

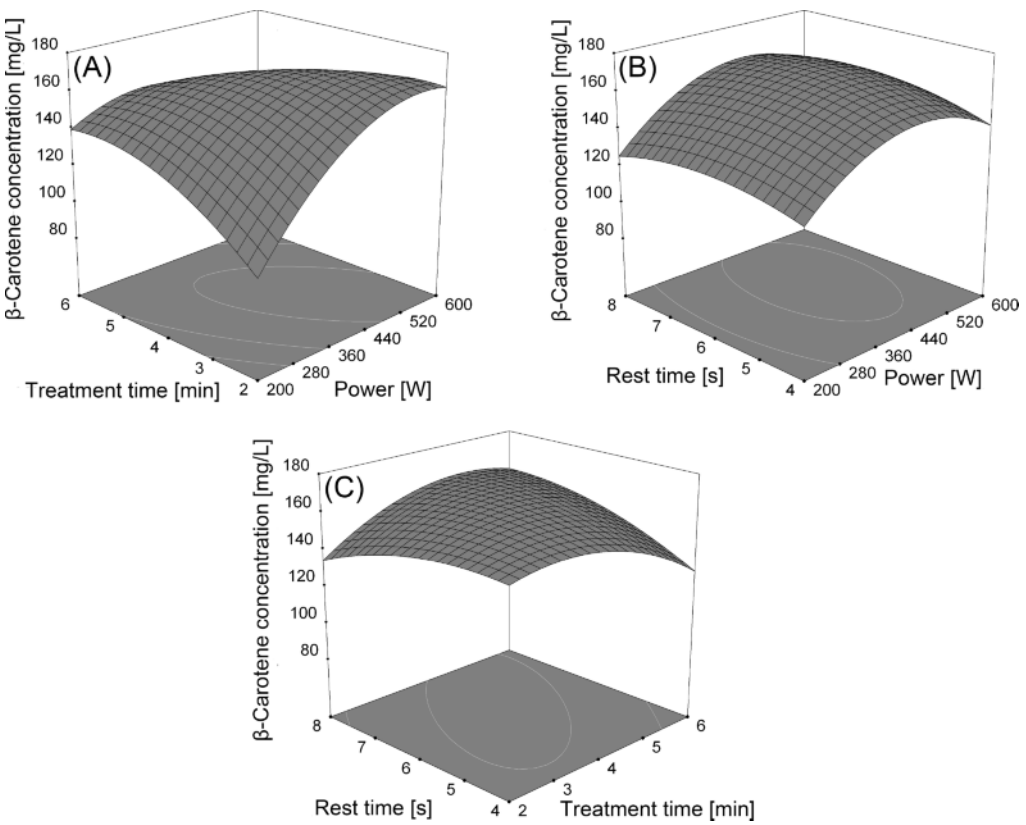

Fig. 2. Three-dimensional response surface plots for the β-carotene yield: (A) effects of power and treatment time on the β-carotene yield; (B) effects of power and rest time on the β-carotene yield; (C) effects of treatment time and rest time on the β-carotene yield.

efficients presented in Table I into equation (1) results in the following equation:

$$C_{\beta\text{-carotene}}\,(\text{mg/L}) = -67 + 0.66X_1 + 28.71X_2 + 9.59X_3 - 0.05X_1X_2 + 5.13\cdot 10^{-3}X_1X_3 + 3.09X_2X_3 - 5.33\cdot 10^{-4}X_1^2 - 3.33X_2^2 - 1.90X_3^2\,, \quad (2)$$

which represents the quantitative effects of the three individual factors and their interactions on the response, $C_{\beta\text{-carotene}}$. The multiple correlation coefficient R^2 of the regression equation for the β-carotene yield obtained by ANOVA was 0.91, indicating that this quadratic equation satisfactorily describes the relationships of the three factors with the response. The established model was found significant ($P = 0.0053$) and the β-carotene yield could be predicted by applying ANOVA [equation (2); Table II]. The P value was used to check the significance of each coefficient. The smaller the P value, the more significant was the corresponding coefficient. $P < 0.05$ suggested that the model terms were significant. The corresponding P value of each coefficient indicates that the power ($P = 0.0136$) had the highest impact on the β-carotene production. With appropriate power, no cavitation occurred in the medium and carotenoid formation was promoted. Power beyond the suitable range would seriously damage the cells and even lead to the death of the fungus.

The graphs in Fig. 2 illustrate the effects of individual factors on the carotenoid yield. Figure 2A shows the effects of power (X_1) and treatment time (X_2); the interaction between power (X_1) and rest time (X_3) is evident from Fig. 2B; the effects of treatment time (X_2) and rest time (X_3) on the β-carotene yield are seen in Fig. 2C. Optimal ultrasonic parameters were identified based on the curvature of the 3D plots. By keeping another variable at its middle level, 3D plots of two factors versus β-carotene production were drawn, and the corresponding contour plot was obtained. The optimal conditions for the three ultrasonic parameters obtained from the model were as follows: power, 491 W; treatment time, 3 min; and rest time, 5.8 s. Therefore, the optimal strategy consisted of exposing three-day-old mycelial cultures to a 3-min ultrasonic treatment at a fixed frequency of 20 kHz, power of 491 W, working time of 3 s, and rest time of 5.8 s, repeated four times at a 24-h interval.

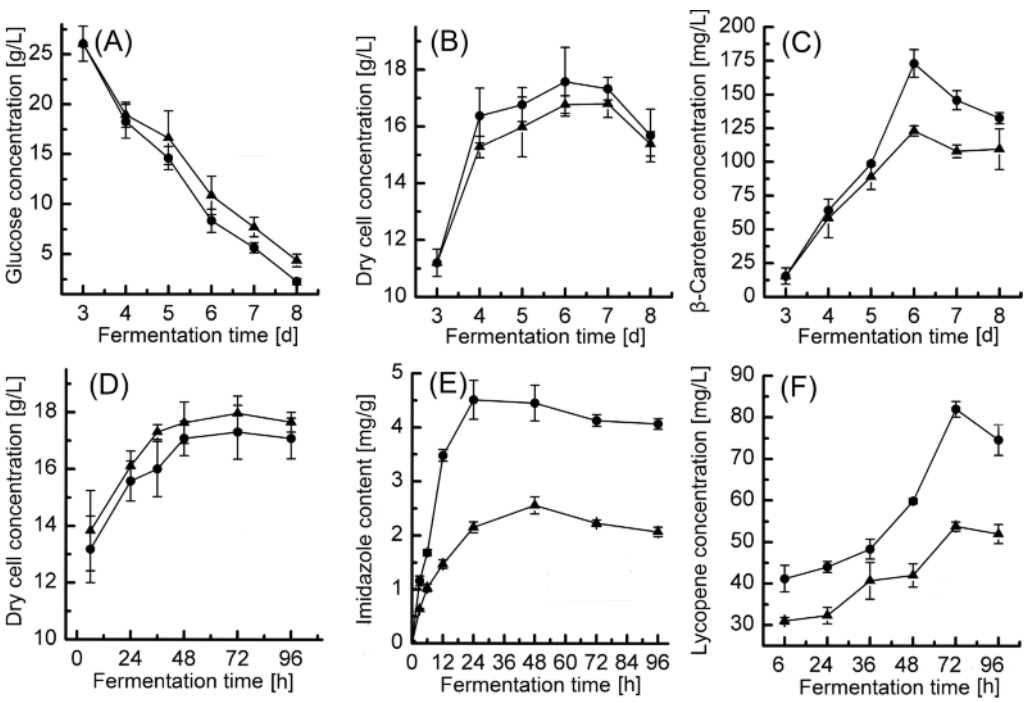

Fig. 3. Effect of ultrasonic stimulation under conditions of β-carotene (A – C) or lycopene (D – F) production: (A) glucose metabolism, (B) biomass, (C) β-carotene production *vs.* fermentation time; (D) biomass, (E) intracellular imidazole content, (F) lycopene production *vs.* fermentation time. ▲ Non-ultrasonic; ● ultrasonic.

Effect of ultrasonic stimulation on β-carotene production

Figure 3A indicates that under ultrasonic stimulation, glucose metabolism was slightly enhanced by about 10%. This increase may provide more acetyl-coenzyme A and reducing equivalents for the β-carotene synthesis. Glucose metabolism was almost completed after 8 d of fermentation. The maximum biomass concentration reached (17.57 ± 1.21) g/L by ultrasonic stimulation and (16.77 ± 0.31) g/L without ultrasonic treatment; these values improved by about 5% after 6 d (Fig. 3B). Dai *et al.* (2003) reported that low-energy ultrasonic treatment increased the biomass concentration of the filamentous fungus *Ecemothecium ashbyii*, and fermentation time was markedly shortened with maximum biomass concentration. However, *B. trispora* growth was not significantly promoted by ultrasonic stimulation. This result was consistent with the results of previous research in that ultrasonic treatment only altered the morphology of mycelia as well as the broth rheology, but without changing the growth of filamentous fungi (Kwiatkowska *et al.*, 2011).

The maximum production of β-carotene obtained experimentally using the optimal strategy reached (173.0 ± 10.36) mg/L, which correlated well with the predicted value of 167.2 mg/L determined by RSM regression. Without ultrasonic treatment, β-carotene production was only (123.1 ± 3.69) mg/L (Fig. 3C). Thus, ultrasonic simulation increased the β-carotene yield by 40.7%. Only when the biomass of *B. trispora* had reached its maximum, the large-scale synthesis of β-carotene did start; therefore, mycelium growth and β-carotene production were not synchronous. Based on our experimental results, glucose metabolism was enhanced slightly, and mycelium growth was hardly affected, while β-carotene biosynthesis was significantly promoted by ultrasonic treatment.

Effect of ultrasonic stimulation on lycopene production

As shown in Fig. 3D, the fungal biomass reached (17.3 ± 0.95) g/L with and (17.97 ± 0.6) g/L without ultrasonic treatment, respectively, after 72 h. Fungal growth slowed down slightly, but not significantly, and the dry weight decreased by only 3.7%. Therefore, ultrasonic treatment did not significantly affect the mycelium growth. Imidazole is known to inhibit the lycopene cyclase activity and thus to increase the lycopene production *in vivo* (López-Nieto *et al.*, 2004). Wang *et al.* (2003) and Kwiatkowska *et al.* (2011) suggested that low-intensity ultrasound can sharply increase the intracellular Ca^{2+} content and promote the cell membrane permeability in *Saccharomyces cerevisiae*. The imidazole content reached maxima of (2.56 ± 0.16) mg/g dry weight after 48 h without and of (4.51 ± 0.36) mg/g dry weight after 24 h with ultrasonic stimulation, respectively. The uptake rate of imidazole into cells was increased by about 2.5-fold by ultrasonic stimulation (Fig. 3E), suggesting a concomitant increase in the inhibitory effect on lycopene cyclase. Lycopene yield reached (82 ± 1.9) mg/L with ultrasonic stimulation and (53.8 ± 1.1) mg/L without. Thus, lycopene yield was enhanced by nearly 52.7% above the control after 72 h (Fig. 3F).

Effect of ultrasonic stimulation on mycelium morphology

The effect of ultrasonic treatment on the mycelium morphology is shown in Fig. 4. Mycelial aggregates were assigned to one of three morphological classes – class 1, $< 100{,}000\ \mu m^2$; class 2, $100{,}000 - 200{,}000\ \mu m^2$; class 3, $> 200{,}000\ \mu m^2$ – on the basis of their projected area (Fig. 4A). As shown in Fig. 4B, on the 6th day of fermentation, when β-carotene concentration reached its maximum, ultrasonication caused a decrease in the percentage of class 3 aggregates from about 60% to about 15%, while that of class 2 aggregates increased from about 25% to about 55%. Thus, the mycelium morphology changed from aggregates with a large projected area to aggregates with a smaller projected area, and the dispersion of the mycelium in the culture increased significantly by ultrasonic treatment. Nanou *et al.* (2007) reported that such morphological change in filamentous fungi is correlated with high oxidative stress, and furthermore, that this oxidative stress in realty stimulated the carotenoid production in *B. trispora*. Poor cell growth and low β-carotene production could be attributed to mass transfer limitation (Jeong *et al.*, 2001; Choudhari *et al.*, 2008). The enhanced mycelium dispersion increased the contact area, facilitating the exchange of nutrients and metabolites between the environment and the cells. This phenomenon reduced the mass transfer limitation and simultaneously enhanced the oxygen uptake rate, which improved the peroxide level of mycelia in submerged fermentation. These results explain the improvement in β-carotene and lycopene production as a result of increased mycelium dispersion.

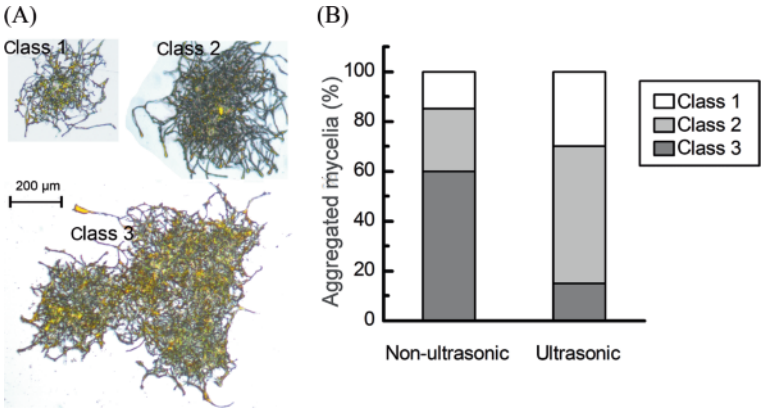

Fig. 4. Effect of ultrasonic treatment on the aggregation state of mycelia on the 6th day of fermentation: (A) different classes of aggregate sizes on the basis of their projected area; (B) percentage of aggregate classes in cultures with and without ultrasonic treatment.

The effect of ultrasound on the fermentation process is very complex. Ultrasound does not appear to affect a single factor which can be linked to the stimulation of a bioprocess (Kwiatkowska *et al.*, 2011). Generally, our study has demonstrated that ultrasound stimulates the glucose metabolism, decreases the mycelium aggregation, and improves the uptake of imidazole into cells. These changes could collectively be conducive to increasing β-carotene and lycopene production. Thus, the stimulation of the glucose metabolism, the decrease in mycelium aggregation, and improvement of the uptake of imidazole into cells are likely to synergistically enhance the accumulation of β-carotene and lycopene in *B. trispora*. Supporting evidence includes the following observations. The mycelium morphology changed from larger to smaller aggregates, and thus the dispersion of the mycelium in the culture increased significantly by ultrasonic treatment (Fig. 4). The enhancement of mycelium dispersion improved the contact area between oxygen, nutrients, and the cells, which may increase the respiration of the fungus. Therefore, the increase in mycelium dispersivity could stimulate the aerobic metabolism of glucose and thus provide more precursor (acetyl-coenzyme A) and reducing equivalents for the biosynthesis of β-carotene and lycopene (Filotheou *et al.*, 2012). The production of β-carotene thus increased by 40.7 % compared with the control (Fig. 3C). Given the change in the imidazole uptake by ultrasonic treatment, the intracellular imidazole content increased by nearly 75 % thus increasing the inhibitory effect on lycopene cyclase (Fig. 3E). The production of lycopene thus increased by 52.7 % compared with the control (Fig. 3F).

Employing ultrasound in the commercial production of β-carotene and lycopene appears feasible. In pilot experiments (unpublished), an independent ultrasonic device was installed outside a 50-L fermentation tank, which could be connected to an ultrasonic device by a circulation pump. Medium containing mycelium was intermittently pumped into the device for ultrasonic treatment, and then returned to the tank for continued fermentation. This method could therefore be used for the large-scale production of β-carotene and lycopene by *B. trispora* with ultrasonic treatment.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (Grant No. 20976065) and the New Century Talents Project of Hubei province.

Choudhari S. and Singhal R. (2008), Media optimization for the production of β-carotene by *Blakeslea trispora*: A statistical approach. Bioresour. Technol. **99**, 722 – 730.

Choudhari S. M., Ananthanarayan L., and Singhal R. S. (2008), Use of metabolic stimulators and inhibitors for enhanced production of β-carotene and lycopene by *Blakeslea trispora* NRRL 2895 and 2896. Bioresour. Technol. **99**, 3166 – 3173.

Dai C., Wang B., Duan C., and Sakanishi A. (2003), Low ultrasonic stimulates fermentation of riboflavin producing strain *Ecemothecium ashbyii*. Colloids Surf. B **30**, 37 – 41.

Filotheou A., Nanou K., Papaioannou E., Roukas T., Kotzekidou P., and Liakopoulou-Kyriakides M. (2012), Application of response surface methodology to improve carotene production from synthetic medium by *Blakeslea trispora* in submerged fermentation. Food Bioprocess Technol. **5**, 1189 – 1196.

Jeong J. C., Lee J., and Park Y. H. (2001), A unique pattern of mycelial elongation of *Blakeslea trispora* and its effect on morphological characteristics and β-carotene synthesis. Curr. Microbiol. **42**, 225 – 228.

Klejdus B., Moravcová J., and Kubáň V. (2003), Reversed-phase high-performance liquid chromatographic/mass spectrometric method for separation of 4-methylimidazole and 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl) imidazole at pg levels. Anal. Chim. Acta **477**, 49 – 58.

Kwiatkowska B., Bennett J., Akunna J., Walker G. M., and Bremner D. H. (2011), Stimulation of bioprocesses by ultrasound. Biotechnol. Adv. **29**, 768 – 780.

López-Nieto M. J., Costa J., Peiro E., Méndez E., Rodríguez-Sáiz M., Fuente J. L., Cabri W., and Barredo J. L. (2004), Biotechnological lycopene production by mated fermentation of *Blakeslea trispora*. Appl. Microbiol. Biotechnol. **66**, 153 – 159.

Mantzouridou F. and Tsimidou M. Z. (2008), Lycopene formation in *Blakeslea trispora*. Chemical aspects of a bioprocess. Trends Food Sci. Technol. **19**, 363 – 371.

Mantzouridou F., Tsimidou M. Z., and Roukas T. (2006), Performance of crude olive pomace oil and soybean oil during carotenoid production by *Blakeslea trispora* in submerged fermentation. J. Agric. Food Chem. **54**, 2575 – 2581.

Miller G. L. (1959), Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31**, 426 – 428.

Nanou K. and Roukas T. (2010), Oxidative stress response and morphological changes of *Blakeslea trispora* induced by butylated hydroxytoluene during carotene production. Appl. Biochem. Biotechnol. **160**, 2415 – 2423.

Nanou K., Roukas T., and Kotzekidou P. (2007), Role of hydrolytic enzymes and oxidative stress in autolysis and morphology of *Blakeslea trispora* during β-carotene production in submerged fermentation. Appl. Biochem. Biotechnol. **74**, 447 – 453.

Nanou K., Roukas T., and Papadakis E. (2011), Oxidative stress and morphological changes in *Blakeslea trispora* induced by enhanced aeration during carotene production in a bubble column reactor. Biochem. Eng. J. **54**, 172 – 177.

Papaioannou E. H. and Liakopoulou-Kyriakides M. (2010), Substrate contribution on carotenoids production in *Blakeslea trispora* cultivations. Food Bioprod. Process **88**, 305 – 311.

Sainz Herrán N., Casas López J. L., Sánchez Pérez J. A., and Chisti Y. (2008), Effects of ultrasound on culture of *Aspergillus terreus*. J. Chem. Technol. Biotechnol. **83**, 593 – 600.

Shi Y., Xin X., and Yuan Q. (2012), Improved lycopene production by *Blakeslea trispora* with isopentenyl compounds and metabolic precursors. Biotechnol. Lett. **34**, 849 – 852.

Sulaiman A. Z., Ajit A., Yunus R. M., and Chisti Y. (2011), Ultrasound-assisted fermentation enhances bioethanol productivity. Biochem. Eng. J. **54**, 141 – 150.

Sun J., Li H., Sun X., and Yuan Q. (2012), Trisporic acid stimulates gene transcription of terpenoid biosynthesis in *Blakeslea trispora*. Process Biochem. **47**, 1889 – 1893.

Wang B., Shi L., Zhou J., Yu Y., and Yang Y. (2003), The influence of Ca^{2+} on the proliferation of *S. cerevisiae* and low ultrasonic on the concentration of Ca^{2+} in the *S. cerevisiae* cells. Colloids Surf. B **32**, 35 – 42.

Xu F., Yuan Q. P., and Zhu Y. (2007), Improved production of lycopene and β-carotene by *Blakeslea trispora* with oxygen-vectors. Process Biochem. **42**, 289 – 293.