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Oxidative Stress Response and Morphological Changes of *Blakeslea trispora* Induced by Butylated Hydroxytoluene During Carotene Production

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Abstract The adaptive response of the fungus *Blakeslea trispora* to the oxidative stress induced by butylated hydroxytoluene (BHT) during carotene production in shake flask culture was investigated. The culture response to oxidative stress was studied by measuring the specific activities of catalase (CAT) and superoxide dismutase (SOD) and the micromorphology of the fungus using a computerized image analysis system. The addition of exogenous BHT to the medium caused changes of the morphology of microorganism from aggregates with large projected area to aggregates with small projected area. This morphological differentiation of the fungus was associated with high oxidative stress as evidenced by remarkable increase of the specific activities of CAT and SOD. The oxidative stress in *B. trispora* resulted in a fivefold increase of carotene production. The highest concentration of carotenes (125.0 mg/g dry biomass) was obtained in culture grown in medium supplemented with 20 mM of BHT.

Keywords Butylated hydroxytoluene · Oxidative stress · Morphology · *Blakeslea trispora* · Carotenes

Introduction

Oxidative stress was defined as the disturbance in the prooxidant–antioxidant balance resulting in potential cell damage. In aerobic metabolism, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\bullet}$), and superoxide radicals ($O_2^{\bullet-}$) are formed during the fermentation. Certain levels of ROS are important for plant physiological functions such as cell wall biosynthesis and cell growth. Excessive ROS, however, can cause lipid peroxidation, DNA damage, inactivation of enzymes and protein, disruption of membranes mutations, and ultimately cell death [1, 2]. For protecting the cells from oxidative injury, aerobic organisms possess both enzymatic and nonenzymatic defense systems. The enzymatic system includes superoxide dismutase (SOD), catalase (CAT),

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glutathione peroxidase, glutathione reductase, alternative oxidase, and thiol peroxidases. The most important nonenzymatic defense systems include carotenoids, glutathione, ascorbic acid, tocopherols, trehalose, ubiquinol, and metallothioneins. They act as radical scavengers, being oxidized by ROS and thereby removing oxidants from solution [2, 3]. The oxidative stress occurs when the concentration of the ROS exceeds the antioxidant capacity of the cells. Factors responsible for the induction of oxidative stress include exposure of cells to ionizing radiation, redox-cycling chemicals, H_2O_2, heavy metals, etc. [4].

The synthesis of carotenes depends on the growth conditions and is stimulated by chemical compounds that increase the ROS concentration [5]. Butylated hydroxytoluene (2,6-di-tert-butyl-4-methyl-phenol, BHT) is a phenolic antioxidant. Smirnova et al. [6] and Faine et al. [7] reported that under certain conditions, BHT induced oxidative stress in winter wheat (*Triticum aestivum* L.) and rats, respectively. Very little published information is available on the oxidative stress in filamentous fungi. Medentsev et al. [8] and Bai et al. [9] investigated the adaptive response of the *Fusarium decemcellulare* and *Aspergillus niger* to the oxidative stress induced by H_2O_2, oxygen enrichment, and elevated temperature. Gessler et al. [5] studied the changes in the activities of SOD and CAT induced by light, menadione and SOD inhibitors in the fungi *Blakeslea trispora* and *Neurospora crassa*. Han et al. [10] examined the effect of oxidative stress in *Penicillium* sp. PT95 induced by $FeCl_3$. Recently, in our laboratory, the role of hydrolytic enzymes and oxidative stress in autolysis of *B. trispora* during β-carotene production in submerged fermentation was studied [11]. In an attempt to increase the carotene production by *B. trispora*, we tried to cause oxidative stress in *B. trispora* induced by BHT. This attempt drove us to the interest on this subject to explain the mechanisms by which the addition of BHT to the medium affected changes in culture morphology and oxidative stress. To our knowledge, this is the first time oxidative stress has been used to increase the carotene-producing capacity of the culture. In examining the processes of oxidative stress in *B. trispora* induced by BHT, we adopted three ways: (1) monitoring the activities of SOD and CAT which are the two key defensive enzymes to oxidative stress, (2) measuring the dissolved oxygen concentration, and (3) using computerized image analysis techniques the morphological differentiation of the culture was investigated.

Materials and Methods

Microorganisms and Culture Conditions

The microorganisms used in this work were *B. trispora* American Type Culture Collection (ATCC) 14271, mating type (+) and *B. trispora* ATCC 14272, mating type (−). Both strains were obtained from the ATCC (Rockville, MD, USA). The strains were grown on potato dextrose agar (Scharlau, 01-483) Petri dishes at 26°C for 4 days for sporulation. The spores were collected with the relevant amount of sterile distilled water to obtain an inoculum concentration of 5.0×10^5 and 1.0×10^6 spores/ml of the strains 14271 and 14272, respectively.

Fermentation Conditions

The fermentation was carried out in 250-ml conical flasks containing 50 ml of the medium with the following composition (grams per liter): glucose (Scharlau, GL 0129) 50, corn steep liquor (obtained from AMYLUM Hellas, Thessaloniki, Greece) 80, yeast extract (Scharlau, 07-079) 1.0, casein acid hydrolysate (Scharlau, 07-151) 2.0, L-asparagine

(Sigma, A8381) 2.0, KH_2PO_4 (Merck, 4873) 1.5, $MgSO_4.7H_2O$ (Merck, 5882) 0.5, thiamine.HCl (Sigma, T-4625) 5.0 mg/l, linoleic acid (Sigma, L1626) 20.0, and Span 20 (Sigma, S6635) 10.0. The medium was supplemented with different concentrations of BHT. The pH of the medium was adjusted to 7.5 with 1 N NaOH and then sterilized at 121°C for 15 min. After cooling, the flasks were inoculated with 1 ml of the spore suspension of each strain of *B. trispora* (obtained as described above). The flasks were incubated at 26°C in a rotary shaker incubator (Lab Line Orbit-Environ Shaker, Lab-Line Instr., Melrose Park, IL, USA) at 250 rpm.

Analytical Techniques

At appropriate time intervals, fermentation flasks were removed and the contents analyzed. Carotene concentration, dry biomass, residual sugars concentration, and the activities of SOD and CAT were determined according to Nanou et al. [11]. In all cases, the specific activity of the enzymes was expressed as units/milligram protein. The protein content was estimated by the method of Schacterle and Pollack [12]. Dissolved oxygen concentration was determined with a microprocessor oximeter (OXI 96, WTW, Germany). The values of the readings were expressed as percentage of the initial level of saturation. The data are the average values of three independent experiments.

The carotenes produced were analyzed by high-performance liquid chromatography (HPLC). The HPLC was performed with a Q-Grad quaternary gradient pump (LabAlliance) and a Diode Array Finnigan Spectral System UV6000LP detector (Thermo). A Grace Vydac 201 TP54 C18 column (250×4.6 mm, 5-μm particle size) was used. The mobile phase of methanol was eluted at a flow rate of 1.5 ml/min. Under these conditions, β-carotene, γ-carotene, and lycopene were eluted within 8.0, 9.5, and 11.0 min, respectively. The detections of β-carotene and lycopene were made at 429, 450, 478 nm and 444, 468, 501 nm, respectively. Standards of the above substances were obtained from Sigma. Since γ-carotene was commercially unavailable, this carotene was identified by its absorbance maxima (440, 462, 492 nm) as indicated by Takaichi [13].

The morphological entities measured were aggregates with different size as shown in Fig. 5. For the determination of big aggregates with projected area higher than 1,000,000 μm^2, an amount of 0.3 g of wet biomass was mixed with 20 ml of sterile distilled water in a Petri dishes and the diluted sample was observed on microscope. Ten amounts of 0.3 g wet biomass were analyzed per sample. For the determination of aggregates with projected area lower than 1,000,000 μm^2, an amount of 1 ml of the fermentation broth was mixed with 39 ml of sterile distilled water and 10×200 μl of the diluted sample were observed on microscope. Ten amounts of 1 ml of the fermentation broth were analyzed per sample. The result was expressed as percentage of aggregated mycelia. Image capture was carried out via CCD video camera (JVC) mounted on a phase contrast microscope (Nikon Eclipse 50i) and digitized by a frame grabber card (LEADEC) installed on a PC. Image analysis was performed with Matrox Inspector 32 image processing program.

Results

The effect of BHT on carotene concentration, dissolved oxygen concentration, activity of antioxidant enzymes, and culture morphology is shown in Figs. 1, 2, 3, 4, 5, and 6. As shown in Fig. 1 in all cases (except the culture grown without the addition of BHT into the

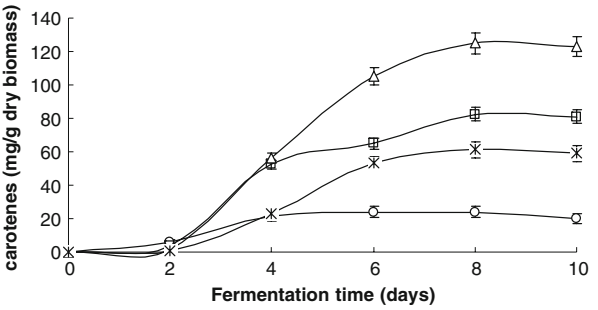

Fig. 1 Effect of BHT on carotene production by *B. trispora* in shake flask culture. *Circle* 0 mM, *square* 10 mM, *triangle* 20 mM, *zhe* 30 mM

medium), the concentration of carotenes increased as fermentation progressed up to 8th day and then remained almost constant. The dry biomass increased rapidly during the first 2 days of the fermentation and then remained constant (data not shown). In all cultures, the maximum dry biomass was 27.0±1.5 g/l. In cultures grown in media supplemented with 10, 20, and 30 mM of BHT, the dissolved oxygen levels were 5–55%, 10–70%, and 20–80% of saturation, respectively, between 2nd and 10th day of the fermentation. On the other hand, in culture grown without the addition of BHT into the medium, the dissolved oxygen concentration was only 2% of saturation at the same time (Fig. 2).

Figures 3 and 4 show the changes in the activities of antioxidant enzymes during fermentation. The specific intracellular activities of SOD and CAT showed two maxima, one in the middle of the exponential phase and another at the end of stationary phase. The minimum between the two maxima was observed after 2 days of incubation for the SOD and 3 days for the CAT. When the maximum concentration of carotenes was observed (after 8 days of fermentation), the carotenes produced were analyzed by HPLC. As shown in Table 1, three carotene compounds (β-carotene, γ-carotene, lycopene) were identified. The major accumulated compounds were β-carotene and γ-carotene, but lycopene also occurred in significant amount.

The dispersed morphology of *B. trispora* consists of aggregates with different sizes (classes 1 to 5) on the basis of their projected area, i.e., class 1 consists of aggregates with projected area up to 100,000 μm^2, class 2 >100,000–200,000 μm^2, class 3 >200,000–500, 000 μm^2, class 4 >500,000–1,000,000 μm^2, and class 5 over 1,000, 000 μm^2 (Fig. 5). The culture morphology data are presented in Fig. 6. The values of morphological parameters measured were based on the change of the projected area of the aggregates during fermentation.

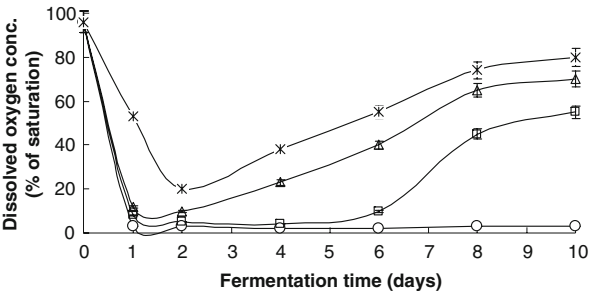

Fig. 2 Effect of BHT on dissolved oxygen concentration (% of saturation) during carotene production by *B. trispora* in shake flask culture. *Circle* 0 mM, *square* 10 mM, *triangle* 20 mM, *zhe* 30 mM

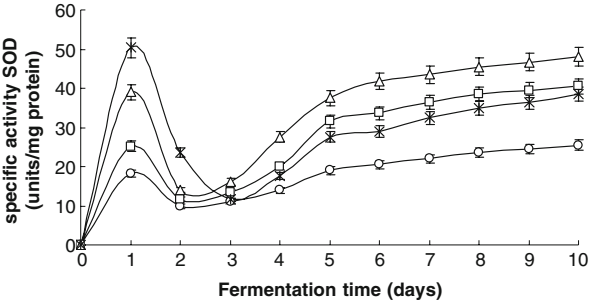

Fig. 3 Effect of BHT on the specific activity of SOD during carotene production by *B. trispora* in shake flask culture. *Circle* 0 mM, *square* 10 mM, *triangle* 20 mM, *zhe* 30 mM

Discussion

Figure 1 shows that carotenes were synthesized in two steps. In the first step (0 to 2 days of fermentation) called the "growth phase", a small amount of the pigments was obtained, and in the second step (2 to 8 days of fermentation) called the "production phase", the biosynthesis of carotenes were carried out. The concentration of residual sugars decreased during the first 2 days of incubation and then remained practically constant (~5.0 g/l). This was accompanied with the rapid increase of biomass concentration observed at the same time (data not shown). As shown in Fig. 1, the concentration of carotenes increased significantly as the concentration of BHT increased up to 20 mM and then decreased. This was due to the decrease of the activities of SOD and CAT as shown in Figs. 3 and 4. The decreased SOD and CAT activities suggest an increased exposure of the fungus to oxidative stress. Gessler et al. [5] reported that the decrease in SOD and CAT activities is associated with inactivation of the enzymes by the high oxidative stress. Thus, when the medium was supplied with high concentration of BHT (30 mM), *B. trispora* possess primarily the nonenzymatic antioxidant system that protects the microorganism from oxidative stress resulting in a decrease in carotene concentration (Fig. 1). Also, the depression of antioxidant enzymes might be due to the prooxidant effect of BHT. It has been demonstrated that several known antioxidants might have both antioxidant and oxidant action dependent on their concentration. Although BHT is known for its antioxidant activity, the administration of higher doses may exert a prooxidant effect on the organisms [7]. In our case, BHT acting as prooxidant interacts with molecular oxygen rather than with $O_2^{\bullet-}$ radical. This reaction yields phenoxyl radical and superoxide anion [6]. Deeper oxidation of BHT may result in formation of BHT quinone methide and other products of BHT oxidation which can also generate superoxide anion [6]. In our experiments, this

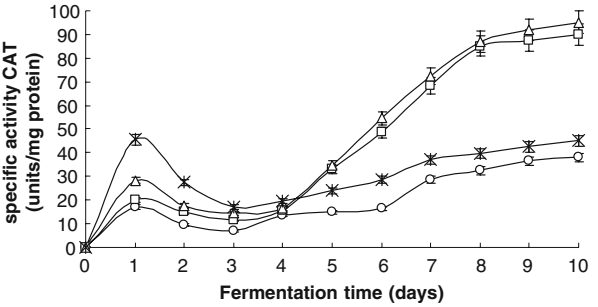

Fig. 4 Effect of BHT on the specific activity of CAT during carotene production by *B. trispora* in shake flask culture. *Circle* 0 mM, *square* 10 mM, *triangle* 20 mM, *zhe* 30 mM

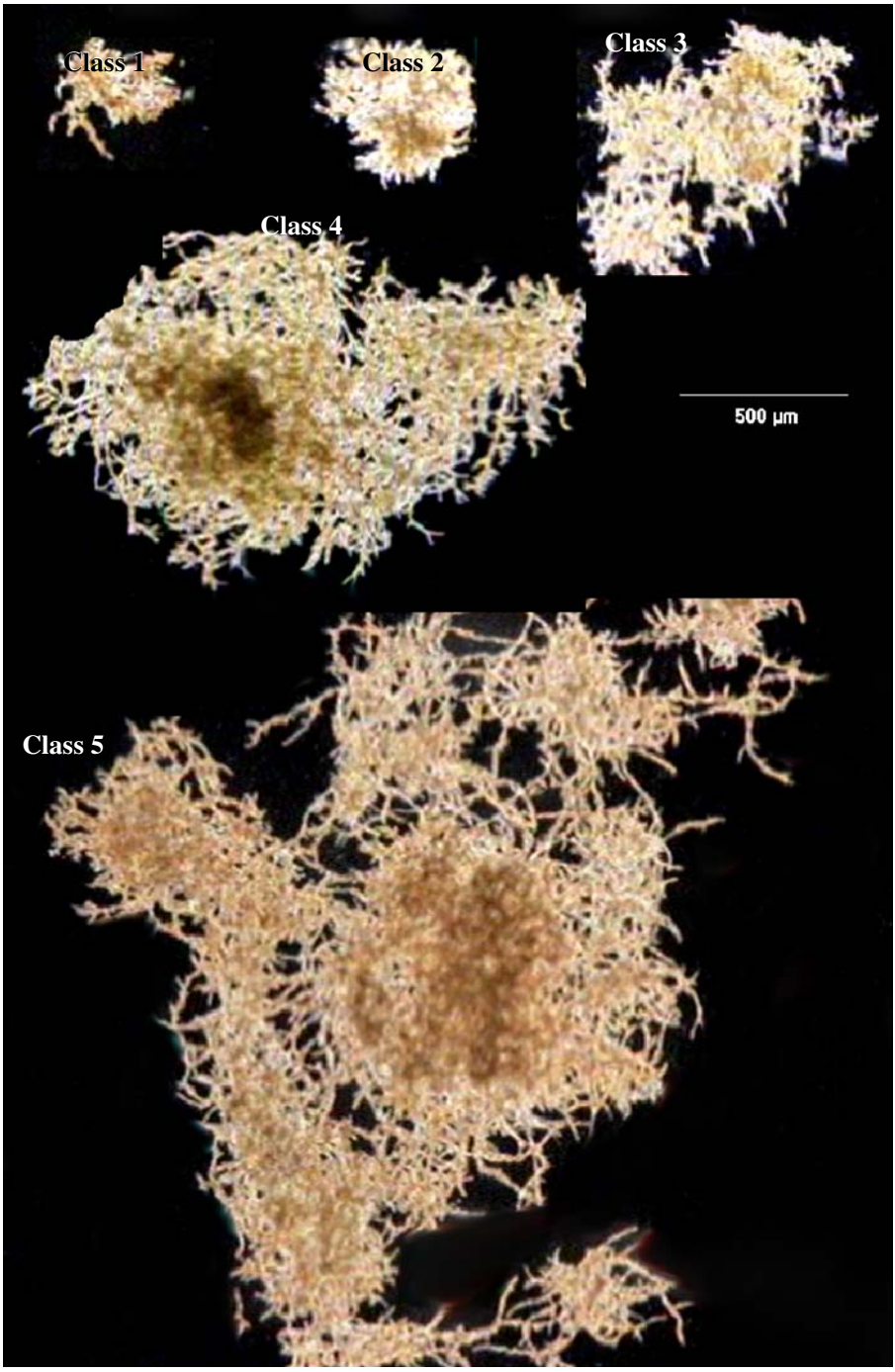

Fig. 5 Photographs showing the classes of the aggregated mycelia of *B. trispora*. Class 1 up to 100,000 μm^2; class 2 >100,000–200,000 μm^2; class 3 >200,000–500,000 μm^2; class 4 >500,000–1,000,000 μm^2; class 5 >1,000,000 μm^2

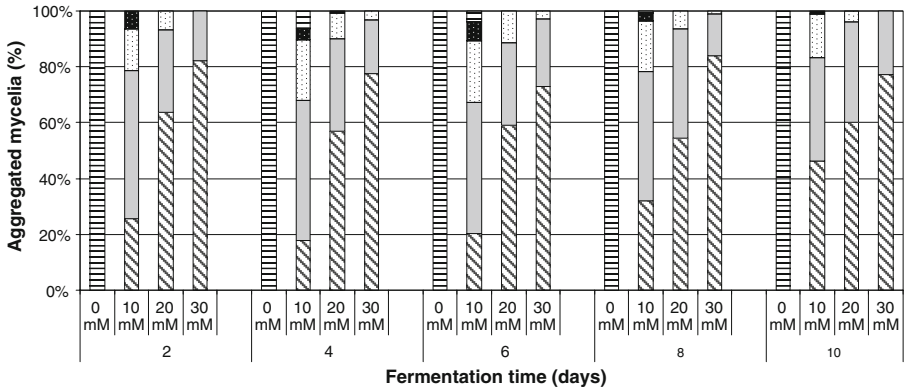

Fig. 6 Effect of BHT on the variation of percentage of aggregated mycelia of *B. trispora* during carotene production in shake flask culture. class 1, class 2, class 3, class 4, class 5. Differentiation of the classes as shown in Fig. 5

suggests that under high concentrations, BHT can behave as a prooxidant. In addition, the decrease in the activities of the above enzymes may be due to the effect of BHT on cell respiration which probably increases the rate of hydrolysis of ATP in the mitochondrial membrane and directly leads to the total depletion of cellular ATP and subsequent inhibition of cellular respiration [14]. Finally, the decrease in the activities of antioxidant enzymes may be due to the toxic effect of BHT on the microorganism. Thompson and Moldeus [14] reported that BHT is a toxic compound and its toxicity appears to be related to its effect on biomembranes and mitochondrial bioenergetics.

Generally, our results indicate that exposure of *B. trispora* to BHT promotes oxidative stress. Supporting evidence includes the following: When the medium was supplemented with different concentrations of BHT, a drastic increase in the concentration of carotenes was observed compared to the control (Fig. 1). This may be explained by the fact that the morphology of microorganism changed from aggregates with large projected area to aggregates with smaller projected area. This change of the aggregates began early during the growth phase and continued until the end of fermentation (Fig. 6). The mechanism by which BHT act on culture morphology is not clear. Thompson and Moldeus [14] reported that BHT affect on lipid membranes with particular emphasis on the mitochondrial membrane. BHT is a phenolic compound and undergoes spontaneous dispersion in aqueous medium. It is incorporated into the phospholipids of the plasma membrane causing in modifications of the membrane properties. Thus, the incorporation of exogenous BHT into the cell membrane caused significant changes in the morphology of the fungus forming aggregates with small projected area (Figs. 5 and 6), and this would result in a perceived stress to the microorganism. Therefore, the change in cell wall morphology might be a

Table 1 Effect of BHT on the composition of carotenes (fermentation time 8 days).

BHT (mM)	β-Carotene (%)	γ-Carotene (%)	Lycopene (%)
0	54.4	36.0	9.6
10	43.8	44.5	11.7
20	46.9	39.7	13.4
30	46.4	32.1	21.5

response to this stress. Aggregates with small projected area could be beneficial to improve the mass transfer characteristics relating substrate, products/byproducts, and oxygen. This change of cell morphology works in both directions; through better diffusion, it helps to maintain a satisfactory supply of nutrients to the cells, while it facilitates the removal of byproducts of catabolism from the microenvironment of the cells. Also, the aggregates with small projected area obtained in the presence of BHT could solve the problems of the mixing and the mass transfer limitation in a large scale fermentation system during carotene production by *B. trispora*. Finally, aggregates with small projected area favor oxygen supply to the cells, and it is especially important to maintain the concentration of ROS at high levels. Thus, BHT may affect the intracellular generation of oxygen radicals causing oxidative stress to the fungus resulting in a significant increase of the carotene production.

The highest concentration of carotenes (125.0 mg/g dry biomass) was achieved in culture grown in medium supplemented with 20 mM of BHT, whereas without the addition of BHT into the medium, the highest concentration of the pigments was 25.0 mg/g dry biomass (Fig. 1). A fivefold increase of the concentration of carotenes was obtained by the addition of BHT into the medium. This indicates that when mycelium was grown in aggregates with small projected area, the production of carotenes was highly enhanced. As shown in Fig. 6 on the 8th day of fermentation in medium supplemented with 20 mM of BHT, the percentage of aggregates with small projected area was high, i.e., 55%, whereas in the control, only aggregates with large projected area were observed. At the same time in medium with the addition of 20 mM of BHT, the specific activities of SOD and CAT were 45.0 and 87.0 U/mg protein, respectively, while in the control, the activities of the above enzymes were 23.0 and 32.0 U/mg protein, respectively (Figs. 3 and 4). Moreover, as shown in Fig. 2, the addition 20 mM of BHT to the medium resulted in an increase of the dissolved oxygen concentration (10–60% of saturation) between 2nd and 8th day of incubation compared to the control (2% of saturation) at the same time. This was due to the higher percentage of aggregates with small projected area observed in medium supplemented with 20 mM of BHT (Fig. 6). The addition 30 mM of BHT to the medium resulted in a high dissolved oxygen concentration (20–70% of saturation) between 2nd and 8th day of incubation (Fig. 2). At high aeration rate, superoxide anion ($O_2^{\bullet -}$) can be formed due to direct interaction of BHT with molecular oxygen [6]; as a consequence, a high oxidative stress was caused as indicated by the decrease in the activities of SOD and CAT (Figs. 3 and 4). On the other hand, the higher DOTs in the cultures grown at high concentrations of BHT might suggest direct inhibition by BHT of cell metabolism hence reduced generation of endogenous oxidative stressors. The results show that there is a critical point between oxidative stress and carotene production over which an increase of the oxidative stress resulted in a significant decrease in carotene concentration. Generally, the above results show that the main role of BHT is to prevent the formation of big aggregates and to create aggregates with small projected area. This resulted in a better diffusion of oxygen into the cells producing high concentration of ROS. Thus, the oxidative stress resulted in a significant increase of carotene production.

Conclusions

Our results demonstrate the possible mechanisms by which BHT may induce oxidative stress on *B. trispora*. These mechanisms include changes of the morphology of microorganism from aggregates with large projected area to aggregates with small projected area. Carotene synthesis was enhanced by oxidative stress until a level of BHT.

High concentrations of BHT resulted in a decrease of carotene production. The antioxidant enzyme system (CAT, SOD) did not protect completely the fungus from oxidative stress. Carotenes acted as a potent antioxidant in *B. trispora* under conditions of high oxidative stress. Considering that oxidative stress plays a significant role on carotene production, the findings of this experiment were very useful for evaluating the mechanisms by which BHT caused oxidative stress.

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