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Oxidative stress response of *Blakeslea trispora* induced by H_2O_2 during β-carotene biosynthesis

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Abstract The cellular response of *Blakeslea trispora* to oxidative stress induced by H_2O_2 in shake flask culture was investigated in this study. A mild oxidative stress was created by adding 40 μm of H_2O_2 into the medium after 3 days of the fermentation. The production of β-carotene increased nearly 38 % after a 6-day culture. Under the oxidative stress induced by H_2O_2, the expressions of *hmgr*, *ipi*, *carG*, *carRA*, and *carB* involving the β-carotene biosynthetic pathway all increased in 3 h. The aerobic metabolism of glucose remarkably accelerated within 24 h. In addition, the specific activities of superoxide dismutase and catalase were significantly increased. These changes of *B. trispora* were responses for reducing cell injury, and the reasons for increasing β-carotene production caused by H_2O_2.

Keywords *Blakeslea trispora* · Oxidative stress · β-carotene · H_2O_2 · Synthetic medium · Shake flask culture

Introduction

Studies on the function of β-carotene and lycopene have drawn significant attention because of their biological functions in human wellness, which lead to their commercialization in functional food, medicine, and cosmetic industries. Compared with other microorganisms, *Blakeslea trispora* exhibits higher potential for β-carotene production [10]. At present, *B. trispora* is the main microbiological subject for industrial production of β-carotene.

The improvement of carotenoid production using *B. trispora* induced by oxidative stress has become a research hotspot. Mantzouridou et al. [11] reported that oxygen transfer rate, H_2O_2 accumulation, and β-carotene synthesis reveal a positive relationship under oxidative stress in *B. trispora*. Nanou et al. [14] indicated that enhanced aeration changes the oxidative stress of *B. trispora* and increases β-carotene production in a bubble column reactor. Xu et al. [25] studied the production of β-carotene and lycopene by combining *B. trispora* with oxygen vectors (*n*-hexane or *n*-dodecane) to increase dissolved oxygen concentration. The oxidative stress responses of *B. trispora* induced by butylated hydroxytoluene (BHT), H_2O_2, liquid paraffin, and iron ions during β-carotene production in shake flask culture were studied [7, 8, 15, 17]. At present, studies have shown that oxidative stress can increase the production of carotenes in *B. trispora* [16, 19]. However, the effects of oxidative stress on carotenoid biosynthesis are mainly confined to the changes of mycelium morphology and scavenging enzyme activity (SOD and CAT) in *B. trispora* [18]. The synthesis of carotenes is closely related with oxidative stress; however, the mechanisms have been rarely reported.

Oxidative stress has been considered as the disturbance in pro-oxidant/anit-oxidant balance, resulting in potential cell damage. Aerobic organisms consume molecular oxygen for aerobic metabolism and energy supply. At the same time, a large amount of molecular oxygen can cause the toxic production of reactive oxygen species (ROS), such as superoxide radicals, hydroxyl radicals, and H_2O_2, highly damaging to cellular constituents, including enzymes, protein, lipids, and DNA injury [2]. Aerobic organisms have both enzymatic

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and non-enzymatic defense systems to protect the cells from oxidative damage. These systems act as radical scavengers, being oxidized by ROS, removing oxidants from the cells [6, 17]. The enzymatic defense system includes catalase (CAT) and superoxide dismutase (SOD) [5]. The non-enzymatic defense system includes antioxidants, such as carotenes.

Compared with other compounds (iron ions, BHT, and liquid paraffin) and oxygen carrier (*n*-hexane or *n*-dodecane) induced oxidative stress, H_2O_2 possesses significant advantages, including low cost, non-environmental pollution, and suitability for mass production. Therefore, this study investigates the response of *B. trispora* induced by H_2O_2 during β-carotene biosynthesis.

Materials and methods

Chemicals and reagents

All chemicals, reagents, and solvents used in this study were of analytical or high-performance liquid chromatography (HPLC) grade.

Microorganisms

The microorganisms used in this study consisted of *B. trispora* ATCC 14060 mating type (+) and *B. trispora* ATCC 14059 mating type (−). Both strains were purchased from China Center for Type Culture Collection.

Culture conditions

The strains were grown on PDA at 28 °C for 3 days, and the spores were used for the inoculation of the culture medium. The spores suspension contained 1.0×10^6 and 2.0×10^6 spores/mL of the strains 14,060 (+) and 14,059 (−), respectively.

Fermentation conditions

The spores suspension was inoculated into 250 mL Erlenmeyer flasks containing 100 mL sterile medium for β-carotene production. Fermentation was conducted at 28 °C on a rotary shaker (200 rpm) for 7 days. The composition of the fermentation medium was as follows (g/L): Span 20 20, glucose 100, yeast extract 12, Na_2HPO_4 1.5, KH_2PO_4 3, and $MgSO_4$ 0.5. The flasks were inoculated with 2 %, v/v of the spores suspension.

Carotenes extraction

The wet biomass underwent vacuum freeze-drying for 48 h, weighed the cell dry weight and ruptured with liquid nitrogen by manual grinding until complete cell disruption [12]. The samples were then subjected thrice to stirring-assisted extraction with petroleum ether at room temperature.

Analytical methods

Analysis of carotenes

The HPLC methods reported by Bononi [3] have been modified was used to analyze the carotenoid content. β-Carotene was separated using an Agilent HPLC system equipped with an Ascentis 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. β-Carotene absorption was measured at 450 nm.

Analysis of glucose concentration and glucose metabolism rate

The 3, 5-dinitrosalicylic acid was used for the glucose analysis to test the reducing sugar [13]. The rate of glucose metabolism was calculated using the following equation: change of glucose concentration (g L^{-1})/time (hours).

Analysis of SOD and CAT enzyme activity

Wet cells (1 g) were disrupted by liquid nitrogen and the enzymes were extracted with 10 ml of prechilled physiological saline (0.9 % NaCl), then centrifuged for 15 min at 4 °C and the supernatant was collected for the analysis of SOD and CAT enzyme activity. Analysis of the enzyme activities was according to the manual of the reagent kits (purchased from Nanjing Jiancheng Bioengineering Institute) [7].

Calculation of specific oxygen uptake rates

Dissolved oxygen concentration was determined with a microprocessor oximeter. Specific oxygen uptake rate (SOUR) is the ratio of oxygen consumption rate (OUR) and dry biomass. OUR was determined using the method reported by Casas López et al. [4].

Real-time PCR

The real-time polymerase chain reaction (PCR) primers are presented in Table 1. The primers of *hmgr*, *ipi*, *carG*, *carRA*, and *carB* were used according to the report by Sun et al. [24]. The real-time PCR cycling conditions were as follows: 95 °C for 3 min followed by 40 cycles at 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 20 s. Measurements were performed in

Table 1 Real-time PCR primers used in this study

Genes	Forward and reverse primers (5′ → 3′)		Amplicon length (bp)
18s	18S-F	TGCTGGCGACGGTCTACTC	149
	18S-R	GCTGCCTTCCTTGGATGTG	
IPI	ipi-F	TCTCACCCCTTAAATACAGCAGATG	161
	ipi-R	CTCGGTGCCAAATAATGAATACG	
carG	carG-F	AATTGTTTTGGCGTGACACCTT	129
	carG-R	CAGTTCCCGATTGACTAGCTTCTT	
hmgR	hmgR-F	AAACGATGGATTGAACAAGAGGG	113
	hmgR-R	TAGACTAGACGACCGGCAAGAGC	
carRA	carRA-F	CTAAAGCCGTTTCACTCACAGCA	129
	carRA-R	ACAAGTAGGACAGTACCACCAAGCG	
carB	carB-F	AGACCTAGTACCAAGGATTCCACAA	92
	carB-R	AGAACGATAGGAACACCAGTACCTG	

triplicate. All results were normalized to the 18S gene of *B. trispora* (Gene Bank: AF157124.1) and are expressed relative to expression of the corresponding control group (non-H_2O_2-added; value = 1), using the comparative method of Livak and Schmittgen [9]. Values are the mean ± standard error of three independent experiments. Values >1 represent over expression compared with the control group.

Statistical analysis

The means of three independent experiments are presented in Figs. 1, 2, 3 and 4. Each data point represents the mean and the standard error. Data was analyzed using student's *t* test. A *p* value between 0.01 and 0.05 was considered as significant (*), and *p* value <0.01 was considered as very significant (**).

Results and discussion

Effect of added amount of H_2O_2 on biomass and β-carotene production

The effect of H_2O_2 on β-carotene synthesis and grown biomass were examined after adding different concentrations in all cases (0, 5, 10, 20, 40, 60, 80, and 100 μM) to the 3-day old cultures of *B. trispora* (Fig. 1). The control (0 μM added) was the culture to which H_2O_2 was not added during cultivation. After 6 days of culturing, dry biomass was slightly decreased from 17.11 to 16.23 g/L when the H_2O_2 concentration ranged from 0 to 60 μM, but remarkably decreased from 16.23 to 14.79 g/L when the H_2O_2 concentration increased from 60 to 100 μM. This result is due to the toxicity of high H_2O_2 concentration, resulting to cell damage [18]. This phenomenon indicates that the addition of H_2O_2 at low concentration (below 60 μM) to the 3-day old cultures of *B. trispora* leads to mild oxidative stress.

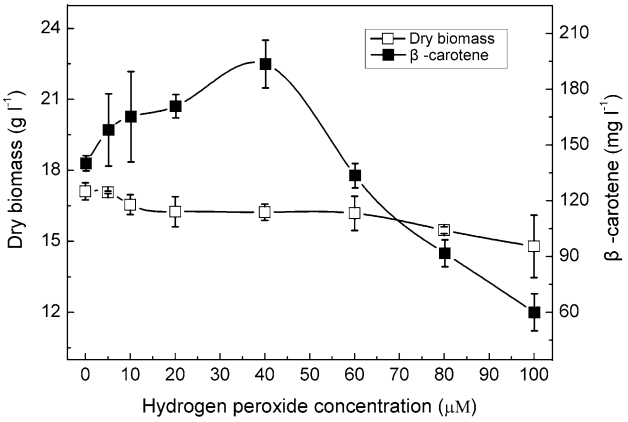

Fig. 1 Effect of added amount of H_2O_2 on biomass and β-carotene production

The production of β-carotene increased significantly from 140.1 to 193.5 mg/L with the increase in the added amount of H_2O_2 from 0 to 40 μM. Therefore, carotenes synthesis may be increased by oxidative stress. Jeong et al. [8] reported that the addition of H_2O_2 (10 μM) to 1.5-day old cultures of *B. trispora* resulted to a 46 % higher β-carotene than that without addition. However, reports on the reasons of increasing the production of β-carotene by mild oxidative stress are rare. The production of β-carotene decreased dramatically from 193.5 to 59.9 mg/L when the H_2O_2 concentration increased from 40 to 100 μM. Carotenes in *B. trispora* served as major antioxidants that protect against cellular injury by quenching the active oxygen species under wild oxidative stress (H_2O_2 concentration above 40 μM). This phenomenon is consistent with the research that the external addition of H_2O_2, in combination with high aeration rates, led to wild oxidative stress and caused significant decrease in β-carotene concentration [14].

Overall, low added concentrations of H_2O_2 (below 40 μM) increased the production of β-carotene, whereas

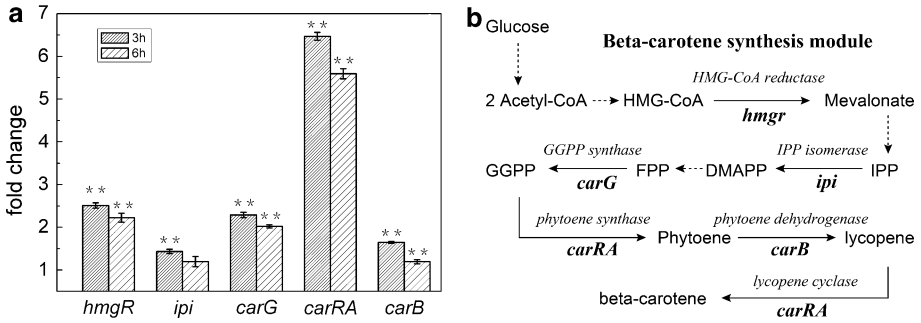

Fig. 2 Fold change of gene transcription after H_2O_2 addition for 3 and 6 h (**a**). β-Carotene biosynthetic pathway (**b**)

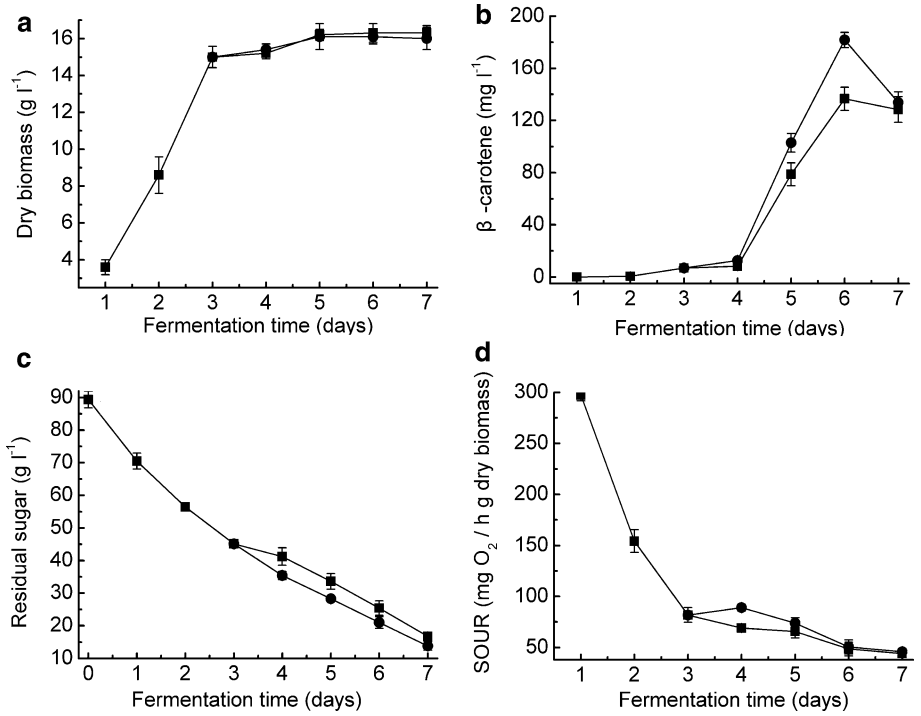

Fig. 3 Effect of H_2O_2 on glucose metabolism (**a**), SOUR (**b**), cell grown (**c**), and β-carotene production (**d**) during carotene production by *B. trispora* in shake flask culture. The cycle and square represent H_2O_2-added and non-H_2O_2-added

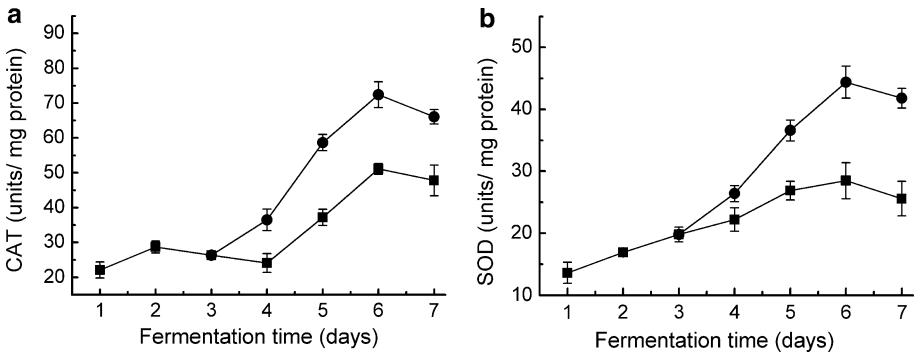

Fig. 4 Effect of H_2O_2 on the specific activity of CAT (**a**) and SOD (**b**) during β-carotene production by *B. trispora* in shake flask culture. The *circle* and *square* represent H_2O_2-added and non-H_2O_2-added

high added concentrations of H_2O_2 (above 40 μM) consumed β-carotene acting as antioxidants to prevent oxidative stress and decreased the production of β-carotene. Thus, 40 μM is an optimal added concentration for enhancing the production of β-carotene.

Effect of H_2O_2 on the expressions of five genes involving the β-carotene biosynthetic pathway

The growth of fungal strains in presence of H_2O_2 shows clear signs of increased oxidative stress [2]. To investigate

the molecular mechanisms of the 3-day-old *B. trispora* under oxidative stress induced by H_2O_2, the gene expression of five enzymes (*hmgr*, *ipi*, *carG*, *carRA*, and *carB*) involving the β-carotene synthesis was analyzed by real-time PCR. The result of transcription level is shown in Fig. 2a, and the β-carotene synthesis pathway is shown in Fig. 2b.

HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase is a rate-limiting enzyme in the mevalonate pathway [21]. Compared with the control group (non-H_2O_2-added), the transcription of *hmgR* increased by 2.51-fold 3 in hours ($P < 0.01$) and 2.23-fold in 6 h ($P < 0.01$) after the addition of H_2O_2. Therefore, the expression of *hmgR* in the cultures remained stable after oxidative stress was induced by H_2O_2. IPP (isopentenyl pyrophosphate) isomerase catalyzes the formation of more reactive DMAPP (dimethylallyl pyrophosphate) from the relatively unreactive IPP [1]. The transcription level of *ipi* improved by 1.43-fold in 3 h ($P < 0.01$) and 1.19-fold in 6 h ($P > 0.05$) after H_2O_2 treatment. The expression of *ipi* in the cultures slightly increased after H_2O_2 treatment. The results show that the transcription of *ipi* was not sensitive to the oxidative stress caused by H_2O_2. GGPP (geranylgeranyl pyrophosphate) synthase catalyzes the formation of FPP (farnesyl pyrophosphate). The expression of *carG* increased by 2.29-fold in 3 h ($P < 0.01$) and 2.03-fold in 6 h ($P < 0.01$) after the addition of H_2O_2. The expression of *carG* in the cultures remained stable after H_2O_2 treatment. *CarRA* and *CarB* genes have been previously identified to be involved in β-carotene biosynthesis in *B. trispora* [20]. *CarRA* encodes a double-functional enzyme with phytoene synthase and lycopene cyclase activity. *CarB* encodes the phytoene dehydrogenase. The expression of *carRA* and *carB* both increased by H_2O_2 treatment. The addition of H_2O_2 led to a 6.45-fold improvement at 3 h ($P < 0.01$) and 5.57-fold enhancement at 6 h ($P < 0.01$) in the transcripts of *carRA*, suggesting that *carRA* transcription was sensitive to oxidative stress by H_2O_2. H_2O_2 may increase the production of β-carotene with increasing expression of *carRA*. The expression of *CarB* increased by 1.65-fold at 3 h ($P < 0.01$) and 1.20-fold at 6 h ($P < 0.01$) after H_2O_2 treatment.

In summary, five genes in the β-carotene biosynthetic pathway (*hmgr*, *ipi*, *carG*, *carRA*, and *carB*) have increased expression after 3 h under H_2O_2 treatment, in which *carRA* was the most sensitive to oxidative stress by H_2O_2 among these five genes. This result demonstrated that the metabolic flow of β-carotene was increased by H_2O_2 treatment. Oxidative stress induced by H_2O_2 appears to increase the production of β-carotene through transcriptional induction of their biosynthetic genes. The increased transcription of five genes is a response for oxidative stress by H_2O_2 treatment in *B. trispora*.

Effect of H_2O_2 on glucose metabolism, SOUR, cell growth, and β-carotene production

The effects on cell growth (a), β-carotene production (b), glucose metabolism (c), and SOUR (d) are shown in Fig. 3, wherein the phases of cell growth and β-carotene biosynthesis were not synchronized. According to the report by Nanou, the process of β-carotene production was divided into three phases: the growth state (0–3 days), production state (3–6 days), and the consumption state (6–7 days), respectively [18].

In the growth state, dry biomass reached up to 15 g/L, and small amount of β-carotene were synthesized. In addition, the metabolic rate of glucose was the highest in the three states, reaching 615 mg/L h, because the rapid metabolism of glucose supplied enough energy for the growth of *B. trispora* in the logarithmic growth phase. SOUR significantly decreased from 297.3 mg O_2/h g dry biomass to 81.8 mg O_2/h g dry biomass for 1-day and 3-day old myceliums, respectively. Mycelium in the early growth state needs large energy consumption for growth, which is closely related to higher glucose metabolism and oxygen consumption.

In the early production state (3–4 days), biomass and β-carotene were constant, the metabolic rates of glucose were 163 mg/L h and 404 mg/L h for non-H_2O_2-added and H_2O_2-added, respectively. Thus, the glucose metabolic rate increased by 148 % under oxidative stress induced by H_2O_2. Furthermore, SOUR was significantly increased, indicating that the aerobic metabolism of glucose was enhanced, but the biomass and β-carotene remained constant. These phenomena showed that increasing the glucose aerobic metabolism is a cellular response to oxidative stress for supplying enough reduced form of nicotinamide-adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH), which may be conducive for alleviating cellular oxidative stress. NADPH was pivotal to the cellular anti-oxidative defense strategies in most organisms [22]. NADPH acts as a key component in cellular antioxidation systems [26]. In Pseudomonas fluorescence, Singh demonstrated a metabolic network promoting NADPH production and limiting NADH synthesis as a consequence of an oxidative stress [23]. The remarkable improvement in the glucose metabolic rate may due to the pentose phosphate pathway shunting the glucose metabolism to supply enough NADPH. Therefore, the aerobic metabolism of glucose was significantly increased for supplying enough NADPH to protect *B. trispora* from oxidative stress. Meanwhile, the strengthening of the aerobic metabolism of glucose may increase intracellular adenosine triphosphate (ATP) and NADPH content. ATP and NADPH are two important cofactors for β-carotene production [27],

and are thus important causes of the increase of β-carotene in the production state.

In the later production state (4–6 days), the biomass remained constant and a large amount of β-carotene was obtained, the metabolic rates of glucose were 329 mg/L h and 300 mg/L h for non-H_2O_2-added and H_2O_2-added, respectively (Fig. 3a). The metabolism rate of glucose decreases slightly due to the reduction on oxidative stress, and does no need to rely on NADPH for resistance to oxidative stress after the enzyme activity of SOD, CAT, and β-carotene was increased in the production state. The highest concentration of β-carotene was 11.3 mg/g dry biomass by H_2O_2 stimulation and 8.4 mg/g dry biomass without H_2O_2 stimulation after 6 days of incubation. The highest biomass dry weight was 16.3 g/L without the addition of H_2O_2, and 16.1 g/L with the addition of H_2O_2. The addition of H_2O_2 at appropriate concentration has no significant change on the cell growth.

In the consumption state (6–7 days), the cell wall of *B. trispora* began to slowly autolyzed, the biomass slightly declined, and the production of β-carotene decreased significantly. Given that SOD and CAT enzyme activity decreased, β-carotene was consumed the main antioxidant.

Effect of H_2O_2 on specific activity of CAT and SOD

The specific activity of catalase (CAT) (a) and superoxide dismutase (SOD) (b) during fermentation are shown in Fig. 4. In two culture systems (H_2O_2-added and non-H_2O_2-added), the intracellular activity of SOD and CAT both reached maximum after 6 days of cultivation, and then decreased. The decreased scavenging enzyme activities indicated an increased exposure of *B. trispora* to ROS. The intracellular activity of SOD and CAT were increased by 18.9 and 51.5 % after 24 h after the addition of H_2O_2. After 6 days of culturing, given the proteolytic degradation of the proteases or the leakage from damaged cells, the activities of SOD and CAT remarkably decreased [18]. This is one of the oxidative stress responses for protecting *B. trispora* from the deleterious effects of ROS. Enzymatic defense system (SOD and CAT) is another strategy in protecting *B. trispora* from oxidative stress. The biosynthetic process of β-carotene was consistent with the formation of SOD and CAT. The improvement of SOD and CAT enzyme activity can reduce the loss of β-carotene by oxidative stress.

Conclusions

Mild oxidative stress can improve the production of β-carotene by *B. trispora*. The reasons for the improvement of β-carotene production induced by H_2O_2 may be attributed to the over expression of key enzyme genes, improvement of ATP, the activity increase of SOD and CAT, and NADH, as well as NADPH. The response of oxidative stress in *B. trispora* against oxidative stress is strongly correlated with the scavenging enzymes (SOD, CAT) and the antioxidant compounds (carotenes). Furthermore, the aerobic metabolic product (NADH and NADPH) may also have a role in the antioxidant defense systems.

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