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Stimulation of the biosynthesis of carotenes by oxidative stress in *Blakeslea trispora* induced by elevated dissolved oxygen levels in the culture medium

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ABSTRACT

The adaptive response of the fungus *Blakeslea trispora* to the oxidative stress induced by elevated dissolved oxygen concentrations during carotene production was investigated by measuring the specific activities of catalase (CAT) and superoxide dismutase (SOD) and the micromorphology of the fungus using a computerized image analysis system. Changes in the ratio of the volume of air (V_a) over the medium and the volume of medium (V_m) in the flask caused changes of the morphology of microorganism from clumps to pellets and increases in the specific activities of CAT and SOD. The oxidative stress in *B. trispora* resulted in a significant increase in carotene production, and a maximum proportion of β-carotene (60%), γ-carotene (50%), and lycopene (10%) (as percentages of total carotenes) was observed at a ratio V_a/V_m of 15.7, 4.0 and 1.5, respectively. The highest concentration of carotenes (115.0 mg/g dry biomass) was obtained in V_a/V_m ratio of 9.0.

1. Introduction

As oxidative stress has been defined the disturbance in the prooxidant–antioxidant balance, resulting in potential cell damage. Aerobic organisms use molecular oxygen for respiration and energy supply, but these processes also result in the formation of potentially damaging reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\cdot}$), and superoxide radicals ($O_2^{\cdot-}$) (Kreiner et al., 2000, 2003; Gessler et al., 2002, 2007; Zhao et al., 2005; Angelova et al., 2005). Cells protect themselves from oxidative injury with enzymatic and non-enzymatic defense systems, such as carotenes, that act as radical scavengers (Gessler et al., 2007; Jamieson, 1998).

Since the production of carotenes is of biotechnological interest (Choudhari and Singhal, 2008; Valduga et al., 2009; Aksu and Tugba Eren, 2005; Malisorn and Suntornsuk, 2009), means of increasing the yields of carotenes produced by organisms are important. One possible way by which increases in carotene production could be achieved is by subjecting cells to oxidative stress. Mantzouridou et al. (2005) studied the effect of oxygen transfer rate (OTR) on β-carotene production by *Blakeslea trispora* in shake flask culture. The results indicated that the carotene concentration was the highest in culture grown at maximum OTR. Xu et al. (2007) who examined the production of lycopene and β-carotene by *B. trispora* found that the production of carotenes was increased when oxygen-vectors, *n*-hexane and *n*-dodecane, were added to the medium. The role of hydrolytic enzymes in autolysis of *B. trispora* as well as the effect of butylated hydroxytoluene (BHT)-induced oxidative stress on *B. trispora* on carotene production in submerged cultures was examined by Nanou et al. (2007) and Nanou and Roukas (2010). It was found that the phenomenon of autolysis was associated with high concentrations of ROS and the addition of BHT to the medium caused changes of the morphology of microorganism from aggregates with large projected area to aggregates with small projected area. To further study the response of carotene-producing *B. trispora* to oxygen stress, batch cultivations in flasks containing different ratios of medium volumes to air volumes were conducted. The activities of superoxide dismutase (SOD) and catalase (CAT), the two key defensive enzymes to oxidative stress, were measured as a function of the dissolved oxygen concentration, and computerized image analysis was used to investigate morphological changes in response to oxidative stress.

2. Methods

2.1. Microorganisms and culture conditions

B. trispora ATCC 14271, mating type (+) and *B. trispora* ATCC 14272, mating type (−) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The strains were grown on potato dextrose agar (PDA) (Scharlau, 01-483) Petri dishes at 26 °C for 4 days for sporulation. Ten milliliters of sterile distilled water was added to the Petri dish and the spores were

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collected by scraping of the medium surface. A spore suspension containing 5.0×10^5 and 1.0×10^6 spores/ml of the strains 14271 and 14272, respectively, was used to inoculate the production medium.

2.2. Fermentation conditions

Cultivation was carried out in 250-ml conical flasks containing 100, 50, 25, and 15 ml of medium in order to obtain a ratio V_a/V_m of 1.5, 4.0, 9.0, and 15.7 where V_a is the volume of the air over the medium and V_m is the volume of the medium in the flask. The medium consisted of (g/l): glucose (Scharlau, GL 0129) 50; corn steep liquor (Sigma, S-4648; sterilized at 121 °C for 30 min) 80; yeast extract (Scharlau, 07-079) 1.0; casein acid hydrolysate (Scharlau, 07-151) 2.0; L-asparagine (Sigma, A-8381) 2.0; KH_2PO_4 (Merck, 4873) 1.5; $MgSO_4.7H_2O$ (Merck, 5882) 0.5; thiamine.HCl (Sigma, T-4625) 0.005; linoleic acid (Sigma, L-1626) 20.0; Span 20 (Sigma, S-6635) 10.0; and butylated hydroxytoluene (BHT) (Sigma, B-1378) 4.4. The pH of the medium was adjusted to 7.5 with 1 N NaOH and the medium was 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*. The flasks were incubated at 28 °C in a rotary shaker incubator (Lab Line Orbit-Environ Shaker, Lab-Line Instr., Melrose Park, IL, USA) at 250 rpm.

2.3. Analytical techniques

2.3.1. Estimation of carotenes, dry biomass, residual sugars, pH, and dissolved oxygen concentration

At appropriate time intervals, fermentation flasks were removed and carotene concentration, dry biomass, residual sugar concentration, and pH were determined according to Nanou and Roukas (2010). Dissolved oxygen concentrations were determined with a microprocessor oximeter (OXI 96, WTW, Germany) and are expressed as percentage of the initial level of saturation. Carotenes were analyzed by high-performance liquid chromatography (HPLC). The HPLC was performed with a P2000 pump, an AS3000 autosampler and a UV6000LP diode array detector. A Thermo HyPurity C18 column (150×4.6 mm, 5 μm particle size) thermostated at 40 °C was used. The diode array detector was operated from 380 to 600 nm. The mobile phase of acetonitrile was eluted at a flow rate of 1.5 mL/min. Under these conditions, β-carotene, γ-carotene, and lycopene were eluted within 12.6, 9.6, and 7.2 min, respectively. The detection of β-carotene and lycopene was done at 453 nm. Standards of the above substances were obtained from Sigma and redivivo™ 10% FS, DSM; Nutritional Products Ltd., Basel, Switzerland, respectively. Since γ-carotene was not commercially available, this carotene was identified by its absorbance maxima (440, 462, 492 nm) as indicated by Takaichi (2000). The data are the average values ±SD of three independent experiments.

2.3.2. Enzyme assays

Approximately 10 ml of a culture was filtered through a Whatman No. 3 filter. The mycelium was washed with distilled water until the filtrate was colorless. The wet biomass (0.3 g) was mixed with liquid nitrogen and pulverized with a pestle to extract the enzymes from the biomass. The pulverized materials were mixed with 2.0 ml of physiological saline (0.85% NaCl, pH 6.0) containing 10 μl of a 0.2 M phenylmethanesulfonyl fluoride (PMSF) solution (Sigma, P-7626) for the inactivation of proteases, and centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant was used for the determination of the activities of SOD and CAT as described by Beauchamp and Fridovich (1971) and Aebi (1984), respectively. The specific activity of the enzymes was expressed as units/mg protein. The protein content was estimated by the method of Schacterle and Pollack (1973).

2.3.3. Image analysis

The clumps and pellets were determined by microscopic image analysis of ten 1.0-ml culture samples diluted 20-fold in sterile distilled water. Images were captured by a 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 the Matrox Inspector 32 image processing program.

3. Results and discussion

3.1. Effect of the ratio V_a/V_m on carotene production and dry biomass

One important factor that influences the production of carotenes in shake flask culture is the ratio of V_a/V_m. The purpose of this experiment was to determine the optimum ratio of V_a/V_m that would result in the highest carotene concentration. Fig. 1a shows the concentration of carotenes in response to various V_a/V_m ratios. In all cultures a continuous increase in carotene production was observed up to 6th or 8th day of incubation. Carotene production was highest at V_a/V_m ratios of 9.0 and 15.7. These results demonstrate that a sufficient supply of oxygen is a prerequisite for producing carotenes. Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, products/byproducts, and oxygen. Aeration results in better mixing of the production medium, thus helping maintain a concentration gradient between the interior and the exterior of the cells. This concentration gradient facilitates the removal of gases and other byproducts of catabolism from the microenvironment of the cells. Air supply favors oxygen supply to the cells and this is especially important for high biomass concentrations. Generally, sufficient aeration seems to be

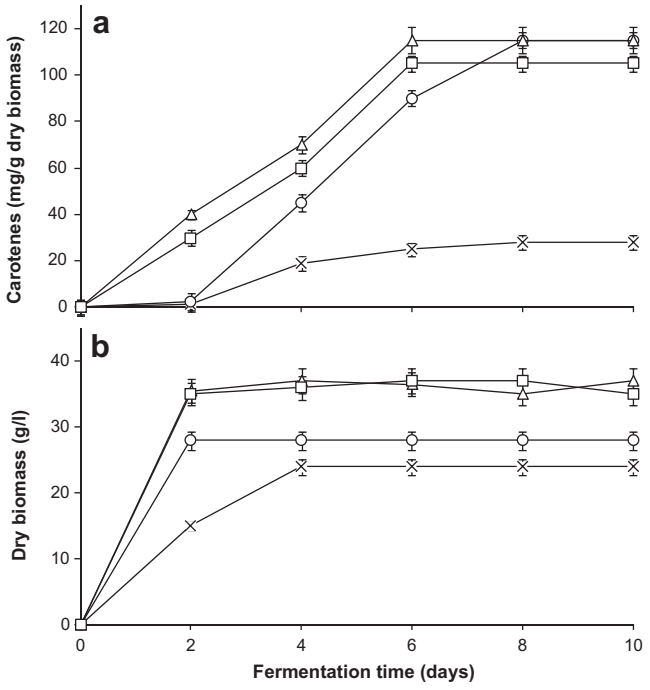

Fig. 1. Effect of the ratio V_a/V_m on carotene production (a) and dry biomass (b) during biosynthesis of carotenes by *B. trispora* in shake flask culture. -Ж- , -○-, -△- , and -□- V_a/V_m of 1.5, 4.0, 9.0, and 15.7, respectively. Error bars represent the SD of the mean.