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Oxidative stress and morphological changes in *Blakeslea trispora* induced by enhanced aeration during carotene production in a bubble column reactor

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ABSTRACT

The oxidative stress and the morphological changes in *Blakeslea trispora* induced by enhanced aeration during carotene production in a bubble column reactor was investigated. Enhanced aeration 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 increase of the specific activities of superoxide dismutase (SOD) and catalase (CAT). The oxidative stress in *B. trispora* resulted in a significant increase in carotene production. The highest concentration of carotenes (55.0 ± 2.5 mg/g dry biomass) was obtained at aeration rate of 4 vvm. Very high oxidative stress in *B. trispora* caused a change in the biosynthesis of carotenes resulting an increase in γ-carotene concentration. The maximum proportion of β-carotene (91.68%), γ-carotene (44.67%), and lycopene (11.54% of total carotenes) was observed at aeration rates of 4, 5, and 5 vvm, respectively. The external addition of butylated hydroxytoluene (BHT) and hydrogen peroxide in the medium did not improve the production of carotenes.

1. Introduction

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 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, and ultimately cell death [1,2]. For protecting the cells from oxidative injury, aerobic organisms possess both enzymatic and non-enzymatic defense systems. They act as radical scavengers, being oxidized by ROS and thereby removing oxidants from solution [2,3].

Very little published information is available on the oxidative stress in filamentous fungi [4–7]. Aksu and Tugba Eren [8], Malisorn and Suntonsuk [9], and Valduga et al. [10] studied the production of carotenes by *Rhodotorula mucilaginosa, Rhodotorula glutinis*, and *Sporidiobolus salmonicolor*, respectively. Goksungur et al. [11] studied the production of β-carotene from beet molasses by *Blakeslea trispora* in stirred-tank and bubble column reactors. Mantzouridou et al. [12] examined the effect of oxygen transfer rate on β-carotene production by *B. trispora* while Xu et al. [13] studied the production of lycopene and β-carotene by *B. trispora* using oxygen-vectors to increase dissolved oxygen concentration. Recently, in our laboratory, the role of hydrolytic enzymes in autolysis of *B. trispora* and the oxidative stress response of *B. trispora* induced by butylated hydroxytoluene (BHT) were studied [14,15]. In this work, we induced oxidative stress in *B. trispora* by enhanced aeration using a bubble column reactor in order to achieve high concentration of carotenes. This attempt drove us to the interest on this subject to explain the mechanism by which the enhanced aeration affected changes in culture morphology and oxidative stress.

The bubble column reactor is one of the most promising devices for gas–liquid mass transfer and is being considered especially for fermentation processes. Bubble column reactor is an elongated non-mechanically stirred reactor with an aspect ratio of height/diameter through which there is a unidirectional flow of gases. Bubble column reactor compared to the conventional stirred tank fermentor has several advantages. There is no mechanical agitation, and the air bubbles forced through the air compressor are responsible for the induced turbulent liquid mixing and the accompanying mass transfer. The fermentation process can be controlled more easily. The required bulk mixing and mass transfer is more cost-effective and requires less energy [16,17]. The disadvantage of the bubbe column reactor is that the reactor performance decreases sharply with increasing gas velocity due to the formation of large gas bubbles. In addition, when viscosity of the liquid phase in the reactor is relatively high, the increases in bubble size causes a sig-

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nificant reduction in the interfacial area between the gas phase and the liquid phase, thereby resulting in a decreased mass transfer rate.

In examining the processes of oxidative stress in *B. trispora,* we adopted three ways: (1) monitoring the activities of superoxide dismutase (SOD) and catalase (CAT) which are the two key defensive enzymes to oxidative stress, (2) measuring the dissolved oxygen concentration, and (3) using computerized image techniques to determine morphological differentiation of the culture.

2. Materials and methods

2.1. Microorganisms and culture conditions

The microorganisms used in this work were *B. trispora* ATCC 14271, mating type (+) and *B. trispora* ATCC 14272, mating type (−). Both strains 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 ml of sterile distilled water was added to the Petri dish and the spores were collected by scraping off the medium surface. The 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 medium.

2.2. Fermentation conditions

Fermentation was carried out in a 1.4-l glass bioreactor (height 60 cm, diameter 5.5 cm) with a working volume of 0.7 l. The substrate consisted of (g/l): glucose (Scharlau, GL 0129) 50.0; corn steep liquor (Sigma, C-4648; sterilized at 121 °C for 30 min) 30.0; 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; and Span 20 (Sigma, S-6635) 10.0. The pH of the medium was adjusted to 7.5 with 10 N NaOH and then sterilized at 121 °C for 20 min. After cooling, the medium was inoculated with 7.0 ml of the spore suspension of each strain of *B. trispora* (obtained as described above). The reactor was incubated at 26 °C in a controlled temperature chamber. The air was supplied from the bottom of the column with an air pump at rates of 3, 4, and 5 vvm (2.1, 2.8, and 3.5 l/min). In order to study the effect of antioxidants and oxidants on carotene production, the medium was supplemented with different concentrations of butylated hydroxytoluene (BHT) (Sigma, B-1378) and hydrogen peroxide (Sigma, H-1009). In this case the aeration rate was 4 vvm. Hydrogen peroxide was added in the medium on the second day of fermentation.

2.3. Analytical techniques

Every 2 days, fermentation broth was removed from the reactor and analyzed. Carotene concentration, dry biomass, pH, and sugar concentration were determined according to Nanou et al. [14]. 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 carotenes produced 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 mm × 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 were 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 [18]. The data are the average values ± SD of three independent experiments.

2.3.1. Enzyme assays

Approximately 10 ml of fermentation broth was filtered through a Whatman No. 3 filter. The mycelium was washed with distilled water until the filtrate was colorless. 0.3 g of wet biomass was mixed with liquid nitrogen and the mixture was pulverized with a pestle to extract the enzymes from the biomass. The particles were mixed with 2.0 ml of physiological saline (0.85% NaCl, pH 6.0) and the liquid was centrifuged at $10{,}000 \times g$ 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 [19] and Aebi [20], respectively. The physiological saline was supplemented with 10 μl of phenylmethanesulfonyl fluoride (PMSF) solution 0.2 M (Sigma, P-7626) for the inactivation of proteases. In all cases, the specific activity of the enzymes was expressed as units/mg protein. The protein content was estimated by the method of Schacterle and Pollack [21].

2.3.2. Image analysis

The morphological entities measured were aggregates with different size. For the determination of aggregates, an amount of 0.1 g of wet biomass was mixed with 20 ml of sterile distilled water in a Petri dish and the diluted sample was observed microscopically. Ten amounts of 0.1 g wet biomass 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.

3. Results and discussion

3.1. Effect of the aeration rate on carotene production and dry biomass

One important factor that influences the production of carotenes in a bubble column reactor is the aeration rate. The purpose of this experiment was to determine the optimum aeration rate that would result in the highest carotene concentration. The effect of the aeration rate on carotene concentration is shown in Fig. 1a. In all cases the carotenes were synthesized into two steps. In the first step (0 to 2–4 days of fermentation) called "growth phase", and in the second step (2–4 to 8 days of fermentation) called "static phase" (Fig. 1a and b). The carotene concentration increased significantly as aeration rate increased up to 4 vvm. On the other hand, a further increase of the aeration rate at values over 4 vvm, resulted in a decrease in carotene concentration. The highest concentration of carotenes (55.0 ± 2.5 mg/g dry biomass) was obtained in culture grown at an aeration rate of 4 vvm after eight days of incubation and then decreased. The decline in the concentration of carotenes was due to the oxidation of the pigments in the relevant isomers and epoxides by the oxidative enzymes of the fungus [22]. At aeration rates of 3 and 5 vvm, the highest concentration of carotenes was 28.5 and 30.0 mg/g dry biomass, respectively. On the other hand at aeration rates of 1 and 2 vvm, the maximum concentration of carotenes was low (20.0 and 26.0 mg/g dry biomass after 14 and 10 days of incubation, respectively) (data not shown). At low aeration rates the cells of the microorganism were not uniformly dispersed in the medium and precipitated at the bottom of