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Enhancing beta-carotene biosynthesis and gene transcriptional regulation in *Blakeslea trispora* with sodium acetate

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ABSTRACT

The mold *Blakeslea trispora* is one of the most promising and economically attractive natural sources of β-carotene. In this study, the effects of sodium acetate (NaAC) on β-carotene content and production in mated *B. trispora* were investigated. The results show that the addition of acetate into mated medium could enhance β-carotene accumulation in *B. trispora*. The highest β-carotene content (59.91 mg/g dry biomass) and production (2130 mg/l) were obtained as adding 35 mM of NaAC at stationary phase, which were 77.7% and 80.5% increments compared with that of the control, respectively. Furthermore, the effects of NaAC on expression levels of carotenogenesis genes in mated *B. trispora* were investigated. The results show that addition of NaAC in mated medium caused the induction of five carotenogenesis genes expression (*hmgR*, *carG*, *ipi*, *carRA*, and *carB*) and promoted de novo synthesis of β-carotene. The induction of five genes expression exhibited sequential gene expression profiles and the five gene expression were ranging from 1.8 to 3.8 folds increment as early as 24 h after NaAC addition. We demonstrate that NaAC stimulation of β-carotene biosynthesis in mated *B. trispora* involved in change at genes transcriptional levels. Such regulatory mechanism provides an explanation for effect of NaAC on the biosynthesis of β-carotene in mated *B. trispora*.

1. Introduction

The β-carotene is a carotenoid of orange-red terpenoid pigments biosynthesized by microorganisms and plants and has been wildly applied in the pharmaceutical, nutraceutical, cosmetic, and animal feed industries [1–4]. The commercial β-carotene has been mainly produced using carotenogenic microorganisms, such as *Blakeslea trispora* [5], *Rhodotorula glutinis* [6], *Sphingomonas* sp. [7], and *Phycomyces blakesleeanus* [8]. One of the most promising natural commercially utilized sources of β-carotene is derived from the zygomycete mold *B. trispora*. This heterothallic zygomycota possess two mating types, termed "plus" and "minus", which are well known to produce β-carotene on an industrial scale during the mating of (+) and (−) strain mycelia [9].

In *B. trispora*, β-carotene biosynthesis is derived from the mevalonate biosynthetic pathway [10]. As shown in Fig. 1, the biosynthesis of β-carotene from 3-hydroxy-3- methy glutaryl-coenzyme A (HMG-CoA) in *B. trispora* requires at least five crucial enzymatic activities, which are catalyzed by enzymes encoded by the genes *hmgR*, *ipi*, *carG*, *carRA*, and *carB* [11–15].

While the functions of encoded genes used for β-carotene biosynthesis in fungi and yeast have been identified, the regulatory mechanisms remain mostly unknown. Several studies have shown a strong correlation between the carbon source present in the medium and the amount of carotenoid biosynthesis in yeast [16]. Glucose or other fermentable sugars are metabolized through the glycolytic pathway, where a high glucose concentration serves as the carbon source and strongly inhibits carotenoid biosynthesis. Whereas, small carboxylic acids or alcohols, such as acetate or ethanol are converted to acetyl-CoA through the citric acid cycle, which is the substrate for the synthesis of isoprenoids by the mevalonate pathway, ultimately leading to carotenoid biosynthesis [16]. However, previous studies have also reported that small quantities of acetate and short-chain carboxylic acid could stimulate zygospore production and inhibit carotene production in *B. trispora*,

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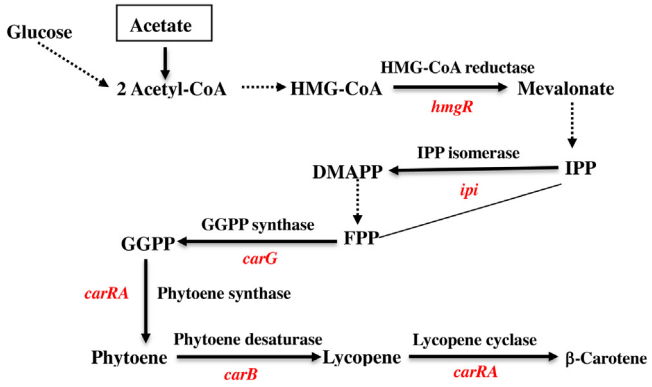

Fig. 1. Scheme of the carotenoid biosynthetic pathway in *B. trispora*. HMG-CoA reductase (encoded by the *hmgR* gene) catalyzes the reduction of HMG-CoA to mevalonate; PP isomerase (isopentenyl pyrophosphate isomerase, encoded by the *ipp* gene) catalyzes the isomerization of IPP to dimethylalyl pyrophosphate (DMAPP); GGPP synthase (geranylgeranyl pyrophosphate, encoded by the *carG* gene) catalyzes the production of farnesyl pyrophosphate (FPP); Phytoene synthase (encoded by the A domain of the *carRA* gene) links two GGPP molecules to form phytoene; Phytoene desaturase (encoded by the *carB* gene) catalyzes the production of lycopene; Lycopene cyclase (encoded by the R domain of the *carRA* gene) form β-carotene.

with β-carotene content dependent on the acetate concentration [17]. These studies suggest that ambiguity still remains regarding the metabolic regulatory mechanisms and the correlation between the addition of acetate and carotenoid biosynthesis in *B. trispora*. Therefore, it is necessary to study the effect of acetate on β-carotene biosynthesis and the expression levels of carotenogenesis genes in *B. trispora*.

In this study, we attempted to increase β-carotene production using various concentrations of sodium acetate (NaAC) as a precursor in mated *B. trispora* cultures. Additionally, the transcription levels of the β-carotene biosynthetic genes (*hmgR*, *ipi*, *carG*, *carRA*, and *carB*) in *B. trispora* were investigated in two cases, i.e., with and without the addition of sodium acetate.

2. Materials and methods

2.1. Microorganism and culture conditions

B. trispora ATCC 14271, mating type (+), and ATCC 14272, mating type (−), were obtained from the American Type Culture Collection (ATCC). The strains were cultured on potato dextrose agar (PDA) (BD Difco) Petri dishes at pH 7.0 and 28 ± 0.5 °C for 5 days for sporulation. A pre-cultures containing 5.0×10^6 spores/ml of the strains 14271 and 14272, respectively, were inoculated in seed medium composed of the following (g/l): 40 glucose, 10 corn starch, 0.5 $MgSO_4$, 2 KH_2PO_4, 0.05 $CaCl_2$, 0.05 $MnSO_4 \cdot H_2O$, and 0.01 $CuSO_4 \cdot 7H_2O$. Spores were grown at 28 ± 0.5 °C on a shaker at 200 rpm for 60–72 h and then the pre-cultures were used for the inoculation of the fermentation medium.

2.2. Fermentation conditions

The batch cultivation was carried out in a 500 ml Erlenmeyer flasks containing 50 ml medium with the following composition (g/l): 45 g glucose, 10 corn starches, 10 yeast extract, 10 soybean oil, 0.5 $MgSO_4$, 2 K_2HPO_4, 2 KH_2PO_4, and 2 urea, pH 7.0. The medium was inoculated with 15% (v/v) inoculum in a 1:1 (volume ratio) mixture of strains 14271 and 14272 of *B. trispora* at 200 rpm and 28 ± 0.5 °C for 6 days to 8 days in the dark. Different sodium acetate concentrations were added into the medium (adjusted to pH 7.0 with 1 mM NaOH) at every 24 h in fermentation process.

2.3. Collection of biomass and determination of residual glucose concentrations

The biomass was collected by centrifugation (12,000 rpm) the collected wet cells were washed thoroughly three times with distilled water to remove residual salts. One portion of collected wet biomass was used for β-carotene extraction and analysis, another portion was dried at 45 °C nuder 0.08 MPa vacuum for 48 h. The dried cells were weight to determine dry biomass. The supernatant was collected for analysis residual sugar concentrations with the DNS method [18].

2.4. β-Carotene extraction and analysis

Wet cell were collected as described above, about 20 mg of the collected wet biomass was mixed with 1 ml ethyl acetate and disrupted by a bead-beater (MM400, Retsch, Germany) for 6 min. The β-carotene was extracted with ethyl acetate at room temperature until the organic extract was clear, with β-carotene content analyzed by high-performance liquid chromatography (HPLC, Finnigan Surveyor, Thermo, Scientific, Germany) as previously described [19]. An YMC30 RP-30 column (4.6 mm × 250 mm × 5 μm) with 1 ml/min of mobile phase at 25 °C was used, with the mobile phase as follows: 3% ddH_2O in methanol containing 0.05 M ammonium acetate (solvent A); 100% TBME (solvent B). Both solvents contained 0.1% (w/v) butylated hydroxytoluene and 0.05% (v/v) triethylamine. Elution was carried out according to the following program: isocratic at 3% B for 2 min followed by a linear gradient from 2% to 38% B in 1 min, isocratic at 15% for 12 min, a linear increase to 68% B in 1 min, isocratic at 68% for 6 min followed by a linear decrease to 3% B in 4 min. β-Carotene was detected by measuring the absorbance at 450 nm and a β-carotene standard was used for quantification (Sigma-Aldrich, No: 22040).

Table 1
Real-time PCR primers used in this study.

Genes	Forward and reverse primers (5′ → 3′)		Amplicon length (bp)
tef1	*tef1*-F	AACTCGGTAAGGGTTCCTTCAAG	138
	tef1-R	CGGGAGCATCAATAACGGTAAC	
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	

F: Forward, R: Reverse.

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