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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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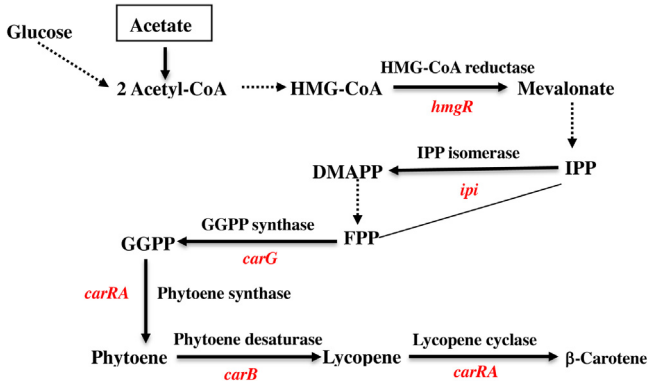

Fig. 1. Scheme of the carotenoid biosynthetic pathway in *B. trispora*. HMG-CoA reductase (encoded by the *hmgR* gene) catalyzes the reduction of HMG-CoA to mevalonate; PP isomerase (isopentenyl pyrophosphate isomerase, encoded by the *ipp* gene) catalyzes the isomerization of IPP to dimethylalyl pyrophosphate (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.

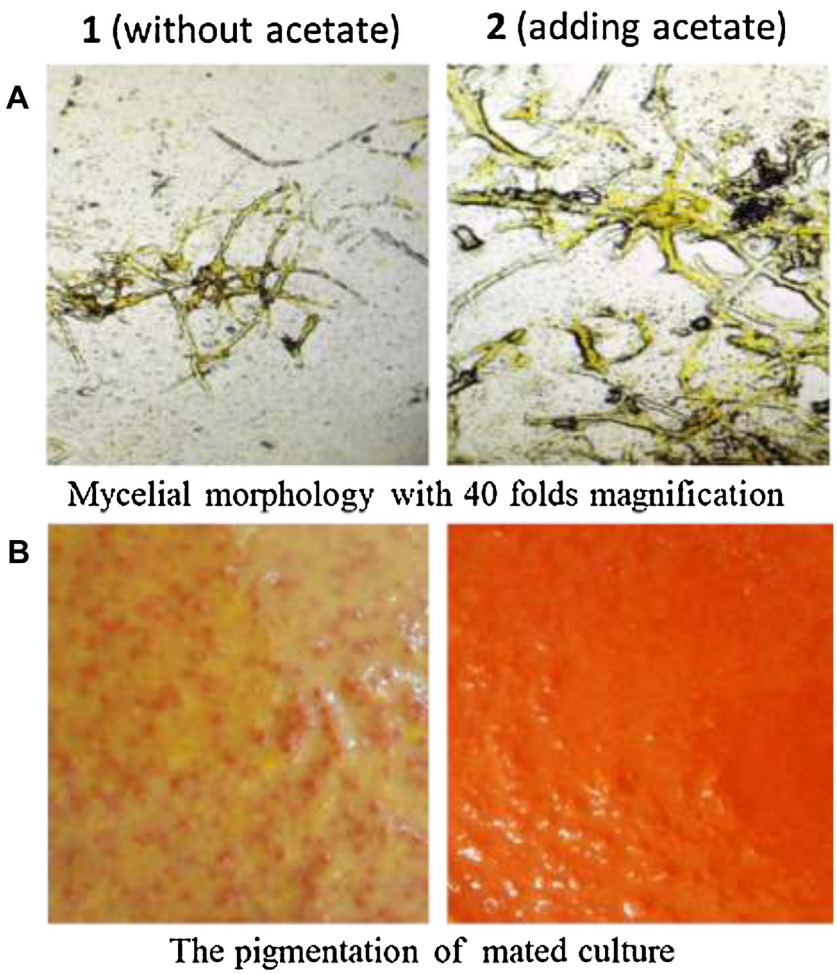

Fig. 2. (A) Mycelial morphology of mated cultures of *B. trispora* grown on 100 ml medium with (A2) or without 35 mM sodium acetate (A1) for 48 h of cultivated time. (B) The pigment of fermentation broth influenced by acetate: mated cultures of *B. trispora* grown on 100 ml medium with (B2) or without 35 mM sodium acetate (B1) for 48 h of cultivated time.

2.5. RNA extraction and reverse transcription PCR

To determine relative gene expression during different times following the addition of sodium acetate, wet cells were processed as described above. Total RNA was extracted using the TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Japan) according to the manufacturer's instructions. Briefly, 10 μl of total RNA was used for cDNA synthesis with 1.0 μl MultiScribe™ Reverse Transcriptase, 2.0 μl RT Buffer, 2.0 μl Primer Mix, and 0.8 μl dNTP Mix (100 mM) in a final volume of 20 μl. cDNA was stored at −20 °C until use.

2.6. Quantitative real-time PCR (qPCR)

Transcriptional levels of five carotenogenesis genes were determined using a Step One™ Real-Time PCR system (Applied Biosystems, Singapore) and a reaction mixture containing 2 μl of the reverse transcription reaction, 0.4 μl of each primer, and 10 μl of SYBR Premix EX TaqII (2×), and 0.4 μl ROX reference dye (50×, Takara, Japan) in a final volume of 20 μl. Primers for *tef1*, *hmgr*, *ipi*, *carG*, *carRA,* and *carB* (Table 1) were used as previously reported [20,21]. The qPCR 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. Each sample was performed in triplicate, with results normalized to *tef1* expression and compared with the corresponding control group without sodium acetate (value = 1) using the comparative $2^{-\Delta\Delta Ct}$ method [17,22].

3. Results and discussion

3.1. Pigmentation and mycelial morphology of mated culture responses by acetate

The (+) and (−) mating strains (1:1 volume rate) of *B. trispora* were inoculated on fermentation broth with or without 35 mM sodium acetate for 72 h cultivated time at 28 °C. The presence of moderate amounts of sodium acetate in the media remarkable modified the mycelial morphology and pigmentation in fermentation broth of *B. trispora* (Fig. 2). The (+) and (−) mycelium of mated culture were more mature and stronger, more well-dispersed growth, and more conductive to zygospores with adding sodium acetate than that of without sodium acetate (Fig. 2a). In the absence of sodium acetate of mated culture, the (+) and (−) mycelium were growth slender and trended to clustering mycelium, and (+) and (−) mycelium clustering were the disadvantage of zygospore formation. This is also supported by several studies, which have demonstrated that small amount of sodium acetate may be signals to stimulate zygospore production [17]. The pigmentations of mated cultures were much more abundant and appeared earlier in the presence of sodium acetate than in the absence of sodium acetate (Fig. 2b). The mated culture showed orange-red in the pres-

Table 2
Dry biomass, β-carotene yield and content of *B. trispora* with 35 mM (final concentration) sodium acetate added at various time points.

Time of addition (h)	Dry biomass (g/l)	β-carotene yield (g/l)	β-carotene content (mg/g dry biomass)
Control (without acetate)	35.08 ± 0.55	1.18 ± 0.11	33.71 ± 0.20
0	35.91 ± 0.69	1.62 ± 0.05	45.11 ± 0.07
24	35.32 ± 0.43	1.75 ± 0.05	49.54 ± 0.11
48	35.51 ± 0.82	1.88 ± 0.07	52.94 ± 0.09
72	35.55 ± 0.85	2.13 ± 0.08	59.91 ± 0.19

The cells were harvested at 144 h. Values are means of a triplicate ± standard derivation.

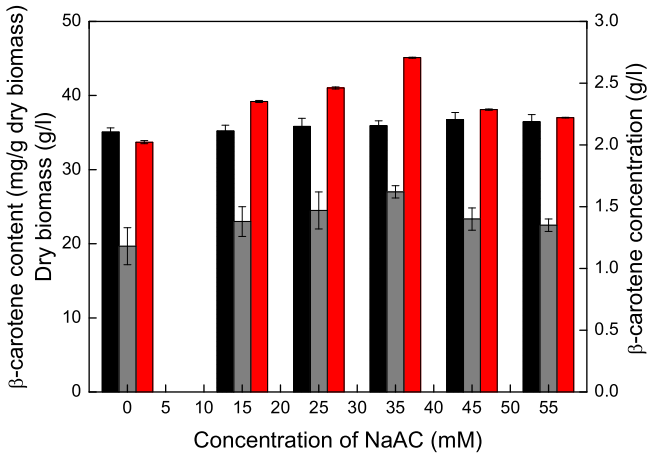

Fig. 3. Dry biomass, β-carotene production and content of B.trispora with different concentrations of sodium acetate (NaAC) added at the start of incubation. Black, gray and bars represent dry biomass, β-carotene concentration and β-carotene content, respectively. Error bars represent the SD of the mean.

ence of sodium acetate and yellow in the absence of sodium acetate. This result indicated that the mated culture of *B. trispora* in the presence of sodium acetate increased to biosynthesis pigmentation due to enhance carotene biosynthesis. However, the high concentration of sodium acetate simulating carotene biosynthesis in mated cultures obtained from this study is inconsistent with the findings of Kuzina et al. [17], which reported the small amount of sodium acetate (1–10 mM) in mated cultures increased zygospore formation and prevent sexual carotenogenesis. The possible reasons for these discrepant results could be due to differences in the concentration of sodium acetate and cultural medium. In the presence of high concentration of sodium acetate in mated culture medium, acetate may first be converted to acetyl-CoA and then increased carotenoid biosynthesis by the mevalonate pathway.

3.2. Effects of acetate concentrations on cell growth and β-carotene production

Small carboxylic acids, such as sodium acetate, exhibit strong signaling effects during zygospore formation and have a profound influence on carotenoid biosynthesis in *B. trispora* [16,17]. To investigate the effect of sodium acetate concentration on *B. trispora* growth and β-carotene production, various sodium acetate concentrations (0, 15, 25, 35, 45, and 55 mM) were added into mating *B. trispora* cultures at the initiation of culturing. As shown in Fig. 3, the biomass value slightly raised with increased sodium acetate concentration, reaching a maximum concentration of 45 mM (35.75 g/l, 4.9% higher than that of control). These results suggested that the high concentration of sodium acetate has no significant effect on cell growth. When a high glucose concentration (250 mM) is present to serve as a carbon source in mated cultures, the sodium acetate may not be utilized as a carbon source, but instead is converted into acetyl-CoA to alter carotene metabolism [16]. The addition sodium acetate of different concentration in the mated medium could increase the β-carotene production and content in *B. trispora* relative to the control. The highest β-carotene production and content reached 1620 mg/l (37.3% higher than that of control) and 45.11 mg/g dry biomass (33.8% higher than that of control), respectively, while 35 mM sodium acetate was added into the mated medium at the start of fermentation. These results demonstrated that the addition of sodium acetate into a mated culture promotes β-carotene biosynthesis in *B. trispora*.

3.3. Effects of acetate addition time on cell growth and β-carotene production

In fungi, β-carotene biosynthesis usually occurs at the stationary stage of cell growth [9], with different sodium acetate addition time possibly affecting biomass and β-carotene production in *B. trispora*. In this work, the sodium acetate was added into the mated medium at various cultivated times. The sodium acetate addition at the different cultivated time caused an remarkable increase in β-carotene content and yields relative to the control (without sodium acetate), and the β-carotene content increased 17.1% and 10% as adding at the log (48 h) and early-log (24 h) growth phase relative to addition at the start of cultivation, respectively (Table 2). The highest production of β-carotene (2130 mg/l, 80.5% higher than that of control) and content (59.91 mg/g dry biomass, 79% higher than that of control) were obtained, when sodium acetate was added into the stationary phase (72 h), after 6 days post-fermentation and was higher than that of the addition at 0 h, 24 h, and 48 h of culturing time (Table 2). These results suggested that different sodium acetate addition times could significantly influence β-carotene biosynthesis in *B. trispora*. The β-carotene content and production increased with addition time of sodium acetate following 0 h, 24 h, 48 h and 72 h of cultivated time, with the optimal addition time being at stationary phase (72 h) of *B. trispora* growth.

Subsequently, kinetic curves for cell growth and β-carotene production with and without the addition of sodium acetate were investigated. When comparing kinetic curves for cell growth and β-carotene production, the results showed that β-carotene is typical a secondary metabolite and begins to massively accumulate at stationary phase, which is consistent with previous reports [9,23]. As shown in Fig. 3, the process of β-carotene biosynthesis in *B. trispora* is divided into three stages, including the cell growth phase (0–3 days), the formatting phase (3–6 days) and the consumption phase (6–8 days). In the cell-growth phase, biomass significantly increases, with small amounts of β-carotene accumulation (Fig. 4a and b). Following 3 days of fermentation, the highest biomasses reached 35.55 g/l with and 35.08 g/l without sodium acetate addition, with the 35 mM sodium acetate exhibiting no significant effect on biomass. Moreover, media residual sugar concentrations decreased rapidly during the first 2 days of fermentation, following a slower rate of decrease from 2 to 4 days (Fig. 4d).

In the formatting phase, a dramatic increase in β-carotene production was noted leading into the stationary phase. The maximum β-carotene content (59.91 mg/g dry biomass) was obtained with adding sodium acetate after 6 days of fermentation relative to 33.71 mg/g of β-carotene content without sodium acetate (Fig. 4c).

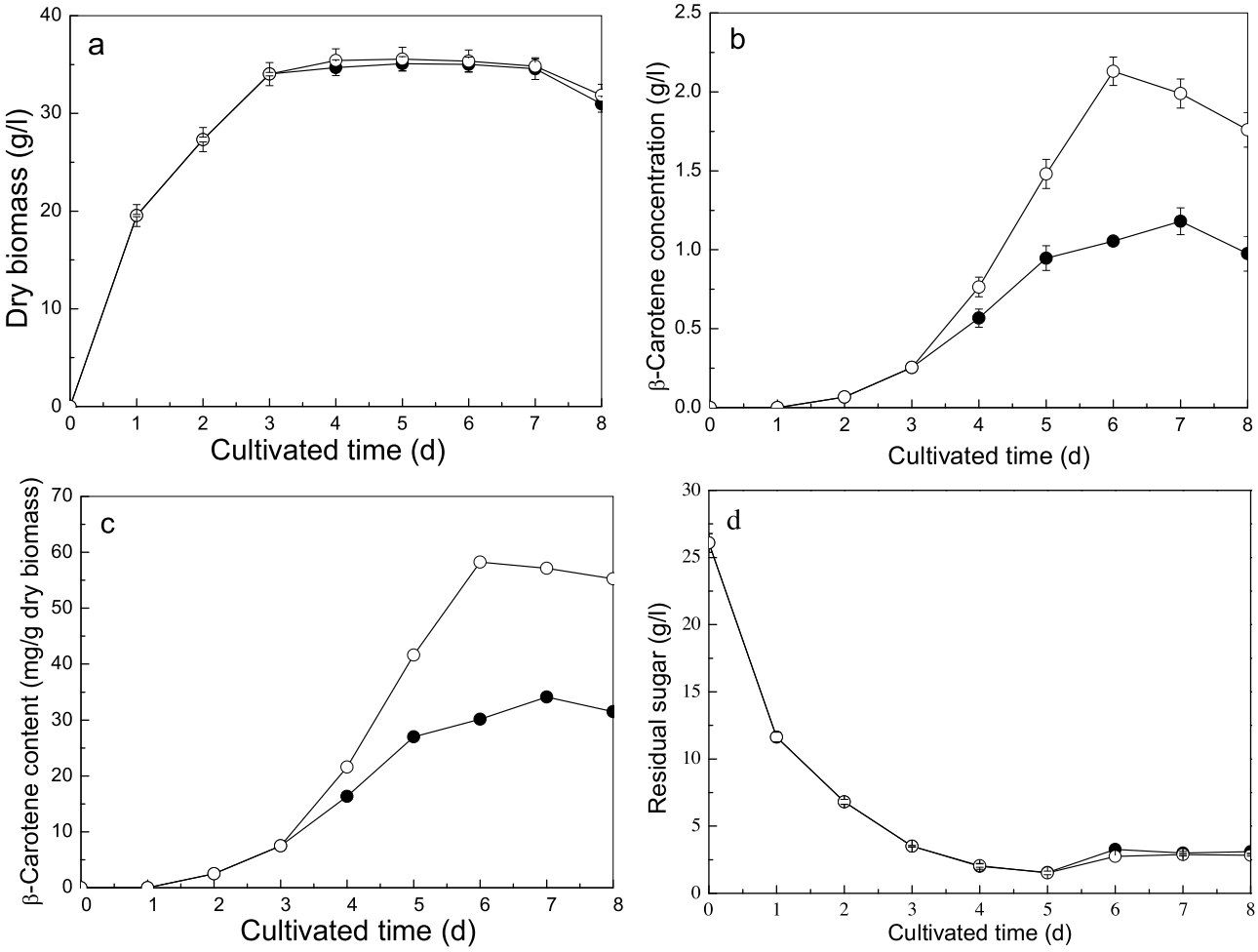

Fig. 4. Time-course profiles of cell growth (a), β-carotene yield (b), β-carotene content (c), and glucose metabolism (d) during *B. trispora* fermentation after 72 h of cultivated with and without adding 35 mM sodium acetate. The white circle and black circle represent adding acetate and the without acetate, respectively.

Table 3
comparison of the performance of β-carotene content, production of *Blakeslea trispora* with that obtained in the related reports.

Operation strategies	β-carotene content (mg/g dry biomass)	β-carotene production (mg/l)	β-carotene productivity (mg/l/d)	References
Shake flask culture	21.5	790	98.8	[24]
Bubble column reactor	12.0	360.2	45.1	[25]
Shake flask culture	15.0	375	46.9	[5]
Bubble column reactor	55.38	775.6	96.8	[26]
Bubble column reactor	44.5 ± 0.32	645.8 ± 37	80.6	[2]
Shake flask culture	12.1 ± 0.5	715 ± 25.6	89.4	[27]
Stirred tank reactor	45.23	1357	169.6	[28]
Shake flask culture	36.6 ± 0.15	1500 ± 56	187.5	[29]
Shake flask culture	58.6	1583	197.9	[30]
Shake flask culture	63	2016	336	[31]
Shake flask culture	59.91 ± 0.19	2130 ± 80	355 ± 13	This study

The highest β-carotene yield of 2130 mg/l reached 6 days of fermentation with sodium acetate addition while samples lacking sodium acetate reached a yield of 1180 mg/l after 7 days of fermentation (Fig. 4b). These results indicate that the addition of sodium acetate could shorten the β-carotene formation time relative to the control, the addition of sodium acetate potentially beneficial in the carotenoid industry. In the consumption phase, the cells began to die rapidly and β-carotene yield declined significantly. These findings indicate that the decreased β-carotene yield was due to cell death and potentially attributed to a lack of available glucose as a carbon-source.

Moreover, when compared with the batch cultivation, the β-carotene content obtained from 35 mM sodium acetate adding in mated medium enhanced around 79%, which was within the upper range of β-carotene content reported in previous literature (e.g., 12–63 mg/g dry biomass) (Table 3). As a result, the almost constant biomass with high β-carotene content led to the ultimate enhancement of β-carotene production with 35 mM sodium acetate adding in mated medium. The highest β-carotene production obtained from medium adding sodium acetate reached to 2130 ± 80 mg/l after 6 days of incubation and shortened two days to reach highest value than that of most of related studies (Table 3), which was nearly 80% higher than that obtained from medium without sodium acetate (Table 2). Further, the highest β-carotene productivity with 35 mM sodium acetate adding was 355 ± 13 mg/l/d, which was not only around 110% higher than that without sodium acetate

medium, but also higher than the result in most of related studies (range from 45.1 to 336 mg/l/d) (Table 3). Therefore, the addition of sodium acetate in mated medium is indeed a very effective way for increasing β-carotene productivity and enhancing β-carotene production of *B. trispora*.

3.4. Effect of acetate on the expression of carotenogenesis genes

Previous studies have demonstrated that adding sodium acetate to *B. trispora* cultures could promote β-carotene production, but the regulatory mechanisms of this phenomenon had not thus far been established. To investigate the molecular mechanisms of sodium acetate regulation in relation to β-carotene biosynthesis in *B. trispora*, we characterized the effect of sodium acetate on the expression of five carotenoid biosynthetic genes (*hmgR*, *ipi*, *carG*, *carRA,* and *carB*; Fig. 5). These results found that upon the addition of sodium acetate, a 1.8-fold increase in the level of *hmgR* mRNA expression was noted relative to the control, with a maximum inducing value reached after 3 h cultivation (Fig. 5a). Moreover, the addition of sodium acetate caused up to a 2.0-fold and 3.8-fold increase in the level of *ipi* and *carG* mRNA expression relative to the controls, respectively, with a maximum induction level reached at 9 h post-fermentation (Fig. 5b and c). Furthermore, the level of *carRA* and *carB* mRNA expression increased gradually, reaching up to a 1.83-fold and 3.98-fold increase at 24 h post-fermentation compared to the control, respectively (Fig. 5d and e). These results indicate that sodium acetate could cause a significant increase in the expression of the five genes required for β-carotene biosynthesis from the mevalonate pathway in *B. trispora*.

After adding sodium acetate to mated cultures, the transcriptional level of five key genes (*hmgR, ipi*, *carG*, *carRA,* and *carB*) involved in β-carotene biosynthesis exhibit a sequential gene expression profile (Figs. 1 and 5). The *hmgR* gene, which encodes a metabolic upstream enzyme catalyzing the reduction of HMG-CoA to mevalonate, reached a maximum expression level at 3 h after adding sodium acetate. The *ipi* and *carA* genes, which encode midstream enzymes for GGPP biosynthesis, reached a maximum level at 9 h post-fermentation and the *carRA* and *carB* genes, which encode downstream enzymes for β-carotene biosynthesis, reached a maximum level at 24 h post-fermentation. These data demonstrate that sodium acetate promotes β-carotene production through the transcriptional induction of carotenoid biosynthetic genes.

In order to further analyze the correlation between carotenoid biosynthetic gene expression and sodium acetate addition, the effect of sodium acetate on early carotenoid production was examined. For this purpose, we investigated β-carotene biosynthesis in a short time (24 h) after the addition of sodium acetate, thus enabling a more direct correlation. In this experiment, *B. trispora* cells were grown in mated cultures for up to 72 h, at which point the cultures were divided into two aliquots. Sodium acetate was added to one of the aliquots to a final concentration of 35 mM and the remaining aliquot was left untreated (control). Subsequently, aliquots from these cultures were collected 3, 9, 12, and 24 h after treatment, and the biomass concentration, β-carotene production and content in each sample were determined. We found that the addition of sodium acetate caused a slight increase in biomass, 52.9% increase in β-carotene production, and 49.2% increase in β-carotene content compared to the control (Fig. 6a–c), which was consistent with the results described above (Fig. 4). As shown in Fig. 2, the (+) and (−) mycelium of mated culture became more mature and stronger, more well-dispersed growth after addition of sodium acetate compared to the without sodium acetate. The well-dispersed mycelium is obviously benefited to mass transfer of dissolved oxygen and cell growth compared to the clustering mycelium. Importantly, the effects were detectable as early as 24 h post sodium acetate treatment, which correlated with changes in carotenoid biosynthetic gene mRNA levels.

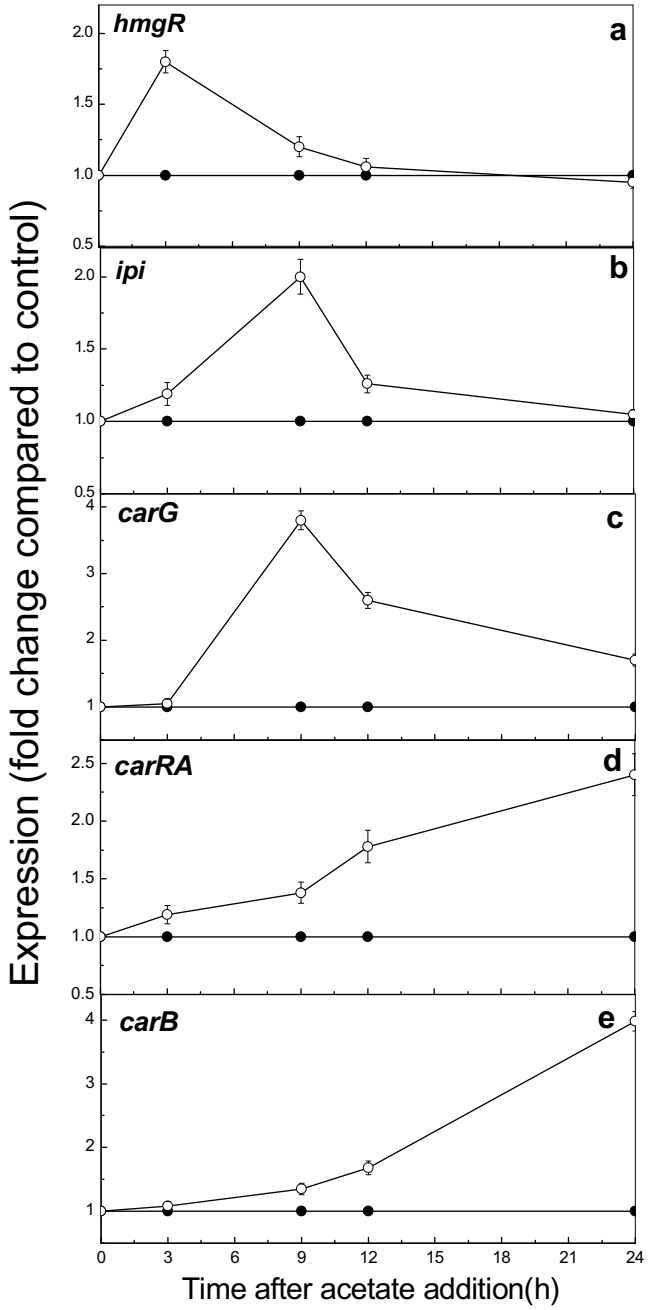

Fig. 5. Effect of acetate on carotenoid biosynthetic gene expression. The gene expression kinetics in the wild-type strain after 72 h of cultivated time adding sodium acetate (35 mM final concentration, white circle) was determined relative to without acetate (black circle) for caroteogenesis genes and for the *hmgR* (a), *ipi* (b), *carG* (c), *carRA* (d), and *carB* (e). The error bars correspond to standard deviation (n = 3).

Carotenogenesis in *B. trispora* is a complex process with regulatory mechanisms that have not been fully elucidated. Several study have reported that small carboxylic acids or alcohols, such as ethanol or sodium acetate could cause the induction of carotenogenesis expression and promote biosynthesis of carotenoid in yeast [16]. But small quantities of acetate (<1 mM) could inhibit the transcripts of carotenogenesis gene (*carB* and *carRA*) and carotene production by *B. trispora* in mated culture [17,32]. Contrary to the previous literature [17], the results obtained in this work show that the high concentration of sodium acetate (35 mM) improved

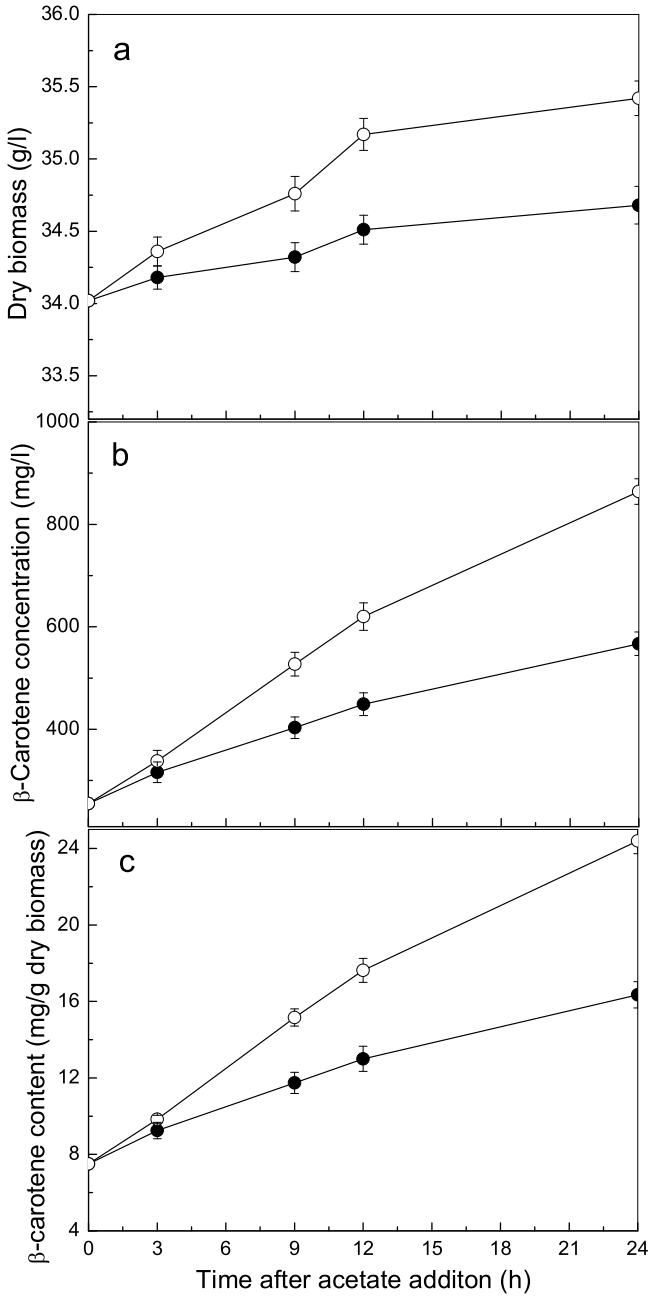

Fig. 6. Effect of acetate on cell growth (a), early β-carotene yield (b) and β-carotene content in *B. trispora*. Dry biomass, β-carotene concentration and content were determined for the whitout sodium acetate (untreated, black circle) and adding sodium acetate (35 mM, white circle).

carotene production and expression level of several genes in *B. trispora* 24 h after treatment. The mRNA levels of the *car*G (catalyzed the biosynthesis of GGPP) increased dramatically when 35 mM sodium acetate was added in mated culture. Moreover, the *hmg*R and *ipi* genes were induced by sodium acetate, as these are responsible for reduction of HMG-CoA to mevalonate and DMAPP biosynthesis. In addition, we found that adding sodium acetate to the mated medium caused an increase in the mRNA level of all of the carotenogenesis genes (*carB* and *carRA*) involved in the synthesis of β-carotene from GGPP. In these experiments, the effect of sodium acetate reached its maximum between 3 and 24 h after adding sodium acetate in mated medium. By 9 h after sodium acetate addition, mRNA levels of *car*G and *ipi* reached a maximum level. Thereafter mRNA levels of carotenogenesis genes (*carB* and *carRA*) reached maximum at 24 h after addition. These findings suggests that sodium acetate could be first converted into acetyl coenzyme A, with the latter becoming substrate for the synthesis of GGPP by the mevalonate pathway, and then GGPP could be transferred into β-carotene by carotenoid pathway in *B. trispora* (Fig. 1). This result is in agreement with previous reports confirming that addition of mevalonate and ethanol causes an increase in carotenoid production in yeast [16,33], probably because of their direct conversion into isoprenoid precursors by the mevalonate pathway. However, the low concentration of acetate (<1 mM) could not match the acetyl-CoA made from the abundant glucose in the medium, and the effect on the transcript of carotenogenesis genes (*carB* and *carRA*) is abolished by acetate in *B. trispora*. Under high concentration of sodium acetate (35 mM) condition, the acetyl-CoA conversion from acetate become majority compared to from the glucose and therefore the express level of these genes increased after addition of high amount of sodium acetate in mated medium.

4. Conclusion

The effects of adding sodium acetate into mated medium on biomass concentration, β-carotene production and content of *B. trispora* were investigated under the batch cultivation. The addition of 35 mM of sodium acetate at stationary phase (72 h of fermentation) was more favorable for β-carotene accumulation in *B. trispora*. The acetate regulation of β-carotene biosynthesis in *B. trispora* involves expressional changes in five carotenoid biosynthetic genes (*hmgR*, *ipi*, *carG*, *carRA,* and *carB*) and five genes exhibited characteristics of sequential gene expression. These results provide the first molecular explanation for the role of acetate stimulation in carotene production in *B. trispora*. Adding 35 mM sodium acetate into mated medium was proved to be an effective method to enhance the β-carotene production, giving the highest β-carotene content, titer an productivity of 59.91 mg/g dry biomass, 2130 mg/l, and 355 mg/l/d, respectively, which are higher than most of the previously reported values.

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