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Adaptive laboratory evolution of *Blakeslea trispora* under acetoacetanilide stress leads to enhanced β -carotene biosynthesis

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Blakeslea trispora is an important microbial producer of natural β -carotene, a valuable compound with significant nutritional and industrial applications. In the present study, an adaptive laboratory evolution (ALE) approach was applied to increase β -carotene production by exposing wild-type and UV-mutant *B. trispora* strains to increasing concentrations of the biosynthetic stressor acetoacetanilide. Over 95 serial transfers spanning 16 months, the adapted strain A₂78 showed a 45% increase in β -carotene yield (54 ± 1.95 mg/L) compared to the wild type (21.6 ± 2.11 mg/L), without a major compromise in biomass accumulation. Quantitative RT-PCR analysis revealed the upregulation of key carotenogenic genes, particularly *hmgR*, *carRA*, and *SR5AL*, in the adapted strains. Additionally, morphological changes, unsaturated fatty acid content, and altered antioxidant enzyme activities were investigated. The results show that chemical stress in the ALE strategy is effective in increasing metabolite production and stress tolerance of filamentous fungi and can pave the way for improving industrial strains.

Keywords *Blakeslea trispora*, β -Carotene, Adaptive laboratory evolution, Acetoacetanilide

Adaptive laboratory evolution (ALE) is a powerful tool for improving microbial performance by natural selection in a controlled environment. This approach involves cultivating a microbial population under specific selective pressure, such as nutrient limitation or the presence of inhibitors. Over time, strains with advantageous traits like increased growth rate, enhanced tolerance, and faster substrate consumption become more prevalent due to their superior fitness. These adapted strains are then repeatedly isolated to obtain a final optimized population^{1–3}. Adaptation occurs through a process called “adaptive walking,” where beneficial mutations spread through natural selection over generations. While new mutations can drive adaptation, this depends on chance and may be limited in smaller populations. This highlights the crucial importance of genetic diversity for successful evolution^{1,2}. The process of adaptation is influenced by evolutionary history, which depends on genetic variation generated by mutation or recombination. Currently, ALE has been employed to enhance the production efficiency of various microbial products in engineered strains, such as lactic acid by *Escherichia coli* SZ470, β -carotene by *Saccharomyces cerevisiae* GSY1136 strain, ethanol by *S. cerevisiae* CEN.PK113-7D and L-ornithine by *Corynebacterium glutamicum* ATCC 13032, which are used to increase the production of metabolites².

β -Carotene is the most active carotenoid, known for its ability to convert into vitamin A. This potent compound effectively combats diseases like heart disease and cancer, while strengthening the body's immune system. While most β -carotene currently comes from chemical synthesis, microbial production is emerging as a strong competitor due to several advantages. Natural β -carotene, free from harmful compounds and with consistent molecular structures, offers clear benefits over chemically synthesized alternatives^{4–6}. Carotenogenic fungi, especially strains selected for overproduction after mutagenesis, are ideal sources of carotenoids. *Blakeslea trispora*, a bisexual mold from the Zygomycetes family, stands out as a promising source for commercial β -carotene production. This microorganism, recognized as safe for consumption by the Food and Drug Administration (FDA), is known for its high production of both beta-carotene and lycopene^{7,8}. β -carotene biosynthesis in *B. trispora* occurs via the mevalonate pathway. The biosynthesis pathway involves key enzymes such as phytoene synthase, lycopene cyclase, and phytoene dehydrogenase, which are encoded by genes like

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carRA and *carB*. In fact, carotenoid biosynthesis is controlled by specific enzymes, and compounds that inhibit these enzymes are used to adapt and enhance the expression of the relevant enzymes⁹. These compounds are applied to adapt and enhance the expression of enzymes involved in related pathways in desired strains. This strategy could improve the production of carotenoids, which are essential for various ecological functions and industrial applications. Understanding the regulation of these genes and the corresponding enzyme activities is essential for maximizing β -carotene yields^{10,11}. Incorporating regulatory chemical compounds in culture media effectively boosts isoprenoid precursors and carotenoid production. Additionally, using carotenoid biosynthesis inhibitors in culture media can lead to valuable intermediate products^{12,13}. An effective class of inhibitors that target the carotenoid biosynthesis pathway in *B. trispora* comprises compounds that interfere with acetyl-CoA synthesis. This includes analogs of acetate, propionate, and butyrate. acetoacetanilide stands out as a highly effective acetate analog that plays a crucial role in inhibiting acetoacetyl-CoA synthetase (AACS) during the initial step of the carotenoid biosynthesis pathway¹⁴. Its significance in this pathway cannot be overstated, as it directly impacts the synthesis of valuable carotenoid compounds. Also, a comparative study of the inhibitory values of schiff bases and their complex compounds with acetostanilide compellingly demonstrates that these complexes show significantly enhanced antimicrobial activity¹⁵.

Thus, in the present study a series of acetoacetanilide concentrations were applied in *B. trispora* cultures during 16 month and the influence of the compound acetoacetanilide was evaluated on the expression levels of genes associated with the enzymes hydroxymethylglutaryl coenzyme A reductase (*hmgR*), lycopene cyclase, Isopentenyl pyrophosphate isomerases (*ipi*), phytoene synthase (*carRA*), phytoene dehydrogenase (*carB*), geranylgeranyl pyrophosphate synthase (*carG*), and steroid 5 α -reductase (*SR5AL*). Furthermore, the influence of acetoacetanilide on β -carotene production, biomass and morphology of the fungus, fatty acid profile, superoxide dismutase and catalase activity were evaluated.

Materials and methods

Strains, growth conditions and spore preparation

Blakeslea trispora wild-type strains (DSMZ 2388; PTCC 5278 minus type) and (DSMZ 2387; PTCC 5277 plus type) were obtained from Persian Type Culture Collection (PTCC). The strain was then cultivated on MEA medium agar plates [6% (w/v) malt extract, 2% (w/v) agar, pH 7 at 28 °C for 120 h. After complete growth, spores were harvested by rinsing the fully grown agar plates with a solution of 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20¹⁶. The cultivation and adaptation experiments were carried out using modified solid medium (CM17) composed of glucose 3 g/L, yeast extract 0.1 g/L, L-asparagine 0.2 g/L, MgSO₄ 0.05 g/L, KH₂PO₄ 0.15 g/L, citric acid 0.1g/L and agar 20 g/L (pH=7) with an addition of acetoacetanilide at gradient of increasing concentrations.

Population diversification using mutagenesis

Before conducting adaptive evolution experiments, a diverse population of wild type strains with enhanced β -carotene production capacity were established using classical mutagenesis techniques and UV exposure. The optimum dosage of UV for mutant screening was determined through finding *Blakeslea* survival rate, which should be around 10%. For an efficient mutagenesis, we carefully removed a 200 μ L aliquot from an initial concentration of 10⁶ spores/mL and spread this onto CM17 plates. The plates were then exposed to ultraviolet light (320 to 380 nm) from a distance of 50 cm for 180 s. After exposure, they were incubated in complete darkness at a stable temperature of roughly 28 °C for 6 to 8 days to identify microorganisms capable of overproducing β -carotene. Selection and comparison of high-producing β -carotene mutants were determined on CM17 solid medium containing acetoacetanilide. Colonies exhibiting a rich dark orange color were carefully selected and refined through multiple rounds of subculturing (two to four times) using spores from individual colonies. The selection of carotene-producing mutants was accomplished based on colony color intensity. At first, spores from mutant strains grown on CM17 were transferred to solid MEA as an enrich culture medium. Afterward, colonies of mutant strains M1, M2, M4, and M7 exhibiting a deeper orange-yellow pigmentation compared to the parent strain were selected through visual screening. This phenotypic characteristic was used as a preliminary indicator of enhanced β -carotene production potential⁸. Among them, strain M1 was subsequently selected for further testing.

Determining *Blakeslea trispora* tolerance to inhibitor concentration

Blakeslea trispora wild-type strain was first exposed to a range of certain concentrations of the acetoacetanilide stressor (0, 100, 200, 300, 500, 1000, 1200, 1500, 2000, 3000 mg/L) in CM17 broth culture medium. This was done in order to determine the tolerance threshold of the strains used in this research against the stressor. To initiate the adaptation experiment, the minimum concentration of the stressor (800 mg/L acetoacetanilide) that significantly inhibited growth but still allowed survival of the strain was selected.

Adaptation condition and selection of adapted strains

Adaptive experiments were performed using wild-type strain W78 and mutant strain M1 on CM17 solid medium containing gradually increasing concentrations of acetoacetanilide as a stressor (800 to 2000 mg/L, in 100 mg/L incremental steps). After incubation at 28 °C for 5 days, 10⁶ spores were transferred to fresh CM17 plates containing the same concentration of acetoacetanilide. This procedure was repeated 8 times for each concentration^{17–19}. The criterion for transition from one concentration to the next was the number of spores that reached 1% survival rate and maintained their population size during successive passages at a given acetoacetanilide concentration. Transfer of spores maintains adaptive genetic traits throughout a defined cycle of mold growth. The cultures underwent 95 serial transfers over a period of 16 months, achieving consistent growth at a concentration of 2000 mg/L acetoacetanilide. At higher concentrations, the strains were unable to grow or survive; thus, the experiments were terminated at this concentration. At the final adaptation concentration (2000

mg/L of acetoacetanilide), the most intensely pigmented colony from wild-type strain 78 and mutant strain M1 was selected and designated A₂78 and A₂M1, respectively. At the end of each exposure cycle, a portion of the resulting population was carefully collected and preserved at – 80 °C in 20% (v/v) glycerol. These preserved populations serve as a frozen snapshot of evolutionary progress and can also be used to restart the experiment in case of unforeseen interruptions or unavoidable events (Table 1).

Culture conditions for β-carotene production

Selected strains from the adaptive evolution stage and the parental strains were cultured on MEA agar plates. Plates were incubated for 96 h at 28 °C. The colonies were then cultured in a 50 mL liquid culture medium containing glucose, soybean powder, BHT, Span 20, KH₂PO₄ and MgSO₄¹⁶, and incubated for 120 h for total carotenoids quantification. The highest producer from each population was selected for further analysis. Given that *B. trispora* is a heterothallic fungus and exhibits enhanced β-carotene production upon mating of compatible (–) and (+) strains, the present study evaluated β-carotene levels not only in the individually evolved (–) strains but also following their mating with the wild-type (+) strain W77. For this purpose, each adapted (–) strain was co-inoculated with strain W77 at a 2:1 ratio in the production fermentation medium. The resulting β-carotene concentrations were compared to those obtained from the reference mating of wild-type strains 78 (–) and 77 (+), serving as the control condition²⁰. At the end of the cultivation period, the resulting biomass was harvested using Whatman filter paper and stored at – 20 °C for subsequent analyses. The evaluations included measurement of dry cell weight, morphological observation of the strains, comparison of carotenoid production levels, analysis of fatty acid profiles, determination of superoxide dismutase (SOD) and catalase (CAT) activities, and assessment of gene expression levels.

Analytical method
Carotenoid extraction and determination

To extract β-carotene, 0.2 g of biomass, which was powdered using glass beads, was mixed with 10 mL of tetrahydrofuran solvent. A 5 µL aliquot of the extracted solution was then injected into a Knauer high-performance liquid chromatography (HPLC) system (Knauer, Berlin, Germany). The column used was a C9, set at a temperature of 40 ± 2 °C. An isocratic program was applied to elute the carotenoids, using a mobile phase composed of a methanol to water ratio of (98:2)^{16,21}.

Detection of biomass and morphological analysis

The mycelium pellet was carefully extracted from the culture medium using Whatman filter paper No. 1. Following this, the pellet was washed twice with distilled water to ensure its purity. The fresh weight of the mycelium was then measured. A portion of the biomass was frozen at – 20 °C for storage, intended for β-carotene assay and other analyses. The remaining portion was dried slowly at 60 °C until it reached a constant weight, which typically took around 24 h. Finally, the dry cell weight was accurately measured using the gravimetric method²². To investigate how adaptation influences the morphology of mold mycelium, The adapted and parental strains were observed under a light microscope while growing in liquid culture. Also, micromorphological analysis of mycelium was conducted using SEM, (TESCAN Co. Czech Republic) imaging at a magnification of 500×. The objective was to illustrate changes in the structure and growth patterns of both the parent and adapted samples and provide insights into their adaptive characteristics^{23,24}.

Fatty acids profile analysis

B. trispora mycelia were harvested after being cultured for 96 h. The cultures were centrifuged at 5000 rpm (Beckman, GS-6, Beckman Coulter Inc, Fullerton, CA, USA) for 10 min, and the biomass was washed with a 0.9% (w/v) NaCl solution. The mycelia were ground using glass pestles and mortars in the presence of liquid nitrogen. A precise 200 mg of purified powder was mixed with 1.5 mL methanol. The mixture was then centrifuged at 10,000g for 10 min, and the supernatant was carefully collected for subsequent procedures²⁵. Free fatty acids in the supernatant were transformed into fatty acid methyl esters (FAMES) using a highly efficient methanolic boron trifluoride (BF₃) transesterification method²⁶. FAMES were extracted using hexane and were analyzed with a Shimadzu Nexis 2030 GC instrument equipped with an FID detector and a Dikmacap-2330 column (60 m × 0.25 mm ID × 0.20 µm). The column temperature was programmed using a gradient mode. It started at 60 °C and was maintained for 2 min. Then, the temperature gradually increased to 200 °C at a rate of 10 °C per minute. Following this, it progressively rose to 240 °C at a rate of 5 °C per minute, maintaining this temperature for 7 min. The injector and detector temperatures were set at 250 °C and 260 °C, respectively. Samples of 1 µL were injected with a split injection ratio of 60:1. Hydrogen was used as the carrier gas. Fatty acid identification was achieved by comparing their inhibition times with FAME standards and the results were expressed as a

Strain	Origin	Description	References
W78	Wild-type	Original <i>B. trispora</i> strain (DSMZ 2388)	Durakli et al. ⁶
W77	Wild-type	Original <i>B. trispora</i> strain (DSMZ 2387)	Durakli et al. ⁶
M1	Mutagenized from W78	Selected mutant with high β-carotene production	This study
A ₂ 78	Adapted from W78	Evolved under acetoacetanilide stress for 16 months	This study
A ₂ M1	Adapted from M1	Evolved mutant (M1) under acetoacetanilide stress for 16 months	This study

Table 1. List of *B. trispora* strains.

percentage. The identification of fatty acids was achieved by comparing the inhibition time of the fatty acids with that of pure fatty acid standards, and the results were expressed as percentages²⁷.

Determining superoxide dismutase content and catalase activity

50 mL of the fermentation broth from the strains were filtered using Whatman No. 1 filter paper. The remaining biomass on the filter was washed with distilled water. Then, 0.5 g of the wet biomass were mixed with liquid nitrogen and ground using a glass pestle and mortar to extract the internal enzymes of the mycelium. The lysed cells were then mixed with 1 mL of saline (0.85% NaCl, pH 6.0) and centrifuged at 10,000g for 10 min at 4 °C²⁸. The supernatant was used to determine the activities of superoxide dismutase (SOD) and catalase (CAT) enzymes. The assay was performed according to the manufacturer's instructions using a Zellbio GmbH kit, employing a photometric method combined with an ELISA reader to ensure accurate and reliable results. The results were expressed in units of SOD and CAT activity per milliliter (U/mL).

Quantitative real-time PCR (qPCR)

Samples were harvested after 56 h fermentation, freezed in liquid nitrogen immediately and then, stored at −80 °C. Total RNA was extracted using RNX Plus Solution (Sinnacolon, EX6101). The concentration of RNA was measured at A260 nm²⁹.

The cDNA synthesis reaction was performed using RealQ Plus 2 × master mix green without ROX™ according to the manufacturer's instructions. The relative quantification was performed using the SYBR Green real-time PCR master Mix (TOYOBO) on a Rotor-Gene Q (QIAGEN, Germany). The real-time PCR primers are presented in (Table 2). The real-time PCR cycling conditions were as follows: 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s. Measurements were performed in triplicate.

Primers related to the gene sequences involved in the β-carotene biosynthesis pathway of the *B. trispora* strain were available³⁰. Changes in the transcriptional expression of key genes within this pathway were investigated using quantitative reverse transcription PCR (qRT-PCR). Specifically, the expression levels of genes associated with the enzymes hydroxymethylglutaryl coenzyme A reductase (*hmgR*), lycopene cyclase, Isopentenyl pyrophosphate isomerase (*ipi*), phytoene synthase (*carRA*), phytoene dehydrogenase (*carB*), geranylgeranyl pyrophosphate synthase (*carG*), and steroid 5α-reductase (*SR5AL*) were compared between the parent and the adapted strains^{30–32}. Measurements were performed in triplicate. The transcriptional levels of five key genes (*hmgR*, *ipi*, *carG*, *carRA*, *carB*) and *SR5AL* obtained by qRT-PCR were normalized to that of the *tef1* gene. *tef1* was used as the reference gene. The results were presented as relative to expression of the corresponding group using the comparative method of Livak and Schmittgen³³.

Results

Abiotic stresses include various factors that affect microbial life, forcing them to adapt. The production of β-carotene in the adapted isolated strain was measured and compared to that of the parent populations (The methodology and results are summarized in Fig. 1).

In general, the isolated adapted strain produced a significantly ($p < 0.05\%$) higher amount of β-carotene compared to the parent population's average. The A₂₇₈ strain was one of the highest producers, producing about 45% percent more β-carotene (54 ± 1.95 mg/L) with dry biomass of 23.14 ± 1.85 g/L compared to the W78 (21.6 ± 2.11 mg/L) with dry biomass of 25.08 ± 3.37 g/L.

The highest β-carotene producers (A₂₇₈) were chosen for further molecular analysis to identify potential mechanisms for increased carotenoids production in these strains (Fig. 2).

Morphological assessment

The SEM images and micro-morphology analysis were conducted on the samples, with images captured at a magnification of 500x. The mycelium parent strains (W78, M1) exhibited a short, linear pattern of hyphae

Genes name	Gene abbreviation	Forward and reverse primers (5'→3')	Amplicon length (bp)
Translation elongation factor – 1	<i>tef1</i>	<i>tef1</i> -F <i>tef1</i> -R	138
Isopentenyl pyrophosphate isomerise	<i>ipi</i>	<i>ipi</i> -F <i>ipi</i> -R	161
Gernyl gernyl pyrophosphate	<i>carG</i>	<i>carG</i> -F <i>carG</i> -R	129
3 Hydroxy-3-methylglutaryl coenzyme A reductase	<i>hmgR</i>	<i>hmgR</i> -F <i>hmgR</i> -R	113
Lycopene cyclase phytoene synthase	<i>carRA</i>	<i>carRA</i> -F <i>carRA</i> -R	129
Phytoene dehydrogenases	<i>carB</i>	<i>carB</i> -F <i>carB</i> -R	92
Steroid 5α-reductase-like gene	<i>SR5AL</i>	<i>SR5AL</i> -F <i>SR5AL</i> -R	180

Table 2. Real-time PCR primers. F forward, R reverse.

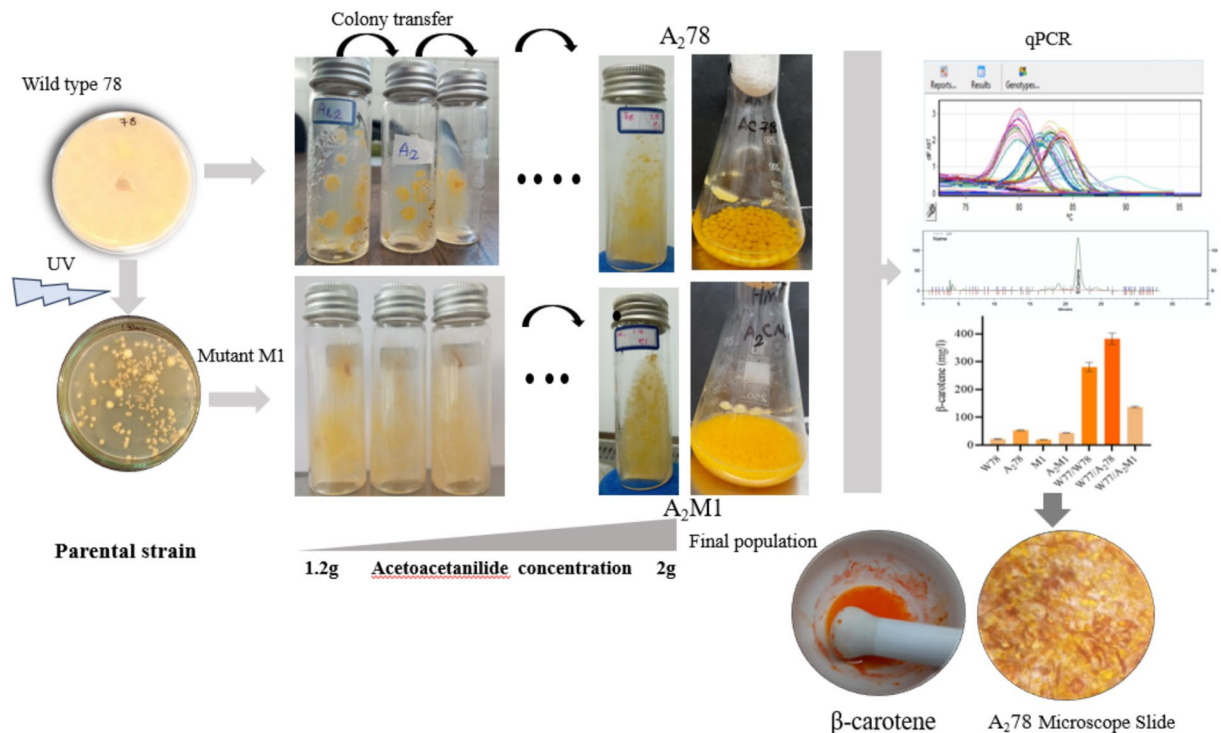


Fig. 1. Overview of the Adaptive Laboratory Evolution (ALE) process applied to the *Blakeslea trispora* wild-type strain W78 and UV-induced mutant strain M1. Serial colony transfers were performed in CM17 medium with increasing concentrations of acetoacetanilide (1.2–2.0 g/L) as a selective stressor. This process led to the development of acetoacetanilide-adapted strains A₂78 (from W78) and A₂M1 (from M1). Subsequent qPCR analysis and β -carotene quantification revealed enhanced production in the evolved strains, particularly A₂78. Microscopic examination of A₂78 cells is also shown.

(Fig. 3a,b). In contrast, the adapted strains (A₂78, A₂M1) displayed relatively denser and longer hyphae (Fig. 3c,d). Additionally, the images of the samples revealed variations in spore count and porosity.

Fatty acids profile analysis

The comparison of the fatty acid profiles produced by the parental and adapted strains in the fermentation medium revealed differences in the fatty acids generated (Table 3). The level of palmitic acid, one of the saturated fatty acids, was found to be reduced by 39% in the adapted strain (A₂78) compared to the wild strain (W78). Notably, linoleic acid, an omega-6 fatty acid with a double bond, is the predominant unsaturated fatty acid, making up 50% of the fatty acid profile. Unsaturated fatty acids dominate the fatty acid profile in all strains, ranging from 72.64 to 80.66%. Mutant (M1) and adapted strains (A₂78 and A₂M1) showed a significant decrease in saturated fatty acid content and a corresponding increase in unsaturated fatty acids compared to the wild-type strain (W78). Among them, strains A₂78 and M1 exhibited the highest levels of unsaturated fatty acids ($p < 0.001$), indicating a favorable shift in lipid composition as a result of mutagenesis and adaptation (Fig. 4).

Superoxide dismutase content and catalase activity

This study demonstrated that treatment with the inhibitor during adaptation resulted in changes in the activity levels of the enzymes superoxide dismutase (SOD) and catalase (CAT). In the adapted strains, The CAT content has increased by 14–19% compared to the control strain, while the amount of SOD decreased significantly (P value < 0.05) in the adapted strains compared to the control strain (Fig. 5). In the mated strains, the amount of (SOD) and catalase (CAT) increases significantly in the adapted strains mated with the positive wild type strain (W77)³⁴.

Quantitative analysis of transcript levels of genes

Enhanced expression of carotenoid biosynthetic genes, resulting from copy number amplification or mutations in promoter regions, could significantly boost carotenoid production. To explore this potential, we employed qRT-PCR to compare the expression levels of *hmgR*, *ipi*, and *carB* genes. Notably, when examining the *carG*, *carRA*, and *SR5AL* genes, it was found that the *carRA* gene exhibited a greater expression in mutants than in the control group. In the adapted strain (A₂78), the expression levels of the *hmgR* and *carRA* genes have risen by 2 times and 1.5 times, respectively, compared to the parent strain (W78). In the adapted mutant strain (A₂M1), the expression of the *hmgR*, *carRA* and *SR5AL* genes has increased by 6 times, 4.5 times, and 2 times, respectively, compared to their parent strains (M1). This compelling transcription data clearly demonstrated the

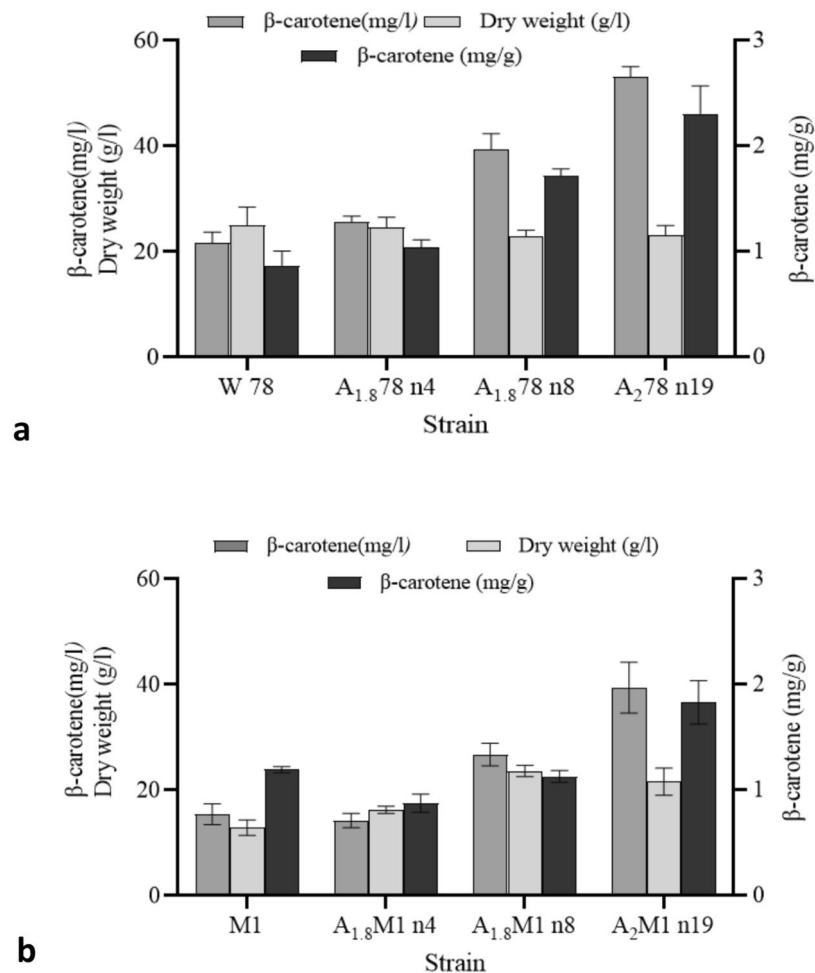


Fig. 2. Comparison of β -carotene production among (a) acetoacetanilide-adapted strains (A78) and the parent strain W78, (b) acetoacetanilide-adapted mutant strain (AM1) and M1 parent mutant strain. Data represent mean values with standard deviations. Significant differences ($p < 0.05$) are indicated. A: acetoacetanilide-adapted; 1.8 and 2: Stressor concentration (g/L); n: Subculture number at the corresponding concentration.

differences in carotenoid gene expression between the overproducing strains and the W78 strain, underscoring the importance of these findings in advancing our understanding of carotenoid biosynthesis (Fig. 6).

Upregulation of key carotenoid biosynthesis genes (*hmgR*, *carRA*, *SR5AL*), morphological changes observed via microscopy, and shifts in fatty acid composition indicate a thorough cellular adaptation at both molecular and physiological levels. Future work may focus on whole-genome sequencing of the adapted strains to identify specific mutations responsible for the enhanced phenotype, and the application of this ALE strategy to other filamentous fungi or biosynthetic pathways.

Discussion

This study aimed to enhance β -carotene production in *B. trispora* through ALE under stress induced by acetoacetanilide. Acetoacetanilide, an acetate analog, inhibits acetoacetyl-CoA synthetase¹⁴, which is a key enzyme in the early stages of the mevalonate pathway. However, this inhibition does not necessarily lead to a long-term reduction in β -carotene synthesis. Under continuous selective pressure, the organism can develop genetic or regulatory adaptations that restore or even enhance pathway function. Notably, the A₂78 strain demonstrated a 45% increase in β -carotene production without a corresponding rise in biomass, indicating a shift in metabolic allocation that favored carotenoid biosynthesis.

Expression levels of biosynthesis pathway genes in *B. trispora* were measured relative to the transcript levels of the translation elongation factor 1 α , encoded by the *tef1* gene, which is independently transcribed. The increased expression of key genes in the carotenoid pathway, specifically *hmgR*, *carRA*, and *carB*, observed in the adapted strains suggests that transcriptional regulation significantly contributes to the phenotype of the improved strain. Notably, the upregulation of *hmgR*, which encodes HMG-CoA reductase, a rate-limiting enzyme in the mevalonate pathway, supports previous findings highlighting its crucial role in carotenoid overproduction under metabolic pressure. Given the high levels of β -carotene produced by this adapted strain, these findings suggest that these genes play a crucial role in the production of β -carotene in *B. trispora*.

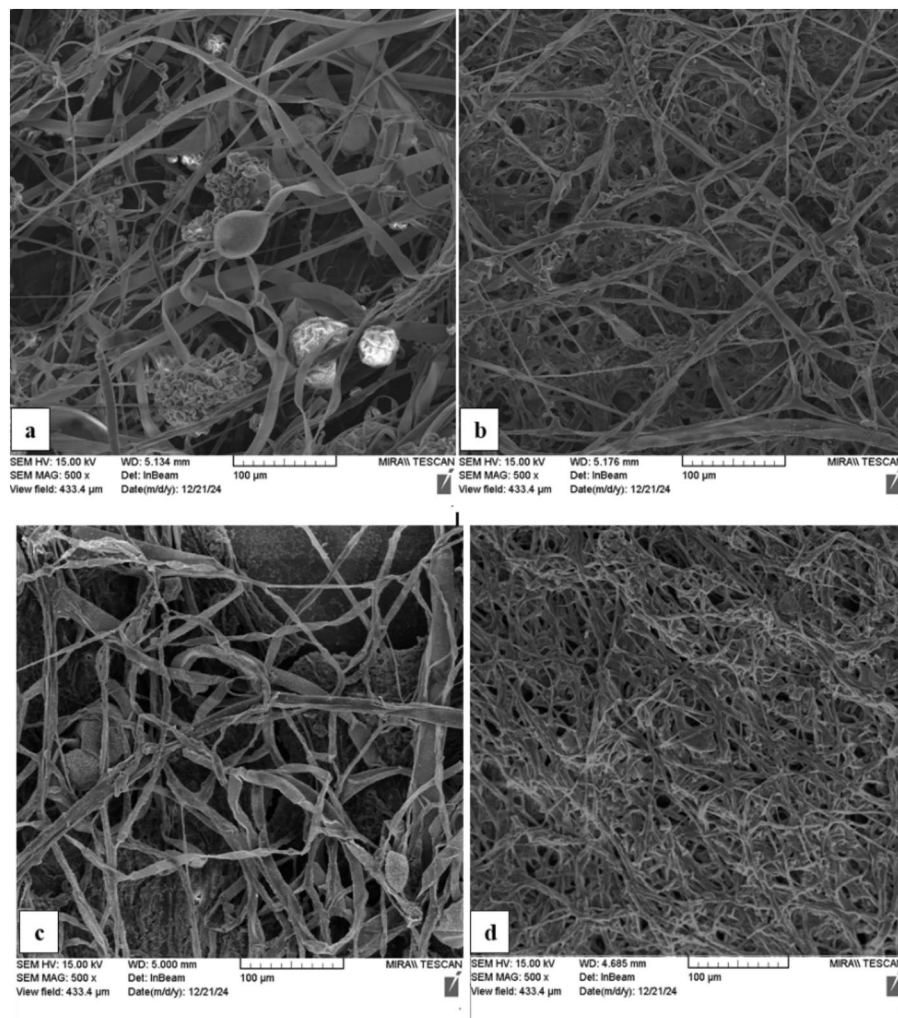


Fig. 3. Morphological characterization of mycelium. SEM image of mycelium of *B. trispora* (scale bar is 100 μ m) (a) Wild78. (b) Adapted strain A₂78. (c) Mutant M1. (d) Adapted strain A₂M1.

The results offer valuable insights into the regulatory mechanisms governing terpenoid biosynthesis in the presence of acetooacetanilid. Additionally, the findings from the superoxide dismutase and catalase enzyme assays indicate that the adapted strains efficiently cope with stressful conditions by favoring the production of β -carotene over enhancing the levels of these enzymes. This suggests that adaptation to imposed stress drives the mycelium to synthesize increased amounts of the nonenzymatic antioxidant β -carotene as a protective mechanism.

Scanning electron microscopy (SEM) images showed distinct morphological differences between the parental and evolved strains. The adapted strains (A₂78 and A₂M1) exhibited longer, denser, and more branched hyphae, in contrast to the wild-type (W78) and UV-resistant mutant (M1) strains, which displayed more linear and scattered hyphal structures. These changes likely enable more efficient nutrient uptake and metabolic distribution, which could lead to an increased synthesis and storage of hydrophobic carotenoids, such as β -carotene. Microscopic comparisons are consistent with findings from ALE studies in fungi and yeasts, where morphological changes, such as increased vacuolization and branching, are linked to enhanced production of secondary metabolites. Similar morphological changes were observed in strains under stress conditions, such as *Aspergillus niger* and *Yarrowia lipolytica*, during the overproduction of citric acid and lipids, respectively^{35–37}. Decreased superoxide dismutase (SOD) activity in the adapted strains (A₂78 and A₂M1), compared to the parental strains (W78 and M1), suggests a decline in enzymatic antioxidant defense. This reduction indicates that the adapted strains may rely less on enzyme-based pathways to neutralize reactive oxygen species (ROS), such as superoxide radicals, and instead may be shifting toward alternative antioxidant strategies that primarily involve non-enzymatic mechanisms. In this context, β -carotene, due to its strong free radical-scavenging capability, likely compensates for oxidative stress. This phenomenon mirrors adaptive responses in *Escherichia coli* and *S. cerevisiae*, where cells under chronic oxidative stress accumulate carotenoids or glutathione to counteract ROS damage^{38,39}. Fungi are known to accumulate lipids during periods of metabolic stress²². β -carotene, which is highly unsaturated and contains 11 conjugated double bonds, exhibits a degree of unsaturation that exceeds

Fatty acid	Concentration (%)			
	W78	A ₂ 78	M1	A ₂ M1
Caprylic acid (C8:0)	0.13	0.18	0.18	0.12
Capric acid (C10:0)	0.2	0.31	0.24	0.32
Luric acid (C12:0)	1.09	1.84	1.28	1.61
Myristic acid (C14:0)	1.49	1.18	1.50	1.82
Myristoleic acid (C14:1)	0.04	0.15	0.05	0.15
Pentadecanoic acid (C15:0)	0.08	0.04	ND	ND
Palmitic acid (C16:0)	15.33	10.26	9.53	13.4
Palmitoleic acid (C16:1)	0.51	0.19	0.35	0.69
Margaric acid (C17:0)	0.09	0:08	0.10	0.09
Heptadecenoic acid (C17:1)	0.08	0.07	0.05	0.07
Stearic acid (C18:0)	6.75	4.58	4.53	4.8
Oleic acid (18:1)	26.74	26.67	25.1	25.91
Linoleic acid (18:2)	42.83	50.28	53.24	46.45
Linolenic acid (18:3)	2.04	1.55	1.61	2.13
Arachidic acid (C20:0)	0.30	0.32	0.36	0.32
Gadeloic acid (C20:1)	0.15	0.19	0.15	0.17
Eicosadienoic acid (C20:2)	ND	ND	0.05	0.09
Heneicosylic acid (C21:0)	0.13	0.14	ND	ND
Behenic acid (C22:0)	0.63	0.69	0.66	0.58
Erucic acid (C22:1)	0.20	0.28	0.027	0.20
Docosahexaenoic acid (C22:2)	0.05	0.05	0.04	0.28
Lignoceric acid (C24:0)	0.28	0.3	0.27	0.21
Total	100	100	100	100

Table 3. Fatty acid profile composition and percentage of the extracted total lipids of parent strains (W78 and M1) with the adapted strains (A₂78 and A₂M1) using GC–MS analysis.

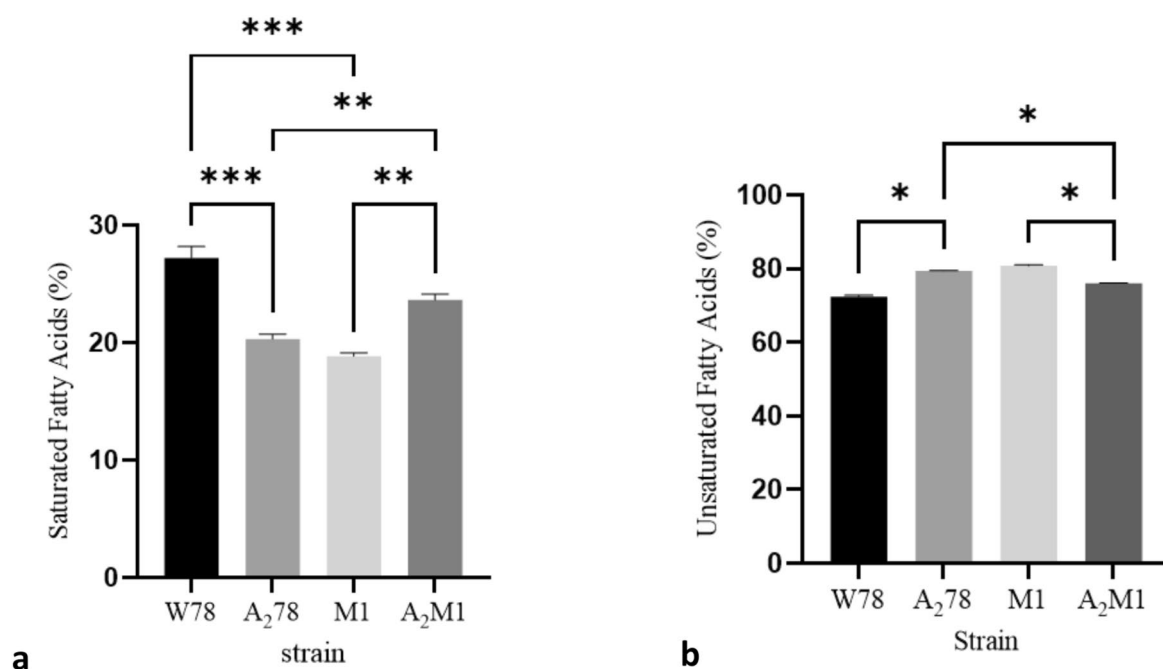


Fig. 4. Variation in fatty acid composition (mean $\pm$ SD, $n = X$) among *B. trispora* wild-type strain (W78), mutant (M1), and adapted strains (A₂78 and A₂M1). (a) Saturated fatty acid percentages, (b) unsaturated fatty acid percentages.

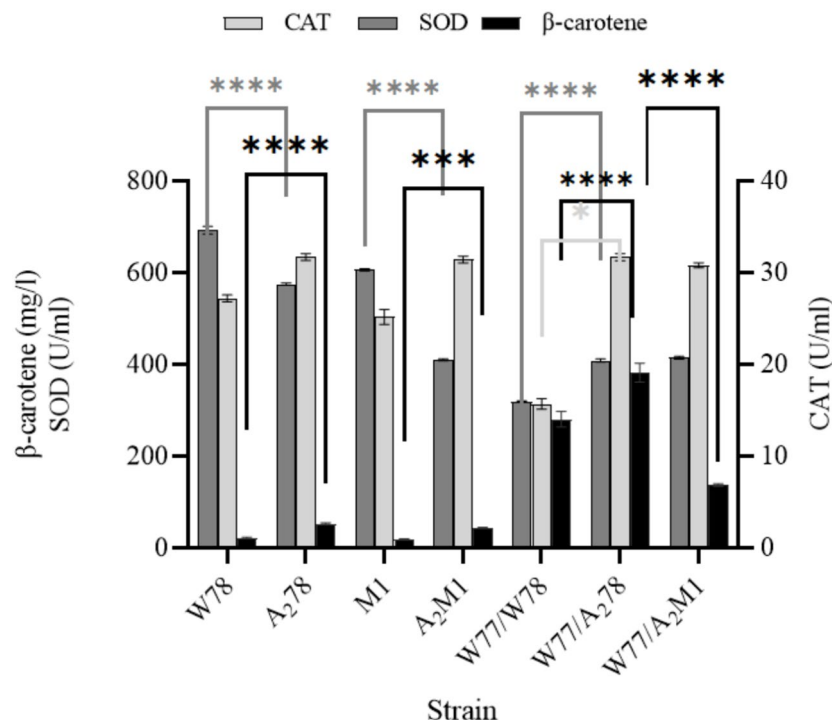


Fig. 5. Comparison of the production of catalase, superoxide dismutase, and β -carotene in acetoacetanilide adapted strains (A₂78) and (A₂M1) with the parent strains (W78) and (M1), as well as the adapted strains mated with W77 (W77/A₂78, W77/A₂M1) with their mated parents (W77/W78).

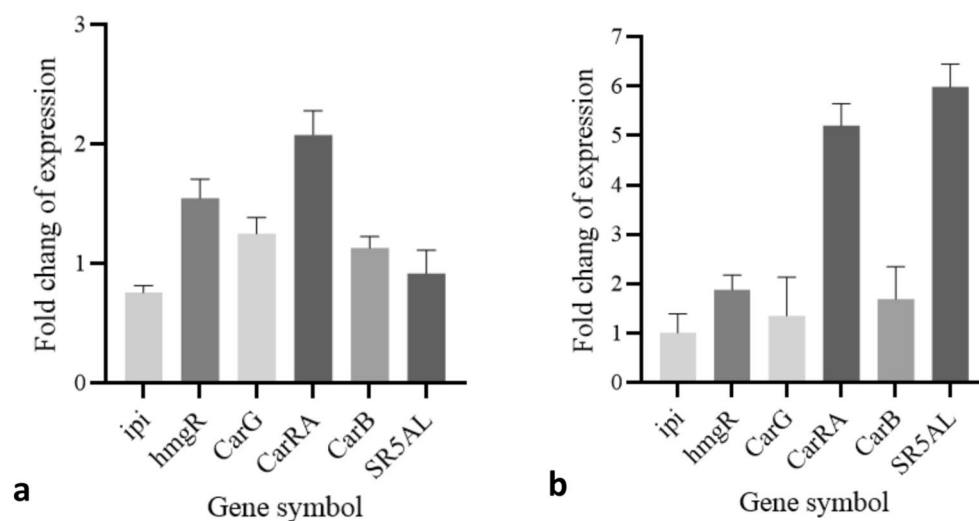


Fig. 6. Gene expression level between (a) Acetoacetanilide-adapted strains (A₂78) and the parent strain W78, (b) (A₂M1) and M₁ mutant strains. All results were normalized to the *tef1* transcript level and relative to the transcript level of the corresponding gene in the control (value = 1). Values are the mean $\pm$ standard error of three independent experiments.

that of intracellular lipids. The random distribution of β -carotene molecules within the hydrophobic interior of the lipid bilayer plays a crucial role in enhancing membrane fluidity. Therefore, the increased unsaturation of intracellular lipids is advantageous for the solubility of β -carotene^{25,40}. This reduction suggests that palmitic acid, being a metabolic precursor, might be redirected to enhance the production of compounds in the carotenoid biosynthesis pathway⁴¹. Fatty acids play a crucial role as essential components of the cell membrane. They can undergo oxidation via β -oxidation to produce acetyl-CoA or be integrated into storage lipid formations like lipid bodies⁴². Acetyl-CoA serves as a common precursor for the synthesis of both carotenoids and lipids.

In the adapted strain, the content of unsaturated fatty acids, such as linoleic acid, was significantly higher than that of other components. This increased level of unsaturated fatty acids not only boosted the fluidity of the cell membrane but also facilitated the accumulation of greater amounts of β -carotene, demonstrating a clear advantage for the cell's functionality²⁵. Furthermore, the substantial presence of linoleic essential fatty acid significantly boosts the nutritional value of this mold, making it a remarkable choice for animal and poultry feed.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Conceptualization: D. Z. Methodology: H. M. Data analysis, interpretation of results: D.Z. and M. A. and H. M.; Writing first draft and revision final manuscript: All Authors; Corresponding authors, responsible for communication with the journal: D. Z.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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