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Evaluating the effect of microbial stimulation and oxidative stress on increasing β-Carotene production in *Blakeslea trispora*

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Abstract

β-carotene is a lipophilic pigment that belongs to the carotenoid family, produced by plants and microorganisms as a secondary metabolite, and is the most widely utilized carotenoid in the industry. *Blakeslea trispora* is one of the most significant industrial sources of β-carotene production among microorganisms. The present study aims to investigate the effect of *K. rhizophila* as a microbial stimulant and butylated hydroxytoluene (BHT) as oxidative stress on increasing β-carotene production in *B. trispora* .*B. trispora* was cultivated in the production medium with and without butylated hydroxytoluene (BHT), and after 24 hours, 10 % *K. rhizophila* cultures with 10^{11} CFU/mL were added to each medium and incubated for another 4 days. The percentage of carotenoid isomers produced in each sample was determined using high-performance liquid chromatography (HPLC). *K. rhizophila* and BHT, each alone, could increase carotenoid production by 2.3 and 2.4 times (respectively) compared to the control. The maximum concentration of carotenoids (793 mg/L) was found in samples containing both BHT and *K. rhizophila*, representing a 7.5-fold increase over the control sample. HPLC analysis of carotenoids showed two prominent peaks, including β-carotene and γ-carotene. The main carotenoid was β-carotene and was found in all samples, followed by a lesser amount of γ-carotene. Overall, microbial stimulation and oxidative stress were effective strategies for increasing β-carotene production in this microorganism.

1. Introduction

β-carotene is a natural pigment available in most biological systems as an *all-trans* isomer (Papadaki & Mantzorido, 2021). This pigment serves a variety of biological functions in the human body. However, since human bodies are unable to produce it, it should be consumed through food or supplements (Bogach Radovamska & Harasym, 2018). β-carotene has a wide range of industrial and biological applications, such as use in cosmetics and pharmaceuticals, natural food coloring, animal feed, dyeing (textiles), light receptors, skin protection, anti-cancer properties, anti-cholesterol, anti-aging, and cataracts, as well as a source of the plant hormone abscisic acid (Eman, 2019; Thakur & Azmi, 2013). Among the various roles of β-carotene in the human body, the most essential is its role as a vitamin A precursor (Bogach Radovamska & Harasym, 2018). Vitamin A is required for embryonic development, cell differentiation, eyesight, immunity, and reproduction (Coria Filo et al.,

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2019). Wackenroder was the first to extract β-carotene from carrots as red crystals in 1831 (Ribeiro et al., 2011).

β-carotene is an inhibitor of the onset of many diseases in which free radicals play a role, including atherosclerosis in cardiovascular disease, cataracts, multiple sclerosis, and, most importantly, cancer (Thakur & Azmi, 2013). Indeed, β-carotene is a micronutrient that, through its antioxidant properties and raising the number of T cells in the human body, reinforces the immune system against a variety of diseases, including viral diseases like the coronavirus, which have a pandemic effect on the global health and economy (Papadaki & Mantzouridou, 2021). Naturally derived β-carotene can be used as anti-angiogenic, anti-oxidant, anti-aging, anti-inflammatory, cancer prevention, pigmentation in animals, and immune functions (Wang et al., 2022). The worldwide carotene market was valued at 314.14 billion dollars in 2021, and it is anticipated to rise to $380.37 billion by the end of 2026 (Forecast Data Market, 2021).

B. trispora is an appropriate and cost-effective natural resource for commercial β-carotene production (Jing et al., 2016). *B. trispora* is a heterothallic zygomycota with mating types (plus and minus). It is known to produce β-carotene on an industrial scale and is the only fungus that produces all-*trans* β-carotene (Zare et al., 2002; Jing et al., 2016). *B. trispora* has received interest for use in β-carotene production due to its properties such as abundant biomass production and the lack of special environmental conditions for growth (Lou et al., 2020; Papaioannou & Liakopoulou- Kyriakides, 2010). In addition, it can successfully transform low-cost raw materials and some industrial, agro and food wastes, and residual crops into high-value end products like carotenoids while at the same time moderating environmental pollution and reducing manufacturing costs (Hu et al., 2012; Papaioannou & Liakopoulou- Kyriakides, 2012; Papadaki & Mantzouridou, 2021; Nanou et al., 2016). The worldwide carotene market was valued at $314.14 billion in 2021, and it is anticipated to rise to $380.37 billion by the end of 2026. This product's combined annual growth rate from 2021 to 2026 is 3.9% (Forecast Data Market, 2021).

By increasing worldwide concern about the hazardous effects of artificial food coloring, some efforts have been made to produce β-carotene from microbial sources, such as *B. trispora*. Numerous methods have been used in the culture medium to optimize the production of carotenoids, particularly β-carotene in *B. trispora*, including optimization of cultivation media, metabolic engineering, the addition of various inducers and inhibitors, and carbon to nitrogen regulation (Sun et al., 2012; Shariati et al., 2018; Nanou & Roukas, 2016; Jing et al., 2016; Chaudhari et al., 2008). The use of microbial cells (microbial stimulants) to stimulate biosynthesis is one of the effective measures to improve the development of this natural color in *B. trispora* (Ciegler et al., 1964; Zare & Azin, 2003). The findings of these investigations revealed that microbial cells increase the production of β-carotene in *B. trispora*.

The interaction among microbial cells, including fungi and bacteria, occurs in several ways, such as antibiotic interactions, signaling reactions, nutritional communications, physicochemical alterations in the environment, chemotaxis and cellular communications, metabolic and acid transfer, and metabolic cooperation, or protein secretion and gene transfer. (Frey- Klett et al., 2011). Since β-carotene is produced in the *B. trispora* by the mevalonate pathway, adding bacteria like *Kocuria*. sp, which creates carotenoids by the same pathway, increases β-carotene production. *Kocuria*. sp members primarily include carotenoid colors and produce enzymes such as catalase, superoxide dismutase, protease, lipase, etc. Moreover, some strains, such as *K. rhizophila*, are salt-tolerant and phenol degrader (Ramos et al., 2021; Takrada et al., 2008; Shi et al., 2021). *K. rhizophila* possesses a variety of valuable features, such as small genome size, ability to grow rapidly with high cell density, strong cell adaptability to various growing circumstances, and high tolerance to a range of

chemical solvents (Takrada et al., 2008). The members of the genus have significant potential for producing enzymes, bio surfactants, antibiotics, and pigments (Timkina et al., 2022). *Kocuria* members interact positively with other microorganisms, resulting in a range of technical advantages in co-cultivated forms. For example, increasing proteolytic activity of *K. variants K4* by *Staphylococcus xylosus* isolated from fermented meat products in co-culture form has been reported by Ramos et al. (2021). Considering the properties mentioned about members of the genus *Kocuria*, this genus of bacteria may increase carotenoids in the *B. trispora* by degrading phenolic compounds in the culture medium, such as the breakdown of butylated hydroxytoluene (BHT) or by producing various enzymes such as catalase and superoxide dismutase. However, multiple studies have demonstrated that oxidative stress could increase carotenoid production in *B. trispora* (Nanou & Roukas, 2011; Roukas, 2015; Nanou & Roukas, 2016). According to these reports, increasing oxidative stress enhanced the specific activity of two enzymes in *B. trispora*, catalase, and superoxide dismutase, followed by a considerable rise in the production of carotenoids, particularly β-carotene. Based on studies and the desirable effects of oxidative stress and microbial stimulation on enhancing carotenoid production, it is probable that combining these two strategies results in a considerable increase in β-carotene levels in *B. trispora*.

Thus, in the present study, the effect of *K. rhizophila* in co-culture form and BHT as oxidative stress was evaluated on β-carotene yield in *B. trispora* in separate and combined forms. Furthermore, the effect of these two factors on the percentage of β-carotene and γ-carotene was evaluated by high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Chemicals

Span 20, HPLC grade β-carotene, and lycopene were prepared from Sigma Aldrich (St. Louis, MO, USA). HPLC and analytical grade solvents such as methanol, tetrahydrofuran chemicals such as BHT, and medium components were obtained from Merck (Darmstadt, Germany). Soybean powder was prepared as explained by Shariati et al. (2019).

2.2. Microorganisms and culture media

B. trispora PTCC 5277 mating type (+), *B. trispora* PTCC 5278 mating type (-), and *K. rhizophila* (PTCC 1110) were gifted by the Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST). Mating types (plus and minus) of *B. trispora* were cultivated in slant media containing 5% malt extract. Trypticase soy agar (TSA) was applied to cultivate and maintain the bacterium *K. rhizophila*.

2.3. Examining the effect of microbial stimulation on carotenoid production in B. trispora

The surface of slant cultures comprising both mating types was scraped and washed separately with sterile distilled water, and the resulting suspension was inoculated in pre-culture media (Shariati et al., 2018). After 48 h, 5 mL of each strain were added to 50 mL of a production culture media consisting of (g/L): KH2PO4 (1.2), Mg2SO4 (0.4), glucose (50), soybean powder (10.34), BHT (4.5), span 20 (2), thiamine (0.01), asparagine (0.1), and 10 mL/L soybean oil while the pH was adjusted to 8. The media were incubated for 24 h in the gyratory shaker incubator (Clim-O-Shaker, Adolf Kuhner, Birsfelden, Switzerland) at 28 ° C and 200 rpm. K. rhizophila was also inoculated in a 50 mL trypticase soy broth (TSB) culture medium, and the Erlenmeyer's (250 ml) were incubated at 28 °C at 200 rpm in the gyratory shaker incubator. Then, 5 mL of bacterium culture was inoculated to 24-h cultures of B. trispora and incubated for an additional 120 h at 28 °C and 200 rpm. A culture medium without inoculation of the bacterium was considered as a control. After the mentioned time, the biomass was filtered and

maintained in a freezer at -20 °C for β-carotene extraction.

2.4. β-carotene assessment

To determine the β-carotene, 0.2 g of wet biomass was pulverized with glass powder using mortar and pestle, then 10 mL ethanol, hexane, and water were added to the solution. The mixture was then centrifuged for 5 min at 5000 rpm. Finally, the supernatant was separated, and its absorbance was recorded at 450 nm using a UV–vis spectrophotometer (Unicam 8620, Thermo Spectronic, Cambridge, UK). β-carotene was calculated using the following formula:

$$\beta\text{-carotene (mg/g dry biomass)} = A*V*10^3/E^{\%1}_{1cm}*100*g \quad (1)$$

Where A is the sample absorption at a wavelength of 450 nm, V is the solvent volume based on mL, g is the weight of biomass in grams, and $E^{\%1}_{1cm}$ is the extinction coefficient, which is 2600 for β-carotene (Shlomai et al., 1991).

2.5. Evaluating the effect of BHT on carotenoid production in *B. trispora*

The phenolic antioxidant BHT was utilized as an oxidative stress causer (Nanou & Roukas, 2016). The culture media described in section 2-2 was prepared, and 4.5 g/L BHT was added to evaluate the influence of this chemical on carotenoid production in *B. trispora*. Furthermore, in the same culture medium (50mL), 5 mL of bacterium suspension was added to assess the combined effect of BHT and *K. rhizophila* on carotenoid production. The samples were then incubated in the gyratory shaker incubator for another 4 days, according to previous protocols, and the carotenoid content of each was assessed.

2.6 Investigating the effect of BHT and microbial stimulation on pH

After the incubation period, all samples were filtered through a Whatman No. 1 filter paper, and the supernatant was applied to evaluate the influence of *K. rhizophila* and BHT on the pH of the culture medium. A pH meter (ISTEK- Eco Met, Korea) was used to determine the pH of the liquid during filtration.

2.7 Evaluating the effect of BHT and microbial stimulation on cell mass and the amount of residual sugar

After filtering, the collected biomass was kept at 90 ° C for 24 h to determine dry biomass weight. The sugar level of each sample was also estimated using a glucose test kit (PARS AZMUN Glucose Kit, Iran) (Clarke & Cowan, 1952).

2.8 Evaluation of β-carotene and γ-carotene isomers using high-performance liquid chromatography (HPLC)

The quantity of β-carotene and γ-carotene produced was measured using high-performance liquid chromatography (Knauer, Berlin, Germany). Carotenoids were extracted from 0.1 g of each sample. Briefly, tetrahydrofuran was applied to extract carotenoids, 5 μL of which was injected by chromatography (Shariati et al., 2018). The mobile phase was 100% methanol, and the device was a Knauer liquid chromatography apparatus with a K2501 detector (UV/vis) at a wavelength of 450 nm. The column was also C18, and the temperature was 40 ° C.

2.9 Statistical analysis

Data were reported as the average values of three replicates. Data were analyzed using Minitab V.16 (Minitab Inc., State College, PA, USA) software. One-way analysis of variances (one-way ANOVA) was applied to test the significance between means. A Tukey multiple comparison test with a 95% confidence interval was applied for post hoc analyses.

3. Results and Discussion

3.1. The effect of microbial stimulation on carotenoid production by *B. trispora*

Figure 1 depicts the investigation's findings on the influence of microbial stimulation on carotenoid production. The findings of the present study demonstrated that *K. rhizophila* increased the production of carotenoids in the *B. trispora*. In comparison to control samples, the mixture culture of this microbial stimulant with *B. trispora* increased carotenoids by 2.3 times in mixed samples. The findings of this study, like those of Ciegler (1964), suggest that adding bacterial biomass to the culture medium could increase the production of carotenoids in *B. trispora*. Microbial cells can act as a stimulant to produce enzymes for primary and secondary metabolism or by directly affecting secondary metabolism using enzymes and precursors, as many carotenogenic enzymes may be similar to other microorganisms (Lampila et al., 1985). However, few reports are available on the mechanism of action in the production of metabolites in mixed cultures. According to Liu et al., *Bacillus thuringiensis/cereus L2* increased *Rhodobacter sphaeroide* biomass and carotenoid production by supplying a substrate and decreasing peroxidase enzyme activity in *Rhodobacter sphaeroide* (Liu et al., 2015). Furthermore, Duponnois and Garbaye (1989) demonstrated that certain soil bacteria might induce the growth of ectomycorrhizal fungus through two processes. In the first mechanism, the bacterium improved the growth of fungi, and in the second mechanism, the polyphenolic compounds produced by the fungi, which were toxic to them, were metabolized by the bacteria, eliminating the toxicity of the culture medium to the fungus (Duponnois & Garbaye, 1989). Regarding the mechanisms mentioned above and based on the *K. rhizophila* bacterium, this microbial stimulation may increase carotenoids in the *B. trispora* by phenolic compounds degradation mechanisms in the culture medium such as the BHT degradation or by producing various enzymes such as catalase and superoxide dismutase.

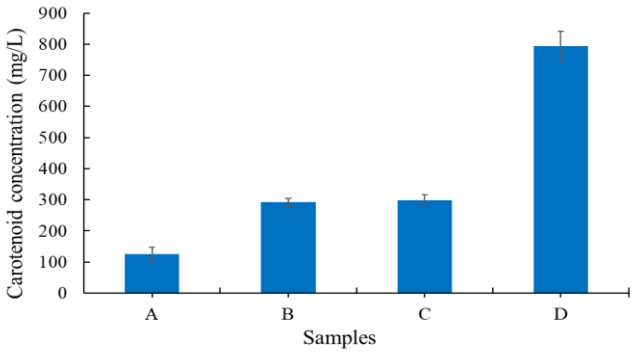

Figure. 1. Evaluating the effect of the microbial stimulant on carotenoid production in *B. trispora*: A) *K. rhizophila* and butylated hydroxytoluene (BHT)-free culture (control), B) Culture containing *K. rhizophila* and BHT-free, C) Culture with BHT and *K. rhizophila* -free, and D) Culture with *K. rhizophila* and BHT.

3.2. Evaluating the effect of BHT on carotenoid production by *B. trispora*

The phenolic antioxidant BHT was utilized as an oxidative stress agent in *B. trispora*. Additionally, BHT by itself, without the addition of a microbial stimulant, was able to increase carotenoid production in *B. trispora* to 298.89 mg/l Fig 1, a 2.4-fold increase over the samples without BHT and bacterium. As a result, one of the stimuli for the production of β-carotene in *B. trispora* is oxidative stress produced by BHT. The findings of this study agree with those of Nanou and Roukas (2011), Roukas (2015), and Nanou and Roukas (2016). By being contained in membrane phospholipids, BHT alters the fungal shape and causes tiny pellets, which may signal stress on the microorganism. Pellet contraction can aid in improving the speed of substrates transmission, oxygen, precursors, and products while also maintaining high levels of reactive oxygen species. As a result, BHT may affect the intracellular production of oxygen radicals, causing oxidative stress and increasing carotene production in the fungus (Nanou & Roukas, 2016).

In addition, in large dosages, BHT functions as a peroxidation agent, interacting with oxygen molecules rather than oxygen radicals to produce phenoxyl radicals and superoxide anions (Nanou & Roukas, 2016). Deeper oxidation of BHT yields BHT quinone methide and other BHT

oxidizing products, which can result in the production of superoxide ions. These ions produce oxidative stress in the fungus and increase catalase and superoxide dismutase enzyme activity (Roukas, 2015; Nanou & Roukas, 2016). However, as indicated in Fig. 1, the maximum level of carotenoid production in *B. trispora* was achieved in the culture medium containing BHT and *K. rhizophila*. BHT and *K. rhizophila* both enhance the amount of carotenoids in the mold separately. However, when these two strategies were combined, the carotenoid production rate was 49.08 mg/g of dry mold weight, which was 7.8 times greater than when neither of these two elements was added to the cultures (Fig. 2). As the findings indicated, microbial stimulants and oxidative stress factors can be utilized as important and effective inducers of carotenoid production in *B. trispora* cultures. Consequently, it is possible that the bacterium *K. rhizophila*, like *B. trispora*, has increased the quantity of its enzyme catalase or other metabolites in response to BHT stress and thus improved carotenoid production as well.

Alternatively, by decomposing BHT, the studied bacterium may have caused more oxidative stress and increased carotenoid production in the B. trispora cultures. Further research into the metabolites produced in the simultaneous cultivation of bacteria and fungus provides more information on the causes of their carotenogenic effects. These findings demonstrate that increasing the production of carotenoids in this mold, particularly β-carotene, may be possible by optimizing various factors that cause oxidative stress in the cultivation of *B. trispora* with *K. rhizophila*, and this issue is of great importance in the industrial production of β-carotene by this mold. Given the hazardous effects of β-carotene from chemical agents, more studies into enhancing natural β-carotene production by microorganisms might be a huge advance for researchers and industry.

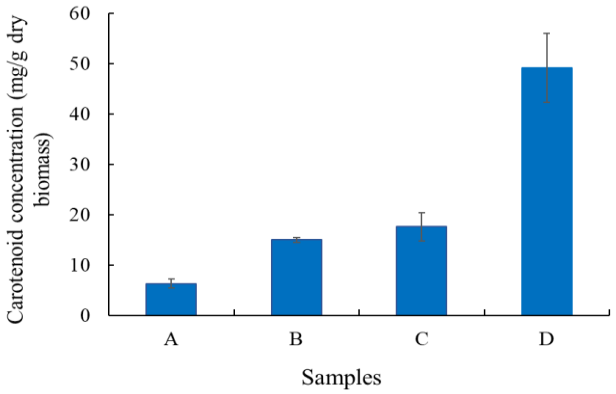

Figure. 2. Studying the effect of BHT and the microbial stimulant on carotenoid concentration in *B. trispora*: A) *K. rhizophila* and butylated hydroxytoluene (BHT)-free culture (control), B) Culture containing *K. rhizophila* and BHT-free, C) Culture with BHT and *K. rhizophila* -free, and D) Culture with *K. rhizophila* and BHT.3.3. Effect of adsorbent weight

3.3 Evaluating the effect of BHT and the microbial stimulant on pH

The pH was evaluated toward the completion of incubation time to find the relationship between crop yield and pH variations. The pH of the culture medium at the end of the incubation time was used to evaluate the results acquired from the examination of the influence of BHT and *K. rhizophila* on pH. The pH level in the samples containing BHT and a microbial stimulant was lower than in the control sample, which did not include any of these inducers (Fig 3). The pH dropped the most in samples containing both BHT and bacterium. These findings showed that carotenoid production and ambient pH have an inverse relationship. Similarly, Zare and Azin reported that the final pH of the culture media decreased when bacterium cells were added to the *B. trispora* cultures, associated with an increase in carotenoid production (Zare & Azin, 2003). The reduction in pH might be caused by the production of trisporic acid or other unknown acidic chemicals (Papaioannou & Liakopoulou-Kyriakides, 2010).

Trisporic acid is a chemical hormone that promotes carotenogenesis and zygophore

production in two mating types of *B. trispora* in mixed cultures (Papaioannou & Liakopoulou-Kyriakides, 2010). However, when Gessler et al. analyzed the early stages of trisporic acid production in the *B. trispora*, they found that in the presence of oxidative stress, β-carotene can be oxidatively degraded to β-apo-13-carotenone, and this compound leads to a significant level of trisporic acid (Gessler et al., 2002). Furthermore, in *B. trispora*, trisporic acid has been demonstrated to improve metabolic flux by increasing carotenoid and fatty acid production while lowering protein production (Sun et al., 2012).

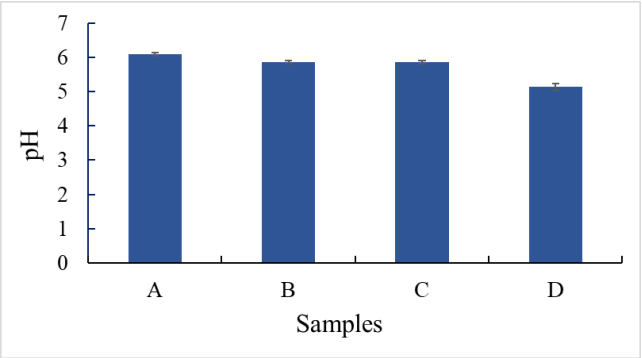

Figure.3. The effect of microbial stimulation and BHT on pH:A) *K. rhizophila* and butylated hydroxytoluene (BHT)-free culture (control), B) Culture containing *K. rhizophila* and BHT-free, C) Culture with BHT and *K. rhizophila* -free, and D) Culture with *K. rhizophila* and BHT.

Therefore, considering the above-mentioned data and the obtained results, the *K. rhizophila* increased carotenoid production, especially β-carotene, which may be due to degrading BHT and increasing oxidative stress. Subsequently, the obtained β-carotene is degraded during the existing stress, resulting in carotenoid production as positive feedback by producing more trisporic acid in the mold.

3.4. Examining the effect of BHT and a microbial stimulant on cell mass and the amount of residual sugar in culture medium

The amount of cell mass collected and the amount of carotenoids extracted from *B. trispora* can be substantial due to intracellular carotenoid accumulation. As a result, the dry weight of each sample after the incubation period was used to examine the effect of bacterium cells and BHT on the quantity of cell mass. As displayed in (Table 1), compared to the control, all samples containing bacterium cells and BHT had lower biomass. The greatest cell mass (20.07 g/l) was found in a control sample without bacterium cells and BHT. Although the concurrent use of microbial stimulation and BHT in the samples resulted in the lowest amount of cell mass, these samples yielded the maximum quantity of carotenoids. The cell mass decreases because most acetyl-CoA flows in the mevalonate pathway (carotenoid production pathway), and thus the level of acetyl-CoA entering the basic metabolism is reduced, impairing the conditions required for cell growth (Wang et al., 2022). The findings of this investigation were consistent with those of a prior study that examined the effect of BHT on increasing β-carotene production in *B. trispora* in the presence of *Serratia marcescens* (Azizi et al., 2020). Additionally, Wang et al. (2022) reported that the cell mass decreases as the carotenoid content in B. trispora increases.

3.5. The effect of BHT and a microbial stimulant on the amount of residual sugar in culture medium

Because carbon supply is important and impacts the number of carotenoids produced in mold, 50 g/L glucose was utilized as a carbon source. The amount of residual sugar in the culture medium was determined following the incubation time to

Table 1. The percentage of β-carotene and γ-carotene produced in *B. trispora* under the effect of the microbial stimulant and BHT

Specimens	Biomass (g/L)	Residual sugar (g/L)
Control (without bacterium and BHT*)	20.07±0.71	0.17±0.03
Bacterium-contained and BHT-free	19.47±0.49	0.49±0.25
Bacterium-free and BHT-contained	17.01±1.84	0.46±0.08
Bacterium and BHT-contained	16.16± 1.39	1.34±0.23

*Butylated hydroxyl toluene (BHT)

evaluate the effect of BHT and the microbial stimulant on the carbon source in the culture medium. The findings of this investigation revealed that the amount of sugar in all samples decreased dramatically after the culture time. On the contrary, as shown in Table 1, the control sample had 0.17 g/L of residual sugar, which was lower than all other samples. The medium containing BHT and *K. rhizophila* had the highest quantity of residual sugar at 1.34 g/L. Therefore, existing BHT and microbial stimulants slowed the carbon source's degradation. BHT and *K. rhizophila.*, as previously discussed, may increase the trisporic acid in the mold, and trisporic acid could affect glucose, fatty acid, and amino acid metabolism (Sun et al., 2012).

3.6. The effect of the microbial stimulant and BHT on the percentage of β-carotene and γ-carotene

Two major peaks with carotenoid spectra were found in carotenoids analyzed using high-performance liquid chromatography. β-carotene and γ-carotene, extracted at 22 and 25 minutes, respectively, were among the carotenoids separated using this approach. Standard β-carotene was also extracted at 22 min (Fig. 4 A). The results indicate that the levels of β-carotene and γ-carotene in the control sample were 98.75% and 1.25%, respectively (Table 2). Based on the areas under the curve of Table 2, the amounts of these two carotenoids were 3.48 and 2.46 times greater in samples containing *K. rhizophila* and BHT-free, respectively, than in the control sample. In the presence of BHT and without bacterium, levels of β-carotene and γ-carotene were 6.3 and 17 times greater than the control, respectively (Table 2). The highest content of β-carotene and γ-carotene were observed in samples that simultaneously contained bacterium cells and BHT, which increased 13.2 and 100 times, respectively, compared to the control (Fig 4, part B). Based on the results obtained in all samples, β-carotene was the primary carotenoid, followed by γ-carotene at a lower percentage. Nevertheless, in samples containing BHT and the mixed culture, the level of γ-carotene grew more than β-carotene levels. However, when *K. rhizophila* was utilized as the single inductor agent, 99.1% of the extracted carotene was β-carotene. As a result, the presence of BHT and the resulting oxidative stress in the fungus causes the production of carotenoids that are more directed towards γ-carotene. Additionally,

Table 2. The percentage of β-carotene and γ-carotene produced in *B. trispora* under the effect of the microbial stimulant and BHT

Samples	γ-carotene (%)	β-carotene (%)	The areas under the curve	
			β-carotene	γ-carotene
Control (without bacterium and BHT*)	1.25	98.75	591835	7425
Bacterium-contained and BHT-free	0.88	99.12	2062005	18289
Bacterium-free and BHT-contained	3.39	96.61	3738745	127080
Bacterium and BHT-contained	9.52	90.75	7830016	745542

Nanou and Roukas reported that in *B. trispora*, extreme oxidative stress alters carotenoid components, resulting in a large rise in γ-carotene levels (Nanou & Roukas, 2016).

Therefore, in the present study, it is possible that increasing the degradation of BHT and increasing oxidative stress using the bacterium cell results in increasing γ-carotene along with the increase of β-carotene. β-carotene and γ-carotene consist of at least one non-substituted non-anionic ring that can be converted to retinol in human and animal bodies (Berman et al., 2014). Considering the significance of vitamin A, any of these carotenes are valuable; however, because β-carotene is becoming increasingly well-known in the worldwide market, creating a larger percentage of it in industrial research could be more valuable. β-carotene is applied as coloring for natural foods and animal feeds and is commonly added to soft drinks, cheese, and butter (Wang et al., 2022). Based on the percentage of carotenoid isomers produced in each sample, each inducer agent raised β-carotene production in B. trispora, but when these two stimulants were used together, a higher content of β-carotene was produced.

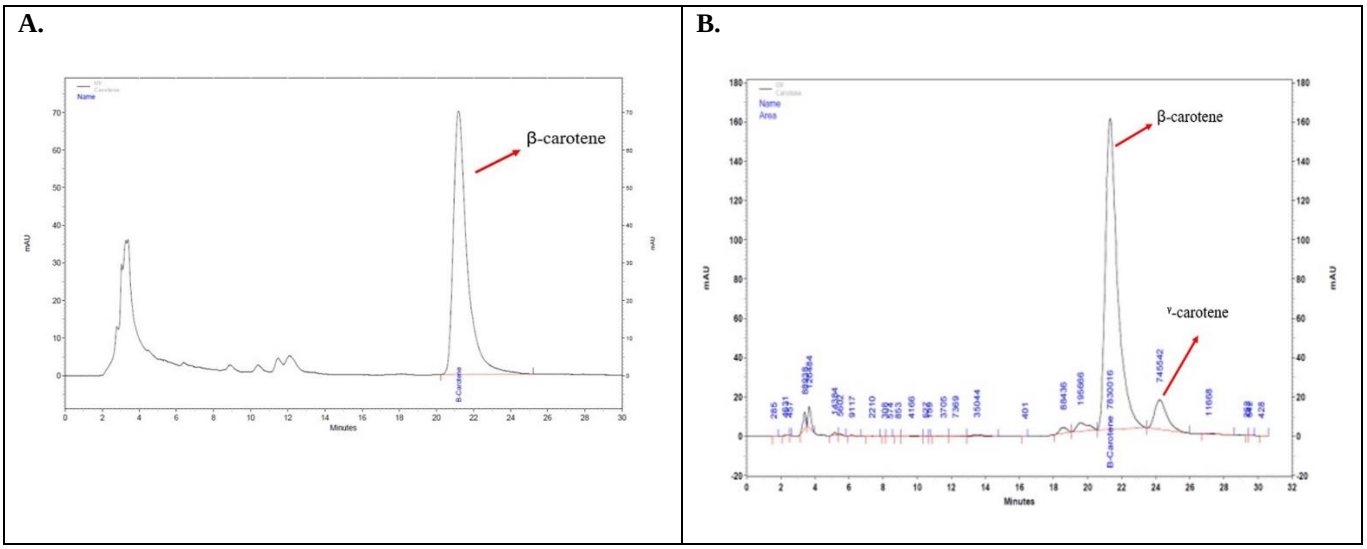

Figure. 4. Chromatograms of the standard β-carotene (A) and a sample containing BHT and *K. rhizophila* (B).

4. Conclusions

The microbial stimulant *K. rhizophila* was utilized to promote carotenoid production in the *B. trispora*. The amount of carotenoids in the targeted mold increased by 2.3 times when this stimulant was used alone. BHT, however, was able to enhance the amount of carotenoids by 2.4 times as oxidative-inducing stress. The amount of carotenoids in *B. trispora* reached 793 mg/L after combining these two stimulants, indicating a rise of 7.8 times compared to the control sample. Nevertheless, HPLC analysis of the collected carotenoid isomers revealed that β-carotene was the predominant carotenoid. The combined use of microbial stimulation and oxidative stress using *K. rhizophila* and BHT could be an effective strategy to increase β-carotene production in *B. trispora* cultures.

Conflict of Interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript**.**

Ethical approval

This article does/does not contain any studies with human participants or animals performed by

any of the authors. This article does/does not contain any studies with human participants or animals performed by any of the authors.

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