بهینه‌سازی تولید و بررسی ویژگی‌های آنتی‌اکسیدانی و سمیت سلولی رنگدانهٔ کاروتنوئیدی *Cellulosimicrobium* AZ

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چکیده

مقدمه: ریزموجودات اکسترموفیل مقاوم به اشعه می‌توانند میزان زیادی کاروتنوئید تولید کنند؛ باوجوداین، بهینه‌سازی تولید متابولیت‌های آنها برای اهداف تجاری با مقیاس وسیع ضروری است. هدف مطالعهٔ حاضر، بررسی تولید رنگدانه‌های کاروتنوئیدی یک سویهٔ اکسترموفیل است که ازنظر فیلوژنتیکی به جنس *سلولزی میکروبیوم* نزدیک است و تاکنون گزارشی دربارهٔ آن ارائه نشده است.

مواد و روش‌ها: اثر منابع کربنی و نیتروژنی مختلف بر زیست‌تودهٔ میکروبی و تولید رنگدانهٔ سویهٔ مطالعه‌شده با استفاده از رهیافت یک-فاکتور-در-یک- زمان بررسی شد. فعالیت آنتی‌اکسیدانی رنگدانه با استفاده از آزمون‌های به‌دام‌اندازی رادیکال‌های آزاد و قدرت احیاکنندگی یون‌های آهن فریک بررسی شد؛ به علاوه، خاصیت ضدمیکروبی و سمیت سلولی عصارهٔ رنگدانه به‌ترتیب به روش‌های انتشار در آگار و سنجش فعالیت متابولیکی سلولی مطالعه شدند.

نتایج: بیشترین رنگیزهٔ کاروتنوئیدی تولیدی سویهٔ مطالعه‌شده در محیط‌کشت تخمیری حاوی ۱ گرم‌برلیتر عصارهٔ مخمر و ۱ گرم‌برلیتر گلوکز (۲۲/۵ میلی‌گرم‌برلیتر) به دست آمد که ۱۰/۷ برابر شرایط غیربهینه (۲/۱ میلی‌گرم‌برلیتر) بود. نصف بیشترین غلظت مؤثر (EC_{50}) رنگدانه به‌ترتیب در آزمون‌های به‌دام‌اندازی رادیکال‌های آزاد و قدرت احیاکنندگی یون‌های آهن فریک برابر با ۱۰/۹۷ میلی‌گرم‌برمیلی‌لیتر و ۶/۰۲ میکروگرم‌برمیلی‌لیتر شد؛ همچنین رنگدانهٔ این سویه هیچ‌گونه فعالیت سمی و مهارکنندگی نسبت به ردهٔ سلولی فیبروبلاست انسانی نشان نداد.

بحث و نتیجه‌گیری: در مقایسه با مطالعه‌های گذشته، عصارهٔ کاروتنوئیدی سویهٔ AZ فعالیت آنتی‌اکسیدانی درخور توجهی از خود نشان داد؛ بنابراین، مطالعهٔ حاضر پایه‌ای برای استفاده‌های بعدی از سویهٔ *سلولزی میکروبیوم* AZ به‌عنوان ریزموجود نویدبخش برای تولید تجاری کاروتنوئیدها در صنایع غذایی یا ضدآفتاب (به‌علت فعالیت آنتی‌اکسیدانی و غیرسیتوتوکسیک رنگدانهٔ کاروتنوئیدی آن) است.

واژه‌های کلیدی: آنتی‌اکسیدان، کاروتنوئید، *سلولزی میکروبیوم*، سمیت سلولی، بهینه‌سازی

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Production Optimization and Evaluation of Antioxidant and Cytotoxic Properties of *Cellulosimicrobium* AZ Carotenoid Pigment

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Abstract

Introduction: The radiation-resistant extremophile microorganisms can produce high amounts of carotenoids. However, the optimization of their metabolite production is critical for large-scale commercial purpose. This study aimed to investigate the production of carotenoid pigments by an extremophile strain phylogenetically close to the genus *Cellulosimicrobium* that has not been reported so far.

Materials and methods: The influence of various carbon and nitrogen sources on the microbial biomass and pigment production of the studied strain were investigated using one-factor-at-a-time-approach. Antioxidant activity of the pigment was evaluated using free radical scavenging and ferric reducing antioxidant power. Besides, the antibacterial and cytotoxicity activity of the pigment extract were investigated using the agar well-diffusion method and cell metabolic activity assay, respectively.

Results: The maximum amount of carotenoid pigment produced by the studied strain was achieved in the fermentation medium containing 1g/L yeast extract and 1g/L glucose (22.5 mg /L) that was 10.7 fold more than the unoptimized conditions (2.1 mg/L). The half-maximal effective concentration (EC_{50}) values of the pigment were evaluated as much as 10.97 mg/mL and 6.02 µg/mL in the free radical scavenging and ferric reducing antioxidant power assay, respectively. Also, the pigments of this strain showed no toxic and inhibitory effect on the human fibroblast cell line.

Discussion and conclusion: As compared to previous studies, the carotenoid pigment extract of strain AZ displayed strong antioxidant activity. Therefore, the present study lays a foundation for future utilizations of *Cellulosimicrobium* strain AZ as a promising microorganism for commercial production of carotenoids in the food industry or sunscreens due to the antioxidant and non-cytotoxic activity of its carotenoid pigment.

Key words: Antioxidant, Carotenoid, *Cellulosimicrobium*, Cytotoxicity, Optimization

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Introduction

Carotenoids, the most common and essential pigments in nature, have a wide application in the food, feed, and cosmetics industries (1). The remarkable properties of carotenoids, such as antioxidant and antimicrobial activity, made these compounds as pharmaceutical products. For example, carotenoids can be effective in treating and preventing cancers by the inactivating and neutralizing of free radicals produced in the cell (2). Besides, some carotenoids are precursors of vitamin A and can be effective in people's vision (3). In addition, some carotenoids have antimicrobial properties, and they can be used as an alternative for antibiotics (4).

Due to the harmful effects of synthetic pigments on human health, natural carotenoids have received more attentions. Plants and microorganisms are two sources of natural pigments. However, plant pigments have many disadvantages, such as instability, poor water solubility, as well as seasonal production. These properties have led to more significant consideration of the microbial pigments recently. The major advantage of microbial pigments is their cost-effective production (5). The reason can be attribute to the rapid growth of microorganisms in the low-cost fermentation media that are not affected by climatic conditions. Besides, the microbial pigments have advantages such as biodegradability as well as significant antioxidant and antibacterial activity (6).

Currently, 1178 natural carotenoids in approximately 700 source organisms, including plants, bacteria, fungi, and algae, have been reported and characterized in the literature. In fact, the natural carotenoids present the most diverse class of natural products concerning structural and physicochemical properties (7, 8). However, efforts to find microbial strains producing new carotenoids are ongoing. Among the microorganisms, the radiation-resistant extremophiles produce amazing primary and secondary metabolites, including carotenoids, which survive these organisms against UV radiation (9).

This study aimed to investigate the production of carotenoid pigments in an extremophile strain phylogenetically close to *cellulosimicrobium* that have not been reported so far. Besides, the antioxidant and cytotoxicity activity of the pigment, as well as the influences of different nitrogen and carbon sources on the pigment synthesis were evaluated.

Material and Methods

Microorganism and culture conditions: The radiation-resistant strain investigated in the present study was previously isolated from oil-contaminated soils at the Biology laboratory of Isfahan University (10). The TGY medium containing tryptone (5 g/L), glucose (1 g/L), and yeast extract (5g /L) was used (pH = 7.2) for isolation of the studied strain (11). To obtain a pure culture of bacteria, 1.5% w/v agar was added to the liquid medium.

Microorganism identification: Morphological and biochemical characteristics of the strain, including gram reaction, spore formation, motility, oxidase, catalase, nitrate reduction, the production of H_2S, indole, urease, as well as carbohydrate utilization (glucose, lactose, mannitol, cellulose, cellobiose, raffinose, rhamnose, trehalose, and salicin) were determined according to the standard methods (12, 13).

To molecular identification of the strain, bacterial genomic DNA was extracted using the boiling method from the pure culture. The 16S rDNA was amplified by PCR using the universal primer 27F (5′-AGAGTTTGATYMTGGCTCA-3′) and 1492R (5'-CGGTTACCTTGTTACGACTT-3'). PCR Master Mix (2×, Cinna Gen), 10 p mole of each primer (0.5 µL), and extracted DNA (1µL) were used as the PCR mixture in the

final volume of 25 µl. The PCR conditions were the initial denaturation at 94 °C (5 min), 30 cycles of denaturing at 94 °C (45 s), annealing at 55 °C (1 min), and extension at 72 °C (1 min), and then a final extension at 72°C (5 min). PCR product was electrophoresed on a 1% w/v agarose gel (10). The gene sequence was determined (Korean Macrogen Company) and compared to the previously reported bacterial sequences available in the Gene Bank (www.ncbi.nlm.nih.gov/Blast.cgi, NCBI). Then, the multiple alignments of data and the phylogenetic analysis were performed using the Muscle program and the maximum likelihood method, respectively (MEGA 6 software package).

Carotenoid pigment extraction: Different solvents, including acetone, methanol, chloroform, ethanol, N-hexane, were used to extract the pigments produced by the strain AZ. The strain was inoculated into the broth medium and incubated at 30°C for five days (120 rpm). The solvent was added to the bacterial pellet after centrifugation at 3584 ×g for 10 minutes. The resulting mixture was vortexed for 10 min and incubated at 10 °C for 45 min. It was then centrifuged at 3584 ×g for 10 minutes. The supernatant was collected, and the solvent was added to the residual biomass again. The mentioned steps were repeated until the biomass became completely colorless. The supernatant was collected, dried at 40°C, and then stored at 4ºC for further analysis (14). The UV/Vis spectra of the carotenoids extract were assayed at 200–600 nm using a spectrophotometer (Biochrom WPA Biowave II, ZEISS Specord, Germany) to confirm that the extraction procedure was an efficient method that did not change the core structure of the natural carotenoids present in the extract. Besides, Thin Layer Chromatography (silica gel GF254 plate; Merck, Germany) was conducted using a suitable solvent system (ethyl acetate: N-hexane in the ratio 2:8) to confirm the presence of the carotenoid pigment(s) in the strain pigment extract.

Optimization of pigment production: To investigate the influence of different carbon sources on the pigment synthesis, the following broth medium containing 1 g/L yeast extract, 0.1 g/L dipotassium phosphate (KH_2PO_4) and 1g /L of each carbon sources such as glucose, sucrose, maltose, starch, xylose, sorbitol, and raffinose was used.

To find the most effective nitrogen sources on the pigment production, 1 g/L of various nitrogen sources, such as yeast extract, tryptone, peptone, casein, ammonium sulfate, calcium nitrate, potassium nitrate, and ammonium nitrate were used separately in a broth medium containing glucose (1 g/L) and KH_2PO_4 (0.1 g/L).

Besides, the influence of different concentrations of whey including 40%, 50%, and 60% w/v, was also studied. Cheese whey was purchased from Pegah Company (Iran). The whey medium was autoclaved at 110°C for 15 min as heat treatment for deproteinization. Subsequently, it was filtered with Whitman paper (No. 40) and centrifuged at 3584 ×g (20 min) for removing the precipitates. The studied carbon and nitrogen sources were added to the primary fermentation medium at this stage. Then, the resulted solution was autoclaved again at 110°C (15 min).

The strain AZ was inoculated at the concentration of 1% v/v in all culture media and incubated for five days (30°C, 120 rpm). Then the amount of biomass and the carotenoid content were evaluated.

Biomass and Total Carotenoids content measurement: To determine the amount of microbial biomass in the fermentation medium, 100 ml of the culture medium inoculated by strain AZ as well as 100 ml of the medium without any inoculation (control) were centrifuged at 3584 ×g for 5

min. Then, the resulting pellets were dried for 72 h at 70°C. Subsequently, both pellets were weighed. The bacterial biomass in the fermentation medium was calculated by subtracting the weight of two pellets according to equation 1:

$$\text{Microbial biomass } (\frac{g}{L}) = \text{DWF} - \text{DWC} \quad (1)$$

In equation 1, DWF was the dry weight of bacterial pellet in fermentation medium and DWC was the dry weight of pellet in the medium without any bacterial inoculation (control medium).The amount of the produced pigment in 10 ml of culture medium was calculated according to the formula provided by the equation 2 (15).

$$Total\ carotenoids\ \left(\frac{\mu g}{L}\right) = \frac{(A_{474}) \times (V_S) \times (10^9)}{(A^{\%1}_{1\ cm}) \times (100)} \quad (2)$$

In equation 2, A_{474}, V_s, and $A^{1\%}_{1\ cm}$ are the total carotenoid maximum absorbance in the solvent, the sample solution volume, and the total carotenoid specific absorption coefficient in a 1 cm cell for a 1% solution, respectively.

Antioxidant activity assay of the pigment: The antioxidant activity of strain AZ pigment extract was evaluated using ferric reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl radicals (DPPH) according to the protocol of previous studies with some modification (16, 17).

Reducing of ferricyanide complex to ferrous form is a rapid and appropriate method to evaluate the reducing power of compounds and can be considered as a marker of its antioxidant activity. Due to the reducing power of an antioxidant, the color of the solution can change from yellow to blue. To evaluate the ferric reducing antioxidant power of strain AZ carotenoid pigment, various concentrations of the pigment were dissolved in 1 mL double distilled water (0.5 to 10 µg⁄mL). Subsequently, sodium phosphate buffer (2.5 mL, 200 mM, pH 6.6) as well as potassium ferricyanide (2.5 mL, 1% w/v) were added to the pigment solution and the mixture incubated at 50ºC for 20 min. Then, trichloroacetic acid (2.5 mL 10% w/v) was added, and the final solution was centrifuged at 896 ×g for 10 min. Subsequently, 1 mL ferric chloride (0.1% w/v) was added to a 10 mL sample mixture (5 mL supernatant and 5 mL sterile double distilled water), and the absorbance of the final solution was measured immediately at 700 nm. The positive control was ascorbic acid.

Besides, the antioxidant activity of strain AZ pigment extract was evaluated using DPPH method. For this purpose, various concentrations of the pigment were dissolved in 1 mL double distilled water (0.05 to 10 mg⁄mL). Subsequently, 1 mL of DPPH methanol solution (0.1 mM) was added to the sample solutions. The mixture were allowed to react in the dark for 30 min at room temperature, then, the absorbance values were measured at 517 nm.

The half-maximal effective concentration (EC_{50}) values of the studied pigment in both methods, were also determined using dose-response linear regression plots.

Cytotoxic Activity Assay: The inhibitory or cytotoxic effect of the studied carotenoid pigment on human cell lines was evaluated using the MTT assay. In this assay, the inhibitory effect is inferred from the ability of cellular NAD(P)H-dependent oxidoreductase enzymes in reducing a tetrazolium dye named MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to its purple insoluble form (formazan) in the presence of carotenoid. The MTT assay was performed using human breast cancer (MCF-7), human cervical cancer (HeLa), and normal human fibroblast (HBF) cell lines in the present study. For this purpose, 10^4 cells as well as 500 µl RPMI 1640 medium containing 10% v/v serum and 1% w/v antibiotic (penicillin

and streptomycin) were added to each well of 96-well plates. Then, the microtiter plate was incubated at 37 °C for 24 h with 5% CO_2. Then, different concentrations of the pigment extract (50, 100, 200, 500 µg/mL) in dimethyl sulfoxide (DMSO) was added to each well. Subsequently, 100 µL MTT was added to each well after 48 h of incubation. The 96-well plate was incubated for 4 h at 37 °C to the formation of insoluble formazan crystals. Then, 150 µL DMSO was added to each well to dissolve the insoluble formazan, and the absorbance of each well was measured at 570 nm. The cell survival rate was calculated based on the absorbance of the treated cell concerning the untreated cells. Besides, the morphology of cells in the presence and absence of pigment extract was also evaluated to study the effect of pigment extract on the cell morphology (18).

Antibacterial Activity Assay: The antibacterial activity of pigment extract was determined using the agar well-diffusion method against some microbial flora, *i.e.*, *Staphylococcus epidermidis* (IBRC-M 10694) and *Escherichia coli* (IBRC-M 11108) in Moller Hinton agar (MHA) plates. The strains were purchased from the Iranian Research Organization for Science and Technology (IROST). For this purpose, 5 ml nutrient broth medium was inoculated by a pure colony of each bacterium and incubated at 37°C to reach an optical density to that of approximately $1–2\times10^8$ CFU/ml, equivalent to a 0.5 McFarland standard. After spreading 100 µl bacterial suspension over the MHA plate and allowing the inoculum to absorb into the agar for 10 min, 50 µl of each concentration of the pigment extract or 50 µl methanol as the control, were added into 6 mm wells which were punched in the agar. The diameter of inhibition growth after 24 h incubation at 37°C was reported as the antibacterial activity of the pigment extract (19).

Statistical analysis: Duncan's multiple range test and Analysis of Variance (ANOVA) with a 95% confidence interval was performed to evaluate the statistical significance of the observed differences between various treatments.

Results

Microbial strain identification and phylogenetic analysis: The strain AZ was gram-positive coccobacilli with small, convex, mucoid, and yellow colony. The results of the biochemical tests of this strain are listed in Table 1.

Table 1- Results of biochemical tests of strain AZ

Test	Result
Catalase	+
Oxidase	+
Nitrate reduction	+
Urease	+
Indole	-
H_2S	-
Cellulose	+
Raffinose	-
Salicin	+
Trehalose	+
Glucose	+
Lactose	+
Mannitol	+
Rhamnose	-
Cellobiose	+

The partial 16S rRNA gene (1380 bp) of the strain was determined, and showed more than 99% similarity to *Cellulosimicrobium funkei* strain W6122, *Cellulosimicrobium aquatile s*train 3bp, and *Cellulosimicrobium cellulans* strain MJMG8.3 according to the results of the Basic Local Alignment Search Tool (BLAST). The 16S rRNA gene sequence of the strain was further deposited in Gene Bank under the accession number of MG602070. To confirm the BLAST results, the phylogenetic tree was plotted using Mega 6 software (Fig. 1). The phylogenetic analysis of the 16S RNA gene sequence showed the strain had a close relationship with *Cellulosimicrobium funkei* strain R6-

420.

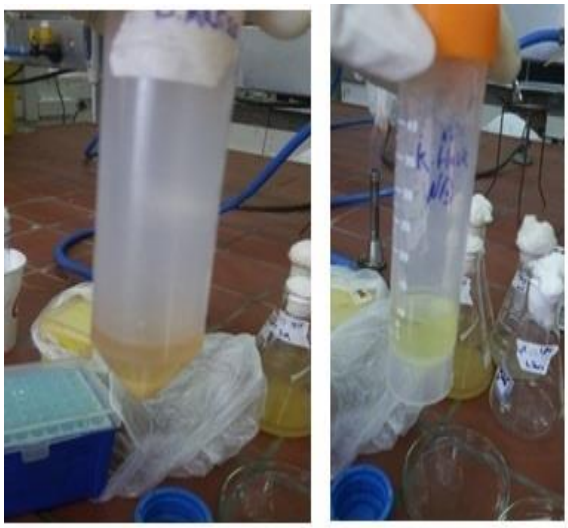

Fig. 1- The carotenoid extract of *Cellulosimicrobium AZ* in methanol

Effect of different carbon sources on pigment production: The results showed that all carbon sources had a significant effect on the pigment production compared to the control, which had no carbon sources. However, only sucrose, glucose, and lactose had a significant impact on the biomass production. The maximum pigment production was obtained in the presence of glucose as much as 7.3-fold. The biomass production in the medium containing sorbitol was not significantly different from the control medium (Fig. 2).

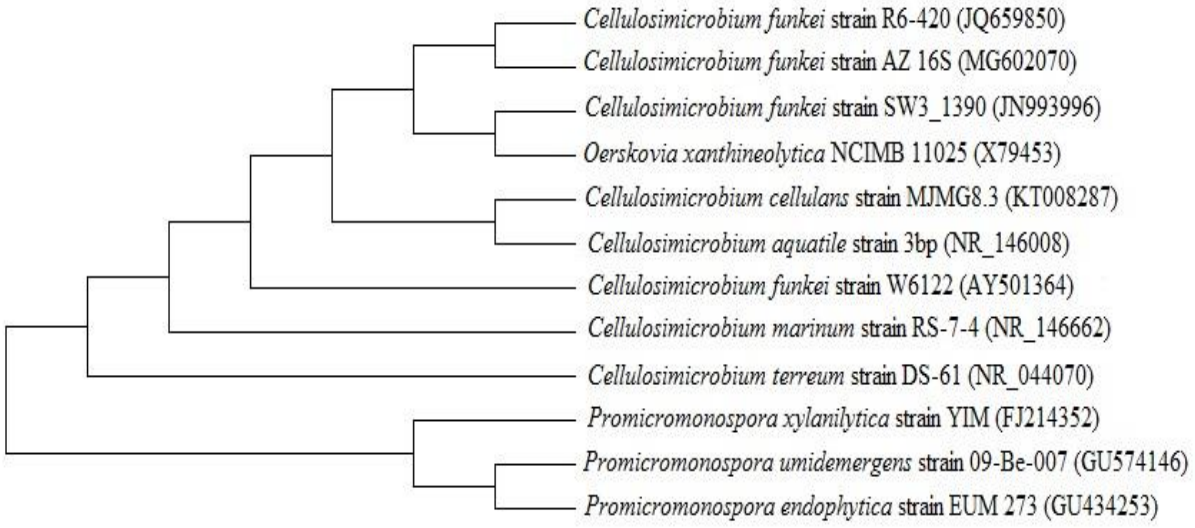

Fig. 2- The phylogenetic analysis of the 16S rRNA sequence of the isolated strain based on the maximum likelihood method. The numbers in brackets showed the accession numbers of the reference strains

Effect of different nitrogen sources on pigment production: All studied organic nitrogen sources had a statistically significant effect on the biomass and carotenoid production of strain AZ. The highest pigment biosynthesis was observed in the presence of yeast extract due to increasing pigment production as much as 22.5 mg/L concerning the control (2.1

mg/L). Besides, the inorganic nitrogen sources also had a positive effect on the strain AZ pigment production. Potassium nitrate and ammonium nitrate increased the pigment production by about 7.4- and 7.1-fold, respectively (Fig. 3).

Effect of different concentrations of whey on pigment production: Whey medium at the concentration of 60% w/v had a better effect on the biomass and carotenoid production of strain AZ (17.5 mg/L). However, other studied concentrations of whey also had a positive influence (Fig. 4).

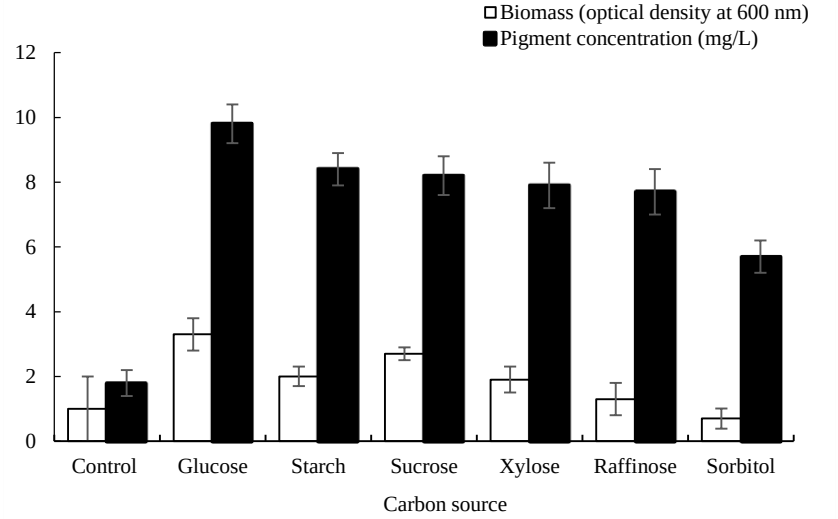

Fig. 3- Effect of different carbon sources on the biomass and carotenoid production by strain AZ

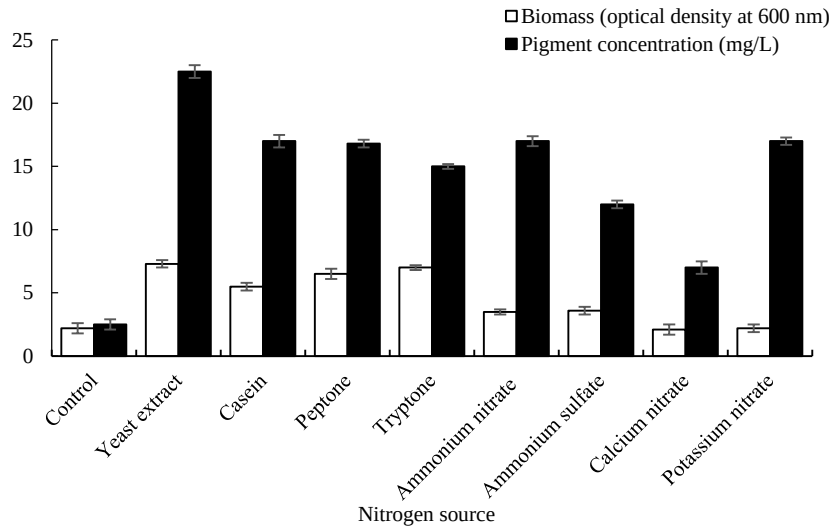

Fig. 4- Effect of different nitrogen sources on the biomass and carotenoid production by strain AZ

Evaluation of antioxidant activity of pigment: The results of the antioxidant activity of strain AZ pigment extract using DPPH and FRAP tests were illustrated in Figures 5 and 7, respectively. The pigment extract showed higher antioxidant activity concerning the ascorbic acid as the control. According to the results, the ferric reducing antioxidant power and free radical scavenging capacity of the pigment were evaluated as EC_{50} = 6.02 µg/mL and EC_{50} = 10.97 mg / mL, respectively (Fig. 6 and Fig. 8). The pigment extract at higher concentrations had more antioxidant activity significantly.

Evaluation of antibacterial and cytotoxic

properties of pigment: The studied carotenoid pigment had no toxic and inhibitory effects on the HFB cell line. The HFB cells had a more survival rate in the presence of strain AZ carotenoid extract concerning the ascorbic acid (Fig. 9). However, there was no anticancer effect on the Hela and MCF 7 cell lines in the presence of the studied carotenoid extract. Besides, the studied carotenoid pigment had not any inhibitory effect on the studied bacterial strain.

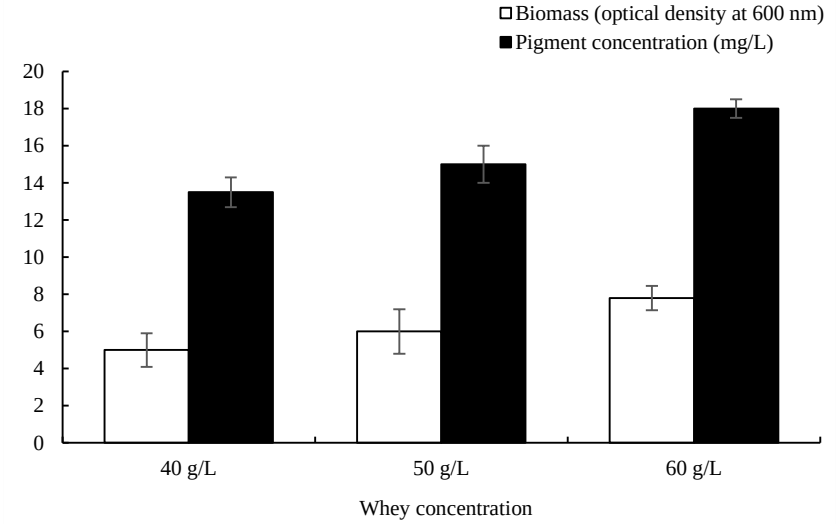

Fig. 5- Effect of different concentrations of whey medium on the carotenoid production by strain AZ

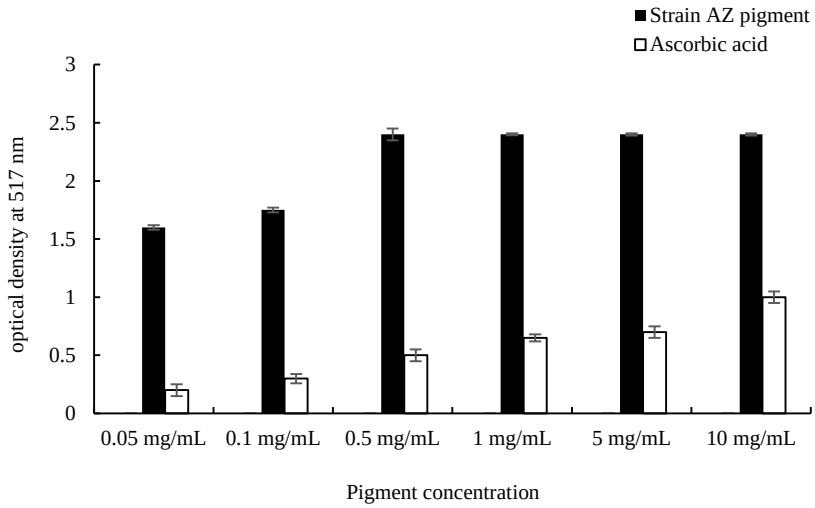

Fig. 6- The antioxidant activity of strain AZ pigment extract using DPPH assay

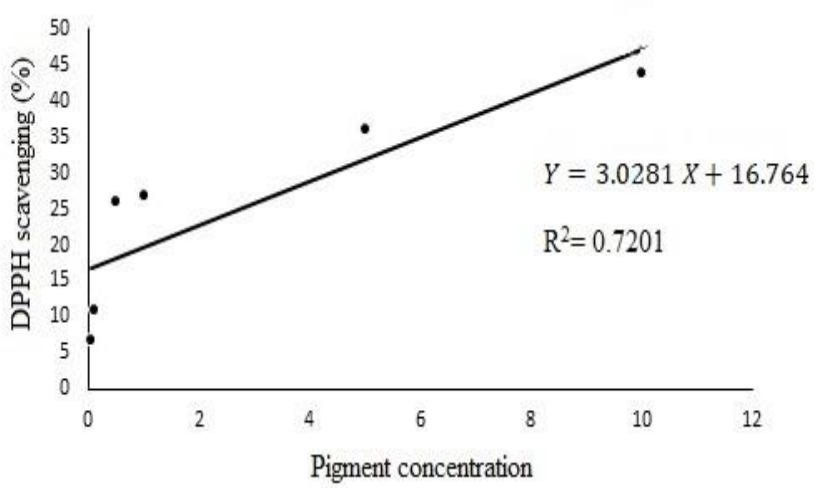

Fig. 7- Half percent effective concentration (EC_{50}) of free radical scavenging capacity of strain AZ pigment extract based on the results of DPPH assay

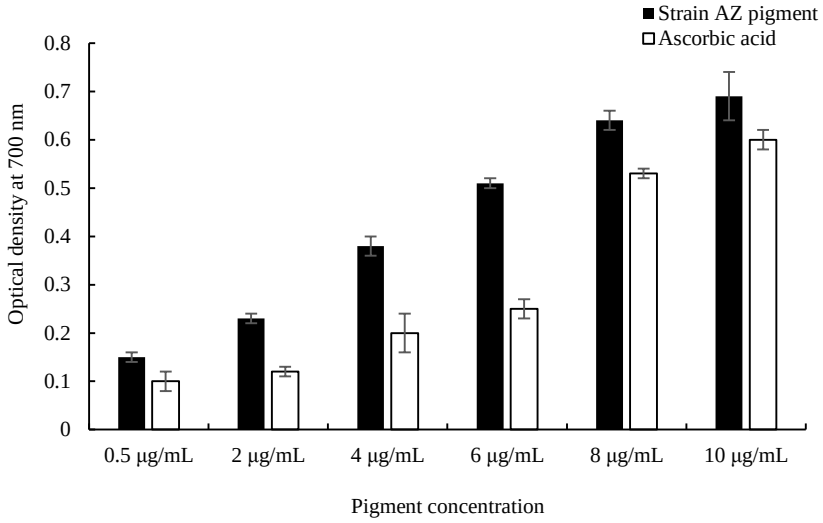

Fig. 8- The antioxidant activity of strain AZ pigment extract using FRAP assay

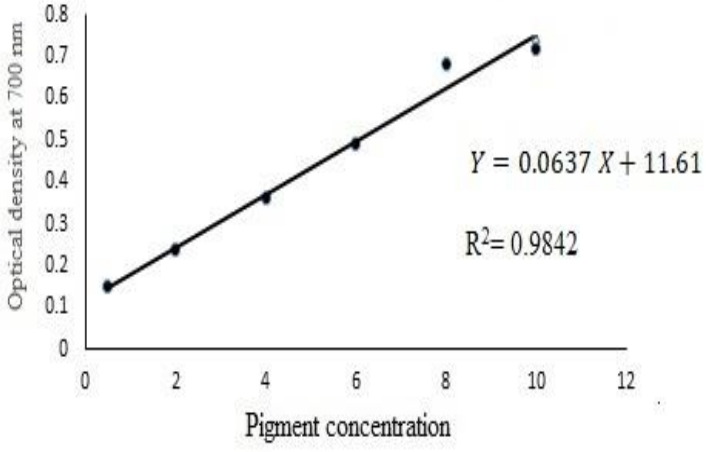

Fig. 9- Half percent effective concentration (EC_{50}) of reducing capacity of strain AZ pigment extract based on the results of FRAP assay

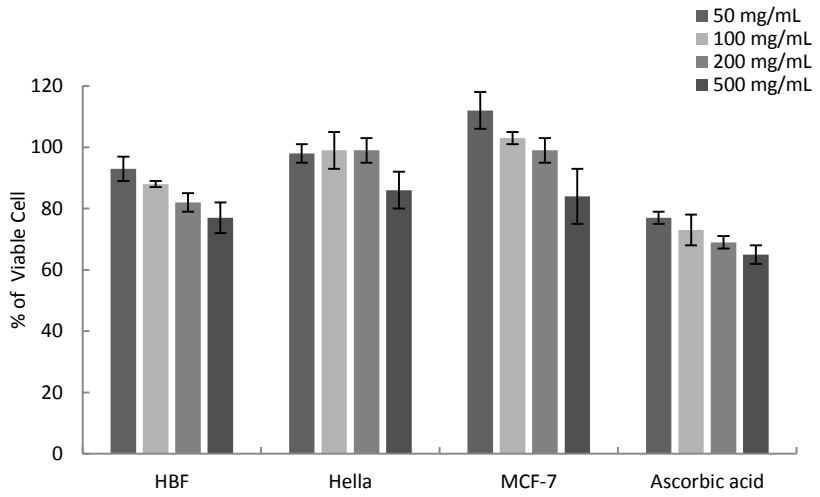

Fig. 10: The effect of strain AZ pigment extract on the normal human fibroblast (HBF), human cervical cancer (HeLa), and human breast cancer (MCF-7) cell lines concerning ascorbic acid based on the results of MTT assay

Discussion

The bacteria in the genus *Cellulosimicrobium* are gram-positive, facultative anaerobes, catalase-positive, non-spore forming, and usually have bright yellow colonies. The genus *Cellulosimicrobium* belongs to the family promicromonosporaceae and the phylum actinobacteria (20). The previous studies reported that *Cellulosimicrobium funkei* is a haloalkaliphilic bacterium that can remove chromium and biodegrade aflatoxin (21, 22). However, there was no report of carotenoid pigment production in this genus previously. In the present study, the carotenoid pigment produced by a radiation-resistant extremophile strain of *Cellulosimicrobium funkei* named strain AZ was investigated in the presence of various sources of carbon and nitrogen.

According to the results, the pigment production of the studied strain was more in the presence of the organic nitrogen sources, and had a direct relationship with the bacterial biomass that confirmed the results of previous study (23). The organic nitrogen sources increased the pigment production in the following order: yeast extract > tryptone > peptone > casein. Among inorganic nitrogen sources, the pigment production was as much as 17.7 and 17.8 mg/L in the presence of ammonium nitrate and potassium nitrate, respectively. However, this difference was not statistically significant. The results also showed that calcium nitrate had less positive effect (7.3 mg/L). Therefore, various nitrate salts may influence the pigment production differently.

Similar to our results, Zhao et al. (2002) reported that the best nitrogen source for *Rhodotorula* sp. RY1801 carotenoid production were yeast extract (24). However, El-Banna et al., (2012) reported that the best nitrogen sources for the production of carotenoid pigment by *Rhodotorula glutinis* was ammonium sulfate (23). Therefore, the optimum conditions for the pigment production for each microbial strain are very different from those of the other strains.

The pigment production also increased by 7.3-, 6.8-, and 6.5-fold, in the fermentation medium containing the glucose, starch, and lactose, respectively. Besides, all studied carbon sources increased the microbial biomass as much as 2-3.5-fold. Therefore, the highest increase in the microbial biomass and the carotenoid synthesis was obtained in the medium containing glucose as the carbon source. This result was in contrast to the results of Gmoser et al., (2018) reported that the carotenoid production of *Neurospora*

Intermedia decreased in the presence of glucose due to the catabolite repression phenomenon (25). However, the catabolite repression has not been reported for the carotenoid pigment production by other microbial strains (24, 26, 27). The pigment production also had a direct relationship with the bacterial biomass in the presence of all studied carbon sources except for starch.

Compared with the maximum amount of carotenoid pigment produced by *Rhodotorula glutinis* (1.9 mg/L), the strain AZ was able to produce carotenoid pigment as much as 22.5 mg/L (23). The reason may be related to the resistance of the strain AZ against UV radiation that confirmed the results of previous study (28).

In the present study, the pigment production of strain AZ was also investigated in the cost-effective whey medium. The studied strain produced carotenoid about 17.5 mg/L in the 60% w/v whey medium. The amount of carotenoid production using strain AZ in the whey medium was more than previous study (29). The whey contains 4.5% lactose and 0.8% casein protein (30). Therefore, a fermentation medium containing 60% w/v whey have about 2.7 g lactose as the carbon source and about 0.48 g casein as the nitrogen source. Besides, the presence of minerals and vitamins in the whey can increase the growth of most microbial strains and thereby enhance the fermentation products. Both lactose and casein alone were able to increase the carotenoid pigment production in the strain AZ, and their availability in a 60% w/v inexpensive whey culture medium resulted in an approximately 8.3-fold increase in the pigment production. Therefore, the whey medium can be used to produce the carotenoid pigments of the strain AZ. On the other hand, the whey is a by-product of the dairy industries that can cause environmental pollution due to its high chemical oxygen demand (COD). Therefore, the whey medium has always been a focus of interest in the various industries as an inexpensive medium.

In the present study, the antioxidant activity of carotenoid pigment extract of strain AZ was measured by DPPH and also by FRAP assay and compared to each other. Some of the cellular damages and the related diseases in humans, such as cancers, Alzheimer and heart diseases have resulted from the free radical generation during cell metabolic activities. By scavenging free radicals, carotenoids can decrease the oxidative stress of the cell resulted from reactive oxygen species (ROS) and reactive nitrogen species (RNS) formed during the cell metabolism and in various biotic and abiotic stresses (31, 32). The antioxidant activity of the studied pigment was higher than ascorbic acid in both methods. The strain showed EC_{50} values of 10.97 mg/ml and 6.02 µg/mL in DPPH and FRAP assay, respectively. As compared to previous studies, the carotenoid pigment extract of isolated strain displayed strong antioxidant activity. It was reported that EC_{50} values in reducing power assay of *Microbacterium esteraromaticum*, *Dietzia schimae*, *Dietzia maris*, and *Deinococcus* sp. were 35.26, 36.13, 28.46 and 20.19 µg/ml, respectively (10, 14). Therefore, the strain AZ carotenoid pigment extract displayed strong antioxidant activity to other UV tolerant bacterial strains and can be considered as an industrial strain for carotenoid production.

Besides, the carotenoid extract of strain AZ was devoid of cytotoxicity effect in the human fibroblast (HFb) cell line according to the results of MTT assay. Although the studied carotenoid extract had no anticancer effects, this property is not critical for introducing a bacterial strain as an industrial strain for the production of a natural dye for the food or sunscreen cream industry. Besides, the studied carotenoid extract had not any inhibitory effect on skin and gut microflora.

Conclusion

All studied carbon and nitrogen sources led to an increase in the pigment production of strain AZ compared to the control. The highest carotenoid pigment production was obtained in the culture medium containing 1 g/L yeast extract and 1 g/L glucose (22.5 mg /L). Carotenoid extract of strain AZ showed significant antioxidant activity based on the results of DPPH and FRAP tests. Also, the pigment of this strain showed no toxic and inhibitory effect on the human fibroblast cell line. The carotenoid extract of strain AZ can be considered as a natural dye for the food or sunscreen industry because the results of the present study showed its antioxidant activity, non-toxicity and inhibitory effects on the human fibroblast cell line, and microbial flora, besides of its color.

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