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Isolation of *Lactobacillus plantarum* subsp. *plantarum* Producing C_{30} Carotenoid 4,4′-Diaponeurosporene and the Assessment of Its Antioxidant Activity S

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Carotenoids are organic pigments with antioxidant properties and are widespread in nature. Here, we isolated five microbes, each forming yellow-colored colonies and harboring C_{30} carotenoid biosynthetic genes (*crtM* and *crtN*). Thereafter, *Lactobacillus plantarum* subsp. *plantarum* KCCP11226, which showed the highest carotenoid production, was finally selected and the produced pigment was identified as C_{30} carotenoid 4,4′-diaponeurosporene. This strain exhibited the highest survival rate under oxidative stress and its carotenoid production was also enhanced after exposure to 7 mM H_2O_2. Moreover, it showed the highest ability to scavenge DPPH free radical. Our results suggested that *L. plantarum* subsp. *plantarum* KCCP11226, which produces 4,4′-diaponeurosporene as a natural antioxidant, may be a functional probiotic.

Keywords: *Lactobacillus plantarum* subsp. *plantarum*, carotenoid, 4,4′-diaponeurosporene, antioxidant, isolation

Carotenoids are natural pigments widespread in plants, some animals, fungi, and photosynthetic or non-photosynthetic bacteria [1]. Their antioxidant activities have also been extensively studied [2]. Carotenoids have interesting biological functions including antioxidant, anticancer, and anti-obesity effects and are widely used in foods, nutraceuticals, and the feed industry [3]. Structurally, carotenoids can be divided into carotenes (*e.g.*, α-/β-carotene and lycopene) and xanthophylls (*e.g.*, lutein, zeaxanthin, fucoxanthin, and astaxanthin) [3]. Several types of carotenoids are produced by microorganisms. The yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) produces astaxanthin [4], and microalgae produce lutein [5]. The fungus *Blakeslea trispora* has been reported to produce β-carotene and lycopene [6].

Lactic acid bacteria (LAB) are a type of gram-positive bacteria that are generally recognized as safe (GRAS) and have had diverse applications in fermentation of various foods for many centuries [7, 8]. LAB exhibit microaerophilic or facultative anaerobic growth characteristics owing to their lack of catalase expression and inability to remove free radicals [8]. To overcome this problem, LAB produce various antioxidants, such as superoxide dismutase and thioredoxin reductase [8, 9]. Moreover, LAB are known to produce carotenoids as a strategy for eliminating reactive oxygen species [10, 11]. The yellow pigment produced by

LAB strains has been identified as 4,4′-diaponeurosporene, one of the C_{30} carotenoids [10, 12, 13]. Indeed, *Lactobacillus plantarum* and *Enterococcus gilvus* express components of a carotenoid biosynthetic pathway and produce 4,4′-diaponeurosporene [10, 12]. Dehydrosqualene synthase (*crtM*) and dehydrosqualene desaturase (*crtN*) play important roles in carotenoid biosynthetic pathways by converting farnesyl-PP (three isoprene units, 15 carbons) to 4,4′-diaponeurosporene (six isoprene units, 30 carbons) [10].

Previous studies have reported that carotenoid production in microorganisms improves stress tolerance [14, 15]. LAB also exhibit tolerance against multiple stresses, such as envelope stress (lysozyme), high temperature (heat), solvent, bile, acid, salt, and osmotic stresses [16–20]. Recently, Hagi *et al.* showed a correlation between carotenoid production by LAB and oxidative stress tolerance [10, 21]. Moreover, the *crtM* and *crtN* genes of *E. gilvus* are upregulated by a variety of stress conditions, leading to carotenoid production [10]. Nevertheless, the antioxidant activity of 4,4′-diaponeurosporene, a C_{30} carotenoid produced by LAB, is still unclear. Therefore, in this study, we isolated C_{30} carotenoid-producing LAB, evaluated 4,4′-diaponeurosporene production by these LAB, and confirmed the antioxidant capacity of the carotenoids produced in LAB, in particular *L. plantarum* subsp. *plantarum* KCCP11226.

Four hundred fifty-six strains were firstly isolated from various fermented foods and obtained from two culture collections, the Korean Culture Center of Microorganisms (KCCM) and the Korean Culture Collection of Probiotics (KCCP). Among them, 79 strains forming yellow-colored colonies were subsequently screened, since the interesting strains in this study should produce carotenoid compounds with the sought-after pigments. Next, five strains harboring carotenoid biosynthesis genes were selected as detected by PCR with the following primer set: crtNM-for (5′-CGC GGAATTCATGAAGCAAGTATCGATTATTGGC-3′) and crtNM-rev (5′-GATCGAATTCTTAAGCCTCCTTAAGGGCT AGTTC-3′) [12, 13]. These strains, carrying carotenoid biosynthetic genes, were identified as *L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *plantarum* by 16S rRNA gene sequencing by employing bacterial universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Fig. S1). Thereafter, the five *Lactobacillus* isolates were cultured in de Man, Rogosa and Sharpe (MRS) media for 24 h at 30°C, followed by extracting the yellow pigments that were finally isolated [22]. Consistent with these findings, various LAB, including *E. gilvus*, *L. plantarum*, *L. fermentum*, *Pediococcus acidilactici*, and *P. pentosaceus*, have been shown to produce deep yellow pigmentation, which could be involved in C_{30} carotenoid compounds, in previous studies [10, 12, 13, 15, 21].

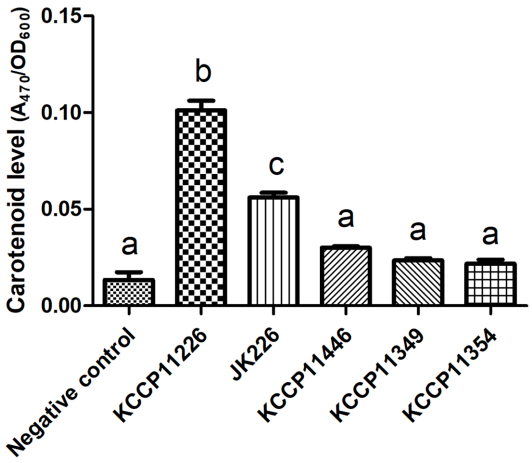

Fig. 1. Comparison of carotenoid production by the isolated strains.

In order to extract carotenoids in the cells harvested from *Lactobacillus* culture broth, cell pellets were introduced in a 15 ml conical tube and extracted with a total volume of 5 ml methanol. After shaking tubes overnight at room temperature, 5 ml hexane and 2.5 ml distilled water was added to the methanol extract containing yellow pigments. After centrifugation at 2,000 ×*g* for 10 min, the carotenoid-containing organic phase was collected. The organic phase was evaporated under O_2 gas, and carotenoids were re-suspended in 1 ml petroleum ether. The amount of pigment in the extract was determined by measuring the absorbance as 470 nm (A_{470}) using a spectrophotometer (Shimadzu, Kyoto, Japan). Separation of the yellow pigments was performed using thin-layer chromatography (TLC) with silica gel (Merck, Darmstadt, Germany) and a mixture of petroleum ether and acetone (9:1, v/v) in a sealed glass chamber. After scraping off the pigment and eluting with petroleum ether to recover the pigment, the absorbance spectrum of the purified pigment was calculated using an ultraviolet-visible (UV-VIS) spectrophotometer (Shimadzu, Japan). The carotenoid pigment level was calculated by dividing the absorbance at 470 nm (A_{470}) by the optical density at 600 nm (OD_{600}). *L. plantarum* KCCP11031 with no operon *crtNM* was used as the negative control strain. The results represent the averages of three independent experiments. Error bars correspond to the standard deviations. Significant means were obtained after analysis of variance (ANOVA) analysis with Tukey-Kramer multiple comparison tests ($p < 0.01$).

The evaluation of carotenoid production level from each of five isolates determined by A_{470}/OD_{600} revealed that *L. plantarum* subsp. *plantarum* KCCP11226 showed the highest carotenoid level (0.10) compared to that produced by other isolates, even reaching 10-fold higher than that in *L. plantarum* KCCP11031, which does not harbor operon *crtNM*, and is considered to be a negative control strain

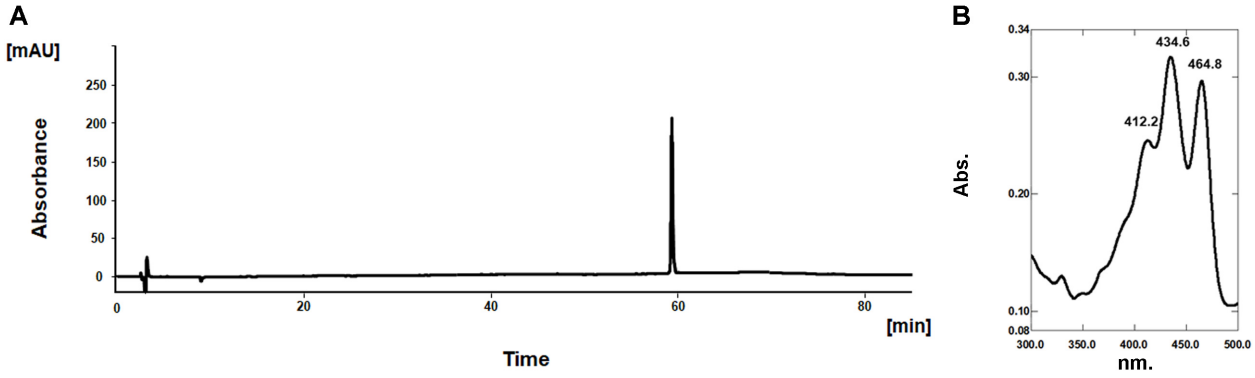

Fig. 2. Analysis of 4,4´-diaponeurosporene produced by *L. pentosus* KCCP11226 using HPLC chromatographic analysis (**A**) and UV-visible absorption spectra (**B**).

The purified carotenoids by preparative TLC were dissolved in EtOAc, and loaded onto a silica gel column. The silica gel column was pre-equilibrated with a 9:1 hexane/EtOAc solvent system. Carotenoids were eluted using the pre-equilibrated solvent system with an increasing gradient of EtOAc and dried using an evaporator. The purified pigments were dissolved in acetone and filtered through a 0.5 μm polytetrafluoroethylene filter, and 50 μl was injected into the HPLC. Analysis of carotenoids was performed by reversed-phase HPLC. The column was a C18 reversed phase column (250 × 4.6 mm I.D., particle size 5 μm). A binary gradient elution system was used to change from 100% of mix A to 100% of mix B at a flow rate of 1.0 ml/min over a period of 65 min. Mix A contained methanol and water for HPLC (60:40; v/v), and mix B contained methanol, methyl tert-butyl-ether, and water for HPLC (28.5:67.5:4; v/v/v). A UV-visible photodiode array detector was used, and detection was carried out at 470 nm.

(Fig. 1). After the extraction of pigments produced from *L. plantarum* subsp. *plantarum* KCCP11226, we separated and isolated the pigments by thin-layer chromatography (TLC) with silica gel plate to tentatively identify the carotenoids produced by *L. plantarum* subsp. *plantarum* KCCP11226. Typical HPLC chromatographic analysis of the purified carotenoids showed a single chromatographic peak at a retention time of 59.4 min (Fig. 2A). In addition, the UV-VIS absorption spectrum of the purified yellow pigments showed maximum absorbance peaks at 412.2, 434.6, and 464.8 nm (Fig. 2B), which are identical to the peaks obtained from 4,4′-diaponeurosporene [23]. This UV-VIS absorption spectra result of the purified yellow pigments was similar with those from other isolates in this study, suggesting that the existence of carotenoid biosynthetic operon *crtNM* harbored in *Lactobacillus* strains could be a biomarker for the biosynthesis of C_{30} carotenoid 4,4′-diaponeurosporene [12, 13].

Although the survival rates of microorganisms were reduced under harsh conditions, carotenoid-synthesizing microorganisms have been shown to exhibit increased carotenoid production under stress conditions, such as anaerobic, thermal, and oxidative stresses [15]. In particular, the LAB-derived carotenoids are thought to play major roles in oxidative stress tolerance similar to other carotenoids including astaxanthin and staphyloxanthin [10, 24, 25] Accordingly, we evaluated the survival rates of three isolates showing high carotenoid production levels under oxidative stress by employing the presence of H_2O_2. Interestingly, *L. plantarum* susp. *plantarum* KCCP11226 exhibited the highest survival rates under oxidative stress conditions, even in the presence of 32 mM H_2O_2 (Fig. 3A). LAB are catalase negative but produce NADH-peroxidase and decompose H_2O_2 [26] and *L. plantarum* subsp. *plantarum* KCCP11226 showed the highest survival rate among the carotenoid-producing strains after H_2O_2 treatment. Carotenoid production by *Rhodotorula glutinis* is significantly increased by oxidative stress [27]. Similarly, carotenoid production by *L. plantarum* subsp. *plantarum* KCCP11226 under stress conditions was higher than that under normal conditions. Moreover, when *L. plantarum* subsp. *plantarum* KCCP11226 was treated with H_2O_2, the carotenoid levels were increased compared with those in the untreated cells, and it showed a suddenly increased carotenoid production level over 7 mM H_2O_2 (Fig. 3B). In detail, the cell mass of *L. plantarum* subsp. *plantarum* KCCP11226 was not significantly decreased until the concentration of H_2O_2 reached 6 mM, and a dramatic decrease was observed in the presence of 7 mM or more H_2O_2. Carotenoid levels can be expressed as carotenoid content relative to the cell mass [15] and were significantly increased owing to the decreased cell mass during treatment with high concentrations of H_2O_2. Indeed, the cell mass of the KCCP11226 strain decreased by 20.5% compared with that under untreated conditions, whereas the production of

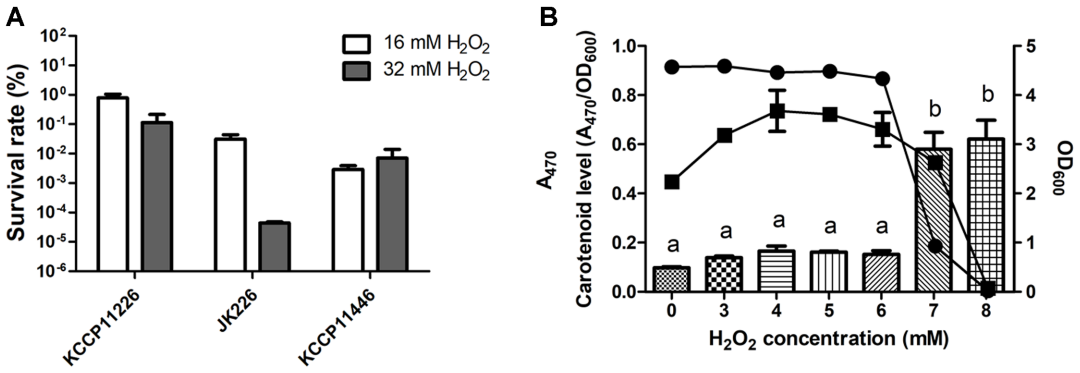

Fig. 3. Survival rate (**A**) of carotenoid-producing *L. pentosus* strains after exposure to H_2O_2 stress conditions and the effect of H_2O_2 stress on carotenoid production (**B**) by *L. pentosus* KCCP11226.

In order to evaluate survival rate (%) in part A, cell pellets were resuspended in saline containing H_2O_2 (16 or 32 mM) and then incubated at 30°C for 90 min. The bacterial suspensions washed with saline were plated onto MRS agar plates, and the number of viable bacteria was determined. Survival rate was calculated using the following equation: Survival rate (%) = (log cfu N_1/log cfu N_0) × 100. where N_1 represents the total viable count of LAB strains after exposure to H_2O_2 stress, and N_0 represents the total viable count of LAB strains before exposure to stress conditions. To determine carotenoid production level under H_2O_2 stress conditions in part B, 1% (v/v) of *L. pentosus* KCCP11226 pre-cultured overnight at 30°C in MRS media was inoculated into a 500 ml Erlenmeyer flask containing 200 ml MRS media with various concentrations (0-8 mM) of H_2O_2 and then cultured for 24 h at 30°C. After cultivation, the carotenoids from the cell pellets of *L. pentosus* KCCP11226 were extracted as previously described in Fig. 1. Closed circles and squares represent OD_{600} and A_{470}, respectively. The results represent the averages of three independent experiments. Error bars correspond to standard deviations. Significant means were obtained by ANOVA with Bonferroni's multiple comparison tests ($p < 0.05$).

carotenoids increased by 117% following treatment with 7 mM H_2O_2 (Fig. 3B). Accordingly, carotenoid production per cell mass in the KCCP11226 strain treated with 7 mM H_2O_2 was increased by 5.9-fold compared with that under normal conditions. A previous study showed that β-carotene production by *Blakeslea trispora* was significantly stimulated by H_2O_2 and that carotenoids served as antioxidants protecting against cellular damage by quenching reactive oxygen species [28]. Additionally, carotenoid production by engineered *Saccharomyces cerevisiae* was dramatically increased in short-term evolution experiments using periodic H_2O_2 shocking [29]. Researchers have also shown that increased carotenoid production in the evolved mutants partially alleviated the oxidative stress observed in the cell. In addition, the expression levels of several genes (*CAB1*, *NSG1*, *ERG13*, *ERG27*, and *CYB5*) involved in lipid biosynthetic pathway by H_2O_2 were increased [29]. Therefore, H_2O_2 may promote the expression of lipid biosynthesis-related genes by *L. plantarum* subsp. *plantarum* KCCP11226, thereby improving carotenoid production.

Reactive oxygen species (ROS) foamed under oxidative stress can react with molecules in organisms to cause cellular damage. On the other hand, antioxidants such as carotenoids are substances that can reduce ROS and prevent macromolecule oxidation [30]. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method has been used to evaluate the antioxidant capacity of carotenoids extracted from microorganisms [31]. C_{50}-carotenoids from *Haloarcula japonica* showed much higher DPPH free radical scavenging activity than β-carotene [32]. Carotenoid pigment content from *Sporobolomyces* sp. has been shown to increase with an increase in the DPPH scavenging activity [33]. Accordingly, in this study, we performed DPPH assays using culture supernatants from *L. plantarum* subsp. *plantarum* KCCP11226 as well as *L. plantarum* subsp. *plantarum* KCCP11446 and *L. pentosus* JK226 to compare the DPPH radical scavenging capacities of carotenoid-producing LABs (Fig. 4). All of three strains of LAB showed higher DPPH free radical scavenging activity than butylated hydroxytoluene (BHT, 50 μg/ml), a traditional antioxidant. In particular, *L. plantarum* subsp. *plantarum* KCCP11226, which produced the most carotenoids as shown in Fig. 1, showed 27.41% of DPPH radical scavenging activity, which was slightly higher than the other LAB isolates, even though KCCP11226 produced total carotenoids prominently higher than JK226 and KCCP11446 (Fig. 1). These similar DPPH free radical scavenging activities in three LAB isolates could be the result of additional non-enzymatic antioxidants such as glutathione, exopolysaccharides and organic acids [34−36] as well as carotenoids present in

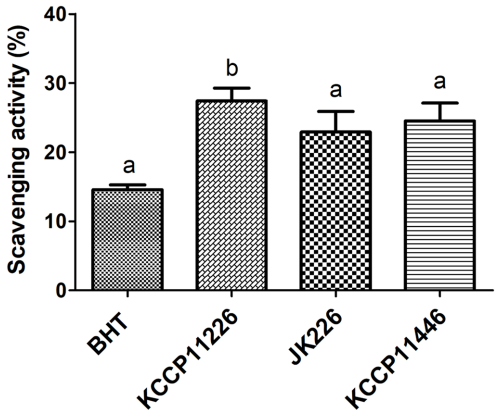

Fig. 4. Scavenging activities of carotenoid-producing *L. pentosus* strains on DPPH radicals.

Freshly prepared DPPH solution (0.2 mM in ethanol; Sigma Aldrich, USA) was mixed with bacterial supernatant. The mixture was incubated in the dark at room temperature for 30 min. The scavenged DPPH radical was then monitored by measuring the decrease in absorbance at 517 nm using a microplate reader. The scavenging ability was defined as follows: scavenging activity (%) = $(1 - [A_{sample} - A_{blank}]/A_{control}) \times 100$. The control contained saline instead of the sample solution. The blank included only bacterial supernatant and ethanol. The results represent the averages of three independent experiments. Error bars correspond to the standard deviations. Significant means were obtained by ANOVA with Tukey-Kramer multiple comparison tests ($p < 0.01$).

Lactobacillus strains. The previous studies describing that the protection from oxidative stress by scavenging free radicals in gram-positive bacteria could be responsible for the carotenoids with their conjugated double bonds, supports our results which showed that C_{30} carotenoid 4,4′-diaponeurosporene, with a chromophore containing no conjugated double bonds produced by *L. plantarum* subsp. *plantarum* KCCP11226, has DPPH radical scavenging activity [12, 25, 37].

In conclusion, the C_{30} carotenoid 4,4′-diaponeurosporene was isolated from *L. plantarum* subsp. *plantarum* KCCP11226 and was found to affect the survival of the strain under oxidative stress. Additionally, 4,4′-diaponeurosporene produced by *L. plantarum* subsp. *plantarum* KCCP11226 could be used as a natural antioxidant, and *L. plantarum* subsp. *plantarum* KCCP11226 may be a potential probiotic with functional applications in foods.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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