

Article

Complete Genome Sequence and Cosmetic Potential of *Viridibacillus* sp. JNUCC6 Isolated from Baengnokdam, the Summit Crater of Mt. Halla

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Abstract: Novel microbe-derived products are gaining increasing attention for their ability to modulate skin conditions. The use of microbial metabolites to improve skin health outcomes is of particular interest because growing evidence points to the importance of natural products without side effects on human health. This study aimed to sequence the genome of *Viridibacillus* sp. JNUCC6 isolated from Baengnokdam, the summit crater of Mt. Halla. We further investigated the potential use of its extract as a cosmetic ingredient in controlling melanogenesis and inflammation. The genome of this strain was sequenced using both Illumina Novaseq 6000 and third-generation sequencing technology (PacBio RSII) to obtain trustworthy assembly and annotation. Different concentrations of the *Viridibacillus* sp. JNUCC6 extract were tested for its anti-melanogenic and anti-inflammatory effects in α -melanocyte-stimulating hormone (α -MSH)-induced B16F10 melanoma and lipopolysaccharide (LPS)-activated RAW 264.7 cells, respectively. The whole genome sequence of the strain contained 4,526,142 bp with 35.61% GC content, one contig, and 4364 protein-coding sequences. Furthermore, antiSMASH analysis of the whole genome revealed three putative biosynthetic gene clusters that are responsible for the production of various secondary metabolites. Our study found that the *Viridibacillus* sp. JNUCC6 extract inhibited the α -MSH-induced melanin production and tyrosinase activity in B16F10 melanoma cells. In addition, it decreased the LPS-induced nitric oxide (NO) production caused by LPS stimulation in a concentration-dependent manner. Therefore, *Viridibacillus* sp. JNUCC6 has potential applications as an ingredient in skin-whitening and anti-inflammatory products and can be used in the cosmetic and medical industries.



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1. Introduction

Natural ingredients are increasingly being used in conventional cosmetics and personal care products. Companies are replacing synthetic chemicals with natural ingredients because of consumer demand and to move toward the use of sustainable materials [1]. Many biological components of plants and various other organisms are the main sources of natural ingredients [2]. Natural cosmetics have long used extracts from plants and animals as basic ingredients. However, microorganisms, such as bacterial cells, are now considered to be potential sustainable sources of functional ingredients or additives to improve the quality of these products. Moreover, these products are commercially easy to produce. Although various microorganisms exist in nature, few are commercially used in the cosmetics industry. Thus, their abundant but underutilized biodiversity represents a potential opportunity for their future biotechnological and cosmetic applications [3,4]. Chemical studies on biologically active compounds isolated from microorganisms have accelerated tremendously in recent years. The growing demand for biomolecules is primarily due to their potential efficacy in cosmetics, pharmaceuticals, fine chemicals, and functional personal care products [5,6]. Microorganisms are advantageous resources because they

produce metabolites, which can be mass produced at a reasonable cost. The main benefit of microbial ingredients is their biocompatibility. Other benefits include simplified processes, improved and consistent product quality, as well as their small environmental footprint. Bacteria secrete many biologically active compounds that are of considerable commercial value. These compounds are applied to a variety of cosmetic products that are used to improve the health of beautification or targets in place of chemical ingredients [7,8].

There are many kinds of whitening functional cosmetics in the global beauty industry, and their market share is expected to expand further. The cosmetics industry has introduced several chemical additives, including hydroquinone and arbutin, to meet the growing demand for skin-whitening agents. These compounds exhibit a good whitening effect. However, some of these drugs are associated with toxic side effects. In an effort by researchers worldwide to solve this problem, whitening active substances derived from natural ingredients have been discovered that are less toxic and have fewer side effects [9,10]. In addition, an increasing number of consumers consider the safety of cosmetics to be important. Moreover, green and environmentally friendly organic products have become popular. Ultimately, research and discovery of safe and healthy skin-whitening ingredients have become a trend in modern development [11,12]. Hyperpigmentation is one of the most common skin diseases that affects both men and women of all ethnic groups owing to a number of factors, such as ultraviolet (UV) exposure and skin inflammation. Local whitening agents have been reported to be the best and least aggressive treatments for hyperpigmentation compared to those performed with instrumental approaches. Hyperpigmentation is associated with reduced melanin production, a major pigment that controls skin and hair color. Excessive production of melanin can cause severe skin cancer by accumulating melanin on the skin and forming freckles; therefore, it is necessary to suppress melanin production to prevent excessive skin pigmentation [9–12]. In addition, a good anti-inflammatory effect may exhibit a synergistic whitening effect by reducing the damage caused by oxidation and inflammation of the skin [13–15].

The *Viridibacillus* genus belongs to the family *Caryophanaceae*, phylum Bacillales, which was first proposed to reclassify three species in the genus *Bacillus*. To date, there are three strains of this genus: *Viridibacillus arenosi* DSM 16319, *Viridibacillus arvi* DSM 16317, and *Viridibacillus neidei* DSM 15031. According to a recent study, *Viridibacillus* is not only used for the production of green silver nanoparticles (G-AgNPs), which have received much attention in the medical community for their excellent effect on multidrug-resistant microorganisms, but also for environmentally friendly, economical, reliable, and controlled production of cadmium sulfide particles [16,17]. Therefore, the genetic, physiological, and ecological information on *Viridibacillus* is insufficient so far, and its application to human health is still in the early stages.

Baengnokdam, the summit crater of Mt. Halla, is one of the representative geosites of the World Natural Heritage Site and Global Geopark on Jeju Island. It spans 3 km in circumference and 500 m in diameter and is surrounded by approximately 360 parasitic cones. At its peak, people can feel like they are floating on the clouds. The present study describes the isolation and characterization of *Viridibacillus* strains from Baengnokdam for the development of microbial ingredients to improve skin health outcomes, which is of particular interest because growing evidence points to the importance of natural products with few to limited side effects on human health [18].

Specifically, whole genome sequencing and bioinformatics tools were used to analyze the genomic DNA and protein sequences of the isolated *Viridibacillus* strains. Moreover, their screening for anti-melanogenic and anti-inflammatory activities was performed in mouse melanoma B16F10 cells and macrophage RAW 264.7 cells.

2. Materials and Methods

2.1. Chemicals and Reagents

Luria-Bertani (LB) and *lactobacilli* MRS media were purchased from BD Difco (Becton, Dickinson and Company, Sparks, OR, USA). Lipopolysaccharide (LPS) from *Escherichia coli*,

Griess reagent, α -melanocyte stimulating hormone (α -MSH), sodium hydroxide (NaOH), arbutin, and L-DOPA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Biosesang (Seongnam, Gyeonggi-do, Korea). Dulbecco's Modified Eagle's Medium (DMEM) and penicillin–streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Merck Millipore (Burlington, MA, USA). All reagents used were of analytical grade.

2.2. Bacterial Isolation

Viridibacillus sp. JNUCC6 was isolated from Baengnokdam, the summit crater of Mt. Halla and was collected in September 2019. Briefly, 0.5 g soil samples were mixed with 0.45 mL of 0.1% tris-buffer (*w/v*) and shaken on a rotary shaker at 180 rpm, 30 °C for 1 h. Then, 100 μ L of the suspension was serially diluted (10^{-5} to 10^{-9}) and spread onto MRS medium containing proteose peptone No. 3 (10 g/L), beef extract (10 g/L), yeast extract (5 g/L), dextrose (20 g/L), polysorbate 80 (1 g/L), ammonium citrate (2 g/L), sodium acetate (5 g/L), magnesium sulfate (0.1 g/L), manganese sulfate (0.05 g/L), dipotassium phosphate (2 g/L), and bacteriological agar 15.0 g/L, at pH 6.5. For routine work, JNUCC6 was cultured aerobically on LB solid medium and LB liquid broth for 1 d at 30 °C. Further, it was maintained in 20% (*v/v*) glycerol suspension at -80 °C.

2.3. Genome Sequence Analysis

The *Viridibacillus* sp. JNUCC6 was inoculated into 100 mL of LB broth in a 250 mL triangular flask and incubated in a rotary shaker at 28 °C and 150 rpm for 24 h. The JNUCC6 cells were centrifuged and harvested at $7000\times g$ at 4 °C for 15 min, and their genomic DNA was extracted using QIAGEN genomic-tip (Qiagen Inc., Shenzhen, China) according to the manufacturer's instructions. The genome was sequenced using a PacBio RSII and Illumina platform at Macrogen, Inc. (Seoul, Korea). The genome was annotated using the NCBI prokaryotic genome annotation pipeline. The genome was screened for the presence of plasmids using PlasmidFinder 2.1. Several bioactive secondary metabolites were identified using antiSMASH.

2.4. Extraction of *Viridibacillus* sp. JNUCC6 Culture Broths

The *Viridibacillus* sp. JNUCC6 was cultured in LB medium under aerobic conditions at 30 °C for 4 d. The culture medium was filtered through a 300 mm filter paper (ADVANTEC, Japan) and partitioned with an equal volume of ethyl acetate (EtOAc, DAEJUNG Chemicals and Metals, Siheung, Korea), and this partition was repeated three times. Further, only the supernatant was collected, concentrated under reduced pressure using a rotary evaporator (Heidolph Laborota 4000, Heidolph Instruments GmbH & Co., Schwabach, Germany), and freeze-dried.

2.5. Cell Culture

B16F10 mouse melanoma cells were purchased from ATCC (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS at 37 °C in a humidified incubator (NB-203XL, N-BIOTEK, Inc., Bucheon, Korea) with 5% CO₂. These cells were sub-cultured every 4 d. RAW 264.7 murine macrophage cells were purchased from the Korean cell line bank (Seoul, Korea). These cells were sub-cultured every 2 d.

2.6. Measurement of Cell Viability

Cell viability was measured using the MTT assay. B16F10 melanoma (0.8×10^5 cells/well) and RAW 264.7 (1.5×10^5 cells/well) cells were seeded and pre-incubated for 24 h. Next, the cells were treated with various concentrations of JNUCC6 extracts (2.3 to 37.5 μ g/mL) for 72 h. Then, the cells were treated with the MTT reagent for 4 h, after which the formazan

crystals were dissolved in DMSO. The absorbance of each well was measured at 540 nm using a microplate reader (Epoch, Biotech Instruments, Vermont, IL, USA).

2.7. Measurement of Intracellular Melanin Content

B16F10 melanoma cells (0.8×10^5 cells/well) were seeded in a 6-well plate and incubated for 24 h. After 72 h, the samples (1.2 to 4.7 $\mu\text{g}/\text{mL}$) were treated with 200 nM of α -MSH. α -MSH was used as a positive control. After removing the supernatant, the cells were washed twice with PBS, and the cell pellets were collected and dissolved in 1 M NaOH at 70 °C for 1 h. Each cell lysate was transferred to a 96-well plate, and the absorbance of each well was determined at 540 nm using a spectrophotometer (Epoch, Biotech Instruments, Vermont, IL, USA).

2.8. Measurement of Tyrosinase Activity

B16F10 melanoma cells were inoculated into a 60 mm plate with 0.8×10^5 cells/plate and pre-cultured for 24 h. Then, the B16F10 cells with treated JNUCC6 extracts (1.2 to 4.7 $\mu\text{g}/\text{mL}$) and α -MSH (200 nM) were incubated for 72 h at 37 °C and 5% CO₂ under humidification conditions. After removing the medium, the cells were washed twice with PBS and the cell pellets were collected and dissolved in a RIPA buffer containing 1% protease inhibitor cocktail. After centrifugation at 13,000 rpm for 15 min, the supernatant was collected, and the protein level of each solution was quantified using a bicinchoninic acid (BCA) kit. Next, 20 μL of each adjusted protein sample was mixed with 80 μL of L-DOPA (2 mg/mL) in a 96-well plate. After incubation at 37 °C for 2 h, absorbance was measured at 490 nm using a microplate reader (Epoch, Biotech Instruments, Vermont, IL, USA).

2.9. Measurement of Nitric Oxide (NO) Production

RAW 264.7 cells were seeded at 3×10^4 cells/well in a 96-well microplate and incubated at 37 °C in a 5% CO₂ incubator for 24 h. The cells were then treated with the culture medium, LPS (1 $\mu\text{g}/\text{mL}$), and serial concentrations of JNUCC6 extracts for another 24 h. Subsequently, the nitrite accumulated in the culture supernatant was determined using the Griess reagent comprising solution A (1% sulfanilamide in 5% phosphoric acid) and equal volume of solution B (0.1% N-1-naphthylethylenediamine dihydrochloride in distilled water). Briefly, 100 μL of the culture supernatant was mixed with the same volume of Griess reagent and incubated for 10 min at room temperature. The absorbance was measured at 540 nm and NO production was calculated using a sodium nitrite standard curve.

2.10. Statistical Analysis

All data are expressed as mean \pm standard deviation (SD; $n = 4$). Each experiment was repeated at least four times. Statistical analyses were performed using one-way analysis of variance (ANOVA) using SPSS software (v. 22.0, SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.001$ (###) were considered statistically significant.

3. Results and Discussion

3.1. Data Description of Draft Genome Sequence

In this study, *Viridibacillus* strains were isolated from the summit crater of Mt. Halla (Baengnokdam) and characterized using 16S rRNA gene sequencing. The *Viridibacillus* sp. JNUCC6 showed the highest 16S RNA gene sequence similarity to *Viridibacillus aroi* DSM 16317(T) (99.79%) and *Viridibacillus arenosi* LMG 22166(T) (99.51%), followed by *Rummeliibacillus pycnus* NBRC 101231(T) (96.69%), *Viridibacillus neidei* NRRL BD-87(T) (96.61%), *Rummeliibacillus stabekisii* KSC-SF6g(T) (96.33%), *Kurthia sibirica* DSM 4747(T) (96.13%), *Kurthia gibsonii* NCIMB 9758 (95.88%), and *Rummeliibacillus suwonensis* G20(T) (95.88%). Whole genome sequencing analysis and genome annotation were performed, and the assembled sequence was submitted to GenBank with the accession ID CP063302. The

complete genome contained a single contig of 4,526,142 bp with a GC content of 35.61%. The genome of *Viridibacillus* sp. JNUCC6 contains 4364 protein-coding sequences, 30 rRNA genes, and 75 tRNA genes. The WebMGA tool classified the predicted protein-encoding genes into 23 functional categories, where 70.44% of the predicted coding sequences (CDSs) were assigned to the cluster of orthologous groups (COG), while 29.56% were not annotated with a known function (Table 1). Plasmids were not detected in the isolated genomes. The genome contains genes responsible for the production of three bioactive secondary metabolites [19]. The organism codes for nonribosomal peptides, polyketide–nonribosomal peptide hybrids, and terpenes. Details of the secondary metabolites are provided in Table 2.

Table 1. Number of genes associated with general Cluster of Orthologous Groups functional categories.

Class	Number	Percentage	Description
J	180	4.32	Translation, ribosomal structure, and biogenesis
A	0	0	RNA processing and modification
K	316	7.59	Transcription
L	164	3.94	Replication, recombination, and repair
B	1	0.02	Chromatin structure and dynamics
D	34	0.82	Cell cycle control, cell division, chromosome partitioning
Y	0	0	Nuclear structure
V	92	2.21	Defense mechanisms
T	169	4.06	Signal transduction mechanisms
M	148	3.55	Cell wall/membrane/envelope biogenesis
N	43	1.03	Cell motility
Z	0	0	Cytoskeleton
W	0	0	Extracellular structures
U	32	0.77	Intracellular trafficking, secretion, and vesicular transport
O	100	2.40	Posttranslational modification, protein turnover, chaperones
C	147	3.53	Energy production and conversion
G	200	4.80	Carbohydrate transport and metabolism
E	371	8.91	Amino acid transport and metabolism
F	97	2.33	Nucleotide transport and metabolism
H	96	2.30	Coenzyme transport and metabolism
I	112	2.69	Lipid transport and metabolism
P	248	5.95	Inorganic ion transport and metabolism
Q	52	1.25	Secondary metabolites biosynthesis, transport, and catabolism
R	332	7.97	General function prediction only
S	1231	29.56	Function unknown
Total	4165	100	

Table 2. List of secondary metabolites in *Viridibacillus* sp. JNUCC6.

Type	From	To	Most Similar Known Cluster	Similarity (%)
NRPS	291,753	338,928	Bacillibactin	46%
NRP-PKS	2,036,725	2,077,792	Iturin	22%
Terpene	4,399,568	4,420,389	-	-

3.2. JNUCC6 Extract Inhibits Melanin Production and Tyrosinase Activity in B16F10 Cells

Melanin is the main pigment in the human skin and plays an important role in protecting the skin from UV rays. Alterations in melanogenesis can cause hyperpigmentation with aesthetic and health consequences. Therefore, suppressors of melanogenesis are considered useful tools for medical and cosmetic treatments [20]. Tyrosinase is a rate-limiting enzyme in melanin synthesis and has been widely investigated as a regulator of melanogenesis. It is a multifunctional copper-containing enzyme that is widely distributed in nature and is

responsible for melanin production and browning in plants and animals. This enzyme catalyzes two melanin formation reactions: the hydroxylation of tyrosine by monophenolase and the oxidation of L-DOPA to o-dopaquinone by diphenolase. These reactive o-quinones undergo nonenzymatic polymerization to form melanin. Accordingly, it has been suggested that melanin production is primarily controlled by the expression and activation of tyrosinase. Therefore, the main strategy is to target tyrosinase, with increasing interest in the use of natural products as tyrosinase inhibitors [11,12]. The established murine B16 melanoma cell (B16F10 cells) line offers a model system with readily quantifiable markers characteristic of differentiation, including melanogenesis [21]. Melanogenesis of B16F10 melanoma cells can be induced by using UV and α -MSH; moreover, the proliferation of these cells is an important factor. Therefore, the viability of B16F10 melanoma cells was monitored along with the melanogenesis levels of these cells at low cytotoxicity concentrations when treated with JNUCC6 extracts. To determine whether JNUCC6 extracts have any cytotoxic effects on B16F10 melanoma cells, we first examined the viability of these cells after treatment with variable concentrations of JNUCC6 extracts (1.3–4.7 $\mu\text{g}/\text{mL}$) for 24, 48, and 72 h using MTT assay. We found that there was no significant difference in viability between the control and JNUCC6 extract-treated cells, except for the treatment with concentrations of JNUCC6 extracts above 4.7 $\mu\text{g}/\text{mL}$ for 72 h (Figure 1a). These results indicated that the JNUCC6 extract used in this study was not cytotoxic to B16F10 melanoma cells up to 4.7 $\mu\text{g}/\text{mL}$ at least. To determine the effect of JNUCC6 extracts on melanin production, we treated the B16F10 melanoma cells with different concentrations of these extracts (1.3–4.7 $\mu\text{g}/\text{mL}$) for 72 h. α -MSH was used as the positive control. The treatment with JNUCC6 extracts resulted in a dose-dependent decrease in melanin levels (Figure 1b), suggesting that it may be used in the treatment of hyperpigmentation disorders. The effects of JNUCC6 on tyrosinase activity in B16F10 cells are shown in Figure 1c. Cellular tyrosinase activity was significantly decreased by JNUCC6 treatment, compared with the untreated control group. Compared with the untreated control, JNUCC6 decreased tyrosinase activity to 26.2% at 4.7 $\mu\text{g}/\text{mL}$. These results are consistent with the effects of JNUCC6 on melanin content in B16F10 cells. Taken together, these results suggest *Viridibacillus* sp. JNUCC6 has potential applications as an ingredient in skin-whitening products and can be used in the cosmetic industry. However, further research is needed to assess its safety and efficacy.

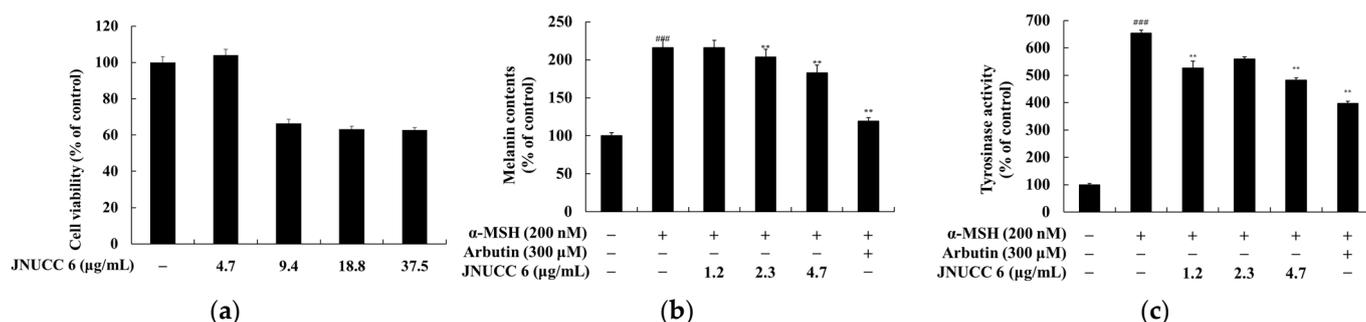


Figure 1. Inhibition of melanin production and tyrosinase activity by JNUCC6 extracts in B16F10 melanoma cells. (a) Cell viability was assessed using the MTT assay and represented compared to the non-treated group as percentage values. To assess the melanin content (b) and tyrosinase activity (c), the cells (5×10^4 cells/well) were seeded for 24 h, and then in the presence of α -melanocyte-stimulating hormone (α -MSH), they were treated with varying concentrations of JNUCC6 extracts (1.2, 2.3, and 4.7 $\mu\text{g}/\text{mL}$) for 72 h. α -MSH (200 nM) and arbutin (300 μM) were used as a positive control and negative control, respectively. For tyrosinase inhibition assay, 3,4-dihydroxyphenylalanine (L-DOPA) was added as a substrate to cell lysates for performing the tyrosinase assay. The results are expressed as mean \pm standard deviation (SD) of data obtained from four independent experiments. ^{###} $p < 0.001$ compared with control group; ^{**} $p < 0.01$ compared with α -MSH-treated group.

3.3. JNUCC6 Extract Inhibits NO Production in LPS-Stimulated RAW 264.7 Macrophages Cells

LPS is a major component of the outer membrane of gram-negative bacteria and serves as a potent initiator of inflammation. LPS activates monocytes and macrophages to produce proinflammatory mediators, such as NO and cytokines. Therefore, in this study, LPS-stimulated RAW 264.7 macrophages were used as an in vitro inflammation experimental model. NO is a reactive free radical that plays an important role in the regulation of inflammatory responses and is released in high amounts during inflammation. Overproduction of NO can cause numerous inflammatory diseases, such as cardiovascular disease, hypotension, vasodilation, apoptosis induction, as well as joint, visceral, and lung-related inflammation [22]. Therefore, the level of NO can be a useful indicator for monitoring chronic inflammation and evaluating the effectiveness of anti-inflammatory treatments in reducing chronic inflammation [23,24]. To determine whether JNUCC6 extracts have cytotoxic effects on LPS-stimulated RAW 264.7 macrophages, the cytotoxic effects of various concentrations of JNUCC6 extracts were determined using the MTT assay. JNUCC6 extract concentrations that exhibited more than 80% cell viability were chosen for further anti-inflammatory analysis, including 2.3, 4.7, and 9.4 $\mu\text{g}/\text{mL}$ (Figure 2a). The anti-inflammatory effects of the JNUCC6 extracts were evaluated by assessing the inhibition of NO production in LPS-induced RAW 264.7 macrophages using the Griess reagent. The results showed that the treatments with JNUCC6 extracts were able to suppress the production of NO in LPS-stimulated RAW 264.7 macrophages in a concentration-dependent manner (Figure 2b). These results indicate that JNUCC6 extract inhibits LPS-induced inflammatory responses in macrophages, supporting the use of the extract as a therapeutic anti-inflammatory agent.

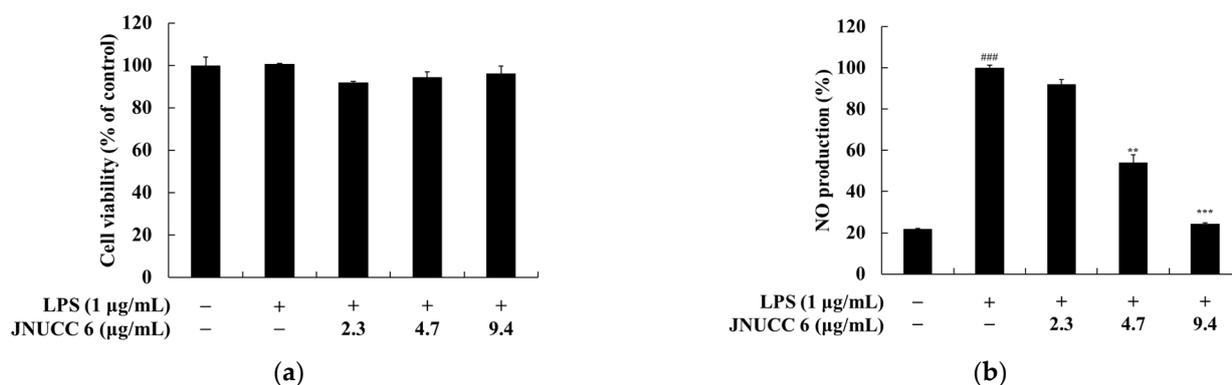


Figure 2. Inhibition of nitric oxide (NO) production by JNUCC6 extracts in RAW 264.7 cells. (a) Cell viability of JNUCC6/treated RAW 264.7 cells determined by MTT assay and represented compared to the non-treated group as percentage values. The cells (1.5×10^5 cells/well) were seeded for 24 h, and then in the presence of lipopolysaccharide (LPS), they were treated with varying concentrations of JNUCC6 extracts (2.3, 4.7, and 9.4 $\mu\text{g}/\text{mL}$) for 24 h. (b) The supernatant of the culture medium was collected and analyzed for NO using Griess reagent. The results are expressed as mean \pm SD of data obtained from three independent experiments. ### $p < 0.001$ compared with control group; *** $p < 0.001$ and ** $p < 0.01$ compared with LPS-treated group.

4. Conclusions

There are many requirements for cosmetics, but most importantly, they should be safe products that have no side effects and have a positive effect on the skin. Recently, microbe-derived ingredients have attracted considerable attention in the cosmetic industry. In the present study, we isolated *Viridibacillus* sp. JNUCC6 from Baengnokdam, the summit crater of Mt. Halla. The genome of this strain was sequenced using the PacBio and Illumina platforms. To further determine whether the microbe-derived ingredients make a good candidate for exploring their cosmeceutical potential, the anti-melanogenic and anti-inflammatory effects of JNUCC6 were analyzed in the present study. JNUCC6 extracts

significantly inhibited NO generation and improved cell viability in LPS-stimulated RAW 264.7 macrophages. In addition, these extracts also inhibited melanin synthesis in α -MSH-stimulated B16F10 melanoma cells by downregulating the intracellular levels of tyrosinase. These results demonstrate that JNUCC6 extracts possess strong in vitro anti-melanogenic and anti-inflammatory effects and can be used in the pharmaceutical and cosmeceutical industries. In the future, further research is needed to assess their safety and efficacy, such as a human patch test and isolation of natural products.

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Conflicts of Interest: The authors declare no conflict of interest.

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