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Short communication

Isolation and characterization of marine-derived *Mucor* sp. for the fermentative production of tyrosol

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ABSTRACT

An investigation of marine red algae surface-associated fungal communities led to the isolation of a previously undescribed *Mucor* strain. Many characteristic features of the genus *Mucor*, including sporangiophores, sporangium, sporangiospores and columellae, were apparent in scanning electron microscope (SEM) images of the isolated strain. The sequence of the internal transcribed spacer (ITS) rDNA revealed that the strain exhibits 97% homology to the genus *Mucor*. In a search for active compounds isolated by an ethyl acetate extract of the strain, tyrosol (2-(4-hydroxyphenyl) ethanol) was identified as a major secondary metabolite present in the culture broth. Remarkably, the tyrosol production level was considerably higher than that of reported tyrosol producing microorganisms. The optimal conditions for the fermentative production of tyrosol by the new strain were identified, including culture media, incubation period, temperature and pH. These findings clearly demonstrate that this novel strain has the potential for development as a natural source of tyrosol for industrial purposes.

1. Introduction

Tyrosol is a monophenolic antioxidant that is present in a variety of natural sources. It has recently gained considerable attention as a fine chemical in the chemical industry and as a bioactive compound in the pharmaceutical industry. The compound itself and its derivatives are industrially important intermediates that can be used for synthesizing several commercially viable compounds, such as betaxolol and metaprolol, which are used to treat several chronic diseases [1,2]. Despite its weak *in vitro* antioxidant power, many recent findings have demonstrated that tyrosol exhibits potent bioactivities in living systems due to its high bioavailability [3,4]. Recently, we discovered that tyrosol exerts neuroprotective activity against MPP^+-induced mitochondrial dysfunction in Cath.a cells, and has great potential for use as a nutraceutical and functional food ingredient [5]. In addition, it is well-known that tyrosol plays an important role in the taste of alcoholic beverages, particularly in sake, beer and wine [6]. Some reports have shown that tyrosol can be used directly as an additive in alcoholic beverages to improve the characteristic sharpness. This is easier than the alternative of increasing the tyrosol content in the product by using tyrosol-producing brewery yeast because many tyrosol producing strains are sensitive to ethanol concentration [7].

Despite its natural abundance, tyrosol for industrial purposes is often produced chemically because of the prohibitive cost of extraction from natural sources. It is known that olive oil, argan oil, wine and sake are the most common natural sources of tyrosol; the content in virgin olive oil ranges from 40 to 180 mg/kg oil. However, extracting tyrosol from olive oil is challenging because tyrosol localizes to the phenolic fraction of the oils as a complex mixture [8–11]. Several attempts have been made to extract tyrosol from natural sources for industrial purposes. The production of tyrosol using microorganisms has been studied, particularly using *Saccharomyces cerevisiae*, because of its ability to convert tyrosine into an extractable form of tyrosol [12]. It is known that fungi produce tyrosol to control the morphological transition from yeasts to hyphae. Alem and colleagues [13] revealed that there is a correlation between tyrosol production and biomass in *Candida albicans* and that biofilm cells secrete at least 50% more tyrosol than planktonic cells. Another study showed that the phytopathogen fungus *Ceratocystis adipose* produces tyrosol as a major secondary metabolite [14]. Applications of metabolic engineering for the production of tyrosol using microorganisms have also been considered, including reports indicate that *Escherichia coli* may be engineered to produce tyrosol from glucose. In addition to microorganisms, plant cell (*Rhodiola crenulata*) suspensions have also been researched as a source of industrial tyrosol. In this paper,

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we present details regarding the isolation and characterization of a novel marine *Mucor* strain and its potential as a natural source for industrial tyrosol production.

2. Materials and methods

2.1. Materials

Extraction of the bioactive compound from the fungal strain was performed using an extraction unit (Dongwon Scientific Co., Seoul, Korea). Silica gel 60 (230–400 mesh, Merck KGaA, Darmstadt, Germany), sephadex LH-20 (Sigma, St. Louis, MO, USA), YMC gel ODS (Octadecyl-Bonded Silica)-A 12 nm S-150 μm (YMC Co. Ltd., Kyoto, Japan) and thin layer chromatography (TLC) plates (Kieselgel 60 F254, 0.25 mm, Merck KGaA, Darmstadt, Germany) were used for column chromatography and analytical TLC, respectively. The culture media: Yeast extract (Lab M Limited, Lancashire, UK), peptone (Lab M Limited, Lancashire, UK), D-(+)-glucose (Yakuri, Sendai, Japan), agar powder (Lab M Limited, Lancashire, UK), glycerol (Sigma, 99%, St. Louis, MO, USA) and penicillin G (Sigma, St. Louis, MO, USA) were of analytical grade. Organic solvents: n-hexane, ethyl acetate (EtOAc), CH_2C_{12}, and methanol (MeOH) (Duksan Pure Chemical, Ansan-si, Korea, 99.5%) were used for the extraction. Coloring reagent used for the visualization of TLC was $Ce(SO_4)_2$ (Sigma, St. Louis, MO, USA). 1H NMR (Nuclear Magnetic Resonance) (400 MHz) and 13C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Tokyo, Japan), using the CD_3OD (3.34 ppm in 1H and 49.86 ppm in 13C NMR) solvent peak as an internal reference standard. Mass spectra were recorded on a JEOLJMS-700 spectrometer (JEOL). BV-2 cells were a kind gift from professor Il-Whan Choi, Inje University, Korea. Lacto phenol cotton blue, glutaraldehyde, osmium tetroxide, 2′,7′-dichlorofluorescin diacetate (DCF-DA), MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], lipopolysaccharides (LPS), and Griess reagent were purchased from Sigma–Aldrich Inc. (Sigma, 99%, St. Louis, MO, USA). Other materials and chemicals used were all of analytical grade as commercially available.

2.2. Isolation, morphological examination and gene sequencing

The fungal strain was isolated and purified from the surface of a marine red alga collected in Guryongpo, Nam-gu, PoHang, Korea (2009), and was grown on agar medium containing 0.5% yeast extract, 0.5% peptone, 1% glucose, and 60% seawater (YPG) at 27 °C for 3 days. For examination under a confocal microscope, a line of lacto phenol cotton blue (LPCB) was dispensed on a slide. A piece of adhesive tape was used to pick up a sample of the fungal strain and was placed on the LPCB line for viewing with a confocal microscope (Leica, Wasteland, Germany). For initial identification, the fungus rDNA; 18S rRNA gene (partial), internal transcribed spacer (ITS) 1, 5.8S rRNA gene and ITS2 were sequenced. For sequence analysis, DNA was extracted from 3-day-old fungus as described previously [15]. Cells were washed with distilled water and digested with an enzymatic lysis solution. The lysate was extracted with a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) three times and purified by adding 1/10 volume 3 M sodium acetate and 1 ml ethanol. The mixture was vortexed and the DNA pellet was obtained. The 18S rDNA sequences and the internal transcribed spacer (ITS) region including the 5.8S rDNA were amplified by PCR with primer pairs 5′-GGATCAGAA-TTCTATTCTGGTTGATCCTGCCAG-3′ and 5′-CTCAGTAAGCTTGATCCTTCCGCAGGTTCA-CC-3′, and 5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′, respectively. PCR reactions were carried out with one cycle of heat treatment at 94 °C for 10 min, a total of 30 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 30 s, extension at 72 °C for 1.5 min, and followed by a final extension at 72 °C for 7 min. Sequencing of the PCR products was performed by Macrogen Korea (Seoul). The nucleotide sequence was aligned with the basic local alignment search tool BLAST® of the National Center for Biotechnology (NCBI).

2.3. Scanning electron microscope (SEM) imaging

The vapor fixation procedure described elsewhere [16] was used to examine the fungal strain by SEM. A 3-day-old culture plate was placed upside down in a well-ventilated fume hood. The petri dish lid was filled with 2% (v/v) glutaraldehyde and incubated for 2 h at room temperature. Next, the dish was exposed to a vapor of 2% (v/v) osmium tetroxide for 2 h. Squares of osmicated black agar blocks (each 5 mm × 5 mm with approximately 1 mm thickness of underlying tissues) were then excised using a razor blade and mounted on a metal stub. They were sputter-coated with gold (approximately 30 nm thick) and examined with an SEM (JSM-5410LV; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV.

2.4. Extraction and structure elucidation of active compounds

Square pieces (1 cm) of the pure fungus cultured on an agar plate containing YPG medium were transferred into YPG culture broth (1 l) for large-scale fermentation and incubated for 30 days (20 l, 25 °C, pH 7.5, 100 rev min^{-1}). To isolate biologically active compounds from the strain, 30-day-old culture broth (20 l) was extracted using analytical grade ethyl acetate (EtOAc) in 1:3 ratio of extract to solvent. The EtOAc extract was then fractionated by column chromatography and eluted with an n-hexane to methanol gradient (starting from 100% hexane and gradually changed to 100% methanol). Thin layer chromatography (TLC) was performed during the purification to rank the fractions. Selected fractions were tested for their ability to inhibit NO and ROS production. The bioactivity-guided fractions were further purified using a series of column chromatography. Pure compounds were obtained from the active fractions, which were further purified with ODS column chromatography followed by high performance liquid chromatography (HPLC). An HPLC System equipped with a C18 column (YMC Co. Ltd., Japan) was used at a 1 ml/min flow rate and the column temperature was kept at 25 °C. The mobile phase was a mixture of methanol and water (60:30). To analyze the structure of the isolated compound, 1H, 13C and 2D NMR of the compound were recorded on a JEOL JNM ECP 400 NMR spectrometer (JEOL, Japan). Mass spectra were obtained from a JEOL JMS-700 spectrometer (JEOL, Japan).

2.5. Bioactivity studies

BV-2 cells were maintained in Dulbecco's modified Eagle's minimal essential (DMEM) medium supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin; the medium was exchanged twice a week. Cells were incubated at 37 °C with 95% humidity and 5% CO_2 and used for experiments when cells reach the desired confluence. The cytotoxicity of the test fractions against cultured cells was measured using an MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) assay. Initially, cells were plated on 96-well plates at a density of 5×10^3 cells/well and incubated overnight. Then cells were washed with fresh medium and incubated in fresh medium containing different concentrations of the fractions dissolved in 10% DMSO. The solvent of the fractions (DMSO) was used as the negative control (blank). The final concentration of DMSO in culture medium was less than 0.1% (v/v). After incubation, the culture medium was aspirated off and 100 μl of MTT solution (1 mg/ml) were added into each