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Antitumor Effects of *Kluyveromyces marxianus* TFM-7 Isolated from Kefir

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Abstract The strain TFM-7, which has an antitumor effect, was isolated from Kefir and identified based on analysis using the API 50 CHL kit and 26S rDNA sequencing. Strain TFM-7 was confirmed to belong to the genus *Kluyveromyces*. Analysis of the 26S rDNA nucleotide sequences found strain TFM-7 to be related to *Kluyveromyces marxianus* NRRL Y-8281T. *K. marxianus* TFM-7 was cultured with potato dextrose broth medium at 27°C for 72 hr, and its inhibition effects on the proliferation of seven tumor cell lines and a normal cell line were assessed using the MTT assay. The antitumor effects and growth characteristics of *K. marxianus* TFM-7 were investigated during a culture period of 7 days. By the 3rd day, *K. marxianus* TFM-7 showed a dry cell weight of 2.39 g/L, a pH of 4.39, an ethanol content of 0.89%, and an inhibition effect on the proliferation of seven tumor cell lines above 50%, except for A-549 tumor cell line. *K. marxianus* TFM-7 was the most effective at inhibiting the growth of Hep-2 cell line among all tumor cell lines tested. Growth inhibition of a normal cell line, NIH/3T3, was less than 35%, suggesting a decreased level of cytotoxicity toward normal cells. These results indicate that *K. marxianus* TFM-7 may have used as a yeast strain with antitumor activity.

Keywords: Kefir, 26S rDNA, *Kluyveromyces marxianus*, MTT assay, antitumor effect

Introduction

Modern medicine has made the treatment and prevention of many diseases possible. Nonetheless, no definite cure has yet been found for cancer, the leading cause of death in Koreans, while cancer patients are increasing each year. As for the causes of cancer, reports have indicated that exposure to chemicals accounts for more than 90% of cancer cases (1), with 40-60% of those related to diet (2). Studies on this subject are being actively conducted not only to uncover the causal agents related to diet, but also to find new anticancer agents in edible plants (3). Furthermore, various studies using natural substances are aiming to develop anticancer agents with a new concept that could overcome the complications and toxicity of previous anti cancer agents (4).

Kefir is a fermented milk beverage originally made in the Balkans, Eastern Europe, and the Caucasus. It is traditionally produced by inoculating milk with grains of Kefir. The starter culture used to produce this beverage is an irregularly shaped, gelatinous white/yellow grain (5). When suspended in milk, the grains swell to form a gelatinous-like product, kefiran, which contains lactic acid bacteria (LAB), yeast, and acetic acid bacteria (6). The majority of bacteria (as much as 80%) belong to the genus *Lactobacillus* (7). Kefir is natural probiotic containing live bacteria, yeast, and the products that these microorganisms produce. It is not clear whether the health-promoting properties of Kefir are due to one particular bacteria or yeast (8). Recently, Kefir has been consumed as a healthy soft drink known for its functional characteristics such as antimicrobial activity, digestion support, and immunological and antitumor activities (9-13).

The objectives of this study were to identify and characterize strain TFM-7, which provides the most potent antitumor effects of Kefir. This was done by analyzing carbon source utilization and 26S rDNA sequencing. The inhibition of the proliferation of various tumor and a normal cell lines and the growth characteristics of strain TFM-7 during a 7 day culture period were also investigated.

Materials and Methods

Grain activation and traditional Kefir production Kefir was produced by adding 20 g of grains to 500 mL of pasteurized market milk at 25°C. The grains were sieved and transferred to 500 mL fresh pasteurized market milk in every 24 hr. This procedure was repeated until the grains were used to isolate the microbial population at specified time intervals.

Cultivation of tumor cells and a normal cell lines A-549 (human lung carcinoma), Farrow (human melanoma carcinoma), HEC-1-B (human uterus carcinoma), Hep-2 (human larynx carcinoma), SK-OV-3 (human ovary carcinoma), SNU-5 (human stomach carcinoma), SW-156 (human kidney carcinoma), and NIH/3T3 (mouse embryo) obtained from the Korea Cell Line Banks (KCLB; College of Medicine, Seoul National University, Seoul, Korea) were stored in a liquid nitrogen tank at -196°C.

A-549, Farrow, SK-OV-3, SNU-5, SW-156, and NIH/3T3 cells were cultured at 37°C in RPMI 10, which is RPMI 1640 medium (Gibco BRL, MD, USA) with 10% fetal bovine serum (FBS, Gibco BRL) and 1% penicillin-streptomycin (Gibco BRL) in a 5% CO_2 atmosphere. HEC-1-B and Hep-2 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) 10, which is DMEM supplemented with 10% FBS and 1%

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penicillin-streptomycin in a 5% CO_2 incubator (MCO-18 AIC; Sanyo, Tokyo, Japan). All cell lines were stored in a liquid nitrogen tank at -196°C before use. After thawing in a 37°C water bath, the cell lines were centrifuged, washed, and cultured in 25 cm^2 tissue culture flasks (Corning Co., Corning, NY, USA) at 37°C in a 5% CO_2 incubator. The cells were split and medium was exchanged 2-3 times a week. At this time, the adhesive cells were collected from the flask bottom after washing in phosphate buffered saline (PBS, pH 7.2) and treated with 0.05% trypsin-EDTA (Gibco BRL) for 3 min. These cells were used for further study by subculturing (14).

Isolation and analysis of strain TFM -7 Aerobic viable cells were counted on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) using the pour plate method (15). Ten colonies selected on the PDA plates by the pour plate method were stained using Gram's method. They were divided into LAB and yeast after Gram staining and microscopic inspection. Biochemical tests on the isolates were carried out with an API 50 CHL kit (Biomerieux, Lyon, France).

Strains TFM 1-10 isolated from Kefir in PDA were incubated in potato dextrose broth (PDB; Difco Laboratories) as the growth medium at 27°C, and stored as stock solution in 20%(v/v) glycerol at -70°C. Working cultures were propagated in PDB with shaking at 27°C. Each strain was inoculated into 70 mL of sterile PDB, and the seed culture (2%, v/v) was then transferred to a jar fermentor (3.5 L working volume, MD-300; Marubishi Co., Tokyo, Japan). After incubation at 27°C for 72 hr, cells were collected by centrifuging at 16,000×g for 2 min (Union 32R; Hanil Science Co., Seoul, Korea), washed, and resuspended in 1 mL of sterile distilled water. An aliquot of the each supernatant was tested for antitumor effects against seven tumor cell lines. Growth characteristics of strain TFM-7 during a 7 day culture period with regard to changes of pH, ethanol content, and dry cell weight (DCW) were also assessed.

MTT assay For the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, 100 μL of cell suspension adjusted to 2×10^4 cells/mL was aliquotted into a 96 well plate. After these aliquots were incubated at 37 °C in a 5% CO_2 incubator for 24 hr to allow the cells to adhere, 100 μL of supernatant from strains TFM 1-10 were added. These mixtures were cultured for 48 hr at 37°C in a 5% CO_2 incubator. Then, 10 μL of MTT (Sigma Chemical Co., St. Louis, MO, USA) at 5 mg/mL in PBS was placed into each well and left at 37°C in a 5% CO_2 incubator for 4 hr. The dark blue formazan crystals formed in intact cells were solubilized with 100 μL of dimethyl sulfoxide (DMSO; Sigma Chemical Co.) and absorbance at 540 nm was measured using a spectrophotometer (Microplate autoreader; Bio-Tek Instrument, Winooski, VT, USA) (16).

Genotypic identification of strain TFM-7 Cell morphology, Gram-staining, API 50 CHL medium, and 26S rDNA were analyzed for the identification of strain TEM-7. The API 50 CHL kit was used to study the carbohydrate use of strain TEM-7. Strain TFM-7 was cultured for 24 hr at 27°C in PDB medium. Cells were collected by centrifuging at 16,000×g for 2 min, washed, and resuspended in 100 μL of sterile distilled water. After rewashing, the suspension was boiled for 10 min and centrifuged at 16,000×g for 5 min. An aliquot of the supernatant was used for 26S rDNA sequencing analysis. Alignment of sequences and 26S rDNA similarity were calculated by using a Kimura 2-parameter correction. Evolutionary distance was calculated using the Jukes and Cantor model, and Neighbor-joining methods within the Program CLUSTALW which generates a phylogenetic tree on the basis of a distance matrix calculated from the sequence data (17-19).

Analysis of strain TFM-7 growth A strain TFM-7 growth was assessed by measuring the dry cell weight (DCW), and pH was measured using a pH meter (MD-15; Fisher Scientific Co., Pittsburgh, PA, USA) (20). Ethanol content was analyzed by using gas chromatography (GC5890; Hewlett Packard, Palo Alto, CA, USA). GC was carried out using an HP-FFAP column (25 m × 0.20 mm, thickness 0.25 μm; Hewlett Packard). The oven temperature was 115°C. The injector and detector were set at 200 and 210°C. Helium was used as the carrier gas and the column flow rate was 1.1 mL/min.

Statistical analysis Data were expressed as means ± standard deviation (SD). The statistical significance of differences between groups was determined by applying Student's *t*-test. Values of $p<0.05$ were considered statistically significant.

Results and Discussion

Isolation of strains To obtain strains with antitumor effects, approximately 300 microbial strains were isolated from Kefir (data not shown). Of the strains which grew well, 20 strains were separated, 10 of which grew rapidly and formed distinct colonies on isolation media. The isolated strains were tentatively named as strains TFM 1-10.

Antitumor activity of strains TFM 1-10 MTT is a tetrazolium salt, which is reduced to formazan (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenyformazan) by living cells via the 'succinate-tetrazolium reductase' system (17). The formazan produced by the cellular suspension is directly correlated with the number of metabolically active cells, and the colorimetric the MTT assay is used to assay cell proliferation. Therefore, we conducted the MTT assay to evaluate the growth inhibition effects of strains TFM 1-10 on various human tumor cell lines and a normal cell line. Table 1 shows the results of the MTT assay, which reveal the effects of strains TFM 1-10 isolated from Kefir on the proliferation of 7 tumor cell lines and a normal cell line.

Proliferation of the SK-OV-3 cell line was inhibited more than 50% by supernatant from strains TFM 1, 2, 3, 7, 8, and 9. Proliferation of the SW-156 and Hep-2 cell lines was inhibited more than 60% by supernatant from strains TFM-3, 7, 8, and 9. Supernatant from all 10 TFM strains (TFM 1-10) inhibited proliferation of the A-549 and HEC-1-B cell lines by less than 35%. The anti-

Table 1. Growth inhibition effects of isolated strains TFM 1-10 on cell lines using the MTT assay

Cell Line	Isolated strain									
	TFM-1[1)]	TEM-2	TFM-3	TFM-4	TFM-5	TFM-6	TFM-7	TFM-8	TFM-9	TFM-10
A-549	13.7[2)]	16.3	21.9	13.5	19.2	20.1	26.1	18.4	26.1	27.9
Farrow	54.3	30.1	21.4	30.9	30.6	38.7	46.1	49.2	50.7	29.5
HEC-1-B	34.6	31.2	19.3	29.1	30.4	29.1	16.1	24.5	23.5	32.1
Hep-2	43.2	39.8	76.5	53.1	24.1	44.8	80.1	62.6	69.2	51.6
SK-OV-3	70.6	50.6	76.2	42.3	46.9	39.2	80.2	51.2	55.2	38.2
SNU-5	38.4	42.6	51.2	60.3	61.2	27.5	51.2*	34.1	26.8	31.6
SW-156	51.2	41.3	61.2	51.9	46.2	41.9	61.1	60.2	61.2	30.2
NIH/3T3	19.2	31.5	32.6	10.2	15.9	24.1	32.1	30.1	19.5	22.1

[1)]Concentration of each test sample is 10 mg/mL.
[2)]Inhibition rate (%).

proliferation effects of strain TFM-7 were found to be especially potent against all human tumor cell lines tested, with greater than 40% inhibition of all except the A-549 cell line. Strain TFM-7 was found to inhibit the proliferation of Hep-2 and SK-OV-3 tumor cell lines 80.1 and 80.2%, respectively, in a dose-dependent manner. Growth of the SW-156 cell line was suppressed by 61.1% after 48 hr of treatment with supernatant from strain TFM-7 at 10 mg/mL. Growth inhibition of less than 35% of the normal cell line NIH/3T3 shows evidence of weaker cytotoxic effects relative to the tumor cell lines, even at relatively high supernatant concentration of 10 mg/mL. Thus, the isolated strains TFM 1-10 show greater cytotoxicity toward tumor cell lines than normal cell lines based on the MTT assay.

Microbial identification of strain TFM-7 Table 2 shows the assimilation of carbohydrates by strain TFM-7. Based on the results obtained using the API 50 CHL kit, strain TFM-7 was identified as *K. marxianus*. 26S rDNA partial sequencing analyses were conducted to confirm the further identification. Figure 1 shows the phylogenetic tree based on partial 26S rDNA sequences of strain TFM-7. The phylogenetic tree shows that strain TFM-7 forms an evolutionary lineage within the cluster comprising *Kluyveromyces* species. The 26S rDNA sequence of strain TFM-7 showed the highest degree of relatedness to *K. marxianus* NRRL Y-8281T, sharing 100% 26S rDNA similarity. From the phylogenetic analysis based on 26S rDNA sequence comparison, strain TFM-7 was identified as a member of the genus *Kluyveromyces*, and most phylogenetically related to *K. marxianus*. Hence, the strain TFM-7 was named *K. marxianus* TFM-7.

Kefir grains contain microorganisms belonging to a diverse spectrum of species and genera including lactobacilli, lactococci, and leuconostoc species (21, 22). Yeasts isolated from Kefir grains include *K. marxianus, Torula kefir, Saccharomyces exiguus*, and *Candida lambica* (23-26). These results revealed the differences in microflora among the grains studied. These differences could be explained primarily by the different origins of the Kefir grains (22). In another study, members of the genera *Kluyveromyces* were frequently isolated from dairy products (27).

Table 2. Carbohydrate assimilation profiles of strain TFM-7 isolated from Kefir

Test	Strain TFM-7
Glucose	+[1)]
Glycerol	+
2-keto-D-Gluconate	+
L-Arabinose	+
D-Xylose	+
Adonitol	+
Xylitol	+
Galactose	+
Inositol	-
Sorbitol	+
α-Methyl-D-glucoside	-
N-Acetyl-D-glucosamine	-
Cellobiose	-
Lactose	+
Maltose	-
Sucrose	+
Trehalose	-
Melezitose	-
Raffinose	+

[1)]Symbols : + , positive reaction; - , negative reaction.

Growth characteristics and antitumor effects of *Kluyveromyces marxianus* TFM-7 *K. marxianus* TFM-7 was incubated in PDB medium with an initial pH of 5.1 at 27°C for 7 days. The DCW, pH, and ethanol content were measured during the 7 day incubation period. As shown in Fig. 2, the DCW was 1.79 g/L at the 1st day and showed a regular increase to 2.565 g/L until the 5th day. After the 6th day, the DCW of *K. marxianus* TFM-7 did

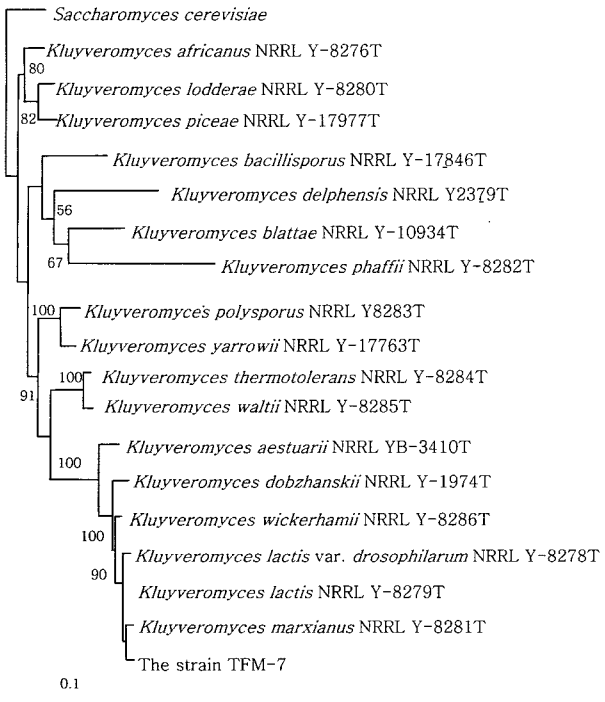

Fig. 1. Phylogenetic tree based on partial 26S rDNA sequences showing the position of strain TFM-7 and other *Kluyveromyces* sp. The phylogetic tree was constructed by using the neighbor joining method: the scale bar indicates 0.1 substitutions per nucleotide position.

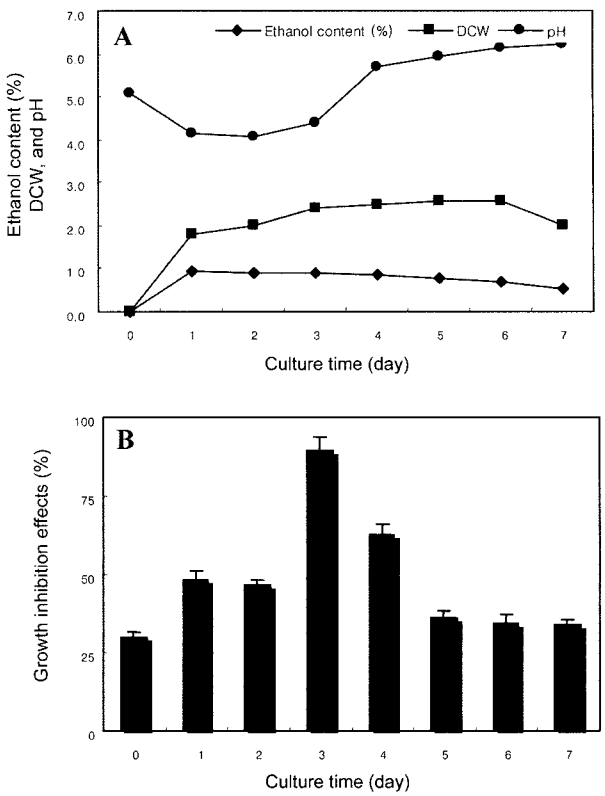

Fig. 2. The growth characteristics of *Kluyveromyces marxianus* TFM-7 (A) and growth inhibition effect on the Hep-2 cell line by *Kluyveromyces marxianus* TFM-7 (B). Concentration of test sample is 10 mg/mL. The values are mean±SD (n=3).

not increase. In another study, cell numbers increased rapidly for 48 hr after which the growth rate dropped. There were no differences in microbial growth in the various media tested such as Nutrient agar (NA, Difco Laboratories), MRS (Difco Laboratories), and PDA (28). The pH declined through the 1st day and reached pH 4.15. The pH then showed a sharp increase during the 2nd to 4th days of incubation and reached pH 6.24 by the 7th day. The pH showed a sharp decline, corresponding with accelerated cell growth until 24 hr. This pH decline was due to organic acids produced by the microorganisms in Kefir during fermentation (28). The ethanol content decreased throughout the 7 days of incubation. During the 1st day, the ethanol content of 0.93% was at its highest point and declined to 0.53% by the 7th day. The alcohologenic capacities of the yeast strains depend on the efficiency of conversion of sugar to alcohol as measured by gram weight of sugar against % alcohol in total volume (29, 30).

As shown in Table 3, *K. marxianus* TFM-7 isolated from Kefir was found to inhibit the proliferation of seven tumor cell lines and a normal cell line during a 7 day incubation period. During the first two days, cell proliferation of all seven tumor cell lines was inhibited more than 30% by 10 mg/mL *K. marxianus* TFM-7 supernatant. Also, proliferation of the Hep-2 cell line during a 7 day culture period was inhibited by more than 30% with 10 mg/mL *K. marxianus* TFM-7 supernatant. The highest degree of inhibition for every cell line was observed on the 3rd day except the A-549 cell line. The other 6 umor cell lines were suppressed by 69.9, 52.7,

Table 3. Growth inhibition effect of *Kluyveromyces marxianus* TFM-7 on cell lines using the MTT assay

Cell line	Culture time (day)						
	1[1)]	2	3	4	5	6	7
A-549	33.7[2)]	30.6	29.1	11.3	34.0	26.1	27.9
Farrow	54.3	60.2	69.9	45.8	30.6	38.7	29.5
HEC-1-B	38.1	35.6	52.7	43.2	42.4	25.6	27.3
Hep-2	48.6	46.4	89.4	62.7	36.0	34.5	34.1
SK-OV-3	50.2	49.8	78.3	41.9	48.5	53.9	11.1
SNU-5	31.5	39.6	59.2	39.2	14.3	13.9	14.1
SW-156	66.2	63.6	68.7	27.1	11.9	15.3	18.5
NIH/3T3	29.2	30.2	32.5	32.1	34.6	28.6	24.8

[1)]Concentration of sample of each day is 10 mg/mL.
[2)]Inhibition rate (%).

89.4, 78.3, 59.2, and 68.7% after 72 hr of treatment with supernatant from *K. marxianus* TFM-7 at 10 mg/mL. *K. marxianus* TFM-7 was found to inhibit proliferation of the Hep-2 tumor cell line by 89.4%, more than any other tumor cell line tested. The NIH/3T3 normal cell line showed weaker growth inhibition by *K. marxianus* TFM-7, which was less than 35%. Therefore, *K. marxianus* TFM-7 was more cytotoxic toward tumor cell lines than a

normal cell line based on the MTT assay.

K. marxianus is a homothallic yeast teleomorph of *Candida kefyr.* The presence of *K. marxianus* has been reported in diary products, fermented drinks, and in aged cheese, in which it seems to play an important role in the development of the functional and sensorial properties of the final products (31). The antitumor activity of Kefir has been shown in a few other studies (11, 13).

In conclusion, these studies demonstrate that *K. marxianus* TFM-7 isolated from Kefir will be very valuable as new therapeutic material because of its antitumor effects. The purification and characterization of antitumor substance(s) in *K. marxianus* TFM-7 is necessary to uncover the nature of this antitumor effect.

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