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Isolation and Characterization of *Penicillium crustosum*, a Patulin Producing Fungus, from Apples

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Abstract Patulin is a food mycotoxin which induces genotoxicity and acute intestinal disease in infants. Patulin mainly originates from fruit putrefactive moulds, especially in apples, which necessitates the maintenance of strong safety standards against patulin for fresh and processed apples. To investigate the patulin producing moulds in Korean apples, 16 morphological types of fungi were isolated from Korean apples and a patulin producing fungus was identified based on a sequence analysis of the region of internal transcribed spacers (ITS5-5.8S-ITS4 region, 505 base pair) and the 26 rRNA D1/D2 region (527 base pair). Morphological analyses were also performed. The isolated patulin producing fungus was found to a representative species of *Penicillium crustosum*. The maximal patulin production ability of the isolated fungus (*P. crustosum*) and the patulin producing standard strain (*P. griseofulvum*, ATCC 46037) in an SY broth medium were 0.32 and 2.46 mg/L, respectively.

Key words: patulin, apple, *Penicillium crustosum*

Introduction

Patulin [4-hydroxy-4H-furan (3,2C)-pyran-2(6H)-1] is a mycotoxin which is mainly produced from several species of *Penicillium, Aspergillus,* and *Byssochlamys* (1-4). Patulin was first isolated from *Penicillium claviforme* by Chain *et al.* (5), and it has also been called clavacin, expansine, mycoin C_3, penicidin, leucopin, and termicin (6). Patulin acts as a mutagen and it also influences the human nervous and immune systems (7, 8). Especially, it causes acute poisoning in the infant digestive system (9, 10). Although it has been detected in various grains and fruits, the most frequent incidences of patulin contamination have been reported in apple and apple juice. Patulin contamination in commercial apple juice has been reported at concentrations of 10-350 μg/L (11, 12). Therefore, the maintenance of strong safety standards against patulin is important for apples and apple juice (13, 14). The quarantine standard of patulin in apple juice is regulated at less than 50 μg/L (15, 16).

For several decades, research attention has focused on aflatoxin as a food mycotoxin (17-22). However, few evaluations of patulin and patulin producing fungi have been conducted. A representative patulin contamination accident in grain resulted in a mass poisoning of milk cows in Japan in 1954. The accident was proven to be caused by a microbial contamination of a patulin producing fungi, *Penicillium urticae,* in the feed of dried malt (23). Since then, some instances of patulin contaminations in apples, juices, jams, and fruits have been reported in Europe (24, 25). Recently, case studies of patulin contamination have been reported in Iran (26), Turkey (27), and South Africa (28).

The cultivation area and the total production of apples in Korea reaches 26,000 ha and 500,000 ton/year, respectively (29). However, few studies have investigated the patulin contamination and/or patulin producing fungi of Korean apples. In this study, therefore, we isolated and characterized a patulin producing fungi from apples as basic information for an evaluation of patulin incidence in Korean apples.

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Materials and Methods

Samples and chemicals One hundred apples (*Malus pumila* var. *dulcissima*) were randomly purchased at 10°C markets in Daejeon (4 markets), Daegu (2 markets), and Jeongup (4 markets), Korea, in November 2005. The samples were stored at 10°C for 2 months prior to isolation of patulin producing moulds. A standard patulin and reagents such as toluene, ethyl acetate, formic acid, acetonitrile, and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of fungi from apples The putrid apple sections were sliced, thoroughly mixed, and then used for preparing dilutions for fungal isolation. Each sample was homogenized for 5 min at 4°C with an equal volume of sterile saline water (NaCl 0.85%). One mL of the diluted samples was spread on a potato dextrose agar (PDA) and plates were incubated for 7 days at 30°C. After a serial subculture of a fungal colony, a single colony was isolated and cultured on a slant media of PDA and stored at 4°C.

Production of patulin from isolated fungi *Penicillium griseofulvum* (ATCC 46037), a patulin producing standard

mould, and the isolated fungi from apples were cultivated on the PDA plates for 7 days at 30°C. The conidia on the plates were washed twice with sterile Tween 80 (0.1%, v/v) solution and centrifuged (2,000×g for 10 min at 4°C) in a refrigerated centrifuge (Vs-5500; Vision Scientific, Co., Seoul, Korea). The conidia pellets were washed twice with sterile saline and suspended to a cell density of 10^7 cells/mL. Fifty mL of sterile SY liquid media (contained 4.0% of sucrose and 2.0% of yeast extract in distilled water) were prepared in a 250 mL flask (30), and 10^5 cells of conidia were inoculated. The cultures were cultivated with shaking and kept stationary in the dark for 14 days at 30 °C.

Extraction and analysis of the patulin The patulin was extracted an analyzed by AOAC method (31). After 14 days of incubation, the cultivated broth in each flask was extracted with 50 mL of ethyl acetate. Each extract was mixed with 20 g of anhydrous sodium sulfate and left at room temperature for 30 min, and then filtered using filter paper (Whatman No. 4). The filtered samples were concentrated at 40°C by a nitrogen stream, cooled at room temperature, dissolved in distilled water (pH 4.0), and then stored at -70°C.

Thin layer chromatography (TLC) was used for qualitative detection of patulin produced from the isolated fungi (31). The TLC plate (Merck silica gel 60) was activated in an oven dryer at 100-110°C for 2 hr. Each 10 μL of the standard reference (patulin 0.1 mg/L solvent) and the extract were loaded on the TLC plate. The plate was developed with toluene, ethyl acetate, and 90% formic acid mixture (5:4:1; v:v:v), and dried at 110°C. Patulin was detected as a characteristic spot in UV light with Rf 0.60, and was visualized with a spray reagent (0.5% MBTH).

High performance liquid chromatography (HPLC) was used for further confirmation and quantitative detection of patulin. A 10 μL aliquot was injected into an HPLC (Waters Associates, Milford, MA, USA) with a built-in UV detector at 276 nm. HPLC analyses were performed with a Shiseido column (3.9 mm i.d., 300 mm length) and the extracts were separated with a distilled water-acetonitrile (95:5) solvent at a flow rate of 0.7 mL/min. The column temperature was maintained constant at 40°C. The patulin produced from the isolated fungal strain was identified using the retention time, and confirmed using the patulin standard.

Identification of the isolated patulin producing mould The nucleotide sequences of the internal transcribed spacer regions (ITS5-5.8S-ITS4 region), including 5.8S rDNA, were directly determined using polymerase chain reaction (PCR) products (32). The nucleotide sequences of the D1/D2 domain of 26S rDNA were directly determined by using PCR according to the methods of Kurtzman and Robnett (33) and Boekhout *et al.* (34). The sequences of the ITS5-5.8S-ITS4 region and the D1/D2 domain of 26S rDNA determined in this study were recorded in the International Nucleotide Sequence Database (INDS) and DNA Data Bank of Japan (DDBJ). Reference sequences used for the phylogenetic study were obtained from the data base. The generated sequence of the ITS5-5.8S-ITS4 region and the D1/D2 domain of 26S rDNA were aligned with the species of the genus *Penicillium* by using the CLUSTAL W ver. 1.74 program (35). The phylogenetic tree was constructed from the evolutionary distance data of Jukes and Cantor (36) by using the neighbor-joining method (37).

A systematical identification test was performed to confirm further the genetic analysis. The isolated strain was transferred to Czapek Yeast Agar (CYA) and 2% Malt Extract Agar (MEA) (38). The plates were incubated in the dark for 10 days at 25 and 37°C. The morphological taxonomic methods of Pitt (39, 40), Robert *et al.* (41) and Hoog *et al.* (42) were used for the species identifications.

Determination of the patulin producing activity of the isolated fungi The pure isolate of the patulin producing fungi in this study and the patulin producing standard strain *P. griseofulvum* (ATCC 46037) were grown in SY, MEB, and 5-GYEP broth media, and the differences in growth rate and patulin producing activity were investigated. Portions of the medium (50 mL) were poured into a 250 mL Erlenmeyer flask and autoclaved. The flasks were inoculated with 10^5 spores/mL of fungal conidia which were then cultivated in the dark for 4 weeks at 25°C with continuous shaking (200 rpm). The fungi growth rates were determined by dry weight measurement. The total mycellia were harvested by filtration (Whatman No. 4 filter paper), washed twice with sterile distilled water, and dried at 60°C for 24 hr, after which the culture weights were measured. TLC and HPLC were routinely used for the qualitative detection of patulin, as described above.

Results and Discussion

Isolation of patulin producing fungi Several kinds of moulds were isolated from the putrid apple sections and grouped into tentative genera according to their colony characteristics and the microscopic morphology of conidiophore. Sixteen isolates were identified as tentative genera: *Penicillium* (4 species), *Aspergillus* (6 species), *Rhizopus* (2 species), and *Mucor* (4 species) (data not shown). The cultures of the isolated moulds in the SY broth media were serially extracted, and the patulin was detected by TLC. TLC is a useful and convenient detection method for aflatoxin, ochratoxin A, CPA, citrinin, penicillic acid, and patulin. In this study, patulin was detected as a patulin-specific, yellow-brown, fluorescent spot in a long-wave UV light with Rf 0.45 (43) and confirmed using chromatography with a standard patulin and extracts of *P. griseofulvum* (ATCC 46037). Among the 16 tentative fungal strains, patulin was detected from only one fungal strain, which was named patulin producing fungi P-1. Patulin, produced from isolate P-1, was further confirmed by HPLC. The peak retention time (5.56 min) for the patulin extract of isolate P-1 was most likely presented by the extract of *P. griseofulvum* and the patulin standard (Fig. 1). Finally, the isolate P-1 was selected as a patulin producing fungi.

Identification of the patulin producing fungi ***Phylogenetic position*:** The taxonomic and phylogenetic position of the patulin producing fungus P-1 was investigated. In

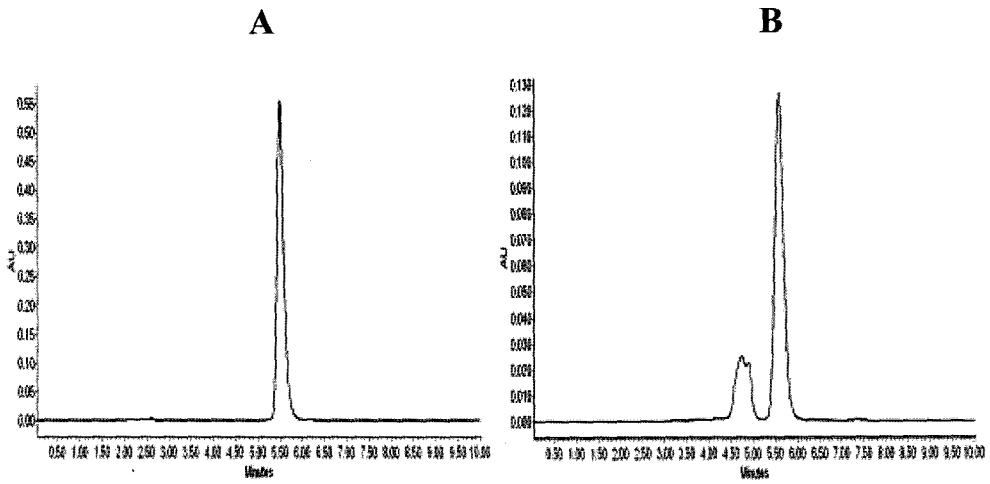

Fig. 1. High performance liquid chromatogram of the patulin extracts of *Penicillium griseofulvum* (A) and isolate P-1 (B). HPLC was performed on a reverse phase column and the wavelength of the UV detector was 276 nm for patulin.

the similarity (Table 1) and phylogenetic tree (Fig. 2) analyses, based on the ITS5-5.8S-ITS4 region sequences (505 base pair), the isolated P-1 strain was located at the same positions as *Penicillium crustosum* FRR 1669 (100 %) and *Penicillium commune* wb193 (100%). Similarly , the isolate P-1 strain was located at the same positions of *P. commune* CBS 311.48, *Penicillium expansum* IFO 8800, *Penicillium mali* NRRL 13719 and *Penicillium farinosum* NRRL 6293 by the similarity (Table 2) and phylogenetic tree (Fig. 3) analyses based on the sequencing (529 base pair) of the D1/D2 region of 26S rDNA. In the two phylogenetic trees, P-1 was perfectly related to 6 strains of *Penicillium* genera, and was also closely related to the other *Penicillium* species (more than 99%). Therefore, it was regarded that the description of the isolated P-1 should not solely rely on nucleotide sequencing and/or phylogenetic data.

Table 1. Similarity analysis of isolate P-1 based on the nucleotide sequences (NT) of the ITS5-5.8S-ITS4 region

Strains	Accession No.	Similarity (%)	NT difference /compared
P. crustosum FRR 1669	AY373907	100.00	0/490
P. commune wb193	AF455527	100.00	0/505
P. aurantiogriseum	AY280956	99.60	2/505
P. solitum FRR 937	AY373932	99.39	3/490
P. camemberti FRR 877	AY373900	99.39	3/490
P. echinulatum FRR 1151	AY373911	99.39	3/490
P. chrysogenum CBS 306.48	AY213669	99.39	3/489
P. dipodomyicola NRRL 13487	AY371616	99.18	4/489
P. aethiopicum FRR 2007	AY373896	98.77	6/489
P. expansum VIC	AY425984	98.60	7/500
P. griseroseum VIC	AY425983	98.38	8/494
P. expansum	AF455466	97.58	12/496

Description of growth and morphological characteristics: Identification of the patulin producing fungus P-1 was further confirmed by morphological and physiological classification. The observed morphological analysis of P-1 (*P. crustosum*) is presented in Table 3 and Fig. 4.

Growth rate: After 10 days at 25°C, the colonies on the MEA (Fig. 4A-1), CYA (Fig. 4A-2), CzA (Fig. 4A-3), and G25n (Fig. 4A-4) agar plates were spread over 1.8, 3.0, 1.6, and 1.5 cm, respectively. After 10 days at 37°C, however, the observations were no growth of CYA, a micro-colony on MEA and CzA, and a 0.15 cm spread on G25n.

Colonies on MEA: After 10 days at 25°C, the surface was powdery, covered with dull green or pale yellow

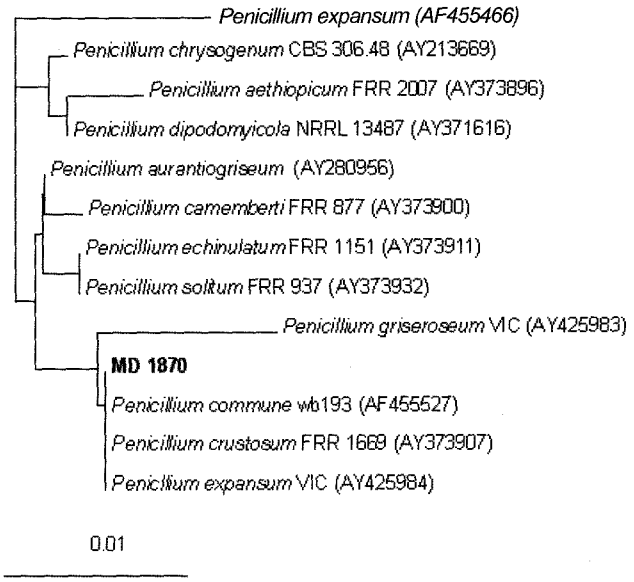

Fig. 2. Neighbor-joining tree based on the ITS-5.8S rDNA-ITS region sequences of the genus of *Penicillium*. *P. expansum* (AF455466) was used as an outgroup. The scale bar indicates 0.1 nucleotide substitutions per nucleotide position.

Table 2. Similarity analysis of isolate P-1 based on the nucleotide sequences (NT) of the D1/D2 domain of 26S rDNA

Strains	Accession No.	Similarity (%)	NT difference /compared
P. commune CBS 311.48	AY213616	100.00	0/528
P. expansum IFO 8800	AB047232	100.00	0/528
P. mali NRRL 13719	AF527056	100.00	0/510
P. farinosum NRRL 6293	AF527057	100.00	0/510
P. sclerotigenum NRRL 3461	AF033470	99.80	1/510
P. polonicum NRRL 995	AF033475	99.80	1/510
P. aurantiogriseum NRRL 971	AF033476	99.80	1/510
P. viridicatum NRRL 961	AF033478	99.80	1/510
P. cordubense NRRL 13072	AF527055	99.80	1/510
P. chrysogenum NRRL 807	AF033465	99.41	3/510
P. griseroseum NRRL 832	AF034449	99.41	3/510
P. viridicatum NRRL 5880	AF033390	99.41	3/510
P. coprophilum NRRL 13627	AF033469	99.22	4/510

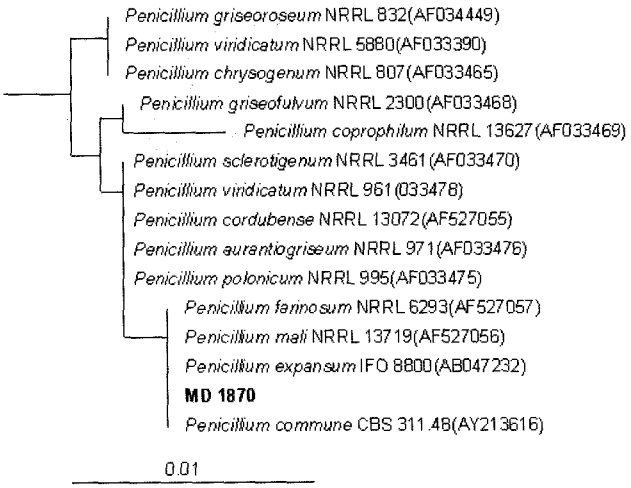

Fig. 3. Neighbor-joining tree based on the 26S rDNA D1/D2 region sequences. *Aspergillus versicolor* (AF433092) was used as an outgroup (data not shown). The scale bar indicates 0.1 nucleotide substitutions per nucleotide position.

powder on the reverse side, showed an inconspicuous mycelium and had no exudates or soluble pigment. Conidiogenesis was very abundant with masses of conidia (Fig. 4A-1).

Colonies on CYA: After 10 days at 25°C, the colony was plane and radially sulcate, while the surface was powdery with a white narrow margin (Fig. 4A-2), covered with dull green powder which changed from yellow at the center to orange brown at the margin in the reverse side, showed a white mycelium and had small exudates but no soluble pigment. Conidiogenesis was heavy over the entire colony area, with a dull green color.

Conidiophores on MEA: Conidiophores arising from the agar surface were from aerial mycelium or funicles, which formed synnemata towards the margin or in sectors in some colonies after 10 days. The stipes were heavily roughened, mostly terverticillate and rarely biverticillate, and 120-280 μm long. One to two rami were branched out

Table 3. Physiological and morphological analyses of the patulin producing fungi isolated from Korean apples

Test parameters		Results
Growth rate	At 25°C	Diameter of the colony: MEA : 1.8 cm, CYA : 3.0 cm, CzA : 1.6 cm, G25n : 1.5 cm
	At 37°C	MEA : micro-colony, CYA : no growth, CzA : micro-colony, G25n : 0.15 cm
Colonies	MEA	plane and low surface : appearing powdery mycelium : inconspicuous, subsurface conidiogenesis : very abundant, forming masses of conidia, breaking off in crusts when jarred, colored dull green exudates : absent soluble pigment : absent reverse : pale yellow
	CYA	plane and radially sulcate, low with a velutinous texture surface : appearing powdery with white narrow margin mycelium : white conidiogenesis : heavy over the entire colony area, coloured dull green exudates : small, clear soluble pigment : absent reverse : from yellow at the center to orange brown at the margins
Conidiophores on MEA		mostly borne from subsurface, closely appressed Stipes : 120-280 μm long, with alls heavily roughened, mostly terverticillate and rarely biverticillate rami : 1-2 per penicillus, often rough walled, 18-27 μm long metulae : 13.0-14.5 μm long phialides : ampulliform, 6.5-12.0 μm long
Conidia		subglobose to ellipsoidal, 3.3-3.8 × 3.2-3.4 μm

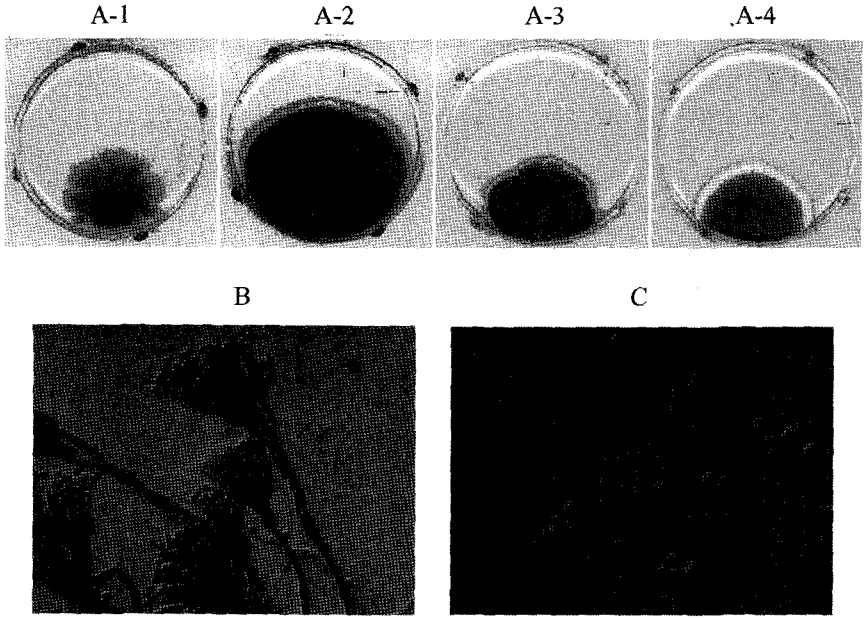

Fig. 4. Colonies, conidiophores, and conidia of the patulin producing fungi isolated from the Korean apples grown at 25°C for 10 days.

per penicillus and they were 18-27 μm long. Metulae were 13.0-14.5 μm long, and the phialides were shaped as ampulliform and they were 6.5-12.0 μm long (Fig. 4B). Conidia were subglobose to ellipsoidal, (3.3-3.8)×(3.3-3.4) μm (Fig. 4C).

The genetic analyses confirmed the patulin producing fungi (P-1) to be a sister species of *Penicillium*. In the taxonomical identification based on the phylogenetic and morphological analyses, the isolated patulin producing fungi P-1 was identified as being a representative species of *P. crustosum*.

Patulin producing activity of the isolated fungi

The growth and patulin producing activity of isolate P-1 (*P. crustosum*) was compared with the type strains of patulin producing fungus *P. griseofulvum* (ATCC 46037) in a patulin inducing broth media (SY broth) (Fig. 5). The maximal concentration of patulin in a culture of *P. griseofulvum* (ATCC 46037) reached 2.46 mg/mL after 2 weeks culture, after which the concentration was gradually decreased. Patulin concentration of P-1 (*P. crustosum*) was 0.135 mg/mL for 1 week culture, peaked at 0.323 mg/mL after 3 weeks, and subsequently decreased with further culturing.

The mycelial growth of *P. griseofulvum* was increased by a log phase after 1 week of cultivation to reach 38.96 mg/mL (dry weight). After 2 weeks, the biomass of the fungi gradually decreased, but then increased again due to the action of the conidia formation. The pattern of the mycelial growth of isolate P-1 was not different from that of *P. griseofulvum*. However, the maximal growth of mycelium reached 14.620 mg/mL after 2 weeks cultivation. In spite of the differenced in growth rate and growth phase, the points at which maximum patulin concentration was reached were synchronized within one week after the maximum mycelial growth level. Therefore, the patulin concentration was found to be increased after the mycelial

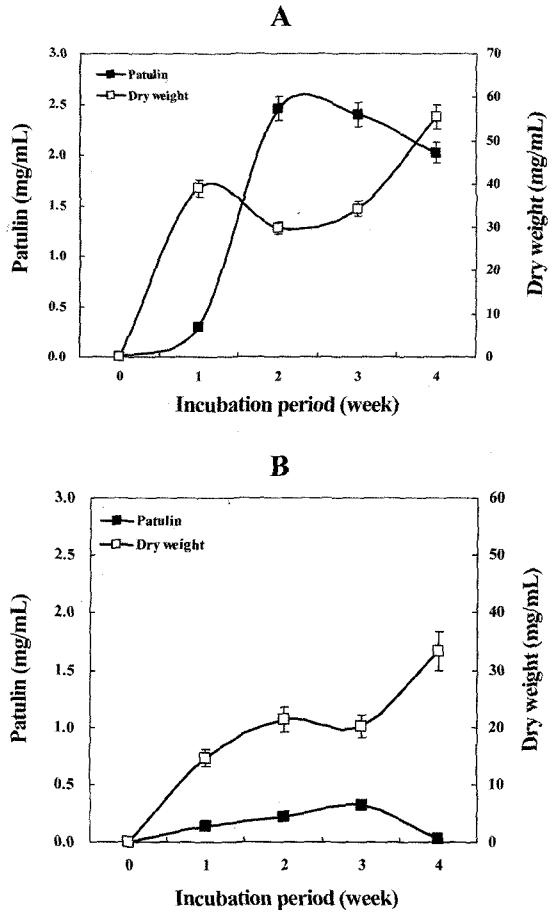

Fig. 5. Comparison of the mycelial growth and patulin producing ability between the patulin producing standard *Penicillium griseofulvum* (A) and *Penicillium expansum* (B) in a sucrose yeast broth during a 4-week incubation at 30°C. Patulin content was analyzed by the HPLC method in a cultured medium.

growth had reached a peak level.

In conclusion, a patulin producing fungus was isolated from Korean apples and identified as a typical species of *P. crustosum*. The patulin producing ability of the isolate was relatively lower than that of *P. griseofulvum* in a SY broth media.

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