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Phylogenic Analysis of *Alternaria brassicicola* Producing Bioactive Metabolites

Dong-Sun Jung[1]*, Yeo-Jung Na[1] and Ki Hyun Ryu[2]

[1]*Department of Food and Microbial Technology*
[2]*Department of Horticultural Science, Seoul Women's University, Seoul 139-774, Korea*

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The fungal strain SW-3 having antimicrobial activity was isolated from soil of crucified plants in Pocheon, Kyungki-Do, Korea. Strain SW-3 was identified as *Alternaria brassicicola* by its morphological characteristics, and confirmed by the analysis of the 18S gene and ITS regions of rDNA. The fungus showed a similarity of 99% with *Alternaria brassicicola* in the 18S rDNA sequence analysis. *A. brassicicola* has been reported to produce an antitumor compound, called depudecin. We found that strain SW-3 produced antimicrobial metabolites, in addition to depudecin, during sporulation under different growth conditions. The metabolite of the isolated fungus was found to have strong antifungal activity against *Microsporium canis* and *Trichophyton rubrum*, and antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aerogenes*. The amount and kind of metabolites produced by the isolate were affected by growth conditions such as nutrients and growth periods.

Key words: rDNA, *A. brassicicola*, fungal metabolites, antimicrobial activity

Most fungi produce secondary metabolites such as mycotoxins or antibiotics. These compounds are synthesized from precursors derived from primary metabolism and can be valuable as antifungal, antibacterial or antitumor agents (Omura, 1992). A variety of bioactive compounds have recently been isolated from several filamentous fungi such as *Aspergillus* (Udagawa *et al.*, 2000), *Xylaria* (Isaka *et al.*, 2000), and *Fusarium* (Park *et al.*, 2000).

Alternaria species are causal agents of black or brown spot diseases of many fruits, vegetables and field crops (King *et al.*, 1984). The disease is spread during the growing season by wind-blown or rain-splashed spores (MacKinnon *et al.*, 1999). The pathogen attacks most parts of the plant and it is thought to induce its chlorotic effect by excretion of phytotoxins. *A. brassicicola* infections in several cruciferous vegetables, has been reported from several countries (Pattanamahakul *et al.*, 1999). The taxonomy of *Alternaria* is primarily based on morpholgical characteristics (Cho *et al.*, 2001), especially on differences in conidium morphology (Simmons, 1995). However, molecular approaches have been used increasingly in taxonomy and systematics of filamentous fungi. The rDNA genes have been employed to analyze major evolutionary events because it is highly conserved, whereas the rDNA internal transcribed spacer (ITS1 and ITS2) is more variable, and has been used for the investigation of species-level relationships (Bruns *et al.*, 1991; Samuels *et al.*, 1995). Thus the ITS region has been used in classifying fungi because of its systematic and taxonomic usefulness (Chillali *et al.*, 1998). This is because ITS regions are of suitable size for PCR amplication, restriction analysis and sequencing procedures, and because ITS regions are variable among species as well.

It has been reported that *Alternaria brassicicola* produces a number of interesting compounds, such as antitumoric depudecin (Matsumoto *et al.*, 1992), antibiotic complex brassicicolin (Ciegler *et al.*, 1969), and phytotoxic brassicicenes (MacKinnon *et al.*, 1999). Depudecin is a C_{11} compound containing two epoxide groups and shows antitumor activity (Matsumoto *et al.*, 1992). Phytotoxic brassicicenes, fusicoccane-like diterpenoids, have been isolated from the culture filtrate of the canola pathogen *A. brassicicola* (MacKinnon *et al.*, 1999).

The published Korean literature on *Alternaria* genus pertains to about 25 species with a special emphasis on occurrence and pathogenicity. Although there are numerous papers on the production of toxins by species of *Alternaria*, the genus *Alternaria* has not been extensively surveyed for its ability to produce useful bioactive metabolites in Korea (Cho *et al.*, 2001).

In this study, we isolated *Alternaria* species from soil of a Chinese cabbage patch in Pocheon, Kyunggi-do, Korea, and found that the isolated strain yielded several bioactive compounds. We analyzed the 18S gene and ITS regions of rDNA of the isolated *Alternaria* to identify its species,

* To whom correspondence should be addressed.
(Tel) 82-2-970-5637; (Fax) 82-2-970-5639
(E-mail) dsjung@swu.ac.kr

and investigated antibacterial and antifungal activities of the metabolites from the isolated fungal strain.

Materials and Methods

Isolation and cultivation of Alternaria

Fungi were isolated from soil of a Chinese cabbage patch in Pocheon, Kyunggi-do, Korea. Among 5 isolates designated SW-1 to SW-5, strain SW-3 with spores characteristic of the genus *Alternaria* was selected for further investigation to test its ability to produce bioactive metabolites.

A slant culture of the isolated strain grown on potato dextrose agar (PDA, Difco Lab, USA) at 20°C for 7 days was suspended in 10 mL of 0.1% Tween 80 solution. The suspended spore solution was inoculated into a 300 mL Erlenmeyer flask containing 100 mL of seed medium (dextrose 0.5%, Bacto peptone 0.5%, beef extract 0.5%, yeast extract 0.25% and NaCl 0.25%). The flask was shaken at 28°C for 2 days on a rotary shaker at 120 rpm. Twenty milliliters of spore suspension were transferred to a 1-liter flask containing 300 mL of potato dextrose broth and incubated for 10-12 days at 20°C. The production medium (PDB) was modified by addition of sodium nitrate (3%), minerals (0.1% of dipotassium phosphate, magnesium sulfate, potassium chloride and ferrous sulfate) or dextrose (2%) to test the effect of nutrients on production of metabolites. Sodium acetate (0.25%) was added by injecting a sterile solution after 7 days incubation. The effect of nutrients on the fermentation of the isolated fungus was determined by the weight of obtained metabolites.

Microscopic Analysis

The fungi were grown on PDA at 25°C for 7-9 days, and the conidia of the isolates were examined under a microscope (Carl Zeiss, Germany). A slide culture technique was also used to observe the morphology of the isolated fungi.

PCR amplification

Genomic DNA was extracted from the suspension culture of *Alternaria* by the cetyltrimethylammonium bromide (CTAB) method as described by Knapp and Chandlee (1996). Amplifications of 18S rDNA and ITS for the isolate were carried out using the general PCR with conserved primers. The 18S UP (5'-GACTGTGAAACTGCGAATGG-3'; AJ242597) and 18S DOWN (5'-TAAGTTTCAGCCTTGCGACC-3'; AJ242598) primers were designed for amplifying a 1,000 bp DNA fragment for the 18S rDNA and ITS-F (5'-GTCCTAACAAGGTTTCCGTA-3'; AJ297952) and ITS-R (5'-TTCTCCGCTTATTGATATGC-3'; AJ297953) primers for 650 bp PCR amplicon for the ITS region of the isolate.

PCR was executed with a 50 μl reaction volume of the following solution mixture: 1 ng of template DNA, 0.2 mM each dATP, dGTP, dCTP, and dTTP, 50 pcM of primer, 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40, 50% glycerol, 1 unit of *Taq* DNA polymerase (Takara, JAPAN), and 5 mM $MgCl_2$. PCR amplifications were performed in a thermal cycler (i-cycler, Bio-Rad, USA) and denaturation was executed at 94°C for 3 min before PCR cycling. The reaction cycle consisted of 40 sec at 94°C for denaturation, 60 sec at 55°C for annealing, and 60 sec at 72°C for extension. A total of 35 cycles was performed. Final extension was done at 72°C for 10 min. PCR products were electrophoresed on an agarose gel and the DNA was eluted from the agarose gel with a Gene Clean kit (Bio101, USA) according to the manufacturer's instructions.

Cloning of PCR products

Purified PCR fragments of 18S and ITS rDNAs were cloned directly into the pGEM-T Easy vector using a TA cloning kit (Promega, USA). The ligation mixture was transformed into competent *Escherichia coli* (strain JM109) cells (Promega, USA). For transformation screening, bacteria were plated onto MacConkey agar plates containing 100 μg/ml ampicillin. Plates were incubated at 37°C for 12-16 h. Selected white colonies were cultured in Luria-Bertani (LB) broth containing 100 μg/ml ampicillin for 12 h and recombinant plasmids were prepared with the alkaline lysis small scale plasmid preparation method (Knapp *et al.*, 1996).

Selected recombinant clones of the 18S and ITS rDNAs of *Alternaria* were determined by *Eco*RI digestion. Sizes of 18S and ITS rDNA inserts in the recombinant clones were estimated using a 1kb plus DNA ladder (GIBCO BRL, USA) as a molecular size marker DNA.

DNA sequencing and phylogenetic tree analysis

DNA sequencing of PCR products was done by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The rDNA homology searches were performed using the BLAST program through the internet server at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, USA). Sequences and accession numbers for compared isolateswere retrieved from the GenBank database. Sequence pair distances among related and different fungi of the isolate were scored with the Clustal X program and phylogenetic tree analysis was performed with the DNASTAR (Burland, 2000) software package (DNASTAR, Inc., Madison, USA).

Isolation of fungal metabolites

Strain SW-3 was cultivated on potato dextrose broth for 10-12 days at 20°C. The mycelial mat was separated from the broth by filtration through a Buchner funnel and dried

for 3 h at 105°C. A crude mycelium extract was obtained after extraction with ethyl acetate and the organic solvent was removed under reduced pressure. The culture filtrate (700 mL) was extracted twice with the same volume of ethyl acetate. The organic layers were combined, dried over sodium sulfate and evaporated under reduced pressure to dryness, yielding 320 mg of a crude extract. The concentrated extract was fractioned on a silica gel 60 F_{254} (Merck, Darmstadt, Germany) and purified by flash column chromatography (chloroform:methanol, 15:1). TLC coated with silica gel $60F_{254}$ analysis was used to visualize fungal metabolites. The elution liquid for TLC was a chloroform-methanol mixture (15:1). Plates were viewed under UV-254 nm or after 2 min heating following spraying with 7% phosphomolybdic acid solution.

Antimicrobial activity

Antifungal activity against *Candida albicans, Microsporium canis, Trichophyton rubrum, Rhizopus japonicus* and *Mucor racenmosus* was performed using a paper disk method. *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Vibrio paraheamolyticus* were tested for antibacterial activity of the metabolites. The fungal metabolite was filtered through a 0.22 μm syringe filter after dissolving in 50% DMSO, and used for the antimicrobial activity test. The filtered metabolites were applied on 6-mm paper disks on the agar plate, which had been inoculated with test culture. The plate was placed in an incubator overnight and the antimicrobial activity was evaluated by measuring the inhibition zone of the bacterial or fungal growth.

Results and Discussion

Morphology of strain SW-3

The isolated fungus corresponded in morphology to *Alternaria brassicicola* described by Wiltshire (1947) and Simmons (1995). Colonies on PDA were velvety and dark olivaceous brown, and spores were produced in branched chains and had no beak. Conidiospores were acropetal (16-51×10-20 μm) with 4 or 5 transverse septa as shown in Fig. 1. The fungus differs from *A. brassicae* in its beakless and smaller conidia (Cho *et al.*, 2001).

PCR amplification, sequence comparison and phylogenetic analysis

To address the molecular taxonomy and relationships, the 18S and ITS rDNAs of the isolate were amplified using the sets of 18S and ITS primers and their sequences were determined in this study. PCR products were successfully amplified with the estimated size (1.0 kb and 0.6 kb for 18S and ITS, respectively) as shown in Fig. 2. Cloned 18S and ITS rDNAs for the isolate were 1,061 and 596 nucleotides long, respectively.

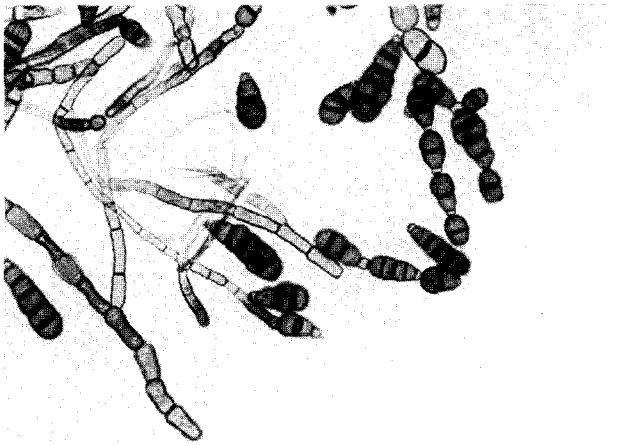

Fig. 1. Acropetal conidiospores and septate hyphae of *A. brassicicola* SW-3 (x400).

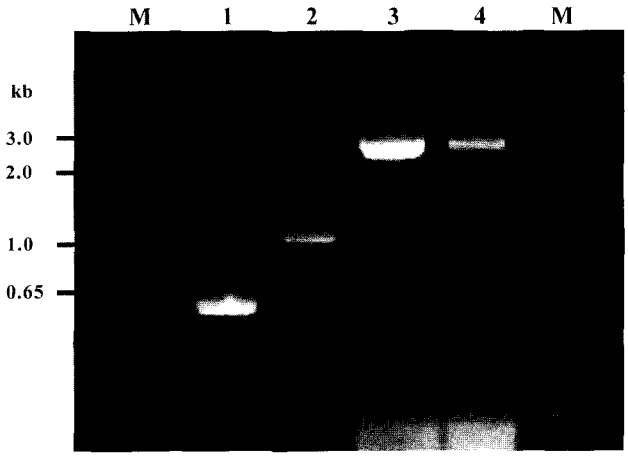

Fig. 2. Agarose gel electrophoresis of PCR products of ITS and 18S rDNA of *Alternaria* and their cloned molecules digested with *Eco*RI. Lane M, 1kb DNA ladder; Lane 1, PCR product of ITS; Lane 2, PCR product of 18S; Lane 3, pAITS digested with *Eco*RI; Lane 4, pA18S digested with *Eco*RI.

These sequences were aligned with the known 18S and ITS rDNA nucleotide sequences of *Alternaria*. Percent similarities of 18S rDNAs between *Alternaria* and other selected fungi ranged from 94.8% (with *Westerdykella cylindrica*) to 99.7% (with other *Alternaria* isolates and *Clathrospora*) (Table 1).

The sequences of 18S rDNAs were aligned and a phylogenetic tree was produced (Fig. 3). As seen in the phenogram, strain SW-3 was grouped with other isolates of *Alternaria*.

The ITS region of strain SW-3 showed 87.4% to 100% nucleotide sequence identities with that of other fungi (Table 2). Strain SW-3 showed 97.2% to 100% identical to *Alternaria brassicicola* isolates. The data matrix of the published ITS sequence of *Alternaria* and the outgroup *Cymbidium* plant produced 1 ITS tree. Discriminatory grouping was observed in the phylogenetic tree from ITS

Table 1. List of percent similarity of nine fungi and one outgroup plant compared to 18S nucleotide sequences from *Alternaria* sample

No.	Species (GenBank accession No.)	Percent similarity
1	*Alternaria brassicicola* SW-3	this study
2	*Alternaria brassicicola* ABc2-(ABU05197)	99.7
3	*Alternaria raphani* (ARU05199)	99.7
4	*Clathrospora diplospora* (CD443464)	99.7
5	*Alternaria alternata* (AF218791)	99.7
6	*Alternaria alternata* AA6 (AAU05194)	99.7
7	*Pleospora herbarum* (PHU43458)	99.6
8	*Alternaria brassicae* AB11 (ABU05196)	99.2
9	*Herpotrichia juniperi* (HDU42484)	95.0
10	*Westerdykella cylindrica* (AY016355)	94.8
11	*Cymbidium goeringii* (AJ271245)	75.3

Table 2. List of percent similarity of ten fungi and one outgroup plant compared to ITS nucleotide sequences from *Alternaria* sample

No.	Species (GenBank accession No.)	Percent similarity
1	*Alternaria brassicicola* SW-3	this study
2	*Alternaria brassicicola* 1 (AF229462)	98.9
3	*Alternaria japonica* (AF229474)	89.5
4	*Alternaria raphani* (ARU05200)	90.9
5	*Alternaria brassicae* 1 (AF392983)	91.6
6	*Alternaria brassicae* 2 (AF392984)	91.6
7	*Alternaria brassicicola* 2 (AF201964)	97.2
8	*Alternaria brassicicola* 3 (AF392985)	97.6
9	*Alternaria brassicicola* 4 (ABU05198)	98.7
10	*Alternaria brassicicola* 5 (AF392986)	100.0
11	*Alternaria radicina* (AF307014)	87.4
12	*Cymbidium goeringii* (AJ300288)	28.9

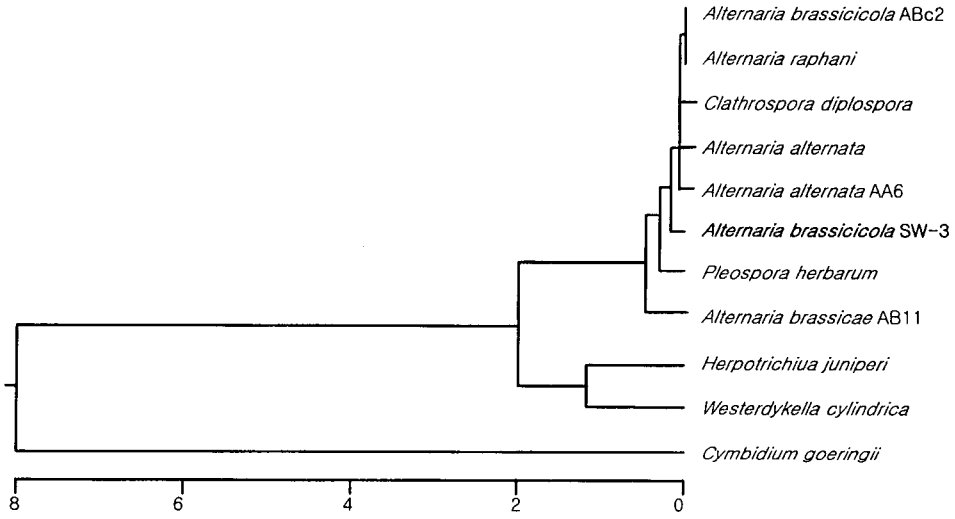

Fig. 3. Phylogeny of 18S rDNAs of selected fungi.

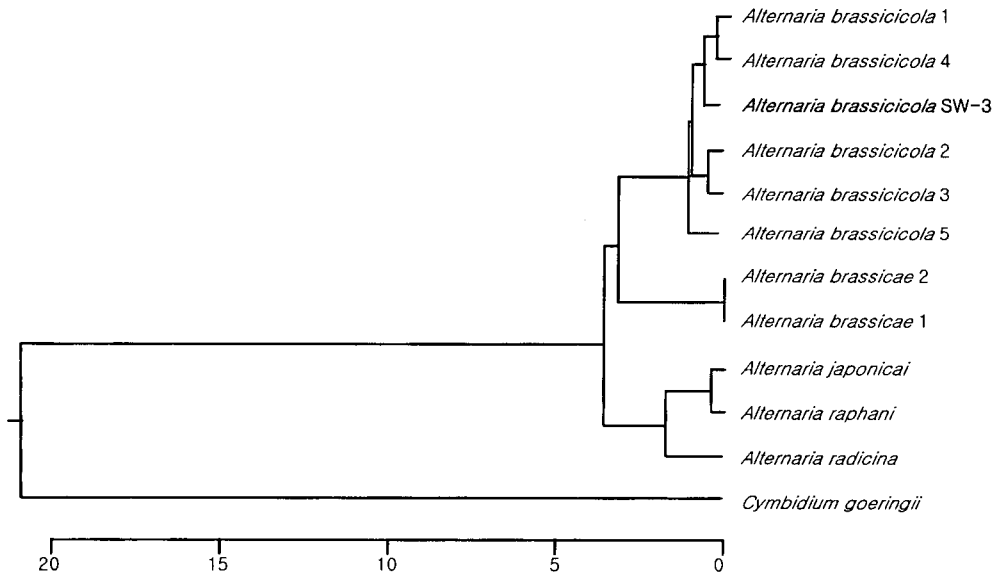

Fig. 4. Phylogeny of ITS rDNAs of selected fungi.

Table 3. Antimicrobial activity of fungal metabolite against various microorganisms

Test microorganism	Inhibition zone size (mm)
Escherichia coli KCCM 11234	-
Pseudomonas aeruginosa KCTC 1636	17.5
Vibrio paraheamolycus KCCM 11965	21.0
Staphylococcus aureus KCCM 11335	19.6
Candida albicans KCCM 50235	8.5
Microsporium canis KCTC 6591	24.5
Trichophyton rubrum KCTC 6345	24.0
Rhizopus japonicus	29.5
Mucor racenmosus	28.7

-: negative activity

analysis (Fig. 4). The phenogram based on the ITS region showed that strain SW-3 was grouped with *A. brassicicola* and distinguished from other *Alternaria* species. Kusaba *et al.* (1995) also reported that the species of *Alternaria*, which had been distinguished morphologically, were clearly separated from one another on the basis of variation in ITS of rDNA. Therefore, the ITS analysis clearly indicates that the SW-3 isolate is a strain of *Alternaria brassicicola.*

Production of bioactive metabolites

Ethyl acetate extract of dried mycelia from submerged cultures of strain SW-3 was tested for antimicrobial activity. Antimicrobial activity was not found in mycelium of the isolate. The crude metabolites from the culture filtrate exhibited antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aerogenes* and

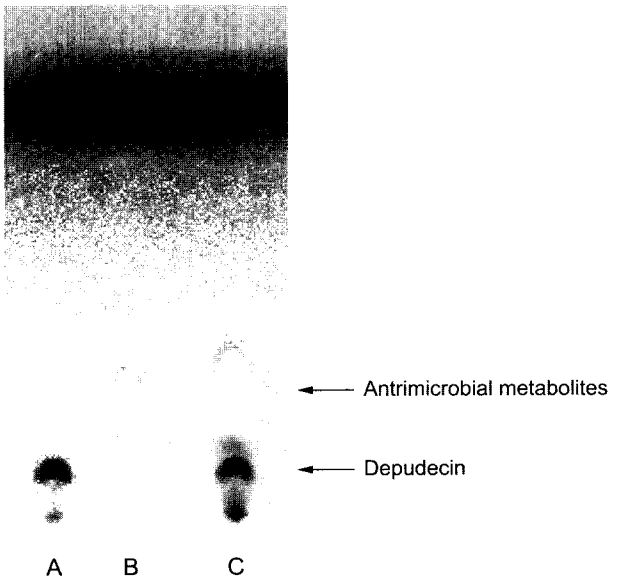

Fig. 5. TLC chromatogram of the fungal metabolites produced by *A. brassicicola* SW-3. A, purified depudecin; B, unknown metabolite; C, crude extract.

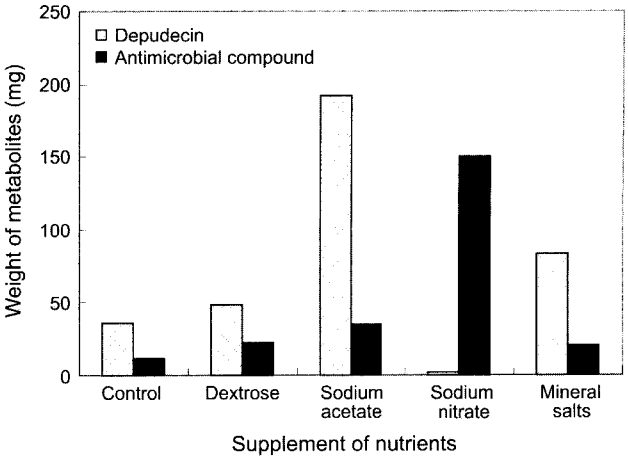

Fig. 6. Effect of nutrients on production of bioactive metabolites from *A. brassicicola* SW-3. Potato dextrose broth (PDB) was used to cultivate *A. brassicicola* SW-3 as a control medium.

Vibrio paraheamolyticus (Table 3).

The crude EtOAc extract of strain SW-3 was visualized to several spots by TLC analysis (Fig. 5). The ethyl acetate extract (320 mg) was further partitioned by silica gel flash column chromatography (chloroform:methanol, 15:1) and 2 subfractions were obtained. Fraction I (Rf=0.25, 20 mg) of the crude extract was isolated and identified as depudecin which exhibited cytotoxic activity against several cancer cell lines, as described in the previous report (Na *et al.*, 2001). Fraction II (Rf 0.37, 125 mg) exhibited potent inhibitory activity against fungi such as *Microsporium canis, Trichophyton rubrum*, *Rhizopus* and *Mucor* species. Standard disk assays yielded inhibitory zones of 24.5 mm and 24.0 mm against *Microsporium canis* and *Trichophyton rubrum*, respectively, at 0.5 mg per disk.

Production of bioactive metabolites by *Alternaria brassicicola* SW-3 was affected by growth condition and nutritional factors. Production of the antimicrobial component increased when potato dextrose broth supplemented with 3.0% of sodium nitrate or 2% of dextrose was used as a growth medium. However, biosynthesis of depudecin decreased by cultivating on potato dextrose broth supplemented with sodium nitrate or sugar, but increased by addition of a low concentration (0.25%) of sodium acetate as a limiting precursor (Fig. 6).

Therefore, we concluded that phytopathogenic fungus *A. brassicicola* is a good source of bioactive secondary metabolites, and production of useful metabolites by strain SW-3 can be controlled by nutrients.

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