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Control of Aflatoxin Production of *Aspergillus flavus* by Inhibitory Action of Antagonistic Bacteria

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Abstract Aflatoxin B_1 is known as the most potent mycotoxin produced by several fungi. It has been demonstrated to be not only carcinogenic but teratogenic and mutagenic as well in humans. To prevent or inactivate aflatoxins, several chemical or physical methods were tested for ammoniation, using insecticides as an example, but they were unsuitable for food products. On the contrary, biological control by antagonistic microorganisms is an ideal method. In order to control aflatoxin B_1 biologically, the antagonists #07, #63, #75, #74, and #61 were separated from various samples by using the antagonistic activity test. Among them, culture filtrate part A (non heat-treated) of #63 and #74 had a higher inhibitory activities compared to part B (heat-treated) on fungi growth. In fact, both showed aflatoxin B_1 degradation activities. The degradation effects of antagonist #63 and #74 on aflatoxin B_1 produced by *Aspergillus flavus* were shown to be 95% and 75%, respectively. Based on the morphological characteristics, #63 was deduced as an *Azospirillum* sp.

Key words: Aflatoxin B_1, *Aspergillus flavus*, antagonist, biological control

Fungi that has a capability of producing mycotoxins are identified to be widespread contaminants of foods and agricultural commodities. The production of mycotoxins in food and feed by several mycotoxic fungi, along with the occurrence of mycotoxicoses in both human and animals, who ingest mycotoxin-contaminated food and feeds, are well documented. Recently, the pathogenic fungi have been documented as etiologic agents of human disease. There are a number of articles on the antifungal activities of microorganisms and new antifungal agents, but the focus has been mainly on their fungicidal activities. For example, chitinase and chitosanase hydrolyze chitin or chitosan and also break down the mycelial wall. Yoon *et al.* [32] purified a thermostable chitosanase from *Bacillus* sp. and the enzyme was stable after heat treatment at 80°C for 10 min. Lee *et al.* [19] reported that polygodial caused fragmentation of the cellular membrane and leakage of intracellular metabolites by destroying cellular membrane. According to Park *et al.* [24], when the nonpathogenic yeast form of *Candida albicans* was transformed to a pathogenic hyphal form, the transition was severely inhibited in a dose-dependent manner by the addition of deer antler extract. Some analogue peptides showed powerful antifungal activity with little or no hemolytic activity [18].

Aflatoxins, one of the most potent mycotoxins, are known as secondary metabolites of the fungi *Aspergillus flavus*, *A. parasiticus*, and *Penicillium puberulum* which can cause mortality or reduced productivity in farm animals. In addition, they have been demonstrated to be carcinogenic, teratogenic, and mutagenic in humans [4, 8, 13]. Based on the results from the study of their chemical [23] and carcinogenic [29] features, aflatoxin B_1 is the strongest oral carcinogen, being 3,750 times more potent than dimethylnitrosoamine, which has been known as the most harmful material in the world. Aflatoxins are produced in cereal grains, peanuts, corns, and seeds which were cultivated in various places throughout the earth [14, 28, 31]. Moreover, human exposure to aflatoxins can occur indirectly by consumption of products from animals taking in mycotoxin-contaminated feeds (e.g. aflatoxin M in milk) [1, 2, 6, 27]. In the latter half of the 70's in Korea, there was a case where the aflatoxins remained in as much as 200–300 ppb in imported peanut dregs needed for livestock. Because Koreans routinely eat traditionally fermented soybean products which utilize some kind of fungi, it is possible that they are also exposed to contaminated food items in a certain extent. In fact, one of the *doenjang* samples which were gathered from Pusan consisted of 84 ppb of aflatoxin content, which was 2.8 times higher than WHO recommendations at that particular time [15].

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Hence, it is highly desirable to prevent aflatoxin formation or to inactivate aflatoxins in contaminated foods. Several chemicals have been used to detoxify aflatoxins. Ammoniation was an effective method for eliminating aflatoxins in contaminated cottonseed meal [22]. Volatile organic acids, such as acetic and propionic acids, have been suggested for prevention of fungal growth and aflatoxin formation in corn [30]. Barium completely inhibited aflatoxin formation at 1 ppm with no effect on mycelial growth [20]. Some insecticides and herbicides have been found to suppress fungal growth and aflatoxin formation [10, 11]. The extract oils of many commodities have been reported to have inhibitory activity [3, 5, 7, 9].

Unfortunately, most of the antifungal chemical agents are incompatible with food or feed, along with the fact that they might show some side effects on the environment. On the contrary, biological control strategy by the antagonistic microorganisms seems to be an ideal method, because of its reduced side effects. Therefore, the authors isolated antagonistic microorganisms from nature by testing for their antagonistic activities, and examined their inhibitory activities on fungal growth and aflatoxin production for controlling the aflatoxin production in the early stage by antagonists. The authors also made some additional observations on these antagonistic activities in soybean agar plates, to confirm the application of *Meju* or traditionally fermented soybean product, *Doenjang*.

MATERIALS AND METHOD

Microorganism

Aspergillus flavus ATCC 15517, the aflatoxin B_1-producing fungi, was obtained from KCCM, and aflatoxin B_1 production during its growth was closely observed. *Aspergillus flavus* was inoculated in a PDB (Potato Dextrose Broth) and was incubated at 25°C. At regular intervals, culture fluid of fungi was extracted and spotted on TLC plates (Silica Gel 60 F-254, Merck, Darmstadt, Germany) with authentic aflatoxin B_1 as a reference. The plate was developed in chloroform-acetone (9:1) [17] and then air dried to examine aflatoxin B_1 by densitometery (SHIMADZU CS-9301PC scanning densitometer, Tokyo, Japan) under 365 nm UV light. Authentic aflatoxin B_1 was purchased from Sigma Co. (St. Loius, U.S.A.) and growth media were obtained from Difco Laboratories (Detroit, U.S.A.). All chemicals were of reagent grade.

Screening and Isolation of Antagonistic Microorganisms

The antagonistic microorganisms included in this study were isolated from soil samples and home-made *Mejus* collected from various regions in Korea. Microorganisms present in environmental samples need to be released for isolation purposes as they tend to become tightly bound to the soil matrix. Therefore, to optimize individual cell recovery, the soil has to be dispersed to separate cells from the organic matter and clay [12]. For screening of antagonistic microorganisms, the samples were mixed with 100 ml of Tris-Cl buffer solution (pH 7.5) and incubated on a nutrient medium at 25°C for 30 min by reciprocal shaking at 110 rpm. The mixture was diluted serially with saline solution (0.85% NaCl) and spread on NA medium. The plates were then incubated at 25°C for 3 days and single colonies were isolated from the solid medium. The isolates were stored at -70°C in NB with 20% glycerol and they were used in further study to determine the antagonistic activity.

The fungi was inoculated in the center of a PDA (Potato Dextrose Agar) plate and incubated at 25°C for 24 h. The screened microorganisms were inoculated at four points of that plate with a square shape while being incubated continually. The plates used for the antagonistic activity test contained the same amount of media, because the difference of the inhibited area might be caused by the difference of the height of the plate. After 7 days, the microorganisms which made an inhibition zone were isolated as an antagonist [26]. For selecting the best antagonistic bacteria, colony diameters which grew against antagonists or by being alone were compared [25]. The expression of inhibition zone is described below. The antagonistic activity test was done three times in triplicate each. In order to select the medium in which the antagonist showed its maximum ability, the antagonistic activity test was carried out on SDA (Soy Dextrose Agar), NA, and PDA media.

$$\text{Zone of inhibition } (\%) = \frac{NT - T}{NT} \times 100$$

NT: colony diameter of no treatment
T: colony diameter of treatment

Preparation of Conidia Suspension and Culture Condition

A. flavus was grown on a PDA slant for 7 days at 25°C. Conidia were harvested by adding 1 ml of sterilized Tween 80 solution (0.1%, v/v) and 5 ml of distilled water, filtering through the filter paper (Whatman No. 3), centrifuging (3,000 ×g), washing three times with sterilized distilled water, and resuspending in sterilized Tween 80 solution. The suspension was adjusted to contain approximately 10^7-10^8 conidia/ml.

PDB medium was used as a basal medium for aflatoxin B_1 production. One ml of the conidia suspension was added to 50 ml of PDB medium in a 250-ml Erlenmeyer flask and the mixture was incubated at 25°C for 7 days. In the case of antagonistic bacteria that was selected by the test, SDBP medium (Soy sauce 3%, Dextrose 5%, Beef extract 0.1%, Proteose peptone 0.1%) was used and antagonists were inoculated in the same way and incubated at 30°C for 3 days.

Preparation of Culture Filtrates of Antagonists
The antagonists were incubated for 48 h on SDBP medium and then centrifuged. The supernatant was divided into two parts. One part (part A) was sterilized by a 0.22 μm nitrocellulose membrane filter (Micron Separation Inc.) and the other (part B) was autoclaved (121°C, 15 min). Parts A and B were used to examine the inhibitory effect on aflatoxin production.

Treatment of Fungi with Antagonists Culture Filtrate
About 1 ml of the antagonists culture filtrate (parts A and B) was poured into the plates in which *A. flavus* was grown for 1 day. These were incubated at 25°C for 7 days and the inhibitory activities of antagonists were carefully observed.

Cultivation of *A. flavus* in the Media Containing Antagonists Culture Filtrate
The media containing the antagonists culture filtrate were made. The fluid media preparation was made as follows; 2.5 ml of double concentrated PDB media in two 15 ml-captubes were autoclaved, the same volume of antagonists culture filtrate (parts A and B) was mixed with that media separately, and the conidia suspension of *A. flavus* was inoculated into that medium. They were then incubated at 30°C for 7 days. The growth and aflatoxin production of fungi were compared with a certain amount of control. The plates containing antagonists culture filtrate were made by following the following rules: With part B, 39 g of PDA powder was dissolved in 1 l of part B filtrate and autoclaved. With part A, the same amount of PDA powder was dissolved in 250 ml of part A filtrate. It was then sterilized, cooled, and the remaining 750 ml of part A filtrate was mixed. *A. flavus* was inoculated in that plate, incubated for 7 days, and the zone of inhibition was shown.

The Inhibitory Effect by Co-Culture
The conidia suspension and antagonistic bacteria seed culture were inoculated in 5 ml of PDB medium at a 1% concentration rate. They were incubated at 30°C by reciprocal shaking, and their growth and aflatoxin B_1 production were examined at regular intervals.

The Inhibitory Effect on Soybean Agar Plate
For studying the antagonistic activity in *Meju* and *Doenjang* which were made from soybean, the inhibitory effect was tested on a SA (Soybean Agar) plate. SA plates were prepared by the traditional *Meju* preparation method. Soybeans was soaked in water for 12 h, boiled for 6 h, and crushed and filtered through a sieve and gauze. The filtrate was mixed with 1.5% of agar powder, autoclaved, and plates were then prepared. The zone of inhibition was detected by examining the growth of fungi against antagonists.

Examination of the Effect of Antagonists on the Aflatoxin B_1 Degradation
The antagonists were inoculated in a 4-day-old culture filtrate of *A. flavus* with aflatoxin B_1 being included. They were incubated at 30°C by reciprocal shaking, and, after 5 days, the level of aflatoxin B_1 remained was assayed by the TLC method.

Identification of Microorganism
For the purpose of making a preliminary identification of the antagonistic bacteria, its morphological characteristics were examined according to the "Bergey's Manual of Systematic Bacteriology" [16].

RESULTS AND DISCUSSION

Aflatoxin B_1 Production by *Aspergillus flavus*
The culture extract of *A. flavus* was analyzed every 24 h, and it was found that aflatoxin B_1 increased as fungi grew up to 4 days. Aflatoxin B_1 production was maximum in 4-day-old culture (Fig. 1). For the test on the degradation of fungi producing aflatoxin B_1, the 4-day-old culture was used as a substrate of reaction.

Isolation of Antagonistic Microorganism
About 500 microorganisms which were screened from soil samples and *Mejus* were grown against *A. flavus* on PDA plates, and approximately 30 microorganisms producing the inhibition zone were selected as antagonists. The maximum zone of inhibition was 43%, and, based on this result, 8 species of the best antagonists were selected for further study (Table 1, Fig 2). These antagonists showed their maximum inhibition activity on PDA medium (Table 2), therefore, the PDA medium was used in the later study.

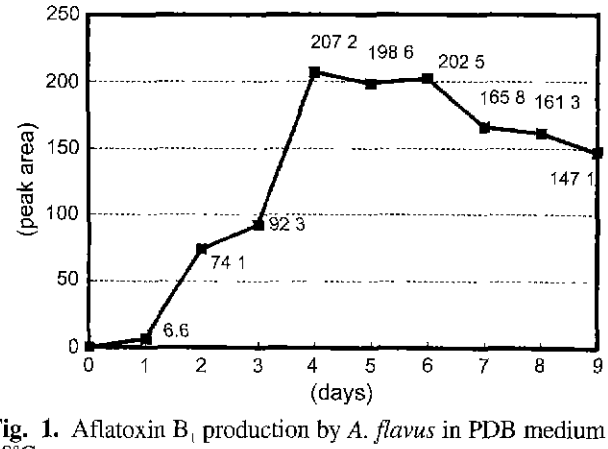

Fig. 1. Aflatoxin B_1 production by *A. flavus* in PDB medium at 28°C.
Each sample was spotted (20 μl) on TLC plate, developed in chloroform-acetone (9 1), and examined under 365 nm UV light

Table 1. Growth inhibition of the each antagonistic bacteria against *Aspergillus flavus* on PDA media for 7 days at 28°C.

	Antagonist							
	#07	#63	#75	#74	#61	#33-2	A#2-2	#59
Zone of inhibition* (%)	43.2	40.5	39.2	37.8	37.8	37.4	36.9	36.5

Zone of inhibition* (%) $= \frac{NT-T}{NT} \times 100$.

NT, colony diameter of no treatment (mm).
T; colony diameter of treatment (mm)

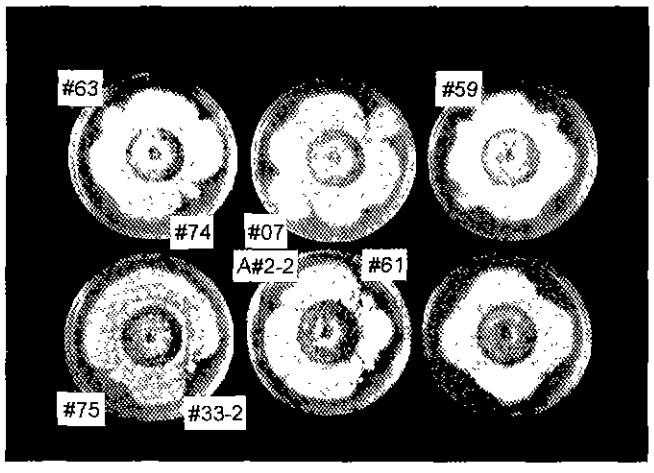

Fig. 2. Inhibition effect of antagonistic bacteria against *Aspergillus flavus* on PDA media for 7 days at 28°C.

Table 2. Growth inhibition of the antagonist #07, #63, #75, #74, and #61 against *Aspergillus flavus* on various media for 7 days at 28°C.

Antagonist	Zone of inhibition (%)		
	PDA	NA	SDA
#07	43.2%	28.6%	19.2%
#63	40.5%	34.3%	27.2%
#75	39.2%	27.3%	25.2%
#74	37.8%	23.4%	16.8%
#61	37.8%	21.4%	18.2%

Table 3. Growth inhibition of *Aspergillus flavus* by the treatment with culture filtrate of antagonistic bacteria #74, #63, #61, #75, and #07. Each antagonist culture filtrate (1.0 ml) was poured into a 1-day-old *A. flavus* plate and incubated at 25°C for 7 days.

Treatment	Zone of inhibition (%)				
	#74	#61	#63	#75	#07
Culture filtrate (part A)	93.3%	26.7%	55.6%	51.1%	46.7%
Heat-treated filtrate (part B)	71.1%	20.0%	71.1%	73.3%	66.7%

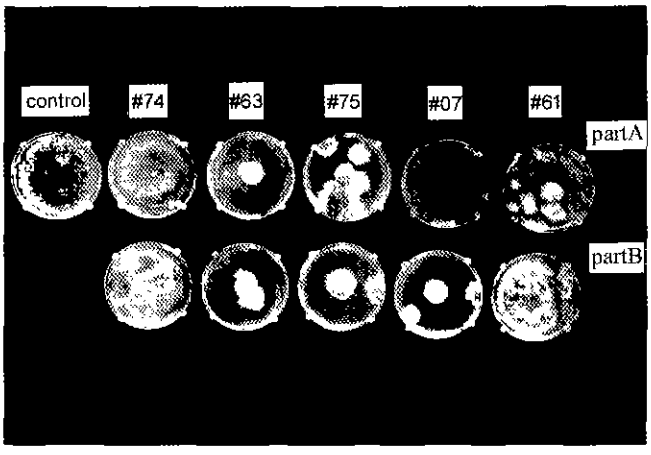

Fig. 3. Growth inhibitory activity of *Aspergillus flavus* by the treatment with culture filtrate of antagonistic bacteria #74, #63, #75, #61, and #07.
Each antagonist culture filtrate (1.0 ml) was poured into a 1-day-old *A. flavus* plate and incubated at 25°C for 7 days.

Treatment of Fungi with Antagonists Culture Filtrate

Antagonist #74 showed 93.3% of the zone of inhibition when its culture filtrate part A was treated. On the contrary, #63, #75, and #07 presented positive effects

Table 4. Growth inhibition of *Aspergillus flavus* on antagonists culture filtrate-containing PDA plates. *A. flavus* was inoculated in the plates containing antagonists culture filtrate and incubated at 25°C for 7 days.

Treatment	Zone of inhibition (%)				
	#74	#07	#63	#61	#75
Culture filtrate (part A)	97.8%	82.2%	71.1%	68.9%	60.0%
Heat-treated filtrate (part B)	62.2%	42.2%	46.7%	37.8%	55.6%

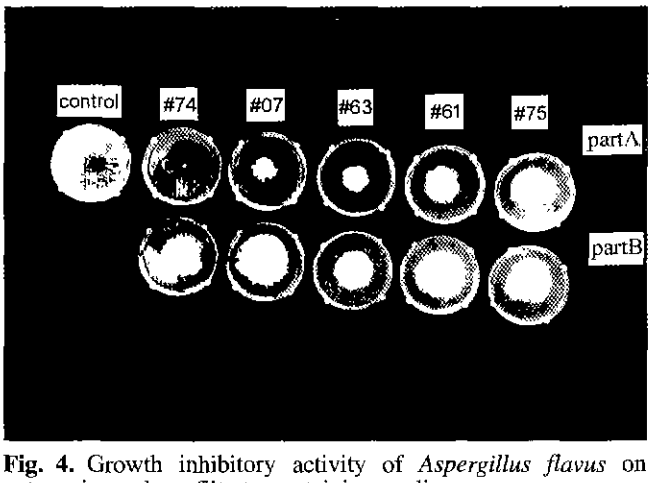

Fig. 4. Growth inhibitory activity of *Aspergillus flavus* on antagonists culture filtrate-containing media.
A. flavus was inoculated in the plates containing antagonists culture filtrate and incubated at 25°C for 7 days.

Table 5. Inhibition of growth and aflatoxin B_1 production in culture filtrate or heat-treated culture filtrate of antagonistic bacteria #74, #07, #63, #61, and #75. *A. flavus* was inoculated in the media containing antagonist culture filtrate and incubated at 30°C for 7 days.

	Growth [DCW/5 ml (%)]		Aflatoxin production [peak area (%)]	
	Culture filtrate (part A)	Heat-treated culture filtrate (part B)	Culture filtrate (part A)	Heat-treated culture filtrate (part B)
A. flavus	11.8 mg (100%)		79.4 (100%)	
#63	0 mg (0%)	0.2 mg (1.7%)	0.4 (0.5%)	0.8 (1.0%)
#75	0 mg (0%)	1.4 mg (11.9%)	1.4 (1.8%)	4.6 (5.8%)
#07	0 mg (0%)	1.4 mg (11.9%)	0.3 (0.4%)	2.1 (2.6%)
#74	0 mg (0%)	0.4 mg (3.4%)	0.8 (1.0%)	1.0 (1.3%)
#61	3.2 mg (27.1%)	3.9 mg (33.1%)	2.1 (2.6%)	4.8 (6.0%)

when treated with the culture filtrate part B (Table 3, Fig. 3). From these results, it was possible that there were not only some enzyme(s) but also some heat-stable material(s) which had suppression activity on fungal growth and/or aflatoxin B_1 production. In the study on the inhibitor of aflatoxin production, *Streptomyces* sp. MRI 142 was found to produce an inhibitor (aflastatin A) of aflatoxin production of *A. parasiticus* and its biological activity suggested that aflastatin A inhibited the aflatoxin production with some morphological changes of the producing organism, without inhibition of the growth [21].

Cultivation of *Aspergillus flavus* in the Media Containing Antagonists Culture Filtrate

When *A. flavus* was cultivated in the PDA plates or PDB medium containing antagonist culture filtrate part A or part B, growth of *A. flavus* was inhibited as much as 60–97% in PDA plates containing part A and inhibited up to 37–62% in plates containing part B. For all the antagonists, culture filtrate part A showed higher inhibitory activity than part B. Part B was shown to contain about 60% of the effect of part A (Table 4, Fig. 4). In PDB media containing antagonist filtrates, the results were much similar to those tested in the PDA plates, and the aflatoxin B_1 production decreased as much as 94–99% (Table 5, Fig. 5). All 5 antagonists showed stronger inhibitory activity with culture filtrate part A compared to part B. Since some inhibitory activity still remained after the heat treatment, it was presumed that some inhibitory substances with different heat-sensitivity factor might existed.

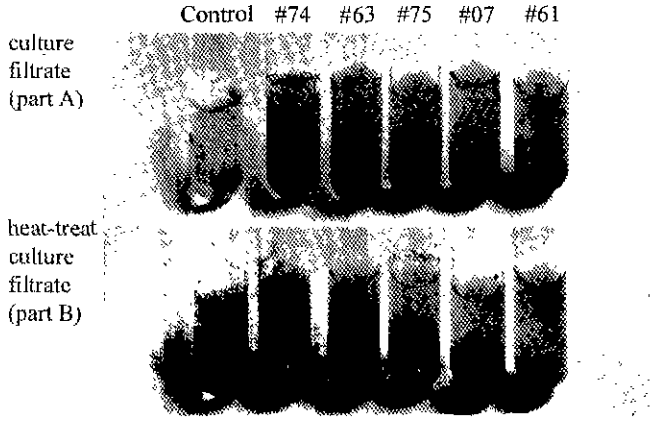

Fig. 5. Inhibitory activity on growth and aflatoxin B_1 production in antagonists culture filtrate-containing media.
A flavus was inoculated in the media containing antagonist culture filtrate and incubated at 30°C for 7 days.

Examination of the Inhibitory Effect by Co-Culture

A. flavus was co-cultured with the antagonists in PDB medium at 30°C. After 4 days, in the co-cultured broth, the maximal production of aflatoxin B_1 was decreased

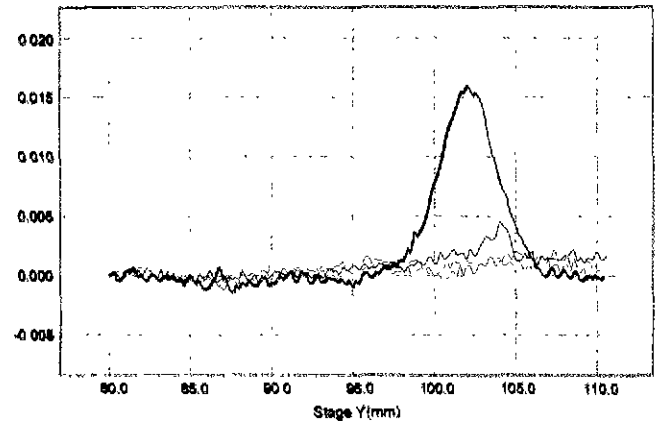

Fig. 6. Inhibition effect of antagonistic bacteria against aflatoxin B_1 production of *Aspergillus flavus* during co-culture.
Aflatoxin B_1 production during co-culture with antagonistic bacteria #07 (blue line), #63 (red line), #74 (pink line), and control (black line).

Table 6. Growth and aflatoxin B_1 production of *Aspergillus flavus* in the co-culture with antagonistic bacteria #63, #74, and #07 for 120 h.

	Growth [DCW/5 ml (%)]	Aflatoxin production [peak area (%)]
A. flavus	32.1 mg (100%)	79.4 (100%)
A. flavus + #63	1.7 mg (5.3%)	2.0 (2.5%)
A. flavus + #74	0.9 mg (2.8%)	2.3 (2.9%)
A. flavus + #07	2.4 mg (7.5%)	5.5 (6.9%)

Table 7. Comparison of the growth inhibition effect of each antagonistic bacteria against *Aspergillus flavus* on SA and PDA media.

Medium	Zone of inhibition (%)				
	#07	#63	#74	#61	#75
SA	40.8%	38.2%	25.1%	22.5%	25.6%
PDA	43.2%	40.5%	37.8%	37.8%	39.2%

compared with the mono-cultured broth of *A. flavus* (Fig. 6, Table 6). It corresponded to 2.0-6.9% of aflatoxin B_1 production in the mono-culture of *A. flavus*.

The Inhibitory Effect on Soybean Agar Plates

On soybean agar, the antagonists also showed some inhibitory activities. Antagonist #07 and #63 had almost the same activity with PDA medium, and #74, #61, and #75 showed somewhat low activities (Table 7). These results indicated that the antagonists suppressed the growth of *A. flavus* and the production of aflatoxin B_1 in *Meju* or *Doenjang*. Therefore, when *Meju* was prepared according to the traditional method, it would be possible to apply some antagonists or their culture filtrates for maintaining *Doenjang*'s hygienical safety and traditional taste and flavor.

Effect of Antagonists on Aflatoxin B_1 Degradation

The antagonists which were grown in the media containing aflatoxin B_1 showed aflatoxin B_1 degraded. Within 5 days of incubation, antagonist #63 showed a 95.5% aflatoxin B_1 degradation rate. Antagonists #07 and #74 showed 80.1% and 75.8% degradation, respectively (Fig. 7, Table 8). According to the results so far gathered, it is obvious that some of the antagonists cells or certain material(s) in the antagonists culture broths not only suppressed the growth of *A. flavus* but also degraded aflatoxin B_1 already formed.

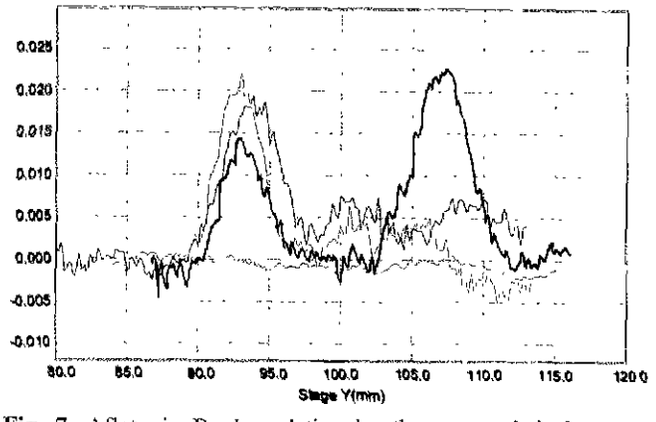

Fig. 7. Aflatoxin B_1 degradation by the antagonistic bacteria #63, #74, and #07.

Aflatoxin B_1 degradation by antagonistic bacteria #07 (blue line), #63 (red line), #74 (pink line), and control (black line).

Table 8. Aflatoxin B_1 degradation by the antagonistic bacteria #63, #74, and #07.

	Antagonist			
	A. flavus	#63	#07	#74
Peak area	114.3	5.1	22.7	27.6
Degradation (%)		95.5%	80.1%	75.8%

However, it was not clear whether the degradation effect was caused by living cells or certain material(s) which was produced by cells. Since there are so many possibilities in determining the types of reaction, simple loss of its fluorescence or uptake by living cell, *etc.* can be seen. An important point is that investigations and reports will continuously be made in the near future.

Among the antagonists #07, #63, #75, #74, and #61 chosen for the antagonist activity test, culture filtrate part A of #63, #07, and #74 showed fairly good effects in the cultivation process of the media containing the culture filtrate. In addition, they contained high activity of fungal growth inhibition and aflatoxin B_1 production, with the aflatoxin B_1 degradation activity of #63 shown to be higher than that of #74. Culture filtrate part A of #75 and #61 also showed good effects in the cultivation process of the media containing the culture filtrate, while those of part B exhibited good effects in treating fungi.

Identification of Antagonist #63

Because the culture filtrate part A of #63 and #74 consistently showed inhibitory effects and #63 had a higher degradation activity, #63 was selected to be the best antagonist. Therefore, the morphological characteristics of #63 were closely examined. The antagonist #63 was determined to be a Gram-negative, motile, non-conidia-forming, and spiral-shaped bacteria. Based on these results, the antagonist #63 was deduced to be a *Azospirillum* sp.

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