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Insecticidal and nematicidal properties of microbial metabolites*

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SUMMARY

Metabolites from 942 microbial isolates were screened for insecticidal and nematicidal properties. The isolates included 302 streptomycetes, 502 'novel' actinomycetes including representatives of 18 genera, 28 unidentified aerobic actinomycetes, 70 fungi and 40 bacteria other than actinomycetes. When diluted 10-fold, the metabolites from 55 isolates caused nearly 100% mortality in mosquito larvae (*Aedes aegypti*) within 24 h. These isolates included 27 isolates of *Streptomyces,* four of *Actinoplanes,* three isolates each of *Actinomadura* and *Streptoverticillium,* two isolates each of *Micromonospora, Bacillus* and *Paecilomyces,* and one isolate each of *Micropolyspora, Nocardiopsis, Streptosporangium, Oerskovia, Thermomonospora, Chainia, Pseudomonas, Fusarium, Monilia* and *Syncephalestrum.* Two fungal isolates could not be identified to the generic level. Extracts from the culture broth of 18 isolates caused 100% mortality in mosquito larvae within 15 min to 24 h at a concentration of 1000 ppm. The LC_{50} of partially purified products from two isolates was 1–2.5 ppm and that of the semipurified preparations from four other isolates was ≤ 50 ppm. Valinomycin was identified as an active component in the culture broth from one isolate. The culture broth from 15 isolates of aerobic actinomycetes and 4 of fungi were toxic to *Panagrellus redivivus* (Nematoda); these included 12 isolates with selective nematicidal properties.

INTRODUCTION

The significance of insects and nematodes to humankind can hardly be overemphasized. A number of synthetic organic and inorganic pesticides have played remarkable roles in increasing crop yields. Compounds such as DDT have been used effectively in managing mosquitoes, lice, flies and other vectors of tropical diseases [6,10]. However, the perpetual emergence of resistant pests and possible long-term harmful effects from pesticides, like carcinogenicity or environmental damage, have provoked serious concerns. Further, certain synthetic pesticides, such as acylamides, phenylureas and phenoxyacetates may break down into more toxic derivatives [22], while others may persist in

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nature as recalcitrant environmental pollutants, injurious to humans and wildlife. Thus, there is need to develop non-polluting safe pesticides. An ideal pesticide should have a number of properties, including selective toxicity, and be decomposable into smaller harmless molecules such as water, carbon dioxide and ammonia. Microbial products, by virtue of their selectivity and well known sensitivity to light, air and other environmental factors, including action by other microorganisms in the ecosystem, may provide such properties. Furthermore, unlike the inherent dangers associated with the production process of synthetic pesticides, the manufacturing process for microbial products are safe, well contained and less polluting.

Microorganisms have been extensively screened for antibiotics for therapeutic use. Except for a limited number of reports [1,17,24], little has been published regarding screening of microorganisms for the production of metabolites toxic to insects or nematodes. In this communication we present results of a systematic screening program aimed at developing commercially exploitable microbe-produced insecticidal and nematicidal chemicals as alternatives to synthetic pesticides. The microorganisms examined include various genera of fungi, bacteria other than actinomycetes and common as well as novel actinomycetes. Special attention was given to novel actinomycetes because the techniques for their isolation and identification are not widely known. Isolation media developed in our laboratory [20] enabled frequent isolation of this group of actinomycetes which are considered 'rare'. Entomopathogens (microorganisms that infect insects) were not included in the present study.

MATERIALS AND METHODS

Isolation of microorganisms from soils

Eighteen samples of soil collected from cultivated fields, indoor potted plants and parks and lawns were examined. Particulars concerning the methods of collection and composition of the media used for the isolation of microorganisms are reported elsewhere [20]. One gram of the soil sample was suspended in 9.0 ml of sterile 0.85% aqueous NaCl and serial 10-fold dilutions were prepared; 1.0 ml of the suspension was mixed with 14 ml of the isolation medium held at 45°C and poured into 100-mm (diameter) petri dishes. Two plates each, of starch-casein agar, NZ amine-A agar, threonine agar and asparagine-biphenyl agar were used for each dilution. These media were formulated to facilitate isolation of a wide range of actinomycetes, including common as well as rarer types [20,21]. Nutrient agar (Difco Laboratories) supplemented with the antifungal antibiotics, cycloheximide and nystatin, and Czapek's agar containing the antibacterial antibiotic chloramphenicol were used for the isolation of bacteria other than actinomycetes and fungi, respectively. Cultures were incubated at 25°C. Colonies of bacteria, fungi and the fast-growing actinomycetes were picked up after 3, 5 and 7 days, respectively and subcultured on YMG agar (yeast extract 4 g, malt extract 10 g, glucose 4 g, bacto agar 18 g, distilled water 1 l, pH 7.2). Colonies of slow-growing actinomycetes usually appeared 2–3 weeks later. After a suitable period of incubation (2–4 weeks), cultures of the actinomycetes were streaked on NZ-amine agar plates to determine their purity and generic identity as described in earlier publications [20,21]. Cultures that required more than 2 weeks of incubation were sealed in sterile polyethylene bags to protect the agar from drying. Chemotaxonomic and physiological characterizations followed the methods of Lechevalier and Lechevalier [14], Mishra and Gordon [18] and Mishra et al. [19].

Bioassay

After identification, cultures were grown in 500-ml baffle-bottomed Erlenmeyer flasks containing 100 ml of liquid YMG or A-9 medium (peptone 5 g, glucose 10 g, molasses 20 g, distilled water 1 l) of Warren et al. [31]. Typically, A-9 medium was used for fungi and the fast-growing actinomycetes while the YMG was used for bacteria and slow-growing or fastidious actinomycetes. The flasks were placed on a rotary shaker at 200 rpm and incubated at 26°C for 5–7 days (fast growers) or 10–14 days (slow growers). The bioassays for insecti-

cidal properties were performed using mosquito larvae, *Aedes aegypti* (Rockfeller strain), reared in the Pesticide Research Center at Michigan State University. The sensitivity of this method has been established in a comparative study employing a number of insects of agricultural importance and commercial insecticides. In this method, ten fourth instar larvae were placed in 90 ml of distilled water in 180 ml clear plastic urinalysis cups. Ten milliliters of culture broth were added to each cup with mild agitation. Controls received 10 ml of the liquid medium. The cups were covered to prevent evaporation and stored at room temperature. The number of dead larvae was recorded after 2, 24 and 48 h. The results were rated Highly Toxic Rapid (HTR) if more than 50% of the larvae died within 2 h and Toxic Rapid (TR) when more than 50% larvae died between 2 and 24 h. Mortalities after 24 h were recorded but the results are not presented here unless the growth and development (GDA) was significantly inhibited or there was clear evidence of molting inhibition. Where positive results were obtained, the bioassay was repeated using 10- and 100-fold dilutions of the culture broth.

The bioassays for nematicidal activities were performed using a culture of the free-living nematode (*Panagrellus redivivus*). Tarjan [27] conducted tests with several species of nematodes, both plant-parasitic and free-living, and several different chemicals, and concluded that *P. redivivus* is suitable for use in nematicide screening.

The nematodes were reared in our laboratory in 250-ml Erlenmeyer flasks containing 5 ml of A-9 medium. A 0.25 ml portion of *P. redivivus* culture (2–3 weeks old) was diluted in 4.75 ml of sterile YMG or A-9 broth. A 45-μl portion of the diluted suspension containing 30–50 nematodes at various developmental stages was transferred into each well (0.7 cm diam. $\times$ 1.0 cm deep) of a 96-well Corning flat-bottomed tissue culture plate. Five microliters of the microbial culture broth were then added to each well and mixed gently. The inoculated plates were held in a glass container at ca. 100% relative humidity to prevent evaporation.

The nematodes were examined with an inverted microscope at a magnification of 40 $\times$. The effect of the test broth was first made by observing motility relative to a negative control that received 5 μl of YMG or A-9 medium. Actively moving nematodes had not been killed and the test broth could be scored as inactive. If all or most of the nematodes were motionless, then a decision had to be made as to whether they were dead or immobile. Usually the decision could be made by making a direct comparison to a positive control that received 5 μl of culture broth of a known nematicide (valinomycin) producer. Mortality and mobility were recorded after 4, 24, 38 and 96 h. Nematodes immobilized by lack of oxygen or exposure to a test chemical would have recovered and become motile by the time the last score was done, but dead nematodes would appear as those in the positive control.

The results were rated Highly Toxic Rapid (HTR) if more than 50% of the nematodes died within 4 h, Highly Toxic Slow (HTS) when more than 50% died between 4 and 48 h and Toxic Rapid (TR) when more than 50% died between 4 and 24 h. Instances of mortalities after 48 h are not presented here. Where positive results were obtained, the microbial culture broth was diluted 10- and 100-fold and the bioassay was repeated.

Extraction and purification

Promising isolates were grown in larger batches in shake culture using 400 ml medium in 2-l flasks which were agitated at 150 rpm, or in 100-l fermentors, containing 70 l medium, aerated at 100 l/min and stirred at 200 rpm. A silicone polymer (antifoam A, Sigma) was used to prevent foaming and the pH was maintained between 6 and 7. After an optimal period of growth (5–7 days for fast growers and 10–14 days for slow growers), the culture broth was centrifuged and the active portion (the cell pellet or the clear broth) was extracted with methylene chloride (with or without 25% v/v methanol) or *n*-butanol and dried in vacuo. Preliminary purification of the crude extracts thus obtained was carried out by flash column chromatography or by solvent extraction. For flash column chromatography, unless otherwise stated, silica gel adsorbent and methylene chloride/methanol solvent mixtures were

used. In the case of solvent extraction, the crude extract was washed first with hexane and the residue was then dissolved in water and partitioned with various solvents. All final purifications were done using thin layer chromatography (TLC) (silica; ethyl acetate/chloroform/methanol, 1:1:2, v/v) or high performance liquid chromatography (HPLC) (C-18 reversed phase; methanol/water, acetonitrile/water solvent systems). Mass spectra (EI, 70 eV) were obtained on a Hewlett-Packard, model No. 5985, computer model 7900 equipped with disc drive.

RESULTS

A total of 942 isolates was examined. These included 70 isolates of fungi, 40 isolates of bacteria other than actinomycetes and 832 isolates of aerobic actinomycetes. The fungi included representatives of the genera *Aspergillus* (16), *Penicillium* (15), *Paecilomyces* (10), *Cladosporium* (10), *Scopulariopsis* (8), *Syncephalastrum* (5), *Monilia* (2) and four unidentified isolates. Of the bacteria, 17 were *Pseudomonas* species, 15 belonged to the genus *Bacillus* and eight isolates were not identified. The study was primarily focused on the aerobic actinomycetes, which included representatives of 19 genera (Table 1). The members of the genus *Streptomyces* accounted for the largest number (32%), followed by *Micromonospora* (14%), *Actinomadura* (6%) and *Actinoplanes* (5%). The remaining 14 genera accounted for 28% of the isolates (Table 1). Twenty-eight isolates could not be identified to the generic level. Most of the streptomycetes and acti-

Table 1

Insecticidal and nematicidal activity of metabolites from microorganisms, as measured by *Aedes aegypti* and *Panagrellus redivivus* bioassays, respectively

Genera represented	Number of isolates examined	Number of isolates with	
		insecticidal activity	nematicidal activity
Streptomyces	302	26	12
Micromonospora	134	2	0
Actinomadura[a]	59	3	0
Actinoplanes	46	4	0
Rhodococcus	26	0	0
Micropolyspora	24	2	1
Nocardia	22	0	0
Nocardiopsis[a]	22	1	0
Streptosporangium	22	1	0
Oerskovia	21	1	0
Thermomonospora	21	1	0
Thermoactinomyces[b]	19	0	0
Streptoverticillium	18	3	1
Saccharomonospora viridis	16	0	0
Promicromonospora	15	0	0
Dactylosporangium	11	0	0
Microbispora	11	0	0
Chainia	8	1	0
Pseudonocardia	7	0	0
Unidentified actinomycetes	28	0	1
Bacteria (other than actinomycetes)	40	3	0
Fungi	70	7	4

[a] Use of these generic names does not imply authors' endorsement regarding their validity.

[b] *Thermoactinomyces peptonomphilus*-like organisms.

nomadurae were isolated on starch casein agar, while asparagine biphenyl medium facilitated greater isolation of micromonosporae and streptomycetes of '*S. hygroscopicus*' type. NZ amine-A agar supported growth of a wide spectrum of actinomycete genera and most of the 'rare' types were isolated on this and on threonine agar.

As is evident from the data presented in Table 1, metabolites from 55 isolates inflicted appreciable mortality (HTR or TR) on mosquito larvae. These included 45 isolates of actinomycetes, seven isolates of fungi and three isolates of bacteria. The actinomycetes included representatives of 11 genera though a majority of the toxigenic isolates belonged to the genus *Streptomyces*. Of the seven fungal isolates, two were *Paecilomyces* spp., and one each belonged to *Syncephalastrum, Fusarium* and *Monilia;* two isolates could not be identified. The toxigenic bacteria included two isolates of *Bacillus* and a *Pseudomonas* sp. Nearly 16% of *Streptoverticillium* isolates were toxigenic, followed by 9% each of *Streptomyces* and *Actinoplanes*. About 5% of *Actinomadura* isolates were rated HTR or TR. Though the frequency of positive effects among the members of other genera was low, some of our promising isolates were identified as *Micromonospora* (isolate 43097), *Micropolyspora* (isolate 536) or *Thermomonospora* (Isolate 32063). The metab-

Table 2

Particulars for 22 promising isolates with insecticidal activity as measured by *Aedes aegypti* bioassay

Isolate number	Identification	Estimated LC_{50} (broth dilution)	Toxicity of crude extract	
			extracted in (solvent)	percent[c] mortality in 24 h (1000 ppm)
31013	*Streptomyces* sp.	10^{-2}	butanol	50 TR
32041	*Streptomyces* sp.	10^{-2}	butanol	100 TR
36011	*Streptomyces* sp.	10^{-1}	ethyl acetate	90 TR
ATCC 31267	*S. avermitilis*[a]	10^{-2}	ethyl acetate	100 TR
04020	*S. griseus*[b]	10^{-4}	methylene chloride/methanol	100 (2 h) HTR
758	*S. griseus*	10^{-2}	butanol	100 (6 h) HTR
502	*S. hygroscopicus*	10^{-2}	butanol	100 HTR
509	*S. hygroscopicus*	10^{-3}	butanol/ethyl acetate	100 TR
45010	*S. hygroscopicus*	10^{-4}	ethyl acetate	100 HTR
577	*S. lavendulae*	10^{-2}	butanol	100 (15 min) HTR
32001	*S. lavendulae*	10^{-4}	methylene chloride/methanol	100 TR
618	*Streptoverticillium*	10^{-3}	butanol	100 TR
40029	*Streptoverticillium*	10^{-3}	butanol	100 TR
32063	*Thermomonospora* sp.	10^{-2}	butanol/ethyl acetate	100 TR
43097	*Micromonospora* sp.	10^{-4}	butanol	100 TR
536	*Micropolyspora* sp.	10^{-2}	butanol/ethyl acetate	100 TR
43099	*Micropolyspora* sp.	10^{-2}	butanol	100 GDA
42031	*Chainia* sp.	10^{-2}	butanol/ethyl acetate	100 TR
44006	*Pseudomonas* sp.	10^{-2}	butanol/ethyl acetate	50 TR
36015	Unidentified fungus	10^{-2}	ethyl acetate	90 TR
39008	*Syncephalastrum* sp.	10^{-3}	butanol	100 (15 min) HTR
43003	*Fusarium* sp.	10^{-1}	butanol/ethyl acetate	100 TR
44013	*Paecilomyces* sp.	10^{-2}	ethyl acetate	100 TR

[a] Known producer of avermectins (included as control).

[b] Produces valinomycin and nikkomycin.

[c] The active compounds are expected to be in very small amounts.

olites from six isolates were rated HTR; five of these belonged to the genus *Streptomyces* and one was a fungus identified as *Syncephalastrum* species.

The culture broth from five isolates affected the growth and development (GDA) of mosquito larvae or inhibited molting. These included one isolate each of *Streptomyces hygroscopicus* (isolate 26159), *Streptosporangium* (isolate 36030), *Actinomadura* (isolate 45020), *Micromonospora* (isolate 28004) and *Micropolyspora* (isolate 43099).

The crude extracts from 22 promising isolates were tested (at a concentration of 1000 ppm) against mosquito larvae. A 100% mortality was observed with 18 isolates, 90% with two and 50% with two other isolates (Table 2). Among the fast-acting were the crude extracts from two isolates that killed all the larvae in 15 min. One of these (isolate 577) was identified as *S. lavendulae* and the other (isolate 39008) as a fungus, *Syncephalastrum* sp. An isolate of *S. lavendulae* (isolate 32001) and a *Micromonospora* sp. (isolate 43097) whose estimated LC_{50} was 10^{-4} broth dilution (Table 2) yielded some unusual results. The mosquito larvae treated with crude extracts from the two isolates were so darkened that they appeared charred. Prior to death, the larvae treated with *Micromonospora* (isolate 43097) showed symptoms of spasm, twisting and curling. The toxin from this isolate also caused 100% mortality when administered (1000 ppm) to the diet of adult house flies, *Musca domestica*.

Valinomycin, an insecticidal compound, was isolated and identified from the mycelia of *Streptomyces griseus* (strain 04020). The identity of these compounds were achieved by spectral means. The IR (in dichloromethane) bands at 1755 (ester), 1660 (amide) and at 1540 (amide) cm^{-1} were characteristic of valinomycin. High-resolution mass spectra of this compound gave the molecular ion at m/z 1110.6311 and analyzed for the molecular formula $C_{54}H_{90}N_6O_{18}$. The ^1H- and ^{13}C-NMR of valinomycin from 04020 were identical to those of a commercial sample of valinomycin (Sigma Chemical Company, St. Louis, MO) [25]. The *S. avermitilis* strain ATCC 31267 obtained from the American Type Culture Collection is a known producer of avermectins and was used as a control. Though the purified preparation from this isolate is extremely toxic, its culture broth appeared less toxic than that from several of our isolates (Table 2). The toxic products from the remaining 21 isolates (Table 2) were not identified as any of the known microbial insecticides. The extracts from four isolates (577, 758, 32001 and 39008) were purified by flash column and the various fractions were bioassayed. The ultraviolet absorption maxima of the semi-purified compounds from *S. lavendulae* (isolate 577) were at 232, 342, 360 and 381 nm, from *S. lavendulae* (isolate 32001) were at 243, 275 and 385 nm, from *S. griseus* (isolate 758) at 260 and 360 (broad peak) nm, and from *Syncephalastrum* (isolate 39008) at 208 nm with shoulders at 226 and 278 nm. The LC_{50} of the semi-purified compounds from these four isolates were ≤ 50 ppm.

Two compounds, A and B, obtained from the mycelia of *S. lavendulae*, isolate 32001, were highly toxic ($LC_{50} < 2.5$ ppm after 72 h). The mycelium of *S. lavendulae* was extracted with methylene chloride/methanol (1:1, v/v). Evaporation of the extract under reduced pressure followed by washing of the dried crude extract with hexane afforded a yellow residue. This was stirred with water and filtered (Whatman No. 42 paper). The filter residue was redissolved in methylene chloride/methanol, filtered, and the residue was discarded. The aqueous filtrate from the water treatment was extracted with methylene chloride. This methylene chloride extract was combined with the previous methylene chloride/methanol extract and evaporated to dryness in vacuo. The crude extract thus obtained was fractionated on a silica gel flash column and eluted with 30% methanol in methylene chloride (fraction I). Other fractions were collected by eluting the column with 40% methanol (fraction II), 50% methanol (fraction III) and 100% methanol (fraction IV). All fractions were bioassayed. Fractions II–IV were not active, hence kept aside. Fraction I was evaporated to dryness in vacuo and further purified on silica gel TLC. Three major bands collected from the TLC had R_f values of 0.75 (band S), 0.19 (band U) and 0.12 (band L). Bioassay results indicated activity for bands U and L. These active fractions were finally purified by HPLC (C-18; meth-

anol/water, 70:30, v/v) and yielded compounds A and B, respectively. Both compounds showed similar ultraviolet absorption maxima, at 243, 275 and 385 nm. However, the EI mass spectra of compounds A and B showed highest mass peaks at m/z 535 and 509, respectively. Their fragmentation patterns in the mass spectra were also different. More analytical work to elucidate the structures of A and B is in progress.

The *Panagrellus redivivus* bioassay revealed activity in the culture broth from 19 isolates (Table 3). These included 15 isolates of aerobic actinomycetes: 12 isolates of *Streptomyces* and one each of *Streptoverticillium*, *Micropolyspora* and an unidentified actinomycete. The toxigenic fungi included one isolate each of *Aspergillus*, *Penicillium* and *Monilia* species, and an unidentified hyphomycete. Metabolites from bacteria and several other genera of actinomycetes were not toxic to the nematodes. The culture broth from 12 isolates showed selective nematicidal property, while the broth from the remaining seven isolates caused 100% mortality among the mosquito larvae within 2–24 h. The active components in the metabolites from a *S. griseus*, isolate 04020, was identified as valinomycin. The anthelmintic agents in the culture broth from other isolates could not be identified as any of the known nematicides.

DISCUSSION

Insecticidal and anthelmintic properties of a limited number of microbial products (secondary metabolites) have been reported during the past few years. These include avermectins [4,24], nikkomycins

Table 3

Particulars about 19 isolates with nematicidal property, as measured by *Panagrellus redivivus* bioassay

Isolate number	Identification	Nematicidal property[a]		Mosquitocidal property
		toxicity	estimated LC_{50} (broth dilution)	
26039	*Streptomyces* sp.	HTR	10^{-1}	HTR
26070	*Streptomyces* sp.	HTS	10^{-2}	None
26167	*Streptomyces* sp.	HTS	10^{-2}	None
42008	*Streptomyces* sp.	TR	10^{-1}	None
42014	*Streptomyces* sp.	TR	10^{-2}	None
04020	*S. griseus* sp.	HTR	10^{-2}	HTR
758	*S. griseus* sp.	TR	10^{-1}	HTR
26151	*S. hygroscopicus*	HTS	10^{-2}[b]	None
26152	*S. hygroscopicus*	HTR	10^{-2}	HTR
30006	*S. hygroscopicus*	HTR	10^{-1}	None
30010	*S. hygroscopicus*	TR	10^{-1}	None
577	*S. lavendulae*	TR	10^{-2}	TR
539	Unidentified actinomycete	TS	10^{-3}	None
618	*Streptoverticillium* sp.	TR	10^{-1}	TR
536	*Micropolyspora* sp.	TR	10^{-1}	TR
43004	*Aspergillus* sp.	TR	10^{-1}	None
43006	*Penicillium* sp.	TR	10^{-1}	None
43010	*Monilia* sp.	TR	10^{-1}	None
44017	Unidentified fungus	TR	10^{-1}	None

[a] HTR (Highly Toxic Rapid), HTS (Highly Toxic Slow), TR (Toxic Rapid).
[b] Kills 100% nematodes in less than 4 h at 1:4 dilution.

[7,9], milbemycins [2,26], tetranectin [1,23] and aureothin [1,23], all of which are produced by members of the genus *Streptomyces.* The avermectins and milbemycins are structurally related and belong to a new family of macrolide antibiotics with activity against a wide range of nematodes and arthropods. Nikkomycins are nucleoside peptide antibiotics that inhibit chitin synthesis of fungi and insects. Tetranectin is a selective miticide and has been in use in Japan since 1974 [17]. Insecticidal properties of several other microbial products described earlier as antimicrobial or antitumor agents have been rediscovered lately. For example, valinomycin, a depsipeptide antibiotic with potent insecticidal and anthelmintic activity [25] was discovered in the 1950s [3] as an antibacterial substance. Similarly, piericidins and antimycins, now recognized as highly active insecticides [1], were initially discovered as fungicides [28]. Some of the amino glycosides like hygromycin [13] and G-418 [29] also appear to be promising anthelmintics.

During the past few decades, a number of entomopathogens have been recognized as promising biocontrol agents for pests. Among the best known are species of the genus *Bacillus*, expecially *B. thuringiensis, B. sphericus* and *B. popilliae,* and several insect pathogenic fungi, such as *Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii* and *Entomophthora* spp. [5,8]. In fact, cultures of *B. thuringiensis* have been marketed since 1958 in the U.S.A. and several European countries for insect control. The idea of using live microorganisms for pest control is, therefore, appealing, as is evident from the concentration of current research efforts on 'improving' the microorganisms through advanced techniques in molecular biology and genetic engineering [16]. This approach has, however, some serious drawbacks. For example, the optimal efficacy of a majority of entomopathogens depends on specific climatic conditions including high humidity and minimal solar radiation. Thus their effectiveness is not always predictable. Second, though inability of a microorganism to cause infection and allergy in humans and animals is apparently a rigid prerequisite to their approval as a biocontrol agent, hardly any data are available regarding their potential to cause infection in subjects with impaired immunity. In this era of stress, organ transplantations, widespread use of corticosteroids, broad-spectrum antibiotics, cytotoxic and other powerful drugs, it is hard to draw a line between the human 'pathogenic' and 'non-pathogenic' species of microorganisms [12,19,32]. If the exposure is massive and the host immunity is severely impaired, the possibility of a fatal secondary complication by any microorganism cannot be ruled out. Third, there is no information about what effect, if any, introduction of a large amount of any one microbial species has on the biological functions of the autochthonous microbial community. Preliminary data from our laboratory indicate a drastic decline in the population density of *Micromonospora* and several fungal species if a large amount of the cultures of *Bacillus* or *Pseudomonas* species is introduced into potted soils. It is noteworthy that *Micromonospora* spp. are known for the production of cellulases and other biopolymer-degrading enzymes [11], and fungi are believed to play a dominant role in the degradation of lignin. In general, the population density of the microbial community is delicately balanced in nature and the microorganisms probably act in concert to recycle the biomass in the ecosystem [15,30]. Therefore, alterations in the natural microbial population may, in the long run, seriously affect the turnover of carbon, nitrogen and other elements or may even alter the biota in the ecosystem. Therefore, Luthy and Arif's [16] caution, 'absence of live microorganisms from the formulated product would be preferable because of safety reasons', deserves consideration. The results of the present study suggest that microbe-produced chemicals, while likely to be more expensive, may produce fewer harmful side-effects.

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