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Staphylococcus aureus, Bacillus subtilis and *Escherichia coli* induced copious production of antibiotics in an overnight co-culture with three soil fungi

Ngwoke, Keneth G; Nwoye, Obioma V; Okoye, Festus BC; Proksch, Peter

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Background: There is need for the development of new antibiotics to combat new resistant strains of pathogens. Currently, majority of the antibiotics in use were derived directly or indirectly from soil organisms because of the oligotrophic condition of the soil which lead to competition and production of antibiotics. Co-cultivation has been used to mimic this condition and the result is promising.

Objectives: To study the effect of co-culture in the production of antibiotic by 3 soil fungi.

Method: Nine fungal strains were isolated from soil sample using soil dilution method. Their antibiotic production potentials were evaluated using agar overlay method. Four isolates with large inhibition zone diameters against some bacteria were fermented in the same medium. The medium was extracted with ethyl acetate after 21 days and the extract tested for antibacterial activities against the same bacteria used earlier. The isolates that showed high potential for antibacterial activities were identified through a series of molecular methods: DNA isolation, PCR amplification, sequencing of the ITS region and nucleotide sequence blasting. Their fermentation extracts were subjected to analytical High Performance Liquid Chromatography (HPLC). The chromatographic peaks were identified by dereplication.

Result: Four isolates (labelled FG2, FG4, FG8 and FG9) had profound antimicrobial activity. Significant differences in the inhibitory activity were however observed between the pure isolates and their fermentation extract as results of inhibition zones in the fermentation extracts were 1-3 mm compared to the overlay method where the inhibition zone diameters ranged from 17-26 mm. The HPLC analysis revealed a diketopiperazine, 6-cyclo-(S-Pro-R-Leu) through dereplication. Diketopiperazines are known to have antibacterial, antifungal and anticancer properties and could have contributed to the observed antibacterial activity.

Conclusion: The co-cultivation of bacteria and soil fungi which mimicked the natural environment led to the copious production of antibiotics in the overlay experiment.

Keywords*: Co-cultivation, co-culture, soil fungi, bacteria, antibiotics antimicrobial production, fermentation.*

Highlights:

- Co-cultivation induced *Trichoderma longibrachiatum* to produce antibacterial metabolites which has not been reported before.
- *T. Longibrachiatum* produces broad spectrum antibacterial metabolites.
- *T. erinaceum* produce metabolites that inhibit Gram-positive organisms.
- *Pseudomonas aeuroginosa* induce the organisms to produce antipseudomonal organisms under 24 hours.

Kenneth G. Ngwoke*
[a]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnmadi Azikiwe University, Nigeria.
Email:kg.ngwoke@unizik.edu.ng
Phone Number: +2349097627744

Obioma V. Nwoye; Festus B.C. Okoye

*Correspondence

Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnmadi Azikiwe University, Nigeria.

Peter Proksch
Institute of Pharmaceutical Biology, Heinrich Heine University, Dusseldorf, Germany

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Introduction

The oligotrophic condition of the soil creates a highly competitive environment for the survival of soil microbiota. These organisms protect themselves by means of antagonism through the production of antibiotics, mycoparasitism and contact inhibition.[1] They have been investigated for their antibiotic production potentials on the basis of their ability to survive in nutrient depleted terrestrial habitats amidst other competing threats.[2] The secondary metabolites produced during these processes have been used as antibiotics and anticancer agents in modern medicine.[3,4,5] For instance, plectasin, an antimicrobial peptide in the defensins class of antibiotics, was isolated from the soil fungus *Pseudoplectania nigrella*.[6]

These organisms produce these antimicrobials to inhibit the growth of other organisms in their ecological niches in order to enhance their own survival.[7,8] This means that when they are cultivated in axenic cultures as it is often carried out in a laboratory environment, some of the antibiotic producing mechanisms are silenced because there is no stress or perceived enemy in the medium which at the same time provides adequate nutrient for growth. Because there is no competition, aggression or nutrient depletion, the organisms end up producing regular molecules that may not have antibiotic properties and when they have antibiotic properties, may not be as potent as those produced in the presence of competing threats.

Co-cultivation has been used to mimic the natural environment to a level. In co-cultivation, at least one or more organisms are introduced and enabled to grow in the culture of a fungus of interest.[9,10] The purpose is to introduce competition between the organisms so that the main organism would see the second as a threat and as such make effort to eliminate it. To do this, it will have to activate silence gene clusters that will be responsible for the production of new antibiotics.[11]Alexander Fleming's experiment could be referred to as an inadvertent co-cultivation experiment. In this study, we report the positive influence of overnight co-cultivation on the antibiotic production of soil fungal isolates compared to antibiotic production of the same organisms in three-week axenic culture condition

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Methods

Test Isolates

Pure cultures of bacterial isolates for the preliminary *in-vitro* antimicrobial assay were obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka-Nigeria. The cultures were maintained on nutrient agar slants prior to test. The microorganisms used for antimicrobial assays in this study were 2 Gram positive and 3 Gram negative human pathogens. The Gram positives were *S. aureus* and *Bacillus subtilis* while Gram negatives were *P. aeruginosa*, *Escherichia coli* and *Salmonella typhi*. The fungi strains were *Aspergillus niger* (mould) and *Candida albicans* (yeast). The cultures were stored at 4 – 8 ºC in Mueller Hinton Agar (Oxoid, UK) for the bacteria and Sabouraud dextrose agar (Oxoid, UK) for the fungi. These are clinical isolates whose susceptibilities to commonly used antibiotics were already established.

Sample Collection

A 100 g of soil samples was collected aseptically from the back of Chemistry Laboratory of the Faculty of Pharmaceutical Sciences, Agulu Campus of Nnamdi Azikiwe University in Anaocha Local Government Area, Nigeria. A sterile spatula was used to collect the soil sample into a sterile sample container. After scraping off the top soil layer, the sample was collected from the top 10 cm of the soil and immediately taken to the Pharmaceutical Microbiology & Biotechnology Laboratory, where it was used.

Isolation and purification of soil fungi

The soil fungi were isolated by the Soil Dilution Techniques (SDT) using the pour plate method described by Warcup[12] on Malt Extract Agar (MEA) (Oxoid, UK).One gram of the soil sample was suspended in 10 mL of double-distilled water and was serially diluted to make microbial suspensions (10^{-1} to 10^{-5}). Dilutions of 10^{-2} to 10^{-4} were taken and introduced into differently labelled sterile Petri-dishes (triplicate of each dilution). Unto the Petri dishes, sterile MEA was added and swirled evenly to ensure homogeneity of the mixture and production of discrete colonies. Chloramphenicol 50 mg/L was added to the MEA medium to inhibit bacterial growth. The Petri dishes were left to set for 30 min on the bench before incubation at room temperature for 7 days in the dark. At the end of the incubation period, the culture plates were inspected. Based on differences and similarities in the colony morphology of the mixed cultures, purification was carried out by sub-culturing onto freshly prepared MEA. This process was repeated until pure cultures were obtained. A

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total of nine pure isolates labelled FG1 - FG9 were successfully obtained.

Assay for antibiotic production by isolates

Method used by Chen et al.[13] was employed to determine the antibiotic production potential of fungal isolates against common human pathogens. Briefly, the culture of each pure fungal isolate was aseptically cut and placed on Mueller-Hinton agar previously inoculated with the different test organisms. These were incubated at 37°C for 24 h, after which the zone of inhibition of each test organism from the overlaid fungal culture was measured in millimeters.

After this assay, isolates lacking antimicrobial activity were discarded while those exhibiting activities were subjected to further investigation. This test was carried out in triplicates.

Small scale fermentation and extraction

This was carried out using the multiple-plate method in which multiple cultures of each fungal isolate were grown in 30 sterile Petri dishes containing MEA for a total of 21 days at 28°C in the dark. At the end of the fermentation period, solid fermentation media were cut into small pieces using sterile spatulas. Exactly 500 mL of ethyl acetate (Sigma-Aldrich, Germany) was poured into appropriately labelled conical flasks and the fungi transferred into their respective flasks and agitated for two days. Thereafter filtration was carried out and the filtrates concentrated using the vacuum rotary evaporated at 45°C.

Antimicrobial assay on the extracts

The antimicrobial assay for each of the crude extract was carried out using the agar well diffusion assay as described by Portillo et al.[14]
Briefly, sterile agar plates were inoculated with the test culture by the surface spreading method using sterile cotton swab which was dipped into each of the inoculums and any excess was removed by rotating the swab several times against the inside wall of the tube above the level of the fluid. This was done to obtain uniformity of the inoculums. A sterile cork borer was used to make six wells (6 mm in diameter) on each of the agar plates.
Aliquots (60 µl) of each extract dilutions, reconstituted in DMSO at concentrations of 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 mg/mL were applied in each of the wells in the culture plates previously seeded with the test organisms. Gentamicin (10 µg/mL), Nystatin (50 µg/mL) and DMSO served as the positive and negative controls respectively. The cultures were incubated at 37°C for 18-24 h (bacteria) and 25-27°C for 48 h(fungi) respectively to allow the growth of microorganisms. The antimicrobial potential for each extract was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). For each extract, three replicates were conducted against each organism. Each extract was tested against all the bacteria and fungi isolates.

High Performance Liquid Chromatography (HPLC)

The extracts of the four isolates were subjected to HPLC for preliminary identification of their components. Exactly 1 mg/mL of each extract was dissolved in HPLC grade methanol, sonicated for 10 min and centrifuged at 3,000 rpm for 5 min. A 1:5 serial dilution was carried out to get 0.2 mg/mL solution and 20 µL of those solutions was analysed in Dionex HPLC system equipped with photodiode array detector (UVD340s, Dionex Softron GmbH, Germany) using 125 mm Eurosphere-10 C18 prefilled column (Knauer, Germany) with 4 mm internal diameter and 5 µm particle size. The mobile phase comprised of nano-pure water adjusted to pH 2 with formic acid and methanol. Separation was monitored at 254 nm and peaks were identified by dereplication.

Identification of fungal cultures

The soil fungal isolates were identified according to a molecular biology protocol by DNA amplification and sequencing of the ITS region. The sequence data have been submitted to GenBank, with accession numbers KY034289 for *Trichoderma erinaceus*, KY034290 for *Trichoderma longibrachiatum* and KY034291 for `*Cladosporium cladosporioides*. Voucher strains (Reference Numbers FG2, FG4 and FG8) were kept in the Institute of Pharmaceutical Biology and Biotechnogy, Universität Düsseldorf, Germany.

Results

Nine fungi isolates were purified from the soil sample, out of which four had profound antimicrobial activity (Table 1). These four were the isolates labeled FG2, FG4, FG8 and FG9. Differences in activity were however observed between the pure isolates assayed in Table 1 and their fermentation extracts (Tables 2-4).
The isolates that showed high potential for antibacterial activities were identified through a series of molecular methods that involves DNA

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isolation, PCR amplification, sequencing of the ITS region and nucleotide sequence blasting. FG2 was identified as T. longibrachiatum, FG4 identified as C. cladosporoides and FG8 as T. erinaceum. Their respective GenBank accession numbers are indicated above. FG2 and FG4 were active against S. aureus, B. subtilis and E. coli in the overlay experiment (Table 1). However, while the extract of FG2 was active against Bacillus only (Table 2), FG4 had activity against Bacillus, Pseudomonas and Candida (Table 3). Sample FG8 initially inhibited the growths of Staphylococcus and Bacillus while its extract was active against Bacillus as well as Candida (Table 4). As could be seen from Tables 1-5, the potency of the isolates in the overlay experiment was far higher than that of the extracts. The inhibition zone diameters in the overlay experiment were generally many times larger than those of the extracts in the later experiment.

Table 1: Results of antimicrobial activity of FG1 - FG9 against the test bacteria in overnight co-cultivation experiment

	Fungi Isolates/inhibition zone diameter (mm)								
Test organisms	*FG1*	*FG2*	*FG3*	*FG4*	*FG5*	*FG6*	*FG7*	*FG8*	*FG9*
S. aureus	0.00	14.5±.05*	0.00	16.5±.12	0.00	0.00	0.00	6.00±.05	0.00
E. coli	0.00	28.0±.13	0.00	24.0±.20	0.00	0.00	0.00	0.00	26.0±.25
B. subtilis	0.00	23.0±.2	0.00	21.0±.03	0.00	0.00	0.00	17.5±.15	24.0±.08
P. aeruginosa	0.00	00.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00

*Standard deviation

Table 2: Results of antimicrobial activity of FG2 extract against the clinical isolates

Test Organisms	Conc. (mg/mL)/ IZD (mm)					Positive controls	
	1	0.5	0.25	0.125	0.0625	Gentamicin (10 µg/mL)	Nystatin (50 µg/mL)
S. aureus	0±0	0±0	0±0	0±0	0±0	17±0	NA
B. subtilis	6±.1	3±.08	0±0	0±0	0±0	17±0	NA
P. aeruginosa	0±0	0±0	0±0	0±0	0±0	19±.01	NA
E. coli	0±0	0±0	0±0	0±0	0±0	19±0	NA
A. niger	0±0	0±0	0±0	0±0	0±0	NA	13±0
C. albicans	0±0	0±0	0±0	0±0	0±0	NA	16±0

*Standard deviation, NA=Not applicable

Table 3: Results of antimicrobial activity of FG4 extract against the clinical isolates

Test Organisms	Conc. (mg/mL)/ IZD (mm)					Positive Controls	
	1	0.5	0.25	0.125	0.0625	Gentamicin (10 µg/mL)	Nystatin (50 µg/mL)
S. aureus	0±0	0±0	0±0	0±0	0±0	17±0	NA
B. subtilis	5±0	3±0	0±0	0±0	0±0	17±0	NA
P. aeruginosa	5±0	4±0	3±0	0±0	0±0	19±.01	NA
E. coli	0±0	0±0	0±0	0±0	0±0	19±0	NA
A. niger	0±0	0±0	0±0	0±0	0±0	NA	13±0
C. albicans	3±0	2±0	0±0	0±0	0±0	NA	16±0

*Standard deviation, NA=Not applicable

Discussion

Microbial secondary metabolites have been reported as one of the best resources for drug discovery. Mimicking the natural microbial environment by co-cultivation allows direct interaction between/among microbes which may lead to the induction/production of new secondary metabolites with antibiotic potentials.[15,16] In this study, we report the positive influence of overnight co-cultivation on the antibiotic production of soil fungal isolates compared to antibiotic production of the same organisms in a three-week axenic culture condition. FG2 was identified in this study as T. longibrachiatum and the antimicrobial activities of extracts or compounds isolated from other strains

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Table 4: Results of antimicrobial activity of FG8 extract against the clinical isolates

Test Organisms	Conc. (mg/mL)/ IZD (mm)					Positive Controls	
	1	0.5	0.25	0.125	0.0625	Gentamicin (10 µg/mL)	Nystatin (50 µg/mL)
S. aureus	0±0	0±0	0±0	0±0	0±0	17±0	NA
B. subtilis	3±0	0±0	0±0	0±0	0±0	17±0	NA
P. aeruginosa	0±0	0±0	0±0	0±0	0±0	19±.01	NA
E. coli	0±0	0±0	0±0	0±0	0±0	19±0	NA
A. niger	0±0	0±0	0±0	0±0	0±0	NA	13±0
C. albicans	6±0	4±0	0±0	0±0	0±0	NA	16±0

Standard deviation, NA=Not applicable

Table 5: Results of antimicrobial activity of FG9 extract against the clinical isolates

Test organisms	Conc. (mg/mL)/ IZD (mm)					Positive Controls	
	1	0.5	0.25	0.125	0.0625	Gentamicin (10 µg/mL)	Nystatin (50 µg/mL)
S. aureus	0±0	0±0	0±0	0±0	0±0	17±0	NA
B. subtilis	4±0	3±0	2±0	0±0	0±0	17±0	NA
P. aeruginosa	6±0	4±0	2±0	0±0	0±0	19±.01	NA
E. coli	0±0	0±0	0±0	0±0	0±0	19±0	NA
A. niger	0±0	0±0	0±0	0±0	0±0	NA	13±0
C. albicans	0±0	0±0	0±0	0±0	0±0	NA	16±0

Standard deviation, NA=Not applicable

of this fungus have been previously reported. For instance, ergokonin A isolated from T. longibrachiatum has been reported for its antifungal properties.[17] In another report, of all the 24 strains of the fungi studied, the methanol extract showed the best anti-yeast property.[18] Interestingly, all these tests have revealed only antifungal properties with little or no antibacterial activity. A plausible explanation could be the absence of inducer organisms in the axenic cultures used for the laboratory experiments in these reports. Our result in Table 1 shows that T. longibrachiatum is capable of producing copious amounts of antibacterial substances in the right environment. Literature search also showed no report of antibacterial properties of C. cladosporioides and T. erinaceum extracts. The reason for their lack of antibacterial properties could be because studies on them have been carried out in axenic culture media where they have no need to produce much antibacterial substance. The genes that are necessary for the production of such secondary metabolites usually remained silent in axenic media.

Apart from co-cultivation in the overlay technique, possible loss of some active principles during extraction as well as slow accumulation of some other active principles could be responsible for the disparity observed. Co-cultivation refers to a situation when micro-organisms of similar or different species are cultured or grown on the same medium. Microorganisms naturally produce secondary metabolites as a means of sustenance in adverse conditions particularly in nutrient depletion.[19] When two or more organisms are grown together, the competition for the available nutrient and survival amongst them usually leads to the production of 'unnatural' secondary metabolites in some organisms.[15,16] This, according to Netzker et al.[20] could result from the activation of previously silent genes leading to the production of new secondary metabolites or up regulation in the activities of already actively expressed genes with resultant accumulation of usually produced secondary metabolites. Bertrand et al.[15] postulated that co-cultivation could serve as a means of generating bioactive natural products for drug discovery. This could be responsible for the activities of the isolated fungi observed in the overlay technique whereby the test bacteria could be regarded as 'inducer organisms" for new or increased metabolite synthesis in the fungi, "the induced organisms".

The difference in activities noted between the fungi isolates and their respective extracts could on the other hand be explained by loss of some active principles during the extraction process or the absence of live fungi, hence the loss of efficacy against previously susceptible organisms and/or susceptibility of previously resistant microbes.

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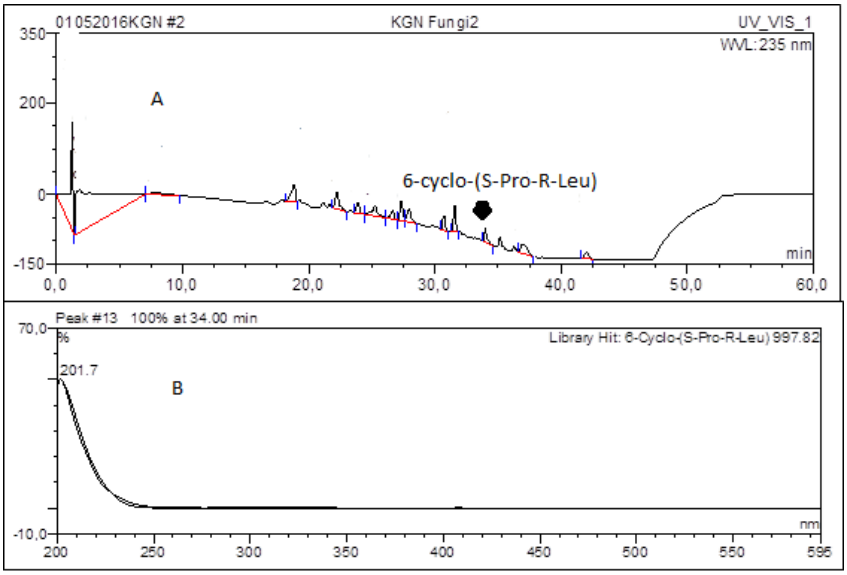

Fig. 1: Chromatogram of FG2 (a); UV spectrum of 6-cyclo-(s-pro-r-leu)(b)

However, in this case, extraction was carried out with ethyl acetate, which has the ability to extract both the polar and the non-polar substances and most antibacterial agents are known to be moderately polar and could have been extracted with ethyl acetate. Evaporation was carried out under pressure at low temperature such that heat labile compounds would not have been lost. We therefore rule out the possibility of loss of active compounds during extraction and concentration. Although it is known that some macromolecules or peptidic metabolites cannot be extracted with solvents,[21] it may not explain the drop in antibacterial potencies that occurred with extracts of the three fungal isolates compared with the overlay experiment. It has been reported that the metabolic pathway of different fungi change according to the environment they find themselves.[22] The presence of bacteria in the overnight overlay experiment and their absence in the solid fermentation media present two different environments that would effectively alter the metabolic pathways and hence composition of their metabolome. This offers a better explanation for the loss of potency observed.

It could be seen from Table 1 that none of the isolates inhibited P. aeruginosa. However, the result of the antibacterial sensitivity testing on their fermentation extracts showed that FG4 (Table 4) inhibited P. aeruginosa. A possible reason for this difference could be that the compounds responsible for antipseudomonal activities do not accumulate quickly. This is because the contact between the organisms in the overlay experiment was only 24 h. It can also be said that Pseudomonas did not effectively induce the production of antipseudomonal compounds like other bacteria did. Some bacteria are known to be capable of inducing the production of antibiotics from other organisms during co-cultivation while others do not have such effect.[23]

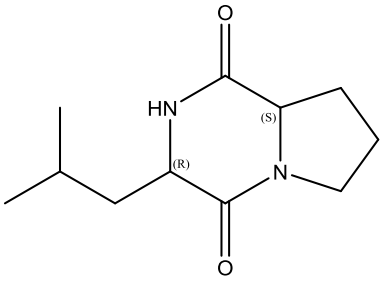

Fig. 2: Structure of 6-cyclo-(s-pro-r-leu), a diketopiperazine

The difference in antibacterial activity between overlay experiment and the fermentation extract experiment strongly suggests that co-cultivation method may lead to the production of very potent antibacterial agent from these fungi. Further work will be carried out to explore this possibility. Table 1 also suggests that FG2 metabolites has a broader spectrum of activity inhibiting both Gram-positive and Gram-negative organisms compared to FG8 metabolites which had no activity against any of

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the two Gram-negative test organisms. It could be seen that only T. erinaceum (FG8) inhibited the growth of C. albicans. Even T. longibrachiatum which has been reported to produce antifungal metabolites exhibited no antifungal activity. [17-18] It is possible that antifungal metabolites were lost or were not secreted since there were no fungal enemies. Compared to other published works cited above, the difference could be attributed to the different fermentation media. While we fermented in MEA, they used rice as their fermentation media. This leads to the production of different metabolites because different media come with different supplements which leads to the biosynthesis of different metabolites.

High Performance Liquid Chromatography analysis of the extracts led to the identification of 6-cyclo-(S-Pro-R-Leu) in FG2 (Figs 1&2) using dereplication. The compound 6-cyclo-(S-Pro-R-Leu) belongs to the 2,5-diketopiperazine group of chemical substances. Diketopiperazines are small organic cyclic dipeptides which generally occur in nature as biologically active substances in some fungi and bacteria. According to literature, 24 the diketopiperazines possess antibacterial and antifungal activities, as well as anticancer activities. Typical examples of drugs in this class include bicyclomycin, a broad-spectrum antibiotic and the immunosuppressant gliotoxin.24 The presence of this compound as well as the other unidentified chemical moieties, could explain the antibiotic effect observed from FG2. Further work will be carried out to completely characterize the components of these extracts as this compound may not be the only antibacterial compound in the extract.

Conclusion

This paper has clearly shown that *Trichoderma sp* and *C. cladosporioides* are capable of copious production of potent antibiotics in a co-culture environment which may not be possible in axenic culture. This could be one of the reasons why further co-culture experiment should be carried to adequately study these potent antibiotics.

Conflict of interest

There is no conflict of interest.

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