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Assessment of *Aspergillus niger* and *Aspergillus flavus* in Inoculant Induced Composting to Remove Antibiotics in Poultry Manure

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ABSTRACT

The extensive use of antibiotics for therapeutic and prophylactic utilization in livestock production has increased the possibility of antibiotic pollution of agro-ecosystem. Manure from livestock production usually serves as raw materials in the production of organic fertilizer and other soil amendments. It is an accepted recycling technique for agricultural waste such as manure turning it into a valuable resource but poses a range of risk to human health, economic productivity, ecosystem services and long-term sustainability of agricultural activity. The study aims to reduce the risk by removing antibiotics in poultry manure through inoculant induced composting. *Aspergillus niger* and *Aspergillus flavus* was screened as to antibiotic resistance, potential for mass production and performance as inoculant during composting to remove tylosin and oxytetracycline. Results showed that both fungal species were resistant to both the antibiotics, has increased growth at various concentration of antibiotic and showed potential for mass production by having luxuriant growth in various substrates. Both species also belong to the primary fungal community present during the time of significant antibiotic removal and may contribute to the treatment of antibiotics in poultry manure.

Key words: *Aspergillus niger, Aspergillus flavus, Composting, Tylosin, Oxytetracycline*

Introduction

Antibiotics administered to animals are excreted in an unaltered state together with their manure (Heuer *et al.,* 2011). It can persist through storage and treatment and find its way to the environment by a variety of mechanisms which includes surface runoff (Pham *et al.,* 2013) (Sun *et al.,* 2013), groundwater seepage and land application (Pham *et al.,* 2013). Since animal manure is almost always used as soil conditioner or fertilizer (Sun *et al.,* 2013) (Zizek and Zidar, 2013), widespread detectable concentration were already reported on soil (Venglovsky *et al.,* 2009) (Sun *et al.,* 2013), surface water (Sun 2013) (Huerta *et al.,* 2013) (Kolpin, 2002) and sediments (Huerta, 2013) (Massey, 2010) within the vicinity of farms or plot of lands where animal manure are regularly applied.

Poultry manure has long been recognized as the most desirable organic fertilizer as it improves soil fertility by adding both major and essential plant nutrients as well as soil organic matter which improves moisture and nutrient retention (Deksissa 2008). However, continuous application of poultry manure poses threat to agroecosystem, water quality and human health because of pathogenic bacte-

ria and emerging contaminant such as antibiotics. These threats may be reduced by developing standard treatment technology which can be used to remove the various contaminant in manure.

Composting is an established technology for stabilization of organic matter, reduction of pathogen and odor for solid waste. Inoculant induced composting is applied when it is aimed to do a more specific task or improved performance. An example of bio-inoculant in composting is the use *Trichoderma* sp. which aims to facilitate the degradation of organic cellulose. Bio-inoculant can also be used to target the removal of specific pollutant in manure such as antibiotics.

Aspergillus flavus is a saprotrophic, mycotoxigenic and pathogenic fungus with a cosmopolitan distribution. It is very well associated with colonizing cereal grains, legumes, tree nuts and causing diseases in corn, peanuts and other crops (Bobson, 2011). *Aspergillus niger* is regarded as a food grade fungus and does not produce aflatoxin. It is responsible for the postharvest decay of fresh fruits and can be isolated from any type of stored commodity (Hocking 2006). It has haploid filaments and extensively used in biotransformation and waste management. It also finds industrial applications such as production of citric acid and various extracellular enzymes such as amylases, pectinases, proteases, oxidases, hydrogenases, cellulases, glucoamylases (Najafpour, 2007). These species can serve as bio-inoculant during composting and target the removal of antibiotics. The primary ingredient of animal feeds are cereal grains which both fungal species typically colonized.

The aim of the study was to assess the possibility of using *Aspergillus flavus* and *Aspergillus niger* as inoculant to remove tylosin and oxytetracycline in poultry manure during composting. Both species will be assessed as to 1. ability to survive in the presence of the target antibiotics; 2. possibility for mass production; and 3. its performance during inoculant induced composting.

Materials and Methods

The following materials and methods were used and carried out to achieve the objectives of the study.

The fungal species were screened as to antibiotic resistance and their growth response in the presence of Tylosin (200 mg/ml; Chemvet Products, Inc.) and Oxytetracycline (200 mg/ml; Universal Robina Corp.) Minimum inhibitory testing was done using different concentration of antibiotics (200 mg/ml, 100 mg/ml, and 50 mg/ml). Disk diffusion method was used to observe the sensitivityto antibiotics. Growth of mycelia during the incubation period of 4 to 6 days were indication of resistance to tylosin and oxytetracycline.

Locally available materials with the high carbon content were evaluated as substrate for the mass production of the fungal species. Rice bran, soybean, corn cob, corn grits, saw dust and mungbeans were evaluated as candidate substrate. Substrate was grinded into smaller part and moistened with water. Substrates were placed separately in a bottle of equal volume. The bottles were placed in an autoclave for sterilization at 121°C for 45 minutes. Substrates were cooled down and separately inoculated with 5 mm mycelia disk of *A.niger* and *A. flavus*. The inoculated substrates were incubated at room temperature and observed for 14 days.

Compost pile was prepared using a ratio of 3:2:0.5:0.5 (v/v) of rice straw, leaf litters poultry manure and carbonized rice hull. The materials were mixed and divided into different treatment. Antibiotics were spiked (630mg of tylosin and 1200 mg of oxytetracycline) by spraying into the composting mass. A total of 42 kg of composting mass per treatment was inoculated with 450 g of inoculant. The materials were placed in a modified compost pile using plastic box with dimensions of 52cm x 39cm x 42cm. The compost boxes were labeled and covered with the lid to prevent the loss of moisture and exposure to sunlight. Weekly turning and watering of the compost piles were done to regulate the moisture, temperature and aeration for microbial activity. Samples was collected at day 1, 3, 9, 24, 40 and 60. A representative sample of 250g was collected and placed in zip lock plastic bag and placed in refrigerator prior to antibiotic analysis. Antibiotic analysis was performed following the method described in USEPA (1694). Harvesting of composts were done after 60 days.

The fungal community during the inoculant induced composting were observed and identified. Genomic DNA extraction was performed using the templates for Polymerase Chain Reaction (PCR). Templates were prepared by taking 3-4 loopfuls of the mycelial growth of the fungal isolates and then placed in 60 microliters of 1x TE buffer. The mixture was placed in a water bath for 5 minutes and frozen

at -80°C for 5 minutes. Freezing and thawing process was done 2 times. The samples were centrifuged for 1 min at 5000-6000 rpm. The supernatant was used as PCR template. Gene amplification of the PCR components include genomic DNA, ITS1 and ITS4 18S primers, Clontech Titanium® Taq Buffer, Titaq DNA Polymerase, and dNTP Mix. Cycling parameters on thermal cycler: 95°C for 1 minute; 30 cycles of 95°C 30 seconds, 59°C for 30 seconds, 68°C for 1 minute; 68°C for 3 minutes and hold at 4°C. Capillary sequencing of the sample was done through the incorporation of fluorescently labeled chain terminator ddNTPs with the components of amplicons, primers and ABI BigDye® Terminator v3.1 cycle sequencing kit. Cycling parameters on Bio-Rad T-100 thermal cycler: pre-hold at 4°C; 96°C for 1 minutes; 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 62°C for 4 minutes. hold at 4°C. Ethanol precipitation was used to remove unincorporated ddNTPs, excess primers and primers dimers. Capillary electrophoresis was used in ABI 370xl DNA analyzer using a 50cm 96-capillary array, POP7TM Polymer and 3730xl data collection software v3.1. base calling on the sequencing Analysis Software v5.4.

Results and Discussion

The results are presented and discussed in the succeeding sections.

A. niger and *A. flavus* was isolated from vermicomposting pile. It was purified, sub-cultured and maintained on potato dextrose agar prior to screening. Vermicompost serve as the source of inoculum to target the removal of tylosin and oxytetracycline during composting. Vermicompost has been shown to be a rich source of beneficial microorganisms as the activity of earthworm enriches the microbial community during the process. Microorganism from the process may help in degrading pollutants of emerging concerns such as antibiotics.

A. niger and *A. flavus* were grown at different concentration of tylosin and oxytetracycline while the control were grown on a media without antibiotic. Increased growth were observed for both species grown at different concentration of antibiotics as compared to the control. Figure 1shows the mycelial growth of the fungal isolates at different concentration of antibiotics.

Growth preference of the two (2) species in various agricultural products and by products such as rice bran, soybean, corn cob, corn grits, sawdust and mungbeanwas shown in Table 2.Both species showed luxuriant growth in almost all substrate tested.

Most fungi can grow and colonize various form of substrates with high lignin content and carbon source, while substrates like saw dust contain high amount of lignin but generally low in protein content. Wheat grains and other cereal bran are known to be rich in protein, carbohydrates, minerals, fats and vitamins. Locally available farm waste were

Table 1. Treatment assignment of composting experiment

Treatment	Material Composition	Treatment	Material Composition
T1	Organic compost (OC) + tylosin	T2	OC + oxytetracycline (OXY)
T3	OC + tylosin + (*A. niger*)	T4	OC + OXY + (*A. flavus*);
T5	OC + tylosin + (*A.niger*)	T6	OC + OXY + (*A.flavus*)

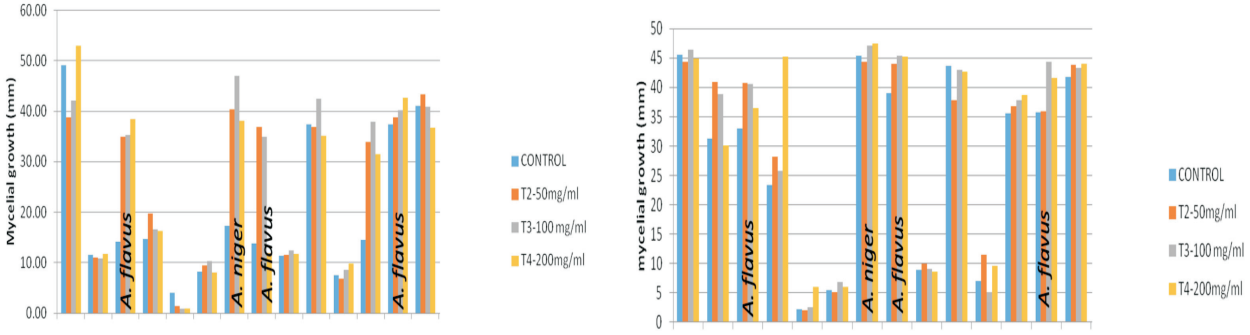

Fig. 1. Mycelial growth of fungal isolate expose to tylosin and oxytetracycline at 5 days

found to provide excellent source of food with varying amount of nutrients for the different species of fungi. Grains are mostly used as substrates because it is easily available, cheaper and has best nutritive media for mass multiplication of many microorganisms (Choudhary, 2010). It also provide maximum spore production due to the higher surface area for the fungi to grow and sporulate (Kumar, 2009) (Sahayaraj, 2008). *Aspergillus flavus* was known to colonized various grains and products and has luxuriant growth in all substrate tested. Both species has luxuriant growth in rice bran, corn cob, and corn grit. *Aspergillus niger* was known to be isolated on any stored commodity including grains and fresh fruits but *Aspergillus niger* shows no growth in saw dust during the experiment. *A. niger* grows well in substrate with high water content such as fresh fruit and high nutrient content such as in grains and nuts. Saw dust have low water and protein content since it comes from dried timber and high in lignin which might be a complex food source for *A.niger*.

Aspergillus niger and *Aspergillus flavus* were mass produced and used as inoculant during composting of poultry manure spiked with Tylosin and Oxytetracycline. Composting of poultry manure using *A. niger* and *A. flavus* as inoculant registered 83-93% and 50-65% percent removal for tylosin and oxytetracycline respectively across treatment after the end of 60 days composting. Similar percent removal was observed between control and the different treatments. Peak of tylosin removal was achieved within 24 days of composting. Tylosin removal follows an increasing trend wherein the highest removal was recorded at the end of composting experiment (Figure 2). The result is slightly higher with the study of

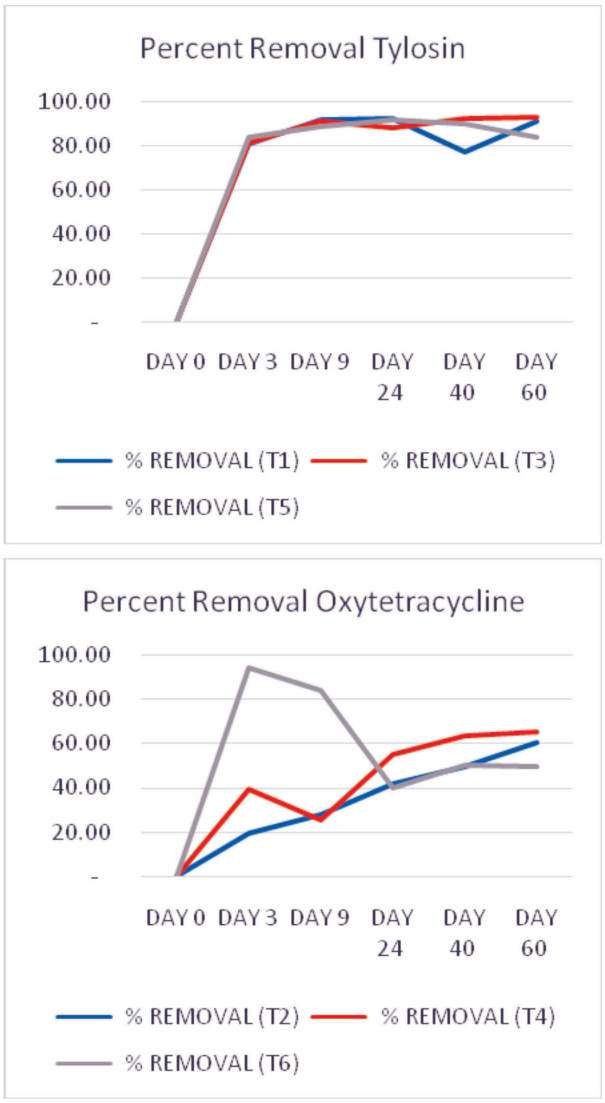

Fig. 2. Percent removal of Tylosin and Oxytetracycline

Table 2. Growth preference of isolates on various substrate.

Isolates	Substrate	Days of Incubation	Mycelial Density
Aspergillus flavus	T1-Rice Bran	6	++++
	T2- Soy Bean	6	++++
	T3- Corn Cob	6	++++
	T4- Corn Grits	6	++++
	T5- Saw Dust	6	++++
	T6- Mung Bean	7	++++
Aspergillus niger	T1-Rice Bran	5	++++
	T2- Soy Bean	5	++++
	T3- Corn Cob	6	++++
	T4- Corn Grits	5	++++
	T5- Saw Dust	7	+

Note: (+) no growth; (++) very thin mycelia growth/spore; (+++) thin mycelia growth/spore; (++++) luxuriant growth.

Dolliver et al. (Dolliver, 2008) which register a 54-76% removal in composting turkey litter for 22 to 35 days. Kim *et al.* (Kim, 2012) studied composting using pig manure and sawdust wherein 20mg/kg concentration of tylosin was reduced below the required guideline of 1mg/kg tylosin. *Ho et al.*(Ho 2013) observed rapid decrease of tylosin up to 98.95% removal during the first 9 days of composting, which was similarly observed in this study wherein 83-93% removal was observed within the same timeframe. Composting beef and dairy manure and biosolid had register conflicting tylosin removal for some studies; wherein a study conducted by Mitchell *et al.* 2015) has recorded 96-98% reduction after 21 days similar with the result of this study but Ray *et al.* (Ray, 2017) reported relatively low removal of tylosin. Both authors studied the effect of thermophilic temperature and management in the removal of tylosin in beef and dairy manure.

The oxytetracycline removal of 50-65% in the study was relatively low compared to other studies. Several studies recorded above 90% to complete removal of oxytetracycline; *Hu et al.* (Hu 2011) recorded 97.3 – 98.5% removal efficiency after 45 days of composting hen and pig manure; *Arikan et al.* (Arikan, 2007) and *Arikan et al.* (Arikan, 2009) register 91-95% removal after 28 days composting beef manure; Wang *et al.* (2015) and *Chai et al.* (Chai, 2016) recorded 100% removal in pig manure after 32 days and 42 days respectively. The primary factor attributed to the removal of oxytetracycline was the thermophilic temperature during composting.

Eleven (11) to twenty one (21) different fungal species were isolated from each treatment. Twenty five (25) fungal isolates from composting were subjected to molecular identification. Based on the result obtained from the molecular identification, the isolates belong to 14 genera of fungi (*Lichthemia, Aspergillus, Trichosporon, Talaromyces, Sarocladium, Rhizomucor, Phoma, Cladosporium, Diaporthe, Culvularia, Chaetomium, Lomentospora, Fusarium, and Myriococcum*) under 3 divisions (Basidiomycota, Ascomycota and Murocomycota). Molecular identification revealed the same species *A. terreus* (I-2, I-17 and I-19), *T. asahii* (I-3, I7 and I-21) and *C. globosum* (I-31 and I-33). Finally, the 25 isolates subjected to molecular identification were reduced to 20 individual fungal species.

The study on identification of fungal communities in producing compost using household waste done by *Dehghani et al.* (2012) had similar number of isolated fungal species. The identified fungal species in their study primarily belongs to Genus *Aspergillus* constituting 34.45%. Other genus isolated which were similar with the present study includes *Fusarium*, *Cladosporium and Culvularia.* Composting of biowaste conducted by Ryckboer *et al.* (Ryckboer, 2003) isolated fungal specie mainly under *Aspergilus* and *Mucor* genera which were detected after the compost were in mesophilic temperature until the end of composting. In composting municipal solid waste conducted by *Rebollido et al.* (Rebollido, 2008), fungal community accounts to 23.1% of microbial community; species of which primarily belonging to *Aspergillus, Trichoderma, Alternaria, Penicillium* and *Ulocladium*. Species belonging to genera *Aspergillus* was isolated in composting a variety of substrate including sugarcane bagasse (Silva 2009); weeds, ayurvedic herbal waste, coir pith and sawdust (Hassena, 2016); household waste (Dehghani, 2012); municipal solid waste (Rebollido, 2008); and biowaste (Ryckboer, 2003). *Aspergillus fumigatus* and *Aspergillus niger* were the primary species found in the above studies. *Aspergillus* species was also found in different potting soils and compost (Haas, 2016). It is also noted that species belonging to genera *Penicillium* and *Trichoderma* were usually isolated in the above stated studies but was absent in the present study. On the other hand, species belonging to genera *Lichthemia,Trichosporon, Talaromyces, Sarocladium, Rhizomucor, Phoma, Cladosporium, Diaporthe, Chaetomium, Lomentospora and Myriococcum*which were isolated in the current study but were not identified in the previous studies.

The primary fungal community during composting was evaluated as to its relation and contribution to antibiotic removal. Figure 3 shows the primary fungal species present during the time the percent removal of antibiotic was measured. For treatment involving tylosin, around 90% removal was achieved within the first 24 days of composting experiment. The primary fungal species present at this stage of composting experiment on all treatment with tylosin was *A. flavus, A. niger, A. fumigatus, A. terreus, T. asahii,* and *L. romosa*. From day 24, no significant increase in the removal of tylosin was observed until the 60th day of experiment wherein the maximum percent removal attained was 93%. The fungal species present at this stage of composting experiment across treatment with tylosin was *A. fumigatus, A. flavus,* and *L. romosa.* It was also ob-

served that *A. fumigatus* was the dominant species at this stage of composting while the percent contribution of *A. flavus*, and *L. romosa* was reduced compared to earlier stage of composting. It was also observed that for treatment 5 & 6 where *A. niger* was used as inoculant, it was present when the peak percent removal was attained and was absent thereafter. During screening *A. niger* showed increased growth in the presence of tylosin. The other fungal species that may contribute to removal of tylosin was *A. flavus, L. romosa* and *T. asahii* as the said species were also present when the peak percent removal was recorded and not necessarily present after. It is also observed that although *A. fumigatus* was present in the whole duration of composting experiment, no further significant increase in percent removal was observed after the 24 day up to the end of the experiment when it is the primary fungal species present. This may be an indication that *A. fumigatus* was not a primary contributor in the re-

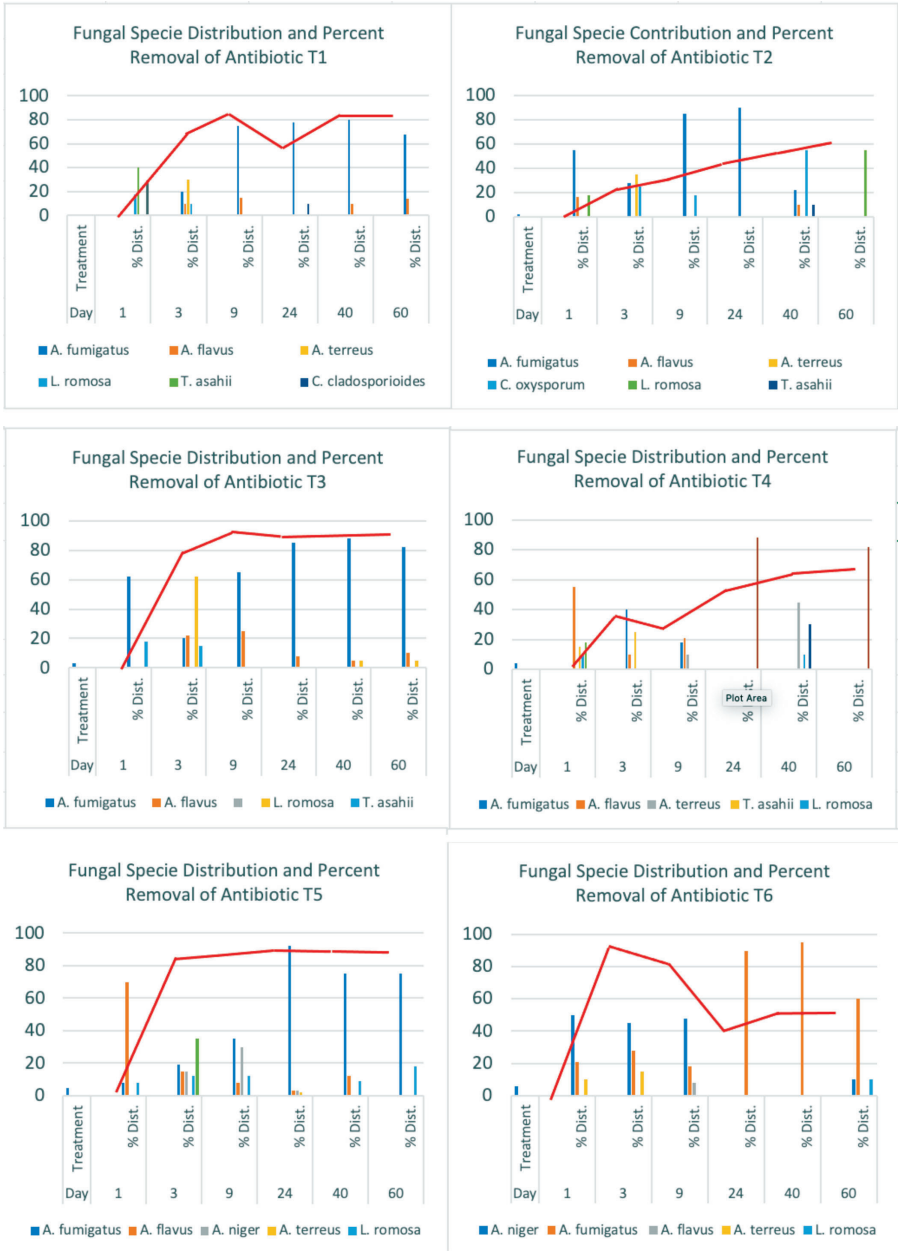

Fig. 3. Primary fungal specie at the time of antibiotic removal.

moval of tylosin and contributing primarily on decomposing organic matter.

A regularly increasing trend of antibiotic removal was observed in treatments involving oxytetracycline. Maximum removal between 50 to 65% was observed in the treatments which was achieved only after 60 days of composting. For the treatment following similar trends of antibiotic removal (T_2 and T_4), the fungal community present at this stage of composting was also similar with the treatment involving tylosin. *A. flavus, A. niger, A. fumigatus, A. terreus, T. asahii, L. romosa,* and *C. oxysporum* were the fungal species observed at this stage with *A. fumigatus* as the typical dominant species. It was observed that *A. fumigatus* was again present in the whole duration of the composting experiment, but since the observed removal efficiency was only 50-65%, it may be playing a role other than the removal of oxytetracycline as the case with the treatment involving tylosin. The fungal species that may have contribution in the removal of oxytetracycline were *A. niger* (T6), *A. flavus* (T4), and *C. oxysporum* (T2) as they are the dominant species during the time oxytetracycline was removed.

The study assessed the potential of *A. niger* and *A. flavus* in inoculant induced composting to remove antibiotics in poultry manure. Both species showed resistance to the target antibiotics and have increased growth in the presence of all concentration of antibiotics. They also have potential for mass production by having luxuriant growth in almost all substrate tested. Inoculant induced composting registered 83-93% and 50-65% removal for tylosin and oxytetracycline respectively. *A. flavus* and *A. niger* as inoculant was able to colonized the treatment they were inoculated and the primary fungal species present during the time of significant antibiotic removal. Other fungal species that may contribute to the removal of target antibiotics includes *Lichthemia romosa, Trichosporon asahii* and *Cladosporium oxysporum.*

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