International Journal of Agriculture Sciences
ISSN: 0975-3710&E-ISSN: 0975-9107, Volume 8, Issue 60, 2016, pp.-3357-3360.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000217

Research Article

ANTIMICROBIAL PROPERTIES OF VERMICOMPOST ASSOCIATED MYCOFLORA OBTAINED FROM DIFFERENT SOURCES

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Received: October 06, 2016; Revised: November 21, 2016; Accepted: November 22, 2016; Published: December 12, 2016

Abstract- In the present study, three samples of vermicompost obtained from different types of wastes were analyzed for antibiotic producing fungi. The entire mycoflora in broth media of all the three samples and the dominant fungi isolated from them were tested for their antibacterial property against *Bacillus subtilis, Escherichia coli*, *Bacillus licheniformis, Paenibacillus polymyxa* and *Klebsiella oxytoca.* Two of the fungal isolates (Vag002 and Vic003) showed strong antibacterial activity and were selected to undergo further characterization by Morphological examination and Lactophenol cotton blue method. The morphological characteristics of the fungal isolates matched with the description for *Trichoderma* spp. and *Aspergillus* spp. Further confirmation by Lactophenol Cotton Blue Test of isolates revealed their identity as *Trichoderma viride* and *Aspergillus niger.* The present findings conclude that these microorganisms could be promising source of bioactive compounds and warrant further study.

Keywords- Antibiotic, Antimicrobial activity, *Aspergillus niger*, Mycoflora, *Trichoderma viride*, Vermicompost.

Citation: Jeswani V., et al., (2016) Antimicrobial Properties Of Vermicompost Associated Mycoflora Obtained From Different Sources. International Journal of Agriculture Sciences, ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 8, Issue 60, pp.-3357-3360.

Introduction

Vermicomposting is the process of aerobic bioxidation by earthworms in which the waste decomposed organic matter is fed to the earthworms which produce castings rich in nutrients and microbial population [1] by converting organic waste into vermicompost that contain eight times as many microorganisms as their feed [2].

According to Dulmage and Rivas [3], soil and compost microorganisms have frequently been screened to discover novel useful metabolites since long time.

Fungi are the important component in vermicompost as they undertake rapid decomposition of lignocellulosic material resulting in the maturation of compost matter. Thousands of fungal species including *Mucor, Aspergillus, Penicillium, Trichoderma, Fusarium, Rhizopus, Chlado sporium* etc. are found in vermicompost.

In recent investigations, the abilities of these fungal strains to produce pigments, biologically active metabolites, immune-suppressants, anticancer compounds and bio-control agents [4-6] have been studied.

In present study, *Aspergillus niger* and *Trichoderma viride* isolated from three vermicompost samples that differed in their source, showed strong antimicrobial activity against test bacteria. *Aspergillus niger* has been reported to show high potency in producing antimicrobial compounds such as tensyuic acid, nigerazine B, tensidol A, orchratoxin [7] and also enzymes having antimicrobial activities such as glucose oxidase [8]. *Trichoderma* species is also one of the most promising microbes for biological control for different bacteria and fungi, due to presence of hydrolytic enzymes namely chitinases, ß-glucanases and proteinases [9,10].

Materials and Methods

Sample Collection

Three samples of Vermicompost were collected out of which two samples were collected from KVK, Kasturbagram, Indore. One of these produced from only agriculture residue (labeled as V_{AG}), and the other obtained from only cow dung and Farm Yard Manure (FYM) (labeled as V_{CD}).

The third sample was collected from College of Agriculture, Indore in which both agriculture residue and FYM were used layer by layer according to Indore compost method of vermicompost production [11]. This was labeled as V_{IC}. Earthworm species used was *E. foetida* in all the samples.

Test Bacteria

The test bacteria used were gram-positive (*Bacillus subtilis – MTCC 441, Bacillus licheniformis - MTCC 8316, Paenibacillus polymyxa* - NAIMCC-B-00318) and gram-negative (*Escherichia coli* (Obtained from School of Life Sciences, DAVV, Indore) and *Klebsiella oxytoca*- MTCC 3030).

The overnight broth cultures of these test bacteria were inoculated in NA (Nutrient Agar–Hi-Media) plates for screening of antimicrobial activity of the dominant fungal population in the test vermicompost samples.

Pure Cultures

Pure Cultures of *Aspergillus niger* and *Trichoderma Viride* were obtained from Maharaja Ranjit Singh College of Professional Sciences, Indore and were used as positive controls in Identification of *A. niger* and *T. viride*.

Antimicrobial activity testing of entire mycoflora of vermicompost samples

0.1g of each sample, namely, V_{CD}, V_{AG} and V_{IC} were dissolved in 10 ml sterile distilled water in 3 sterile 25ml falcon tubes to obtain 1:100 w/v conc. and

centrifuged at 2000 rpm for 5 minutes to separate soil particles. 1ml of this suspension was transferred to the MEB (Malt Extract Broth–Hi-Media) medium and incubated at 30±2˚C for 5 days.

The antimicrobial activity of entire mycoflora is checked after 5 days by disc diffusion method for antimicrobial susceptibility testing as described by Bauer et al. [12]. Briefly, the bacterial culture to be tested was used to lawn NA plates evenly by using a sterile spreader. Sterile filter paper discs (6 mm in diameter) dipped in the above mentioned vermicompost suspensions were placed on agar surface. The plates were then incubated at 30±2˚C for 24-48 hours. All the experiments were performed in triplicate and zones of inhibition in all the plates were measured using calipers and recorded.

Isolation of dominant mycoflora from vermicompost samples

All the three samples of Vermicompost were plated by Serial Dilution Method [13]to isolate the mycoflora on MEA (Malt Extract Agar – Hi-Media) medium. The plates were incubated at 30±2˚C for 5 days and checked frequently to calculate and record the fungal colonies. The dominant fungal colonies were isolated and subjected to screening for antimicrobial activity.

Isolation and screening of fungal strains for antimicrobial activity

The crude distilled water extracts of fungi grown on MEA Plates were subjected to two rounds of preliminary screening via disc-diffusion method for antimicrobial susceptibility testing as described above. The fungi that produced zones of inhibition, thereby indicating an inhibition of the growth of test bacteria, were isolated and sub- cultured on PDA (Potato Dextrose Agar – Hi-Media) and MEA plates to obtain pure slants. Pure fungal isolates obtained from the preliminary selection were subjected to secondary screening via disc-diffusion method. All the plates were incubated at room temperature for 24 hours and the zone of inhibition was observed.

Characterization of pure fungal isolates

Characterization of pure fungal isolates was carried out based on morphological features[14-16]. After determination of their genera, Lactophenol cotton blue method[17]was used to confirm the species of that fungus. The macroscopic and microscopic characters observed were compared with those of the pure cultures obtained from Maharaja Ranjit Singh College of Professional Sciences, Indore.

Antibiotics Susceptibility testing and Comparison with Fungal Isolates

The Bacterial cultures were spread on NA plates and discs were impregnated with four antibiotics, ampicillin (100µg/ml), amoxicillin (100µg/ml), neomycin (100µg/ml) and oxacillin (100µg/ml), dried and placed on same plate approximately equidistant to each other and zone of clearance was measured for each and recorded. The data was then compared with the zone of inhibition obtained by fungal isolates.

Results and Discussion

Antibiotic susceptibility test:

The observations from the standard antibiotic treatments showed that Neomycin was the effective against all test bacteria and gave a significant zone of inhibition with each bacterium. *K. oxytoca* which is resistant to beta-lactam antibiotics, ampicillin as well as oxacillin, being beta-lactamase producing bacterium is particularly interesting bacterium in this study. The average values of zones of inhibition formed during antibiotic susceptibility testing are shown in [Fig-1] and [Fig-2].

Antimicrobial activity testing of entire mycoflora of vermicompost samples

Three vermicompost samples (V_{CD}, V_{AG} and V_{IC}) were studied for the presence of antimicrobial producing fungi using disc diffusion method. The zones of inhibition greater than 10mm were considered significant for this study. This was based upon the minimum measurement of zone of inhibition obtained from standard antibiotic treatment (Ampicillin) of *B.subtilis*. Maximum zone of inhibition was observed using V_{CD} against *B. licheniformis*. V_{IC} showed antimicrobial activity against all the test bacteria. However, the activities against *E. coli* and *K. oxytoca* were not found significant. Both of them are beta-lactamase producing bacteria. Interestingly, the V_{AG} showed a significant zone of inhibition against *K. oxytoca*, which is resistant to both ampicillin and oxacillin. The average values of zones of inhibition are recorded in the [Fig-3] and [Fig-4].

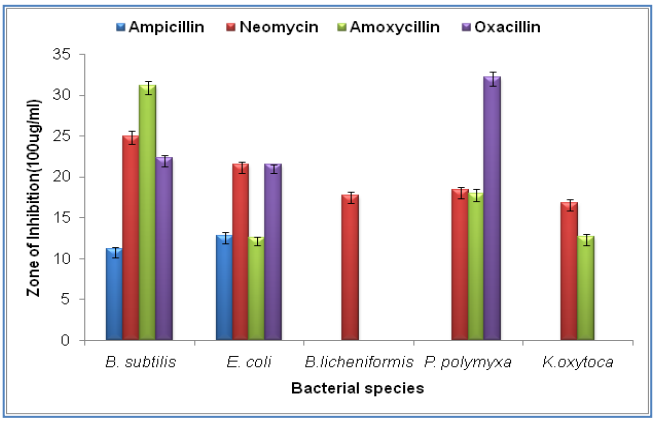

Fig-1 Antibiotics Susceptibility Testing for Test Bacteria

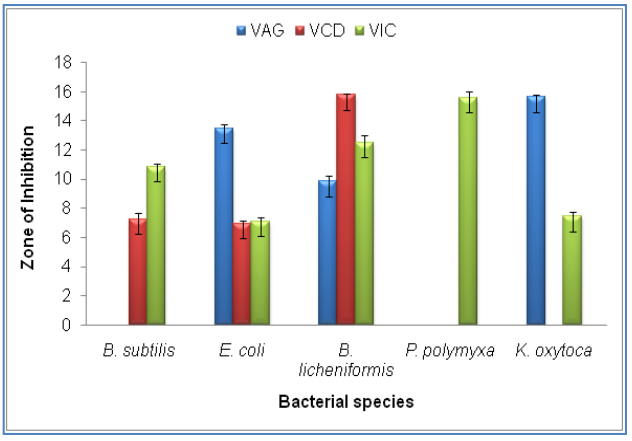

Fig-3 Zone of inhibition formed by entire mycoflora of three different vermicompost samples on different test bacteria.

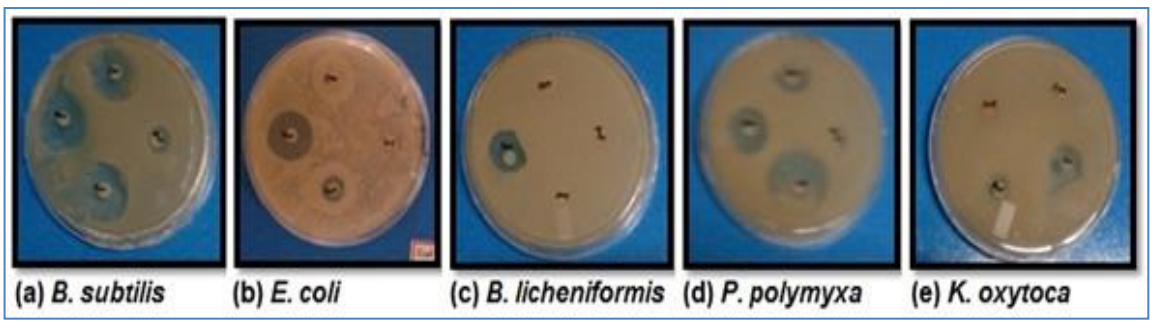

Fig-2 The zone of inhibition formed by action of different antibiotics on the given test bacteria

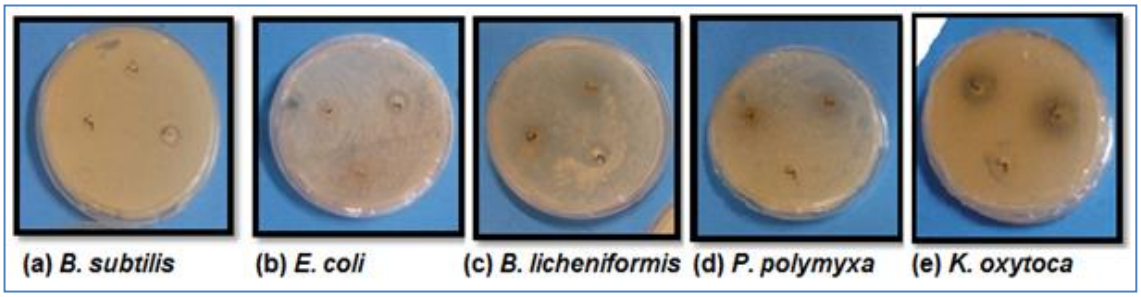

Fig-4 Zone of inhibition formed by entire mycoflora of three different vermicompost samples by Disc-diffusion method

Isolation and antimicrobial activity testing of dominant fungal communities from vermicompost samples

Dominant mycoflora from the vermicompost samples were isolated using serial dilution technique. Eleven different fungal colonies could be successfully isolated from the three vermicompost samples. Four fungal colonies were isolated from V_{AG} sample (Vag001, Vag002, Vag003, Vag004), two colonies were isolated from V_{CD} sample (Vcd001, Vcd002) and five were isolated from V_{IC} sample (Vic001, Vic002, Vic003, Vic004, Vic005). Out of these eleven colonies, six showed zones of inhibition in the first round of preliminary screening. However, only three of these showed zones of inhibition in the subsequent repetitions. They were allowed to undergo secondary screening to further confirm the antimicrobial activities of selected fungal isolates from preliminary screening. In secondary screening, out of the 3 isolates, two isolates (Vag002 and Vic003) showed significant zones of inhibition against the test bacteria. The average values of zones of inhibition formed during secondary screening are shown in [Fig-5] and [Fig- 6].

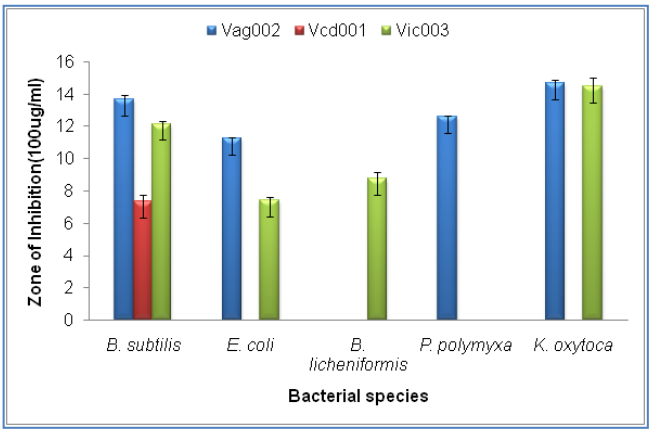

Fig-5 The zone of inhibition formed by crude extracts of fungal isolates during confirmation in secondary screening

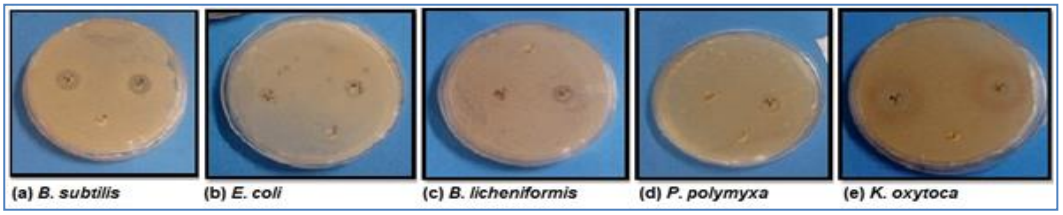

Fig-6 The zone of inhibition for crude extracts of fungal isolates during the confirmation in secondary screening.

Identification of fungal strains showing significant zones of inhibition in secondary screening

Morphological characterization of pure fungal isolates was carried out. The colonies of Vag002 showed black colour from the top, reverse pigment-beige, wooly cottony texture and heaped topography which resembled the characteristics of fungus *Aspergillus niger.* The colonies of Vic003 showed granular appearance with green conidia distributed throughout the plate and some white pustules were also found growing on the green mat of conidia and aromatic odour like coconut which were similar to the characteristics of fungus *Trichoderma viride.* The morphological characteristics of pure cultures of fungi on PDA plates are shown in [Fig- 7 (a) and (b)].

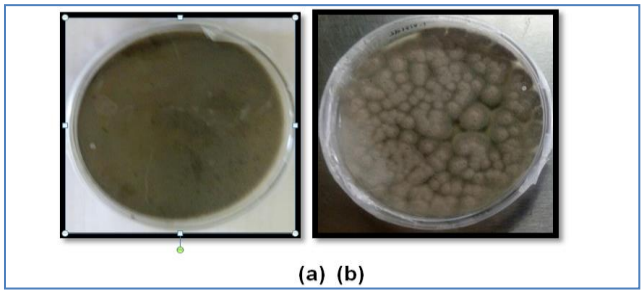

Fig-7 Morphological characters of (a)Vag002 – *Aspergillus niger* and (b)Vic003 – *Trichoderma viride*

Microscopic studies were carried out using lactophenol cotton blue test which confirmed the identity of Vag002 and Vic003 as *Aspergillus niger* and *Trichoderma viride* respectively. The septate hyphae, large, dark conidial head, rough walled conidia, and smooth-walled, dark, hyaline conidiophores confirmed the identity of Vag002 as *Aspergillus niger.* Subglobose to globose conidia were observed of Vic003 to confirm its identity as *Trichoderma viride.* The microscopic observations of lactophenol cotton blue mounts are shown in [Fig-8 (a) and (b)].

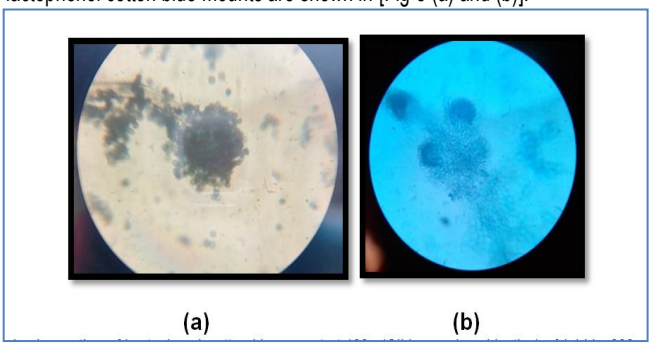

Fig-8 Microscopic observation of Lactophenol cotton blue mount at 100x (Oil immersion objective) of (a) Vag002 -*Aspergillus niger*and (b) Vic003 - *Trichoderma viride*

Discussion

The results of this study revealed antimicrobial activity of some of the dominant fungal communities present in vermicompost samples collected from three different sources namely, V_{AG} produced from only agricultural residue, V_{CD} obtained from only cow dung and Farm Yard Manure (FYM), and V_{IC} in which both

agriculture residue and FYM were used layer by layer according to Indore compost method. The fungal component of vermicompost may have favorable and unfavorable effects in the situations in which the composts are employed. The variations in fungal component of various vermicompost samples are associated with their source as well as method of preparation [18]. When plant material is added to produce vermicompost it has been reported that fungal population is greatly stimulated as the cellulosic material acts as an excellent carbon source for fungal growth [19]. The mycroflora from vermicompost samples showed antibacterial activity against the five different bacterial strains used in this study. In particular, V_{IC} showed activity against all the bacteria, which is expected as it is a combination method. It was observed that V_{AG} showed no activity against *B. subtilis* and *P. polymyxa* when entire mycoflora was tested against bacteria but Vag002 (later identified as *Aspergillus niger*) isolated from V_{AG} sample showed activity against both of them. It could bedue to interference by some components secreted by other fungi in the broth medium that reacted with the antimicrobial compound secreted by *Aspergillus niger*. The activity of V_{IC} and V_{AG} against *K.oxytoca*, which showed resistance to both ampicillin and oxacillin, was especially interesting as there is an emergence of many bacterial strains that have reduced susceptibility to antibiotics. Such increasing resistance to antibiotics has been attributed to haphazard usage of extended spectrum antibiotics. Since extended spectrum β-Lactamase (ESBL) production frequently is accompanied by multi-resistance to antibiotics, therapeutic options have become limited[20]. We attributed the anti *K. oxytoca* activity of the vermicompost samples to their resident fungal communities, as the fungi are known for production of antibiotics. This was corroborated by the isolated strains of fungi, which showed similar activity against the bacteria. The isolated fungal strains were identified as *Trichoderma viride* and *Aspergillus niger* which are known for production of various antibiotics. In this study, we have demonstrated the antibacterial activity of the resident fungal communities of vermicompost. The significance of this study is that it serves as a model for need to analyze various vermicompost samples for their fungal communities that can inhibit soil borne pathogenic bacteria, or even for production of various secondary metabolites.

Conclusion

In present investigation, wider fungal diversity and antimicrobial producing fungi were seen in V_{AG} and $V_{IC,}$ both of which include agriculture residue as substrate for production, as compared to V_{CD}. Therefore, when plant materials are added to produce vermicompost, fungal population is greatly stimulated as the cellulosic material from the plant waste acts as a nutritive material for the faster development of fungi and more diversity. From this study, the two antibiotic-producing fungal isolates (Vag002 and Vic003) were identified as *Aspergillus niger* and *Trichoderma viride* respectively. We may conclude that vermicompost serves as an excellent source of antibiotic producing fungi and can be explored for other useful fungal strains.

Acknowledgement

This work represents a portion of Vandana Jeswani's Masters dissertation work and is funded by Department of Biotechnology, Ministry of Science and Technology, New Delhi.

Author contributions

V Jeswani, SK Sharma, and S Ratnaparkhe have equal contribution in design of study. V Jeswani conducted experiments. Supervision and guidance by S Ratnaparkhe and manuscript finalized by V Jeswani, SK Sharma and S Ratnaparkhe.

Conflict of Interest: None declared

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