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Role of Earthworms and Actinomycetes in Eco-friendly Degradation of Floral Waste via Vermicomposting and Antimicrobial Potential of Actinomycetes

Shobha Shouche[1], Deepika Tiwari[2*]

[1] *Associate Professor, Govt. Madhav Science P.G. College, Ujjain (M.P.), India*

[2] *Research Scholar, Govt. Madhav Science P.G. College, Ujjain (M.P.), India*

**Corresponding author; Email: deepika.sbt@gmail.com*

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ABSTRACT: The utilization of naturally existing microorganisms in bioremediation procedures, which use contaminated areas and dangerous organic chemical residues to be removed, is constantly developing. Actinomycetes have grown in significance since they are crucial to the recycling of organic debris and the creation of new drugs and enzymes. It is possible to use many Actinomycetes genera to bioconvert underused organic waste into highly valuable chemical compounds. This study was conducted to isolate actinomycetes capable of producing waste-degrading enzymes from floral waste vermicompost. In this study, floral waste was decomposed using the technique of vermicomposting. The floral wastes and cow dung in a ratio of 1:1 (50% each) were fed to *Eisenia fetida* earthworms for 45-60 days, keeping 100% floral waste and 100% cowdung as control. The actinomycetes were isolated and characterized morphologically using the dilution technique on starch casein-agar media. A total of six isolates were selected for enzymatic and antimicrobial screening based on their abundance. The amylase, protease, and peptonization-coagulation activities were determined through a screening procedure. All selected strains demonstrated enzyme production. Actinomycetes growth patterns and mycelial coloration were documented. The cultural and morphological analysis identified actinomycetes genera as *Streptomyces*. The actinomycetes isolated from floral waste were found to be promising microorganisms for the production of antibacterial and antifungal antibiotics. Also, the use of a microbial consortium was found to be the best option for faster degradation of waste.

Keywords: Floral Waste, vermicomposting, actinomycetes, enzyme production, antibiotic production, microbial consortium

1. Introduction

Around the world, waste disposal is a huge issue. Problems with waste reduction arise from the diversity of its components. The management of the temple has expressed worry over the safe disposal of flower waste. The floral waste is just dumped into the rivers, oceans, etc., which has a negative effect on both the water quality and the aquatic life that exists there. Due to various religious traditions, this floral debris accumulates at religious locations such as temples, mosques, and

Gurudwaras. It is also produced in regions such as neighborhoods, community centers, and other communities. Vermicomposting is the term for worm-based composting. Vermicomposting uses worms and microrganisms to break down different organic waste products. These creatures are nature's incredibly helpful means of breaking down organic matter. Hence, vermicomposting is a method that speeds up nature's process of breaking down organic waste and yields a very beneficial final product. Vermicompost, the process' ultimate product, is an excellent organic fertilizer because it contains a wealth of water-soluble nutrients. It is a biological process that transforms organic waste into a beneficial soil additive. Earthworms and microorganisms both play crucial roles in the breakdown and transformation of sludge in a vermicomposting system (Yami et al., 2003). Most prominent among these are bacteria, fungus, and actinomycetes. Three-quarters of all known antibiotics are produced by actinomycetes, making them notable manufacturers (Waksman, 1945). The most prevalent genus in soils, making up to 90% of the populations, is *Streptomyces* sp. The use of novel methods for isolating actinomycetes from floral waste vermicompost and analyzing their enzyme activity, however, is crucial for composting floral waste vermicompost. New chemicals are being discovered from compost environments at a faster rate lately. There is a need to seek for and investigate novel actinomycete strains for a variety of biodegradation applications because actinomycetes have been less thoroughly studied for the biodegradation process than bacteria and fungi. Keeping this point in view, the objectives of the present study are:

1. To collect floral waste from the selected temples of Ujjain city.
2. To perform the vermicomposting method of degradation of organic waste as per standard guidelines for the above selected samples.
3. To isolate Actinomycetes bacteria from the degraded waste vermicompost.
4. To characterize the bacterial isolates on the basis of morphology and biochemical tests
5. Physiological characterization of actinomycetes
6. To do antimicrobial testing of isolated actinomycetes based on primary and secondary screening
7. Application of a microbial consortium of isolated strains to test the potency of degradation of waste

Materials and methods

The floral waste (6kg) was collected from nearby temples in Ujjain (M.P.). The 6 kg of cow dung (CD) was procured from a local cowshed and left for 10 days so as to remove excess heat. For the present study, vermibed was made using 10-day-old cow dung and floral waste. For this, collected floral waste (FW), after being chopped into small pieces, was put into plastic cylindrical bins (27h x 21r) in the following ratio: 50:50 (1kg FW + 1 kg CD). Also, 100% FW bed (2kg) and 100% CD (2kg) were taken as controls. All composition beds were prepared in duplicate and labeled A and B. The beds of floral waste composition were partially composted for 10 days so as to remove the excess heat released during the degradation of waste and the aromatic compounds that might harm the survival of the earthworms, as suggested by Singh et al. (2011). The earthworms (*Eisenia fetida*) were purchased from the local organic farming supplier, Agar, Ujjain. After 10 days, earthworms were released on top of partially degraded waste. The windrow compost method was used, in which it was not covered and no ventilation was provided with pipes. The physical parameters such as temperature (25-30^{o}C), pH (6–8), and moisture content (60–70%) were maintained throughout the study by regular sprinkling of

water so as to provide optimum conditions for earthworms to survive and grow (Shouche et al., 2011). The study was carried out during the winter season. The experiment was maintained for 45–60 days until finely granular vermicompost was prepared. When the floral vermicompost was ready, 100g of sample from each bin was collected in sterile plastic bags, labeled, and kept for further study until pretreatment. The rest of the floral vermicompost was also labeled separately and stored in clean, dried, airtight containers at 4^{o}C.

Pretreatment: Collected samples from each of the bins were air dried for 2 days and then heated in a hot air oven for 2 hours at 50ºC prior to isolation (Arifuzzaman et al., 2010). This helps in decreasing the population of gram negative bacteria (Jeffrey, 2008).

Isolation of actinomycetes

Ten grams of each of the pretreated samples were suspended in 90ml of sterile distilled water normal in 100ml conical flasks and shaken well in a vortex mixture. From each of these stock solutions, samples were serially diluted up to 10^{-3} and were used to spread on sterilized Starch Casein Agar (SCA) medium by using an L-shaped glass rod and incubated at 30°C for 1–2 weeks (Kuster and Williams, 1964; Jeffrey, 2008; Anusuya and Geetha, 2012; Iyer et al., 2014). Itraconazole (75 µg/ ml) and chloramphenicol (50 µg/ ml) were added in both media to inhibit fungal and bacterial contamination, respectively (Williams and Davies, 1965; Peela et al., 2017). The media was procured from HiMedia (Mumbai). After 7 days, the plates were observed for actinomycetes colonies based on color, presence, and absence of aerial and substrate mycelium and subcultured on starch-casein agar plates and stored at 4°C for further studies.

Macroscopic Characterization

Morphological characters of isolates such as colony color, size, shape, opacity, pigment production, presence or absence of aerial and substrate mycelium were observed (Duddu et al., 2016)

Microscopic characterization

Microscopy was carried out with simple stain technique using methylene blue was used (Kahasabuli and Kibera, 2014). Nature of hyphae and spore chain was observed and identified according to Shirling and Gottlieb, 1966 and photograph was taken.

Enzymatic screening

Amylase Production

The isolates were cultured on sterile starch media (HiMedia) for 4 days at 30ºC, then it tested for amylase production by flooding bacterial growth by iodine solution. The positive result represented by appearance of clear zone around the isolates surrounding by purple background.

Protease Production

Actinomycetes isolates were grown on casein agar media (HiMedia). The agar plates were streaked by isolates and incubated at 30ºC for 4 days. The formation of clear zone around actinomycetes colonies represented positive results (Salle, 1948).

Peptonization and coagulation of milk

Milk coagulation and peptonization test were carried out with skim milk. The skim milk containing test tubes were inoculated with isolates and incubated at 30ºC for 4 days. The extent of coagulation and peptonization was recorded on 4th day.

Effect of temperature on growth

The ability of the selected isolates to grow at different temperatures was studied at 10 °C, 20°C, 30 °C and 40 °C. The isolates were streaked on starch casein agar slants and incubated at different temperatures (Muiru et al., 2008) and after 7 days observed their growth.

Primary screening for antibiotic production of actinomycetes

The antimicrobial activity of actinomycetes isolated above from vermicompost samples was tested. ATCC strains of *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia*, and *Bacillus subtilis* were used for primary screening, as were human test fungi *Candida albicans*. By using the cross streak method, activities were carried out on Muller Hinton agar for bacteria and Potato Dextrose agar for fungi (Jeffrey, 2008). Each plate was streaked and stabbed with actinomycetes exhibiting positive morphological and biochemical characteristics in the centre of a plate before being incubated at 30°C for 6-7 days. The actinomycetes isolate was streaked perpendicular to a 24 hour subcultured bacteria and a 48 hour old culture of fungi. Then, the plates were incubated for 24 h at 37°C for bacteria and 96 hrs for fungi. After incubation, the isolates showing zone of inhibition against bacteria were selected for secondary screening. Secondary screening of potent strains was further performed by using agar well diffusion method as described elsewhere (Duddu et al., 2016)

Preparation of microbial consortium

All six strains of actinomycetes were selected for consortium formation. Each strain was grown in 3 ml of ISP4 medium broth (HiMedia) at 30˚C for 96 hours to ensure good sporulation. After incubation, the broth cultures of each strain were decanted into a fresh sterile flask, and all the suspensions of ACT 1–6 were pooled together. A stock culture of inoculum was prepared, and it was inoculated in 100 ml of minimal broth containing flower waste. Broth was incubated at 30ºC for 96 hrs. After incubation, this broth was used as a consortium (Kannahi and Babynisha, 2014).

Vermicomposting using microbial consortium:

For vermicomposting, floral waste and cow dung were collected. Floral waste was chopped, and vermicomposting beds were prepared with the following compositions: 1kg of floral waste + 1kg of cowdung + 5 ml of inoculum; 1kg of floral waste + 1kg of cowdung without inoculum; 100 % floral waste + 5 ml of inoculum; and 100% floral waste without inoculum. The vermicomposting beds without inoculum were kept as controls. The waste composition in the beds was allowed to partially decompose so as to remove excess heat before adding earthworms. After 7 days, 20 earthworms were added to each bed.

Results and discussions

From the 6 vermicompost beds, including duplicates, 50 colonies were obtained on starch-casein agar plates showing actinomycetes-like characteristics (Table 1). The isolates from each sample showing different types of morphology were picked and purified on SCA. A similar study was carried out by Pattnaik and Reddy (2012), where actinomycetes were isolated from floral waste vermicompost. All the isolates were also grouped on the basis of colour series. The macroscopic characteristics of isolates observed were big white colonies, blue-white colonies, grey, brownish white and small white cottony colonies (Fig. 1). All the colonies were hard on the agar surface, adhering to the medium and having both aerial and surface mycelium. The variations in the colours of the aerial mycelia of the isolates may be an indication of the diversity or variability of the isolated actinomycetes.

Table1: Actinomycete isolates from each soil sample collected from floral waste

Sample No.	Sample Code	No. of isolates	CFU/g
1	VFW-A	6	$6x10^2$
2	VFW-B	5	$5x10^2$
3	VFWCD-A	11	$11x10^2$
4	VFWCD-B	9	$9x10^2$
5	VCD –A	8	$8x10^2$
6	VCD –B	9	$9x10^2$

Jeffrey (2008) observed similar results with dark grey, brownish white, whitish, grey colonies of actinomycetes isolates on starch casein agar medium. Six isolates were chosen from the purified colonies based on their different colours and subjected to microscopic examination: ACT1 (bluish white), ACT2 (brownish white), ACT3 (grey), ACT4 (dark grey), ACT5 (cottony white), and ACT6 (smooth white) (Fig. 2). The spore chain morphology of each of the six isolates was used to characterize them. ACT1, ACT2, ACT3, ACT5, and ACT6 all had rectus flexibilis spore chains, whereas ACT4 had spira spore chains. Based on spore chain morphology all were isolates were identified as *Streptomyces* species (Shirling and Gottileb, 1966). One of the most important characteristics in actinomycetes identification is spore chain morphology, which varies by genus and species (Shirling and Gottileb). Amylase, protease production, and milk peptonization and coagulation were all found in all six actinomycetes isolates (Table 2). Jeffrey (2008) discovered that approximately 98.4% of the total isolates had one or more enzymatic activities. Natural selection may have resulted in actinomycetes' ability to secrete broad-range enzymes in order to survive in a competitive environment (Boroujeni et al., 2012).

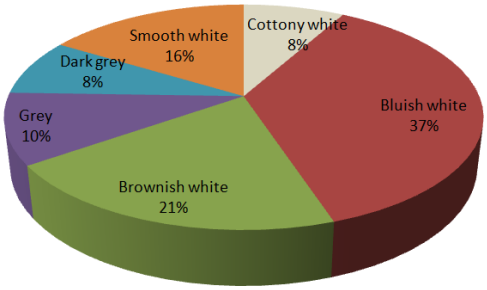
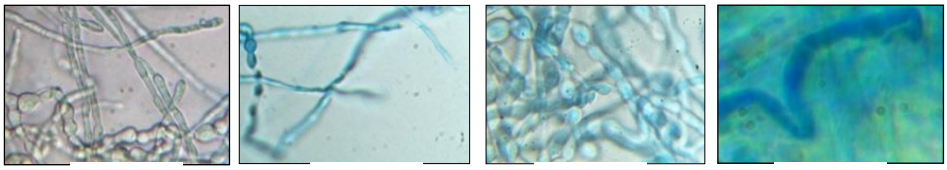


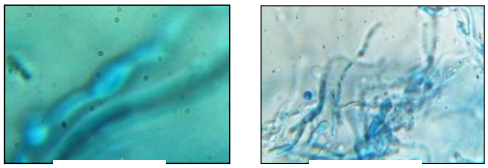
Fig.1. Percentage of colony color observed on starch casein agar

a b c d e f

Fig.2. Colony of actinomycetes a) ACT1 b) ACT2 c) ACT3 d)ACT4 e)ACT5 f) ACT6



ACT11 ACT21 ACT31 ACT41



ACT51 ACT61

Fig.3. Spore Chain Morphology of Actinomycetes

Table2: Enzymatic production ability of Streptomyces isolates

Test	ACT1	ACT2	ACT3	ACT4	ACT5	ACT6
Amylase	+	+	+	+	+	+
Protease	+	+	+	+	+	+
Peptonization &coagulation of milk	+	+	+	+	+	+

Physiological characterization

The results of physiological characteristics indicated that all the selected actinomycetes isolates had shown excellent growth at 30 °C and 40 °C, moderate at 20 °C and poor at 10°C.

Antimicrobial testing

Antimicrobial screening revealed that four of six actinomycete isolates had antimicrobial activity against one or more test bacteria and fungi (Table 3). However, ACT 4 exhibited no antimicrobial activity.

Table3: Zone of inhibition (mm) against test bacteria and fungi via secondary screening

Isolate No.	*Bacillus subtilis*	*E.coli*	*K.pneumoniae*	*S. aureus*	*Candida albicans*
ACT1	11.4±0.05	8.5±0.05	12.3±0.0	12.0±0.0	11.3±0.1
ACT2	10.0±0.05	13.1±0.1	11.1±0.1	13.1±0.05	10.1±0.0
ACT3	12.0±0.1	9.2±0.0	12.2±0.0	12.2±0.05	12.0±0.05
ACT4	-	-	-	-	-
ACT5	9.0±0.1	9.6±0.05	11.0±0.0	9.5±0.05	8.1±0.05
ACT6	11.0±0.05	9.5±0.05	9.1±0.05	12.4±0.1	8.1±0.0

Table 4: Effect of microbial consortium on floral waste degradation

S. No.	Composition	No. of days required to degrade waste
1	1kg of floral waste + 1kg of cowdung without inoculum,	45
2	1kg of floral waste + 1kg of cowdung + 5ml inoculum, and	30
3	100% floral waste without inoculum	60
4	100 % floral waste + 5ml inoculum	35

Results indicated that the use of the test consortium reduced the overall time required for composting in addition to producing the nutrient-enriched compost product. Similar studies were carried out by several researchers (Jadhav et al., 2013; Kannahi and Babynisha, 2014), in which the use of a microbial consortium showed rapid degradation of waste in a short period of time (Table 4).

Conclusion

Vermicomposting technology could be broadly used for the management and recycling of nirmalaya or floral wastes, lowering the bulk and level of pollution at the generation site. It could be the best organic fertilizer for producing organic vegetables, organic fruits, and ornamental plants. Since vermicompost produces a high population of beneficial microflora, its application to cultivated land will also increase their population, increasing soil fertility and reducing pathogenic microorganisms,

and the analysis will let us know the microbial diversity mainly involved in the decomposition process, which will be beneficial to prepare a microbial consortium culture that can be used to achieve even faster decomposition in a short period of time. Apart from bioremediation, actinomycetes also play an important role as biocontrol agents. At present, there is a need to find novel antimicrobial-producing strains as the pre-existing drugs have failed due to the development of resistance among the microorganisms. The present study is also a small contribution towards meeting this need. The study showed that actinomycetes are important agents in turning waste into a resource that can be used to produce antibiotics of medicinal value and enzymes of commercial importance.

Declarations

The manuscript has not been submitted in any other journal or conference.

Conflicts of Interest

There are no conflicts to declare.

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