Analysis on Biological activities of coelomic fluid and microbiome of earthworm, Lumbricina

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Abstract: **Earthworms have been found to exhibit various biological activities such as Hemolytic activity, proteolytic activity, urease production and antibacterial activity and so on. Soils harbor a diverse range of microorganisms, including bacteria, fungi, and protozoa. Balanced nutrient levels nitrogen (N), phosphorus (P), and potassium (K) in balanced proportions. After Using Ammonia and Urea Fertilizer, that can significantly impact soil properties.It can cause damage to their skin, muscles, and internal organs pH imbalance Ammonia can alter the soil pH, making it more acidic. Earthworms are sensitive to pH changes and can be affected by the altered soil chemistry.While earthworms can tolerate some pH changes, extreme pH fluctuations can be detrimental to their health Soil structure alteration Urea can affect the soil structure, leading to changes in the soil's porosity, aeration, and water-holding capacity. These changes can make it difficult for earthworms to move and burrow in the soil.**

Key words: **Lumbricina, Antibacterial Activity, Hemolytic Activity, proteolytic Activity, Urea and Ammonia fertilizer.**

I.INTRODUCTION

Earthworms are important soil invertebrate organisms that participate in nutrient cycling in terrestrial ecosystems and in the formation of the soil profile from the physical, chemical and also microbial point of view. Annelida is a Ubiquitous, common and diverse group of organisms, found in terrestrial, fresh water and marine environments (Maria Capa., et al 2021). Earthworms are used as a model organism in immunology for decades. Their simple body plan consists of two main body cavities: true coelom and digestive tube. Both coelomic cavity and digestive tract represent open systems with permanent contact with soil microorganisms (Lavelle, P. 1988). Two novel pattern recognition receptors (PRRs), Toll-like receptor (TLR) and lipopolysaccharide binding protein/bactericidal permeability-increasing protein (LBP/BPI) were characterized in earthworms. These molecules are expressed in coelomocytes and their production is upregulated after microbial challenge. Moreover, both receptors were detected in digestive tract (Petra Prochazkova, et al. 2019).

Taxonomy of Lumbricina

Kingdom - Animalia
Phylum - Annelida
Class - Polychaeta
Order - Eunicida
Family - Lumbrinerida
Genus - Lumbricina

The coelomic cavity is part of the main body plan of annelids. This fluid filled space takes up a considerable volume of the body and serves as an important site of exchange of both metabolites and proteins. In addition to low molecular substances such as amino acids and glucose and lactate, the coelomic fluid contains different proteins that can arise through release from adjacent tissues (intestine) or from secretion by coelomic cells (Sven Schenk., et al 2020). The earthworms are essential components of the soil biological community. They play a major role in the biogeochemical cycle of terrestrial ecosystems because of their influence on microbial activity, carbon and nitrogen cycles and alteration of soil. Earthworms are the most abundant invertebrate in agricultural lands from temperate regions, where they account for 905 of invertebrate biomass (Marcos Perez., et al 2012). Invertebrates have developed a variety of active immune mechanisms including the production of antimicrobial peptides, coagulations, phagocytosis, and encapsulation reactions. The rapid response is mediated by soluble antibacterial

molecules, such as lysozyme, lysenin, and lumbricin (Alessio Alesci., et al 2023).

Some of the proteins present in the coelomic fluid of earthworms include:

- Lysozyme an enzyme that breaks down bacterial cell walls, providing antimicrobial protection.
- Albumin a protein that helps maintain osmotic balance and transport small molecules (Jamroz, R. C., et al 1993).
- Globulins a class of proteins that include immunoglobulins, which are involved in the immune response (Bilej, M., et al 1995).
- Antimicrobial peptides (e.g., lumbricina): small peptides that exhibit antimicrobial activity against bacteria, fungi, and viruses (Cho, J.H., et al 2002).
- Proteases (e.g., earthworm trypsin) enzymes that break down proteins into smaller peptides or amino acids (Shet, M.S., et al 2005).
- Lipopolysaccharide-binding protein a protein that binds to bacterial lipopolysaccharides, helping to neutralize their toxic effects (Li, Q., et al 2008).
- Cytokines signaling molecules that help coordinate the immune response (Wang, Y., et al 2015). These proteins play important roles in maintaining the earthworm's internal environment, defending against pathogens, and supporting overall health.

The coelomic fluid of earthworms contains various antibacterial molecules that help protect the worm against bacterial infections.

Some of the antibacterial molecules present in the coelomic fluid of earthworms include

- Lumbricina 7.5 kDa antimicrobial peptide that exhibits broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria (Cho, J.H., et al 2002).
- Lysenin a 50 kDa protein that exhibits antibacterial activity against Gram-positive bacteria, including *Staphylococcus aureus* and *Bacillus subtilis* (Nakamura, K., et al 2005).
- Eiseniapore a 2.5 kDa antimicrobial peptide that exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa* (Wang, Y., et al 2011).
- Ficolin a 35 kDa protein that exhibits antibacterial activity against Gram-positive bacteria, including *Staphylococcus aureus* and *Streptococcus pneumoniae* (Li, Q. et al 2013).
- Lysozyme an enzyme that breaks down bacterial cell walls, providing antimicrobial protection against both Gram-positive and Gram-negative bacteria (Dales, R. P. 1978).
- Cecropin a 3.5 kDa antimicrobial peptide that exhibits antibacterial activity against both Gram-positive and Gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa* (Cho, J.H., et al 2004).

The coelomic fluid of earthworms contains various hemolytic molecules that can lyse red blood cells. Some of the hemolytic molecules present in the coelomic fluid of earthworms include:

- Lysenin a 50 kDa protein that exhibits hemolytic activity against mammalian red blood cells (Nakamura, K., et al 2005).
- Eiseniapora a 2.5 kDa antimicrobial peptide that also exhibits hemolytic activity against mammalian red blood cells (Wang, Y.,et al 2011) .
- Lumbricin a 7.5 kDa antimicrobial peptide that exhibits hemolytic activity against mammalian red blood cells, although at higher concentrations than lysenin(cho,J.H.,et al 2004).
- Cecropin a 3.5 kDa antimicrobial peptide that exhibits hemolytic activity against mammalian red blood cells, although at higher concentrations than lysenin (cho,J.H.,et al 2004).
- Coelomic cytotoxin a 60 kDa protein that exhibits hemolytic activity against mammalian red blood cells (li, Q., et al 2013)..
- Hemolysin a 40 kDa protein that exhibits hemolytic activity against mammalian red blood cells (Nakamura ,K., et al 2015).

These hemolytic molecules may play roles in the earthworm's defense against pathogens, although their exact functions and mechanisms of action are not yet fully understood.

II.METHODS AND METHODOLOGY

Collection of Sample

The earthworm (Lumbricina) species were collected from Ilamanoor land form in Ramanathapuram. Gut clearance of earthworm was performed using 1% agar medium for period of 24 hours.

Lumbricina

Gut Clearance of Earthworm

Mature earthworms were washed with running tap water to remove the soil on the earthworm's body surface. Later it was placed in a box containing small pieces of tissue paper and left overnight for the gut clearance. Water was added to the tissue paper for the maintenance of humidity and left overnight for gut clearance. Before using the earthworms, they were put into the distilled water for about 1 hours to get rid of the paper from the earthworm gut.

Extraction of Coelomic Fluid

The coelomic fluid was extracted by using cold shock method. The gut cleared earthworms were washed with distilled water and dried using tissue paper. Thirty earthworms were placed in a sterile petri plate containing 1 ml of Phosphate Buffer Saline and was kept in the freezer for 5 minutes. After incubation, the fluid was collected in a sterile tube and stored at C for future use. The gut cleared thirty earthworms were washed with distilled water and washed and was injected with of Biofertilizers such a Urea and Ammonia. After 24 hours of inoculation of the microorganisms, the earthworms were placed in a sterile petri plate containing 1 ml of Phosphate Buffer Saline which was kept in the freezer for 5 minutes. After incubation, the fluid was collected in a sterile tube and stored at C for future use.

Isolation of Bacteria

1. Spread plate method: The spread plate method is used to isolate bacteria from a mixed culture. A loopful of the mixed culture is spread onto a agar plate, and the plate is incubated at 37°C for 24-48 hours(Atlas, R.M. 2010).

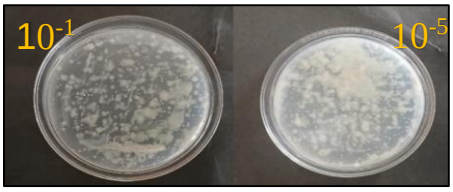

Figure shows Enumeration of bacterial colonies 24 hours incubated from the coelomic fluid of the Earthworm, *Lumbricna*

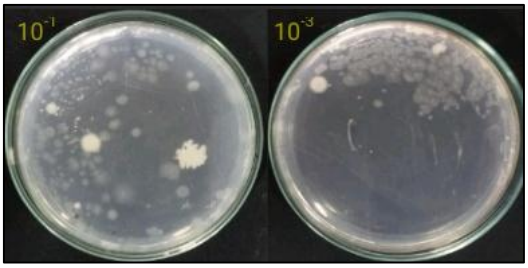

Figure shows Enumeration of bacterial colonies on 48 hours incubated from coelomic fluid of the Earthworm, *Lumbricina*

2. Streak plate method: The streak plate method is used to isolate bacteria from a mixed culture. A loopful of the mixed culture is streak onto a agar plate, and the plate is incubated at 37°C for 24-48 hours(Cappuccino, J.G., et al 2013).

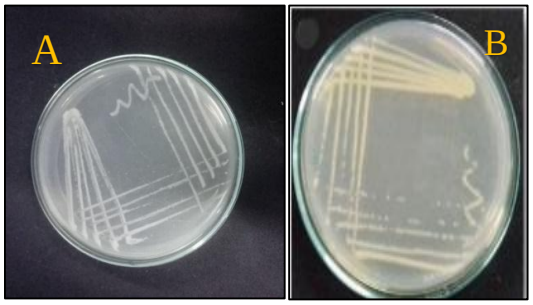

Fig.3: Isolated Bacterial cultures on 24 (A) & 48 (B) hours incubated from Coelomic fluid of Earthworm, *Lumbricina.*

Mass Cultivation

1. Shake flask method: The shake flask method is used to cultivate microorganisms on a large scale. A shake flask containing a suitable medium is inoculated with the microorganism, and the flask is incubated on a shaker at 37°C for 24-48 hours (Cappuccino, J.G., et al 2013).

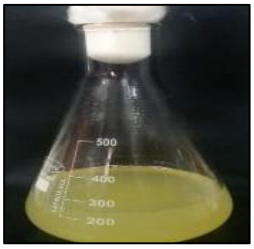

Figure shows that Mass Cultivation

GRAM STAINING

The loopful of the overnight culture was subjected to Gram's Staining. The culture was smeared and heat-fixed on a glass slide and stained using crystal violet for 1 minute followed by a water wash. Gram's Iodine was added to the smear and kept undisturbed for 1 minute after which a water wash was done. Sequentially, the smear was stained with Gram's decolorizer for 15 seconds after which it was again water washed. The smear was counted stained with Saffranin for 1 minute and it was finally water-washed with tap water. The smear was air dried and examined under 100X oil immersion microscope and the results were noted.

Fig.4: Microscopic view of the Isolated Bacterial culture

Biochemical Tests

Indole test: The Indole test is used to detect the ability to produce tryptophanase enzyme, will convert tryptophanase amino acid into indole gas. A few drops of kovac's reagent is added to the test tubes. The Indole gas react with the reagent and the production of red color which indicates a positive result.

Methyl Red test: The Methyl Red test is used to detect the ability to use acid production. The acid production decrease the medium pH that change the color of methyl red from yellow to red color which indicate a positive result.

Voges-Proskauer test: The voges-proskauer test is ability to use acetoin production. Acetoin is the in-between product of this reaction that is identified by using alpha-naphthol and 40 percent KOH. A few drops of barrit's reagent is added to the test tubes, will react with the reagent and production of reddish brown color indicate the presence of positive result.

Citrate Utilization test: The citrate test is ability to use citrate for the carbon source. The presence of citrate utilizing microorganisms which indicate turns into blue color to confirm breakdown of citrate as a positive result (Muhammad Shoaib., et al 2020).

Catalase test: The catalase test is used to detect the presence of catalase enzyme, which breaks down hydrogen peroxide into water and oxygen. A few drops of hydrogen peroxide are added to a bacterial colony, and the production of bubbles indicates a positive result (Cappuccino, J.G., et al 2013).

Oxidase test: The oxidase test is used to detect the presence of cytochrome c oxidase enzyme. A few drops of oxidase reagent are added to a bacterial colony, and the production of a blue color indicates a positive result (Atlas, R.M. 2010).

Urease test: The urease test is used to detect the presence of urease enzyme, which breaks down urea into ammonia and carbon dioxide. A few drops of urease reagent are added to a bacterial colony, and the production of a pink color indicates a positive result (Gerhardt, P., et al 2013).

Table: 1 Biochemical characterization of the isolated Bacterial Culture

BIOCHEMICAL TESTS	RESULTS
Indole	+
Methyl Red	+
Voges Proskauer	+
Citrate Utilization	+
Catalase	+
Oxidase	+
Urease	+

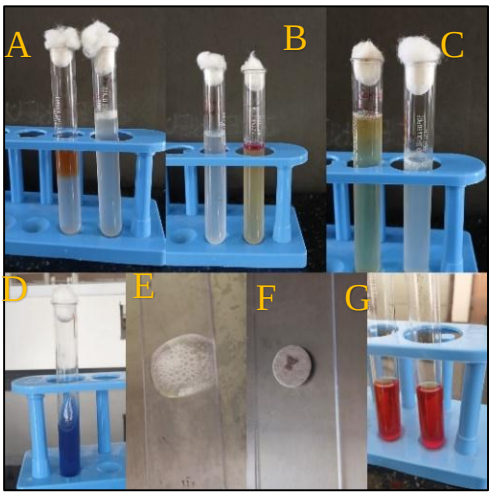

Fig.5: Biochemical Tests of the isolated bacterial culture A. Indole B. Methyl Red C. Voges Proskauer D. Citrate Utilization E. Catalase F. Oxidase G. Urease

Antibacterial Tests

1. Disc diffusion method: The disc diffusion method is used to test the antibacterial activity of a compound. A disc impregnated with the compound is placed on a agar plate inoculated with bacteria, and the plate is incubated at 37°C for 24-48 hours. The zone of inhibition around the disc indicates the antibacterial activity of the compound (Cho, J.H., et al 2002).

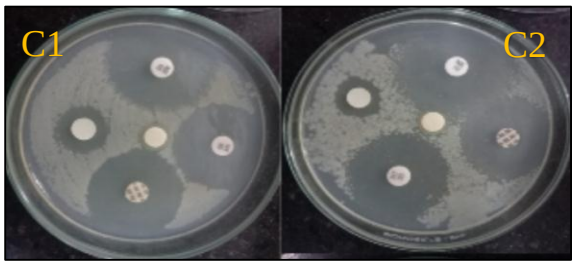

Fig.6: Antibacterial activity of Gram-positive bacterial culture C1,C2. Isolated colonies from Urea and Ammonia.

Antagonistic Activity

Dual culture method: The dual culture method is used to test the antagonistic activity of one microorganism against another. Two microorganisms are grown together on a agar plate, and the plate is incubated at 37°C for 24-48 hours. The inhibition of growth of one microorganism by the other indicates antagonistic activity (Li, Q ., et al 2008).

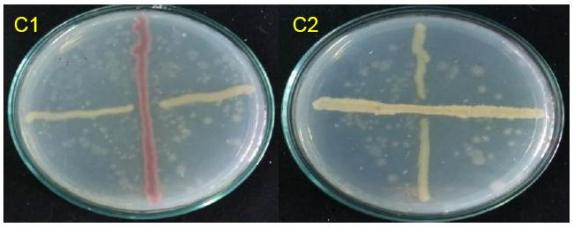

Fig.7: Antagonistic Activity of C1, & C2 Cultures Isolated from Urea and Ammonia Coelomic Fluid of Earthworm Against Isolated culture.

Hemolytic Test

Blood agar plate method: The blood agar plate method is used to test the hemolytic activity of a microorganism. A loopful of the microorganism is streaked onto a blood agar plate, and the plate is incubated at 37°C for 24-48 hours. The production of a clear zone around the colonies indicates hemolytic activity (Nakamura, K., et al 2011).

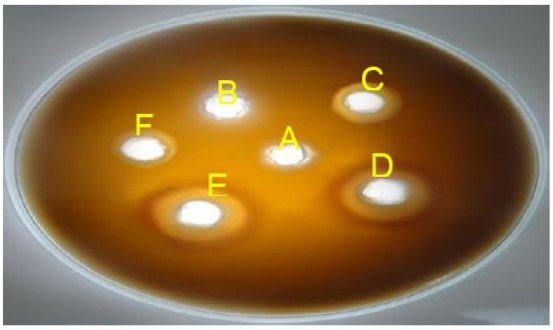

Fig.8: Haemolytic Activity against A, B. Tris Buffer C, D. Isolated colonies *C*ulture Injected Coelomic Fluid of Earthworm (24 & 48 hours incubation) E, F. Isolated colonies Culture Injected Coelomic Fluid of Earthworm (24 & 48 hours incubation).

Proteolytic Test

Gelatinase test: The gelatinase test is used to test the proteolytic activity of a microorganism. A loopful of the microorganism is streaked onto a gelatin agar plate, and the plate is incubated at 37°C for 24-48 hours. The production of a clear zone around the colonies indicates proteolytic activity (Wang, Y., et al 2015).

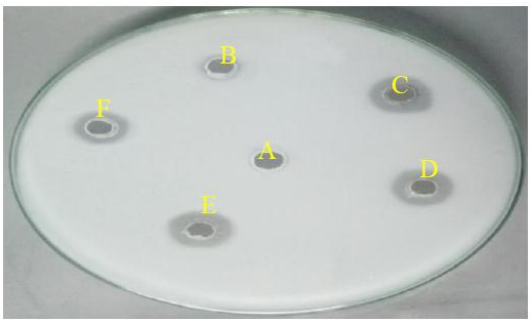

Fig.9: Proteolytic Activity against A, B. Tris buffer C, D. 24 hours incubated isolated colonies Culture Injected Coelomic Fluid of Earthworm (24&48 hours incubated) E, F. Isolated colonies Culture Injected Coelomic Fluid of Earthworm (24&48 hours incubated).

III.CONCLUSION

The present study is mainly focused on the view of secretion of immunological compound within 24 hours of disturbance induced in earthworms from environment. The secreted compounds become active in 24 hours of exposure to foreign substances and exerts biological functions destroying the entered foreign particle after which becomes inactivated after 48 hours. the future studies shows that coelomic fluid and microbiome of Lumbricina may serve as sources for the development of novel antimicrobial ,antioxidant and anti-inflammatory agents,

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