

**Regeneration Augmentation Using the Earthworm,
Perionyx excavatus
Symbiotic Cell Free Bacterial Extract**

PROJECT WORK

Submitted in partial fulfillment of the requirements for the
award of Bachelor of Science Degree in Microbiology

By

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SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
CATEGORY -1 UNIVERSITY BY UGC
(DEEMED TO BE UNIVERSITY)

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **RAMAPRABHA R (41300033)** who have done the Project work entitled "**Regeneration Augmentation Using The Earthworm, *Perionyx excavatus* Symbiotic Cell Free Bacterial Extract**" under our supervision from January 2024 to April 2024.

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ABSTRACT

Regeneration, the complex process by which organisms regenerate injured tissues or missing body parts, keeps generating scientific interest due to its substantial implications in medicine and biotech. The earthworm, *Perionyx excavatus*, which is well-known for its extraordinary regenerating properties, has gained attention in recent years. Understanding the basic principles behind tissue augmentation and healing may be done by using this organism as a model. This project aims to study the symbiotic relationship between *P. excavatus* and certain bacteria, opening up fresh possibilities for biotechnological uses and explaining how these interactions support processes of augmentation and regeneration. We have isolated the bacterial colonies from the gut soil of earthworm, *P. excavatus* which is symbiotically related to them and the bacterial extract is precipitated using the ammonium sulfate method. The bacterial extract which has a wide range of bioactive chemicals is subjected to cell proliferation assay and wound healing assay on 3T3 cell lines (mouse embryonic fibroblast cell lines). The obtained results showed the high cell proliferation rate of 3T3 cells which indicates the enhanced regeneration on the cell lines. This study can open a door for developing novel therapeutics in regenerative medicine.

Keywords: *Perionyx excavatus*, Symbiotic relationship, Cell-free extract, 3T3 cell lines, wound healing

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LIST OF ABBREVIATIONS

LB medium	Luria Bertani medium
lbs	Libra pondo or a pond of weight
DMEM	Dulbecco's' Modified Eagle Medium
PBS	Phosphate Buffered Saline
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
DMSO	Dimethyl sulfoxide

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CHAPTER – 1

INTRODUCTION

1.1. UNDERSTANDING REGENERATION: EXPLORING THE PHENOMENON

Regeneration, a fundamental biological process, refers to the ability of organisms to replace or repair damaged or lost tissues, organs, or body parts, enabling them to restore form and function (Brockes & Kumar, 2008). This remarkable phenomenon spans across various taxa, from simple multicellular organisms to complex vertebrates, demonstrating the versatility and adaptability of life (Alvarado, 2000). Hydra, annelids, mollusks, nemertean worms, planarians, chordates, and vertebrates are some of the well-known examples with diverse regeneration capabilities (Tanaka & Reddien, 2011). Earthworms are one type of annelid that can regenerate whole body segments, much like other invertebrates (Bely, 2006). Two different regeneration phenomena are seen in earthworms: one group of species requires the clitellum to regenerate, whereas the other group of worms may regenerate without it. *Perionyx excavatus* is one of the worms that are not dependent on clitellum for regeneration.

1.2. EARTHWORM, *PERIONYX EXCAVATUS*

The genus *Perionyx* includes earthworms that are widely distributed throughout tropical Asia. These species are epigeic, meaning they inhabit high-moisture, organic-matter-containing soils and organic wastes. Even though several writers have extensively researched *P. excavatus* biology and waste decomposition efficiency (Edwards *et al.*, 1998; Reinecke *et al.*, 1992; Suthar & Ram, 2008).

Perionyx excavatus commonly known as the blue or Indian blue worm, exhibits remarkable regenerative abilities, typically being able to regenerate lost segments of their bodies (Cho *et al.*, 2009) belongs to the following taxonomic classifications:

Kingdom	Animalia
Phylum	Annelida
Class	Clitellata
Order	Haplotaxida
Family	Megascolecidae
Genus	<i>Perionyx</i>
Species	<i>excavatus</i>

1.3. SYMBIOTIC RELATIONSHIP

The digestive system of earthworms contains a vast diversity of microorganisms, but little is known about them since they are soil-dwelling and hence not culturable. It is recognised that bacteria, viruses, and bacteriophages may be found in soil together with prokaryotes and eukaryotes (Adnan & Joshi, 2013) . They all have distinct interactions with one another, form incredibly diverse and intricate connections, and alter the solid, liquid, and gaseous phases of soil (Hornor & Mitchell, 1981) . Along with microbes including actinomycetes, bacteria, fungus, and protozoa, the detritus plants in the soil serve as the primary food supply for earthworms. Since the development of some bacteria is not being inhibited by the gut enzymes, this interaction does not reflect a predator-prey relationship (Lee, 1985). The discovery of bacteria in the digestive tracts of three species of earthworms-*L. terrestres*, *A. caliginosa*, and *A. longa*-is credited to the forefather Parle. The soil and earthworm gut lysate included a variety of bacterial, fungal, and actinomycetes species (Liliensiek *et al.*, 2012) . According to one research, the posterior section of an earthworm's gut contains 473 microorganisms, of which 73% are *Vibrio* and 70% are *Streptomyces limanii*, which is an uncommon species in nature (Gómez-Brandón *et al.*, 2010) . A different research examined the fungi in the intestines of earthworms that

are present in India's tropical areas and found eighteen different species, including *Aspergillus*, *Penicillium*, *Theilavia*, *Fusarium*, *Rhizopus*, *Curvularia*, *Cladosporium*, and *Actinomucor* (Egert *et al.*, 2003) . The primary actinomycete discovered in earthworm guts, *Streptomyces caeruleus*, was shown to have evolved more effectively than soil and to efficiently break down organic debris (Adnan & Joshi, 2013) . The most significant terrestrial ecosystem is soil, which is involved in both nutrient transport and breakdown. It is known that prokaryotes and earthworms are in charge of them. It was discovered that the intestinal mucus of *Octolasion lacteum* and *Lumbricus terrestris* earthworms interacts physically with filamentous bacteria. It was discovered that the hook constructions in the walls held them in place. Thus, it was determined that bacteria had evolved to survive inside earthworm intestines. Numerous additional investigations of the variety of bacteria in the gut have revealed that the *Bacillus* genus predominates there. It has been shown that acidovorax forms a symbiotic interaction with nephridia nodules in the stomach and aids in the breakdown of proteins. The environment in the gut area affects the spread of microorganisms because certain germs may thrive there while others may not. As they go through the intestinal tract, they form a symbiotic association (Schramm *et al.*, 2003).

1.4. AUGMENTATION THROUGH BACTERIAL EXTRACTS

Augmentation through bacterial extracts from earthworms offers a promising avenue for enhancing agricultural practices and environmental sustainability. Earthworms, through their symbiotic relationship with beneficial bacteria in their gut, produce a plethora of bioactive compounds with diverse functions. These bacterial extracts, rich in enzymes, hormones, and growth factors, can be a remarkable potential in promoting plant growth, improving soil structure, and enhancing nutrient cycling. Moreover, their biodegradable nature and low environmental impact make them an attractive alternative to chemical fertilizers and pesticides. By harnessing the bioactive properties of earthworm-associated bacteria, we can pave the way for more eco-friendly and sustainable agricultural practices, thereby contributing to the resilience and productivity of agricultural ecosystems in the face of global challenges such as climate change and soil degradation (Luepromchai *et al.*, 2002)

1.5. THE ROLE OF SYMBIOSIS IN CELLULAR REGENERATION

The role of symbiosis in cellular regeneration, facilitated by bacterial extracts from earthworms, represents a fascinating intersection of biology and biotechnology with profound implications for regenerative medicine and tissue engineering. Earthworms harbor a diverse array of symbiotic bacteria within their gut, which play crucial roles in aiding digestion, nutrient absorption, and overall health. These bacteria produce a myriad of bioactive compounds, including enzymes, growth factors, and signaling molecules, which have been found to possess remarkable regenerative properties.

Firstly, these bacterial extracts contain enzymes such as proteases, lipases, and carbohydrases, which can degrade extracellular matrix components and facilitate tissue remodeling. By breaking down old or damaged tissues, these enzymes create a conducive environment for cellular regeneration to occur. Secondly, bacterial extracts from earthworms are rich in growth factors and cytokines that can stimulate cellular proliferation, migration, and differentiation. These bioactive molecules interact with cellular receptors, triggering intracellular signaling pathways involved in tissue repair and regeneration. For instance, growth factors like fibroblast growth factor (FGF) and transforming growth factor-beta (TGF- β) have been shown to promote the proliferation of various cell types and stimulate the synthesis of extracellular matrix proteins (Farooq *et al.*, 2021)

Furthermore, bacterial extracts from earthworms contain antimicrobial peptides and immunomodulatory molecules that help to maintain a balanced microbial community and regulate the immune response during tissue regeneration. By modulating inflammation and preventing microbial infections, these compounds create a favorable environment for cellular regeneration to proceed unhindered. Moreover, the biocompatibility and biodegradability of bacterial extracts make them ideal candidates for biomedical applications. Unlike synthetic biomaterials, which may elicit immune responses or cause toxicity, bacterial extracts derived from earthworms are derived from natural sources and are generally well-tolerated by mammalian cells and tissues. Additionally, their biodegradability ensures that they can be safely metabolized and eliminated from the body once tissue regeneration is complete.

CHAPTER – 2

REVIEW OF LITERATURE

The stomach of an earthworm is an anaerobic environment that contains a lot of mucus, organic waste, and minerals from the soil. Because of these particular gut environmental conditions, some microbes derived from soil, especially facultative and anaerobes, can grow more readily (Drake & Horn, 2007) . For example, (Wüst *et al.*, 2011) found that the earthworm intestines were significantly stimulated with *Clostridiaceae* and *Enterobacteriaceae*, two families directly linked to the breakdown of saccharides originating from mucus and plants. The intestines of earthworms contain an abundance of copiotrophic bacteria, including *Bradyrhizobium*, *Mycobacterium*, and *Streptomyces*, which are strongly linked to the metabolism of carbon and nitrogen (Fujii *et al.*, 2012; Thakuria *et al.*, 2010). Moreover, other bacteria found in the guts of earthworms could have advantageous roles for plants. For example, endogeic earthworms (*Pheretima guillemi*) have the ability to accumulate a significant number of bacteria that dissolve silicate in their stomachs, which speeds up the weathering of silicon and encourages crops to absorb it (Hu *et al.*, 2020).

Numerous investigations have revealed that the surrounding soil's environmental factors may have a major impact on the microbial communities in the guts of earthworms. Different types of food sources can also significantly regulate the composition of the earthworm gut bacterial community and the dominant species (Hu *et al.*, 2020; Knapp *et al.*, 2009). For instance, earthworm gut microbial diversity was significantly decreased in soils contaminated by triclosan (Ma *et al.*, 2017).

However, using a fully randomised design, the possible removal of DCB by the earthworm *Eisenia fetida* and its symbiotic bacteria in a vermicomposting system over a period of 72 days was investigated by (Zenteno-Rojas *et al.*, 2019) . The contaminated substrate's high amounts of decachlorobiphenyl (DCB) were considerably removed by the vermicomposting system, according to the results. Decachlorobiphenyl at high concentrations can be removed from polluted soils using a vermicomposting system or degrading bacteria from *Eisenia fetida* worms.

The earthworm is a very useful tool for treating skin wounds since its biomolecules can be separated and have a range of bioactivities, such as stimulating cell migration, proliferation, production of extracellular matrix, and secretion of growth factors (He *et al.*, 2021; Wang *et al.*, 2021) . Furthermore, DPPH and ABTS+ free radicals are scavenged by earthworm extract to provide its antioxidant effects (Galkowska *et al.*, 2006) . Earthworm extract may be a source of useful compounds for medicinal purposes, according to the evidences of research. Deng *et al.*, 2018 , examined how earthworm extract (EE) affected the healing process of skin wounds. The information gathered demonstrated that EE had a healing impact on mice's local wounds. Based on macroscopic, histopathologic, hematologic, and immunohistochemical characteristics, it shortened the time it took for a wound to heal and lessened the negative consequences of inflammation.

Of all the natural resources available, earthworms have become a very interesting research topic due to their abundance of bioactive chemicals that have significant effects on cellular regeneration. Exploring the potential of earthworm extracts to improve the regeneration ability of normal cell lines has generated a wave of interest. For example, a study by (Chen *et al.*, 2010) showed that earthworm extracts can be a potential nerve growth factor-mediated neurite outgrowth from PC12 cells as well as the expressions of growth associated protein 43 and synapsin I. Another study by (Wei *et al.*, 2009) reveals that lumbricus extract may have therapeutic benefits for the treatment of peripheral nerve damage in humans by improving sciatic nerve regeneration and function recovery after injury.

Currently, chemotherapy, radiation treatment, and surgery are used to treat cancer. Nevertheless, the quality of life is impacted by these modalities and they have serious negative repercussions. Maximum toxicity levels are attained, which prevents improvement and further treatment intensification. With regard to the oral cancer cell line SCC-9, Augustine *et al.*, 2019 has investigated the cytotoxic potential of earthworm coelomic fluid (ECF) from *Eudrilus eugeniae* (EE), *Eisenia foetida* (EF), and *Perionyx excavatus* (PE). According to the LDH, clonogenic, and comet test data, the ECF of EE, EF, and PE has a strong and varying cytotoxic impact on SCC-9 cells.

Another study investigated by (Liu *et al.*, 2017) showed the potential antitumor effect of *E. foetida* fibrinolytic enzyme on Breast cancer cell line MCF-7.

Integrating the findings from microbial influence, earthworm biology, and external bio-stimulants, the literature survey suggests a probable benefit of cell free extracts from earthworm in augmenting regeneration. However, specific experiments directly assessing these interactions in *Perionyx excavatus* are required to validate these hypotheses.

CHAPTER – 3

AIM AND OBJECTIVES

3.1. AIM

To identify the regeneration augmentation using earthworm, *Perionyx excavatus* symbiotic cell-free bacterial extract.

3.2. OBJECTIVES

- To isolate and characterize fluorescent bacterial species present in the gut of *Perionyx excavatus*.
- To extract the bioactive compounds from the symbiotic relationship between earthworms and bacteria.
- To evaluate the wound healing ability of the extracted bioactive compound using cell culture.

CHAPTER – 4

MATERIALS AND METHODOLOGY

4.1. EARTHWORM CULTURE AND MAINTENANCE

Earthworms were collected from the International Research Centre at Sathyabama Institute of Science and Technology's Molecular Biology and Stem Cell Research Lab. The worms were kept in a plastic container with leaf litter, cow dung, and soil. A diet with an equal ratio of cow dung and jaggery is provided three times per week.

4.2. EARTHWORM SELECTION AND AMPUTATION

For amputating earthworms, matured earthworm species of *Perionyx excavatus* were chosen. Three healthy worms are selected based on the amount of soil present in their gut and subsequently, they are washed in tap water in a glass beaker. Worms are amputated at their 10th segment (head region) using a sterile scalpel. Amputated worms are incubated in separate plastic box with enough moisture content for three days.

4.3. COLLECTION OF SAMPLE

After three days, the amputated worms' wound healed and the worms were washed in tap water in a glass beaker. Using a sterile scalpel worms' gut are dissected and soil present in their gut is collected and dissolved in 1ml sterile distilled water.

4.4. ISOLATION OF SYMBIOTIC BACTERIA FROM EARTHWORM GUT SOIL

4.4.1. Preparation of media

For bacteria to be successfully cultured, they must be grown in the appropriate media. LB, also known as Luria Bertani or Lysogeny broth is a nutrient-rich broth that is standard for culturing bacteria such as *Escherichia coli*, etc., as it allows for quick

growth and high yields. Therefore, the proper preparation of LB will be crucial to maintaining bacterial culture. The addition of agar to LB broth creates a gel and is therefore used for plating bacterial cultures on Petri dishes.

Add 25g of LB broth powder per liter in an Erlenmeyer flask and stir until clumps are gone. Now, add 4g of Agar to the same and stir it. The prepared media is sterilized by autoclaving along with Petri dishes and other requirements such as swabs, tips, etc., at 121°C for 20 mins at 15 lbs pressure. Once the media is cool to touch, add antibiotics and mix by swirling.

Under sterile conditions pour media in plates, covering the surface and avoid bubbles. Allow the media to solidify and now the LB agar plates can be used to swab the soil sample.

4.4.2. Serial dilution

The addition of samples directly to a medium will lead to dense, mixed colonies, from which we will not be able to differentiate the individual colonies and their morphologies. The purification of colonies afterward becomes very difficult. Serial dilution of the samples is a viable technique to isolate microbes as individual colonies and thus differentiate the morphology of isolated bacterial strains. Serial dilution of the soil sample collected from earthworm gut is done as follows:

- 1g of soil sample is dissolved in an Eppendorf tube which has 1000µl sterile distilled water and is considered as stock solution. Eight Eppendorf tubes which have 900µl sterile distilled water and labeled as following dilution factors – 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} (Fig. 4.1)
- 100µl of stock solution is transferred into the first tube containing 900µl of sterile distilled water using a pipette. This becomes the 10^{-1} dilution.
- 100µl from the 10^{-1} dilution tube is transferred into the next test tube containing 900µl of sterile distilled water. This becomes the 10^{-2} dilution.
- This procedure is repeated till the 10^{-8} dilution and 100µl is discarded from the 10^{-8} dilution tube.

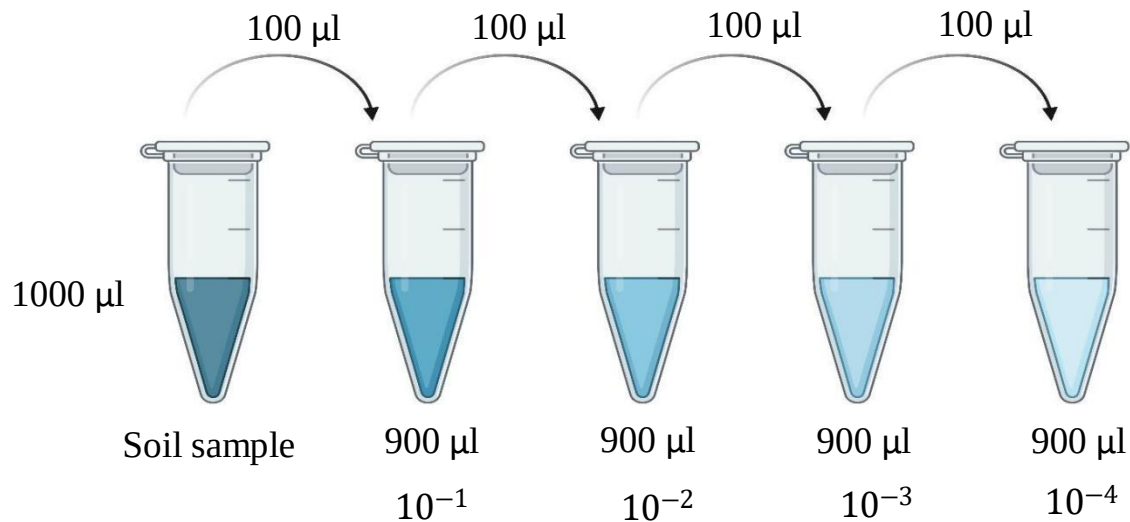


Fig.4.1. Serial dilution technique

- The diluted suspensions (10^{-2} , 10^{-3} , 10^{-4}) were inoculated into sterile LB agar plates, by using a sterile swab (spread plate techniques).
- The inoculated LB agar plates were incubated at 37°C for 24 hours in the inverted position.

4.4.3. Subculture of grown colonies

After 24 hours of incubation, grown colonies were observed under UV vis transilluminator to examine the fluorescent emitting colonies. The bacterial colonies were marked.

LB broth is prepared for 500ml and sterilized along with the other requirements for subculturing the fluorescent-emitting colonies. Under sterile conditions, an inoculating loop is sterilized in a Bunsen burner until it turns red hot. The marked colonies are picked up using the cooled loop and inoculated into LB broth and ensured that the loop touches the bottom to deposit the colony. The loop is gently

swirled in the broth to dislodge the colony and distribute it in the liquid medium. The inoculated broth is labeled and incubated at 37°C for 24 hours.

4.5. EXTRACTION OF BIOACTIVE COMPOUND FROM ISOLATED BACTERIA

4.5.1. Ammonium sulfate precipitation method

One of the most used techniques for removing protein from a solution is ammonium sulfate precipitation. Through the exposed polar and ionic groups, proteins in solution create hydrogen bonds with water molecules. These groups compete with the proteins to attach to the water molecules when high amounts of tiny, highly charged ions, such as ammonium sulfate, are introduced. As a result, precipitation happens because the protein's solubility is reduced and the water molecules are removed. For precipitating the unknown bioactive compound, the following steps are done:

- The grown broth culture is centrifuged at 3000 rpm for 3 minutes at room temperature.
- After centrifugation, the supernatant is collected in a fresh 50ml Falcon tube.
- Now for 50ml, 70% ammonium sulfate solution was freshly prepared in a Falcon tube.
- The freshly prepared ammonium sulfate solution is slowly added to the supernatant collected from the broth culture.
- The mixture is mixed well and stored at 4°C overnight.
- After incubation, the tube is centrifuged at 3000rpm for 3 minutes at room temperature.
- The supernatant is discarded and the pellet is dissolved in potassium phosphate buffer (pH – 7.4) and stored at -20°C for further analysis.

4.6. EVALUATION OF THE IMPACT OF AN UNKNOWN BIOACTIVE COMPOUND ON 3T3 CELL LINES

4.6.1. MTT Assay

In cell biology and drug development, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test is a popular colorimetric method for determining cell viability, proliferation, and cytotoxicity. It is based on the fact that mitochondrial dehydrogenase enzymes in metabolically active cells convert the yellow tetrazolium salt, MTT, into insoluble purple formazan crystals. The quantity of viable cells in culture directly correlates with the amount of formazan generated. In this study, we used MTT assay to determine the toxicity of an unknown bioactive compound extracted from the fluorescence-emitting bacterial colonies on 3T3 cells (Mouse embryonic fibroblast cell lines). The process for assessing the cell proliferation is as follows:

4.6.1.1. 3T3 cell culture and maintenance

To culture 3T3 cell lines, DMEM (Dulbecco's Modified Eagle Medium) medium is prepared and filtered using a 0.22µm filter. The cells were seeded in a T-25 flask containing DMEM medium and incubated in 5% CO₂ incubator to attain full confluence.

4.6.1.2. Collection of cells from T-25 flask

After the flask attains full confluence, the flask is washed with 0% medium three times. Trypsin is added to detach the cells from the flask and 2ml 10% medium is added to the flask. The mixture is collected in 15ml Falcon tube and centrifuged at 900 rpm for 3 minutes. The supernatant is discarded and the pellet is resuspended in 1ml 10% media.

4.6.1.3. Cell counting and seeding in 96 well plate

The collected cells were counted using hemocytometer and the formula for counting cells is as follows:

$$\text{Cell count} = \frac{\text{Required volume of cells} \times \text{Required concentration of cells}}{\text{Available concentration of cells}}$$

The 10 μ l cells were seeded in each well of 96 well plate containing 100 μ l 10% media and the plate was incubated in 5% CO₂ incubator for 24 hours.

4.6.1.4. 3T3 treatment with the unknown bioactive compound

After 24 hours of incubation, 100 μ l of the unknown bioactive compound is added to the 96-well plate. The plate was incubated in 5% CO₂ incubator for 24 hours.

4.6.1.5. Incubation with MTT

MTT is a yellow tetrazolium dye that is soluble in aqueous solutions. It is prepared as a stock solution in phosphate-buffered saline (PBS) at a concentration of typically 5 mg/ml in dark conditions.

10 μ l prepared MTT is added to each well and incubated for 3 hours in dark conditions. After 3 hours of incubation, the mixture in each well is removed. 100 μ l of DMSO is added to the 96-well plate and the plate is read using multi-plate reader at 570nm.

4.6.2. Wound healing assay

A popular in vitro method for examining cell migration and wound closure processes is the wound healing test, sometimes referred to as the scratch assay. It is especially helpful for examining how cells migrate in response to different stimuli like medicines, growth hormones, or genetic modifications. In this study, we used wound-healing assay to assess the ability of cells to heal the wound when treated with the unknown bioactive compound extracted from the fluorescence-emitting bacterial colonies on 3T3 cells (Mouse embryonic fibroblast cell lines).

4.6.2.1. 3T3 cell culture and maintenance

To culture 3T3 cell lines DMEM (Dulbecco's Modified Eagle Medium) medium is prepared and filtered using a 0.22 μ m filter. The cells were seeded in a T-25 flask containing DMEM medium and incubated in 5% CO₂ incubator to attain full confluence.

4.6.2.2. Collection of cells from T-25 flask

After the flask attains full confluence, the flask is washed with 0% medium three times. Trypsin is added to detach the cells from the flask and 2ml 10% medium is added to the flask. The mixture is collected in 15ml Falcon tube and centrifuged at 900 rpm for 3 minutes. The supernatant is discarded and the pellet is resuspended in 1ml 10% media.

4.6.2.3. Cell counting and seeding in 6 well plate

The collected cells were counted using hemocytometer and the formula for counting cells is as follows:

$$\text{Cell count} = \frac{\text{Required volume of cells} \times \text{Required concentration of cells}}{\text{Available concentration of cells}}$$

The 100 μ l cells were seeded in each well of 6 well plate containing 2ml 5% media and the plate was incubated in 5% CO₂ incubator until a primary layer of cells was grown.

4.6.2.4. Treating with unknown bioactive compound and scratching a wound

When a primary layer of cells is grown, 100 μ l of the unknown bioactive compound was added and the wound is created using a sterile tip by scratching on it. The plate with the wound on the primary layer is documented for 8-hour intervals until the wound gets healed.

CHAPTER – 5

RESULTS AND DISCUSSION

5.1. EARTHWORM AMPUTATION AND COLLECTION OF SOIL SAMPLE

P. excavatus is a topsoil earthworm that is habitually subjected to injury by predators, and correlating it with its enormous posterior regeneration ability represents its evolution nature to survey following an injury. Earthworm, *Perionyx excavatus* has an amazing ability to regenerate its lost parts (Banik and Chaudhuri, 2017). *P. excavatus* demonstrates a superior survival capacity compared to other earthworm species. *P. excavatus* is an excellent animal model for studying the central nervous system and tissue regeneration (Banik and Chaudhuri, 2017; Bae *et al.*, 2020). Within two weeks of the amputation, *P. excavatus* achieves complete regeneration, including rebuilding vital organs (Cho *et al.*, 2009; Bae *et al.*, 2020). Recently, Bae *et al.* observed the epimorphosis mode of head regeneration in the earthworm *P. excavatus* (Bae *et al.*, 2020), but the role of morphallaxis in earthworms is vague. Martinez *et al.* (Martinez, Manson and Zoran, 2008) suggest that the earthworm might follow both epimorphosis and morphallaxis regeneration patterns due to high predatory injury, but clear scientific evidence is unclear (Rajagopalan *et al.*, 2023). After day 1 of amputation, the worms amputated at the 10th segment (head region) wound healed and they are healthy. On day 3, the worms were dissected in their gut region which has high soil content. The isolated soil is the place where the symbiotic microorganisms reside in the earthworm.

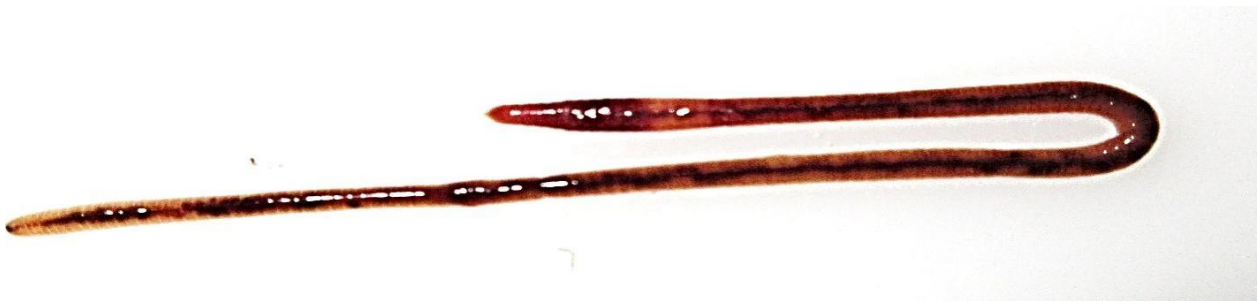


Figure:5.1: Mature earthworm species of *Perionyx excavatus*



Figure:5.2: Worm amputated at 10th segment (head region)

5.2. ISOLATION OF FLUORESCENCE EMITTING BACTERIA

The earthworm receives bioactive compounds like riboflavin from the symbiotic connection in exchange for the bacterium's shelter and nourishment, which helps the earthworm maintain homeostasis throughout the early regeneration stage of starvation (Subramanian *et al.*, 2017). The soil sample collected from earthworms' gut was serially diluted and inoculated in LB agar medium. After 24 hours of incubation, incubated plates were observed under UV vis transilluminator (Fig 5.3). Some colonies emit fluorescence and they were marked. The bacterial colonies emitting fluorescence were smooth and white colored colonies. The marked colonies were inoculated in LB broth and incubated for 24 hours. After 24 hours of incubation, the broth seems to be turbid indicating the growth of the bacterial colonies. According to the report of Mijithra Ganesan, Gram staining results showed that the isolated fluorescence-emitting gut bacterium is Gram-positive and it is rod-shaped. Molecular characterization confirmed that the isolated fluorescence-emitting bacterium is *Priestia filamentosa*. Moreover, two fluorophores responsible for emitting fluorescence, known as unknown fluorescent compound 1 (CF1), and fluorescent compound 2 (CF2) were separated and partially characterized. Anti-inflammatory assays confirm that the fluorophores have very good anti-inflammatory properties. In addition, these fluorophores were expressed in the epithelial cell layer of earthworm, *P. excavatus*,

during regeneration. Notably, bright, intense fluorescence was observed high in regenerating worms compared to control worms (Ganesan *et al.*, 2021).

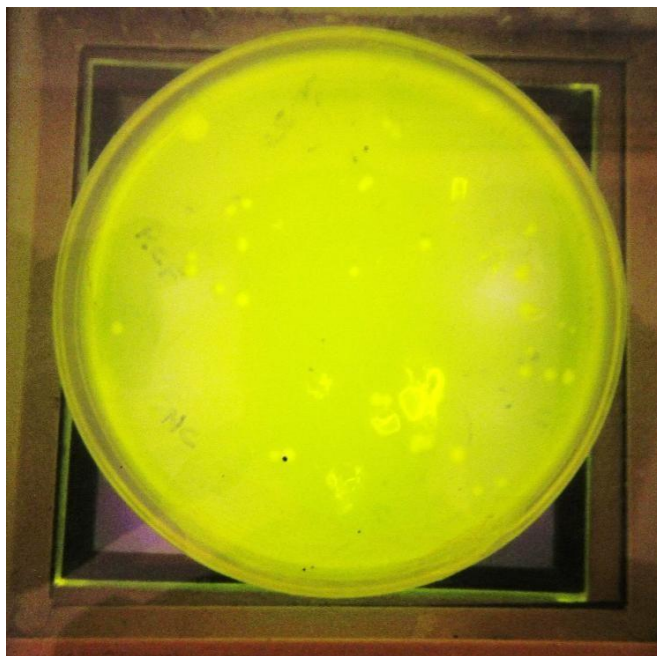


Figure:5.3: Fluorescent emitting bacterial colonies on LB agar medium



Figure:5.4: Broth culture of fluorescent emitting bacterial colonies

5.3. EXTRACTION OF UNKNOWN BIOACTIVE COMPOUND

From the broth culture, by ammonium sulfate precipitation method an unknown bioactive compound was extracted.



Figure:5.5: Unknown bioactive compound

5.4. CELL PROLIFERATION EFFECT OF THE UNKNOWN BIOACTIVE COMPOUND ON 3T3 CELL LINES

Extract from earthworms (*Pheretima aspergillum*) increases osteoblast activity while decreasing osteoclast differentiation at 3 mg/ml (Fu *et al.*, 2014). The extracted bioactive compound was used to study the proliferation effect on 3T3 cell lines (Fig. 5.6) The results were analyzed using MS Excel sheet and graph (Fig. 5.7) shows that control has normal growth of cells, when we increase the concentration of unknown bioactive compound from 5% to 100%, at 25% the proliferation rate is high when compared to control. So we can conclude that 25% of the unknown bioactive compound enhances the regeneration of 3T3 cell lines. Similar studies also conducted by Charng-cherng chyanu. The study conducted by Charng-Cherng Chyau *et al.* aimed to gain a deeper understanding of how the ethanolic extracts of Djulis (*Chenopodium formosanum*, EECF) and its bioactive compounds can potentially inhibit adipogenesis in 3T3-L1 adipocytes. The findings revealed that EECF effectively suppressed the accumulation of OROS-stained material, reduced

triglyceride levels, and inhibited GPDH activity in 3T3-L1 adipocytes. The expression of the key molecules involved in lipid synthesis, including PPAR γ , C/EBP α , and SREBP-1c, was reduced in cells treated with EECF. Based on the analysis using HPLC-DAD and HPLC-MS/MS, it was found that EECF contained rutin, kaempferol, betanin, and nine other compounds. The gene expression of PPAR γ , C/EBP α , and SREBP-1c was reduced, resulting in the suppression of lipid accumulation, thanks to rutin, kaempferol, and betanin. Collectively, these findings imply that the presence of bioactive compounds in EECF may contribute to its ability to inhibit adipogenesis. This suggests that EECF has the potential to be a functional food that can help lower lipid levels (Chyau *et al.*, 2018).

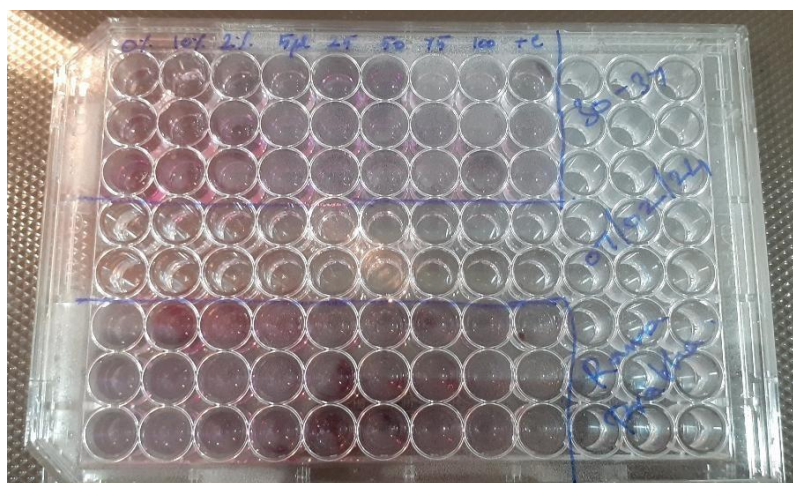


Figure:5.6: 96 well plate after adding DMSO

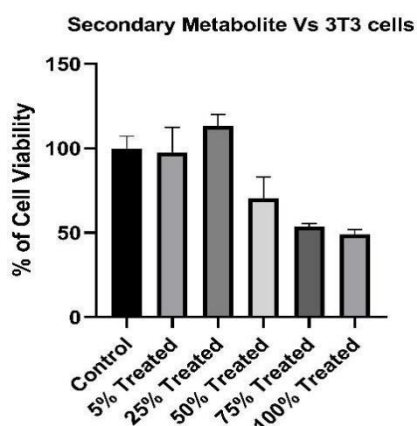


Figure.:5.7: Graph showing the cell proliferation ability of unknown bioactive compound on 3T3 cells in cell proliferation assay

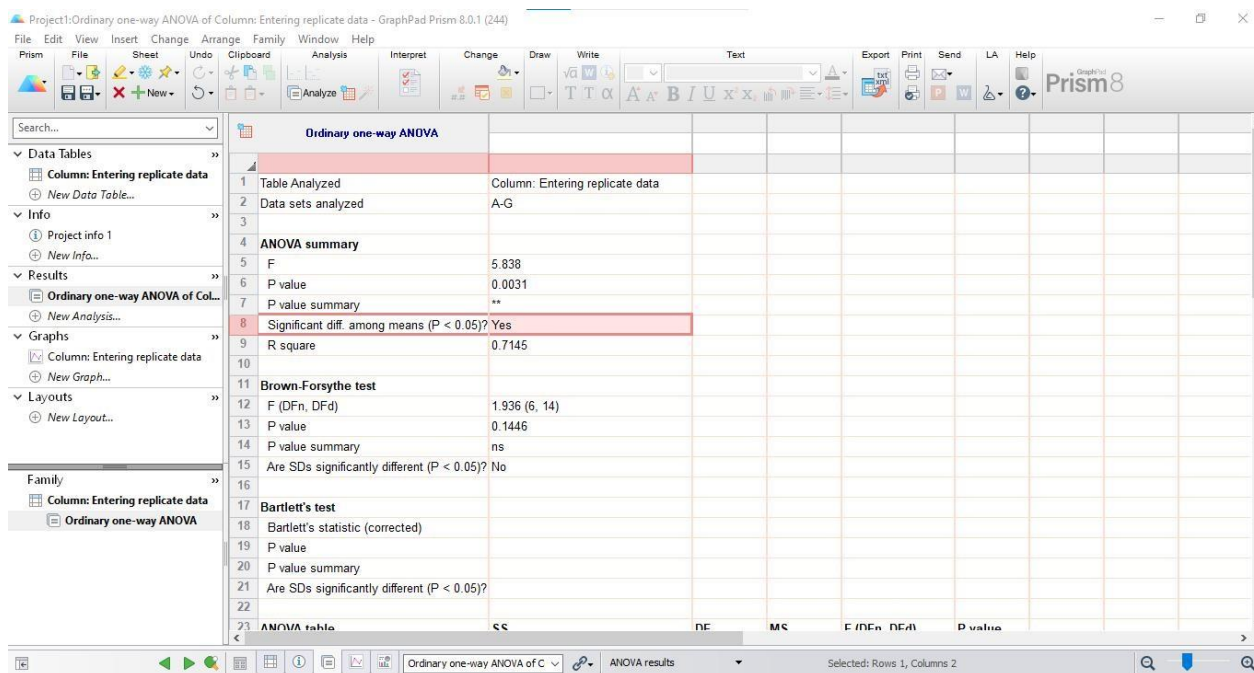


Figure 5.8: Statistical analysis of cell proliferation ability of unknown bioactive compound

5.5. WOUND HEALING ABILITY OF THE UNKNOWN BIOACTIVE COMPOUND ON 3T3 CELL LINES

Three overlapping phases - inflammation, proliferation, and remodeling make up the complex and highly coordinated biological process of wound healing. Earthworm extracts are seemed to stimulate cell migration and regeneration (Chang *et al.*, 2011) . Based on this wound healing assay was conducted and after creating scratch, in control the wound gradually healed whereas in 25% of unknown bioactive compound the wound healed is comparatively high with the control (Fig. 5.9) The cells observed for 8 hours interval showed that the unknown bioactive compound extracted from the fluorescent emitting bacteria provokes the regeneration on 3T3 cell lines. This suggests that there is potential to replace FBS with earthworm extract in animal culture media, but further research is needed to fully explore this possibility in animal cell culture. The coelomic fluid of earthworm, which has been heat-inactivated, is commonly utilized in animal cell culture research as a substitute for FBS (Chellathurai Vasantha *et al.*, 2019). According to the report of Zhen-han Deng *et al.*, pro-wound healing effects of EE on local wound of mice. It decreased wound healing time, reduced the injury caused by inflammation, and improved the quality of healing. The potential mechanisms are probably by accelerated Hyp and TGF- β secretion, increased synthesis of collagen, blood capillary, and fibroblast proliferation. Additionally, it accelerates the removal of necrotic tissue and foreign body by speeding up the generation of IL-6, WBC, PLT, and Gran. Thus enhances immunity, relieves the inflammatory reaction of mechanical injury, reduces the risk of infection, and finally promotes wound healing (Deng *et al.*, 2018).

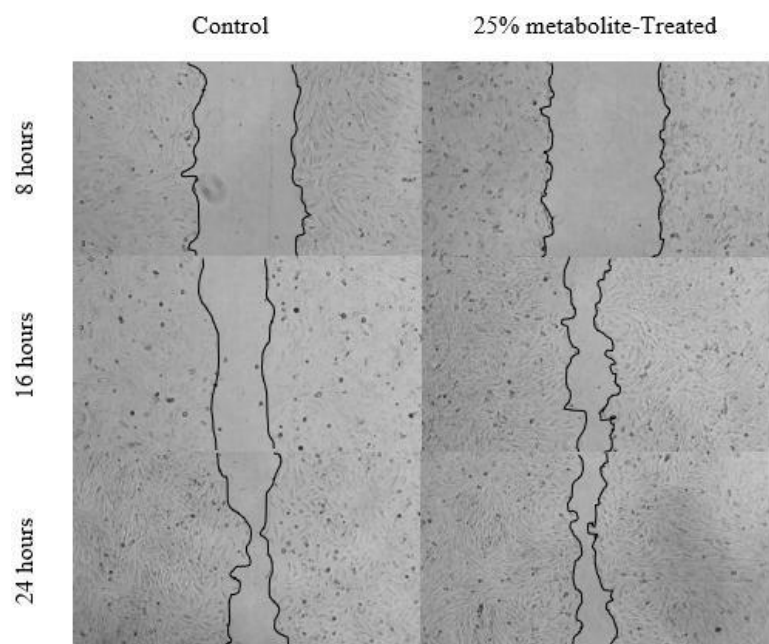


Figure:5.9:Microscopical view of wound healing ability of unknown bioactive compound

CHAPTER – 6

SUMMARY AND CONCLUSION

The regenerative potential of cell-free bacterial extracts obtained from the earthworm, *Perionyx excavatus* symbiotic bacteria was examined in this thesis, along with its impact on mouse embryonic fibroblast cell line 3T3. The primary aim was to investigate ascertain whether they may elicit any advantageous regeneration responses similar to those noted in the host earthworm. In this study, the bacterial symbionts from *Perionyx excavatus* were isolated, and then extracts of the bacteria were made without cells which has numerous unknown bioactive compounds. 3T3 cells were then exposed to different concentrations the extracts. The response of the cell to the bacterial extracts was evaluated using a variety of assays such as Cell toxicity assay, Wound healing assay. In 25% of the extract the proliferation rate is high compared to control and in wound created was healed when compared to the control showed that the cell free extract has potential regenerative effect on 3T3 cell lines. From these findings, we can conclude that the extract from *Perionyx excavatus* has the potential regeneration ability on 3T3 cells. These effects highlight a novel avenue for the development of regenerative therapeutics, leveraging the unique biochemical properties of earthworm symbiotic bacteria. Further by isolating the specific bioactive compounds within the extracts that are responsible for the observed biological effects can increase the efficacy of the extract on 3T3 cell lines.

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