LITERATURE REVIEW

Literature review

I. Introduction to *Eisenia fetida*

Earthworms constitute one of the major invertebrate biomass living in the soil. Among the different species of these oligochaete worms, *Eisenia fetida* (Savigny) is the most renowned one for its ability to efficiently transform organic matters like peels of vegetables, fruits and leaves of plants etc. into vermicompost, the organic manure rich in nitrogen, phosphate and other essential components useful for proper plant growth. This cosmopolitan red worm is also naturally found in the upper part of the soil of mountainous regions and the adjoining terrains of North Bengal where cultivation of famous 'Darjeeling tea' is exercised (Halder, 1999).

The individuals belonging to the genus *Eisenia*, Malm, 1877 (Subfamily- Eiseninae, Family- Lumbricidae, Order- Haplotaxida, Class- Clitellata, Phylum- Annelida) display a reddish colour, epilobous prostomium, closely paired setae, first dorsal pore around annule number 4/5, calciferous gland without pouches in 10, (Bouche, 1972; Gates, 1975; Csuzdi and Zicsi, 2003). There was some heterogeneity and confusion in the somatic characters in the species included in this genus. Many researchers believed that *E. fetida* and *E andrei* were phenotypic variants. Andre (1963) made them conspecific by naming the uniformly pigmented form var. *unicolor* and the striped form var. *typica*. Considering the unavailability of natural hybrids Bouche (1972) gave the uniformly pigmented worms the new subspecific name *Eisenia fetida andrei*. Apart from the difference in pigmentation the two forms cannot be distinguished. Both forms have a mean length of 60-120 mm, a diameter of 3-6 mm and a segment number varying between 80 and 120. The saddle-shaped clitellum covers 6-8 segments. Sims (1983) postulated the uniformly pigmented forms to be derived from the striped forms. Sheppard (1988) recommended two species of *Eisenia* distinguishable by pigmentation. He considered the banded worms to be *E. fetida* (brandling or tiger worms) while uniformly pigmented worms as *E. andrei* (red worm). Sheppard (1988) found that while both forms had similar cocoons laying ability and cocoon viability, but the numbers of progeny per cocoon produced by the two forms were significantly different. The two forms also differed biochemically (Roch *et al.*, 1980; Valembois *et al.*, 1982). Jeanike (1982) electrophoretically proved that at least three loci of the two species differed, indicating reproductive isolation. Jaenike (1982) first separated the two species genetically (Henry, 1999; McElroy and

Diehl, 2001, Perez-Losada *et al*., 2005). The genetic isolation is also correlated with physiological, ecological and behavioural differences, making it easy to distinguish between the two 'forms.

II. Cultivable bacterial diversity associated with *E. fetida*

Eisenia has been observed to continuously devour organic debris or to move ahead to search suitable food. Soil consumption for *E. fetida*, an earthworm is estimated to be 16 mg soil/ individual/day (Shaheen *et al.*, 2010). The nitrogen excretion rate of *E. fetida* has been estimated at 0.4 mg/g/day, which is very high relative to other earthworm species (Stafford and Edwards, 1985). Aerobic intestinal bacterial community structure of earthworm, *E. fetida*, was investigated by Kim *et al*. in 2004 based on 16S rRNA gene analysis. Ninety-one different colonies grown on Brain Heart Infusion medium were randomly isolated under aerobic condition. Based on partial sequence analysis of PCR-amplified 16S rRNA gene for strains, earthworm intestinal aerobic bacteria (EIAB) were divided into 12 groups, and each group was further divided into subgroups. Groups included 6% *Aeromonas*, 3% Agromyces, 31% *Bacillus*, 1% *Bosea*, 6% *Gordonia*, 6% *Klebsiella*, 7% *Microbacterium*, 2% *Nocardia*, 10% *Pseudomonas*, 19% *Rhodococcus*, 2% *Tsukamurella*, and 7% *Streptomyces*, with *Bacillus* being dominant group.

Kim *et al*. in 2010 identified *Brevibacillus agri*, *Bacillus cereus*, *Bacillus licheniformis*, and *Brevibacillus parabrevis* from earthworm viscera by 16S rRNA sequencing. These bacteria may be employed in the conversion of fish wastes into fertilizer. In 2013 Zhang *et al*. investigated change of intestinal bacteria community of *Eisenia* on *E coli* O157:H7 challenge by PCR-DGGE analysis. The result demonstrated that the intestinal bacteria of earthworm had the ability to adjust community structure to eliminate the pathogen *E. coli* within three days, and the amount of bacteria *Bacillus* increased significantly, which might be the positive antagonism to *E. coli*. Kwang-Hee Shin *et al.* in 2004 isolated one hundred different anaerobic bacterial colonies grown on Brain Heart Infusion medium. With the aid of partial sequence analysis of PCR-amplified 16S rDNA, these earthworm intestinal bacteria were identified to be *Clostridium bifermentans*, *C. butyricum*, *C. glycolicum*, *C. celerecrescens*, *C. lituseburense*, *Staphylococcus epidermidis*, *Propionibacterium acnes* (97% or more similarity) and a dominant group (49% of all isolates) of unique pyretic line with 90-95% similarity to *C. subterminale*.

Go´mez-Brando´n *et al*., 2011 found that some specific bacteria are activated and selected than others during passage through the gut. Monroy *et al.* (2011) observed a reduction in the density of total coliforms by 98%, after the passage of pig slurry through the gut of *E. fetida*. This was not related to decreases in bacterial biomass indicating a selection of bacterial group. Bacteria like *Escherichia coli* BJ18 in cattle dung is selectively reduced during passage through the gut of earthworms of the genus *Lumbricus*. Such reduction in the coliform bacteria did not influence other bacterial groups although a shift in the composition of the microbial community is observed. This selective effect on microbial composition of the ingested material through the earthworm gut may be because of competitive interactions between the transiently ingested and the endosymbiotic microbes residing in the gut (Kim *et al.* in 2010).

Phylogenetic distribution of 16S rRNA cDNA sequences obtained from [^{12}C]- and [^{13}C] glucose treatment as Phylogenetic affiliation (total relative abundance of phylum) revealed that the major phyla of earthworm gut contents included *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Nitrospirae, Planctomycetes,* (Alpha-, Beta-, Gamma- and Delta-) *Proteobacteria, Tenericutes and Verrucomicrobia*, taxa common to soils identified as revealed by 16S rRNA analysis. Alpha-, Beta- and Gamma-*Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes* were potentially dominant taxa in the earthworm gut, as determined on the basis of 16S rRNA gene analyses (Furlong *et al.,* 2002; Singleton *et al.,* 2003; Knapp *et al.,* 2009). Several authors have reported bacterial species belonging to the familis *Actinobacteriacae Aeromonadaceae, Comamonadaceae, Enterobacteriaceae, Flavobacteriaceae, Moraxellaceae, 'Paenibacillaceae', Pseudomonadaceae, Rhodocyclaceae, Sphingobacteriaceae* etc. to be present in the alimentary canal of earthworm (Ihssen *et al*., 2003; Horn *et al*., 2005; Byzov *et al*., 2009; Knapp *et al*., 2009). Bacteria of such diverse taxa which if metabolically active in the earthworm gut, might participate in hydrolysis of ingested biopolymers like cellulose, lignin etc. (Martin-Carnahan and Joseph, 2005; Bernadet and Bowman, 2006; Priest, 2009).

III. Culture independent studies of microbial population

Gut bacterial communities of *E. fetida* and *Perionyx excavates* grown on lignocellulosic biomass, was analysed by Singh *et al.,* 2015 16S rRNA gene based

clonal survey of gut metagenomic DNA. Total 67 clonal sequences belonging to *E. fetida* and 75 to *P. excavatus* were taxonomically annotated using MG-RAST. Most of the sequences were annotated to *Proteobacteria* (38-44%), and Firmicutes (9-11%) with a substantial presence of unclassified bacteria (14-18%). *P. excavatus* also had higher abundance of *Actinobacteria* along with *Firmicutes*.

Microbial composition can truly be studied by sequencing the microbiome with modern approaches such as-16S rRNA amplicon sequencing, shotgun DNA sequencing and shotgun RNA sequencing. But, anaerobic microbiome studied by high throughput sequencing strategies had certain biases (Campanaro *et al.,* 2018). This biasness in the abundance of some taxa (*Euryarchaeota* and *Spirochaetes*) was because of the inefficiency of universal primers to hybridize all the templates. Number of hypervariable regions under investigation influences the reliability of the results obtained. Shotgun DNA and 16S rRNA gene amplicon sequencing may underestimate the *Methanoculleus* genus, probably due to the low 16S rRNA gene copy number encoded in this taxon.

Rosselli 2016 commented that DNA-based methods may not provide information on the actual physiological relevance of each taxon within an environment and are affected by the variable number of rRNA operons in different genomes. To overcome these drawbacks an approach was proposed for direct sequencing of 16S ribosomal RNA without any primer or PCR-dependent step. So, the analyses of environmental microbial communities by direct 16S rRNA-seq from bacterial communities were attempted. The method was tested on a microbial community developing in an anammox bioreactor sampled at different time-points.

Development of metagenomics, although only a decade old, has made it possible to investigate microbes in their natural environments

IV. *Eisenia fetida* and microbe interaction

Gut wall-associated bacterial communities of *Lumbricus terrestris* and *L. friendi* (endogeic) were detected by Thakuria *et al*. 2010 using automated ribosomal intergenic spacer analysis of 16S and 23S rRNA genes. Prevalence of specific gut wall-associated bacteria, belonging mainly to the phyla *Proteobacteria, Firmicutes* and *Actinobacteria*, was ecological group dependent. Five different types of food

have been shown to cause shifts in gut wall-associated bacterial community, but the ecological group were same.

Aira *et al.,* 2015 studied effects of earthworms (after passage through its gut) on the taxonomic and phylogenetic bacterial composition of animal manures. They described cast microbiome of earthworm. When *E. andrei* was fed with the cow, horse and pig manures the emitted cast strongly differed in their taxonomic composition, but these differences were markedly reduced once transformed into earthworm cast microbiomes after passage through the earthworm gut. They reported 30 OTUs (2.6% of OTUs from cast samples) to be the core earthworm cast microbiome comprised, of which 10 are possibly native to the earthworm gut. Cast microbiome of earthworm is constituted mainly by the members of Phyla *Actinobacteria* and *Proteobacteria* where as for most other animals core gut microbiomes are composed mainly of Firmicutes and Bacteroidetes. So it seems that earthworms control the microbial composition of cast by selecting from the bacterial pool present in the ingested material.

V. Brief introduction to coelomic fluid and coelomocytes

Cotuk and Dales (1984) examined that the earthworm *E. fetida* has both cellular and humoral defences against bacteria. Later, R. P. Dales and Y. Kalac, 1991 explained that the antibacterial property of coelomic fluid of *E. fetida* is because of three host microbes. *Aeromonos hydrophila, Serratia marcestens, Yersinia ruckeri* are those three microbes which occur naturally in the coelomic fluid of *E. fetida*. Among them, *A. hydrophila* and *S. marcestens*are were known pathogen, and *Y. ruckeri* was later on found to be pathogenic to another earthworm, *Dendrobaena ueneta* (Cotuk and Kala, 1990). Antibacterial substances present in the coelomic fluid of *Eisenia* have been postulated to inhibit growth of some bacteria. Infection of the earthworm leads to enhancement of these substances in the coelomic fluid (Lassegues *et al.,* 1989). Valembois *et al.* (1982, 1986) demonstrated that lipoprotein in the coelomic fluid has antibacterial properties against bacteria which share at least one common antigen with sheep red blood cells, which imparts coelomic fluid a property to lyses RBC. Along with this, coelomic fluid of earthworms *E. fetida exerts* a large variety of biological effects including bacteriostatic, proteolytic, and cytolytic activities (Bilej *et al.,* 1991) Kauschke and Mohrig (1987) described the toxic effect of *E. fetida* coelomic fluid on different cell types, such as chicken fibroblasts, guinea-pig polymorphonuclear leukocytes, and insect haemocytes. They also found that this toxic effect was not

exerted against coelomocytes of other lumbricids as well as the cells of some molluscs, nematodes, and protozoans. The toxic effect seems to be correlated with the haemolytic activity, since 3 out of 7 haemolytic fractions exerted cytotoxic activity. Furthermore, compounds with antitumor activities have been isolated from body homogenates of lumbricids *E. fetida, Lumbricus rubelus,* and *Lumbricus terrestris.* Bilej *et al.,* (1990) examined that the coelomocytes have antigen binding property with akinetic approach., Hanus'ova *et al.,* (1998) suggested that coelomic fluid contains a ubiquitous PLA2-like component which might be involved in inflammatory reactions in earthworms (*E. fetida*). They reported the isolation of a factor from the coelomic fluid of the earthworm *E. fetida* that exerts mitogenic activity on murine splenocytes. This factor, CMF (coelomic mitogenic factor) was found to bind concanavalin A (ConA) and to block ConA-induced spleen cell proliferation. In contrast CMF synergizes with lipopolysaccharide (LPS) to trigger the proliferation of the same cell type. N-terminal amino acid sequencing revealed that CMF displays significant homology with phospholipase A2 (PLA2). CMF-enriched coelomic fluid fraction exerts phospholipase enzymatic activity.

Different authors have classified coelomocytes into types on the basis of morphological, cytochemical criteria and functional attributes (antibacterial property). In general, there are three main types — eleocytes, the free chloragogen cells with functions in nutrition, the other two types are either hyaline or granular amoebocytes, representing effector immunocytes. The amoebocytes seem to be involved in a broad range of immunological functions like phagocytosis. Amoebocytes can engulf materials like inert carbon particles, microbial cells and foreign cells. (Bilej *et al.*,1990). Homa *et al*., 2012 described five types of coelomocytes- leucocytes type I (basophilic) and II (acidophilic), neutrophils, granulocytes and eleocytes. All types of coelomocytes except eleocytes can phagocytose and encapsulate foreign materials.

Kurek *et al.,* (2007) put some light on the origin of amoebocytes from the coelomic mesenchymal lining and eleocytes (chloragocytes) from chloragogen cells that mainly cover the alimentary tract on coelomic side. Amoebocytes recognise and eliminate foreign material, mainly by phagocytosis and encapsulation. They are also involved in clotting, wound healing, cytotoxicity, inflammation, graft rejection, granuloma formation and coelomic fluid coagulation. Eleocytes, also called chloragocytes have numerous spherical granules, the chloragosomes. They have the capacity to store

endogenous materials such as glycogen and lipids as well as pigments including riboflavin. Eleocytes take part in ion and pH balance of the coelomic fluid, in some aspects of nutrition and excretion, but are also associated with immune defence. They are involved in encapsulation and brown body formation. Secretions of eleocytes have bacteriostatic properties. Moreover they take part in detoxification of earthworm tissues and heavy metals accumulation. Different types of stress (i.e. heat, pollution) lead to heat shock proteins expression in coelomocytes.

VI. Modulation of coelomic fluid constituents under bacterial challenge

Coelomic cavity of *E. fetida* communicates with outer environment by dorsal pores. In consequence, the coelomic cavity is not aseptic and always contains bacteria, protozoans and fungi from the outer environment. But, there are efficient mechanisms by which the growth of microorganisms are kept under control (Dales *et al.*,1992). It was reported that coelomic fluid contains naturally occurring bacteria in the magnitude of $6X10^5$ /ml ($0.9X10^5$ /adult individual) while the number of potentially phagocytic cells is more than ten times higher (Ko¨hlerova´, 2004). If the bacterial burden surpasses the optimum limit it may cause disease in earthworm. Smirnoff and Heimpel (1961) reported that when large doses of *Bacillus thuringiensis* invaded the body cavity of the earthworm, it caused an extensive septicemia and eventual death. Hiempel (1966) reported that blister disease of *E. fetida* was found to contain crystalliferous bacteria in all the lesions. Two strains of bacteria were isolated, identified, and typed according to their H-antigen and their vegetative cell esterases. Both strains appeared to be *Bacillus thuringiensis*. Superior number of phagocytes along with various humoral factors prevents the microorganisms from outgrowth (Bilej *et al*., 2000). These phagocytes are coelomocytes which are freely flowing cells in the coelomic cavity. Recently, these coelomocytes have received particular attention (Goven *et al.*, 1987; Rodriguez-Grau *et al.*, 1989; Fitzpatrick *et al.*, 1990). To study immunological processes, techniques for isolation of coelomocytes by dissection, electrical stimulation or withdrawal by fine capillary tubes have been developed. In 1973, Valembois *et al.* have described mechanical and electrical excitation techniques to induce, extrusion of coelomocytes through pores in *E. fetida* integument. A year later, Cooper (1974) proposed the recovery of *Lumbricus terrestris* coelomocytes by puncture through the integument with a sharpened pasteur pipette. A non-invasive extrusion technique for the harvesting of *Lumbricus terrestris*

coelomocytes was proposed by Rodriguez-Grau *et al*. (1989), a technique fully described by Eyambe *et al.* (1991). Its novelty was in part the use of ethanol as an irritating agent and the addition of the mucolytic agent guaiacol glyceryl ether (GGE).

VII. Regeneration in *Eisenia fetida*

Annelids are a large and diverse group of typically segmented worms found in marine, freshwater, and terrestrial environments, with over 17,000 species described (Brusca and Brusca, 2003; Zhang, 2013). The main part of the body is usually composed of a series of repeated segments, ranging from just a few to several hundred (depending on species and age), with an asegmental cap of tissue present at both the anterior and posterior ends. Annelids typically have a large fluid-filled coelom surrounded by a muscular body wall, a complete gut with an anterior mouth (within the anterior asegmental cap) and a posterior anus (within the posterior cap of tissue), and a nervous system composed of paired cerebral ganglia, segmental ventral ganglia, and peripheral segmental nerve rings. Some species possess body wall outgrowths such as lateral, segmentally iterated parapodia (paddle-like outgrowths) used for locomotion; anterior tentacles, palps, or proboscises that aid in sensation, respiration and/or feeding; opercula that seal tube entrances; and lateral or posterior gills that aid in respiration. Based on their body architecture, the most common injuries are expected to involve transverse cuts of the main body (removing head and/or tail) and amputation of the various body wall outgrowths (Bely *et al*., 2014).

Most annelids add segments from a sub-terminal posterior growth zone present ahead to the non-segmental posterior cap (pygidium) to grow (Hyman, 1940; Brusca and Brusca, 2003). Some species reach a fixed number of segments after maturity, but, most others do not. Few annelids appear to grow continuously throughout their life. Under starvation conditions, degrowth in overall size occurs in at least some invertebrates like naidids and degrowth resulting in a reduction of segment number may also occur (Bely, 2014).

Annelid phylogeny was of ever uncertainty until recent molecular studies that have greatly clarified the relationships among annelid groups (Weigert *et al.*, 2014), setting the stage for interpretation of regenerative variation in a phylogenetically. Annelids which are classified into ~100 families fall into the two major clades, the *Errantia* and the *Sedentaria*. Only a few annelid lineages branch outside of these, as basal lineages

(Weigert *et al*., 2014). The *Errantia* includes many mobile species and ancestrally this clade might have pronounced anterior palps and lateral parapodia. The most common groups included in *Errantia* are nereids like, *Platynereis*, *Neanthes*, *Nereis etc*. The *Sedentaria* (meaning sedentary) includes many burrowing species and the ancestor of this clade is said to have reduced palps and parapodia because of burrowing lifestyle. Groups like *Capitellids, Spionids, Terebellids, Serpulids, Sabellids* etc. are included in *Sedentaria*. *Clitellata*, includes groups such as earthworms, aquatic "oligochaetes", and leeches. Annelids vary widely in regenerative ability. Some species can regenerate every part of the body, with extremes which can even regenerate from a single isolated segment. Some are even incapable of regenerating a single lost segment (Bely, 2006). Regeneration of anterior (towards the head) and posterior (towards the tail) segments is well documented in a wide range of annelids.

The ability to regenerate posterior segments is very broadly distributed across the phylum suggesting this ability likely to be ancestral for the phylum. Posterior regeneration is described in as many as 23 annelid families, of both the groups *Errantia* and *Sedentaria*. Only a few groups of *Sedentaria* is reported to be incapable of regenerating posterior segments which may be a secondarily lost character. Anterior regeneration is variable in annelids. Failure to regenerate anterior segments has been shown in many families of Annelida. This may be because of the fact that anterior segment regeneration ability has been lost many times within the phylum (Bely, 2006) or alternatively may have been regained in few groups.

Regeneration of structures like prostomium, feeding palps or operculum has also been shown in many groups of annelids. In species particularly having anterior regeneration capacity can also regenerate the anterior non-segmental tip i.e. prostomium and peristomium along with any head appendages. Such regeneration has been documented in few clitellates. Feeding palp regeneration has been documented in spionids (Lindsay *et al.*, 2008) and operculum regeneration in serpulids (Szabó and Ferrier, 2014). Amputation and regeneration of parapodia have been documented in one species of nereid (Boilly and Boilly-Marer, 1995).

The techniques for assessing the wound healing along with transplantation experiments have earlier been described by Cooper and Roth (1984). They observed healing under a dissecting microscope at 24 hr after wounding or grafting in lumbricid annelids. Well-healed grafts were recognized by a smooth, flat appearance,

homogeneous pigmentation and were outlined by newly formed connective tissue. In the case of well-healed wounds, a tight cicatrix developed on the wound.

Regeneration at molecular level was studied in few groups of annelids, with identification of the genes involved in the regeneration process (Bely and Sikes, 2010). Some annelids exhibit regenerative abilities very similar to planarians (Bode *et al*., 1973; Newmark *et al*., 2000), which can completely regenerate a new organism from small body fragment. However, the mechanisms of regeneration in the two groups are quite different. In planarians totipotent stem cells are widely distributed throughout their bodies that can lead the process of regeneration from every splitted fragment of the body (Redien and Alvaro, 2004). In annelids that do not have such distribution of totipotent cells throughout the body, regeneration is hypothesized to occur primarily by cellular dedifferentiation and redifferentiation (Thouveny and Tassava, 1998). To understand mechanisms of regeneration in annelids, the gene expression pattern during regeneration was studied on model animal *Enchytraeus japonensis* (Myohara *et al*., 2006). Besides the known genes ECM, glutamine synthetase, NICE-5, glucosidase, a new gene, Ejrup1-5 was observed to upregulate during regeneration. Structural analyses of the products of these genes, their functions in transportation and binding, transcriptional regulation, protein interaction and cell adhesion could be speculated. A strong expression of glutamine synthetase gene occuring in the blastemal regions of regenerating *E. japonensis* could also be observed by in situ hybridization (Niv *et al*., 2008). This suggests that at least in *E. japonensis* glutamine synthetase may play roles in regeneration. Cho *et al*., 2009 monitored the expression pattern of three *labial* genes (Pex-la-01, Pex-lab02, Pex-lab03) during regeneration in *Perionyx excavatus*. These genes were found to express only in the head-regenerating tissues. Overall, earthworm provides a unique and valuable model to investigate the mechanism of regeneration because this process is rapid.

VIII. Modulation of coelomic fluid constituents during regeneration

Wound healing in the earthworms is proposed to have use as a biomarker for assessing chemical toxicity (Cooper and Roch, 1986). The wound healing process involves the inflammatory response and various cell types, including coelomocytes. This process was monitored in earthworms like *Lumbricus terrestris* (Cikutovic *et al*., 1999) after exposure to a variety of chloride compounds. The authors found that

increasing concentration and duration of exposure significantly reduced the wound healing process (Ville *et al.*, 1995). Chloride compounds could have interfered with the membrane of coelomocytes suppressing their function in healing process. Some organics (polychlorinated biphenyl, pentachlorophenol, chlordane) suppress phagocytosis of coelomocytes in earthworm. Cell division, which is important in the wound healing process, might be affected with heavy metal (such Cd^{2+} or Cu^{2+}) pollution in soil (Cikutovic *et al.*, 1993). Such metallic ions can interfere with an enzymatic pathway in coelomocytes. Production of superoxide (O^{2-}), required for killing phagocytosed microorganisms may be inhibited. Injured animals are more susceptible to pathogens, so the chemicals that suppress the healing process may have actual effect on immunoactive cells responsible for phagocytizing and killing microorganisms.

The illumination of the regeneration process in annelid is valuable to us to explore strategies to enhance the regenerative capabilities in vertebrates as both these groups, being triploblastic eucoelomates, have similarity in basic body plan. The cellular events like muscle dedifferentiation which occur during wound healing, are similar to those that take place during tail regeneration of lizards. Additional studies in this area would enable us to model the complex mechanism of regeneration of vertebrates.

IX. Ethical permission for experiments on animal

This study was carried out following the recommendation from the Committee for the Purpose of Control And Supervision of Experiments on Animals (CPCSEA), New Delhi. The study did not use any endangered or protected species. Application for permission for animal experiments had been filed to the Institutional Animal Ethics Committee (IAEC), Department of Zoology, University of North Bengal, Siliguri 734013, West Bengal, India (Registration No. 840/ac/04/CPCSEA dt. 01/10/2004).

Eisenia fetida **e**arthworms were collected from Vermiculture Facility, Centre of Floriculture and Agribusiness Management (COFAM), Department of Biotechnology, University of North Bengal, Siliguri-734013, West Bengal, India. Detailed study plan had been given while describing of all surgical procedures (including methods of asepsis) and post-operative care.

X. Objectives of the study.

The present work had been undertaken with the following objectives-

1. To enumerate cultivable bacterial population in gut and coelomic space of *Eisenia fetida*.
2. To accomplish whole-metagenome-analyses of the gut content of *E. fetida* (by Next Generation Sequencing) leading to the evaluation of microbial diversity along with metabolic diversity with the aid of suitable bioinformatics workflow.
3. To enumerate population of different species of *Bacillus* in the coelomic fluid and to study the dynamics of indigenous *Bacillus* species in coelomic fluid in the face of challenge (by forced injection) by *Bacillus thuringiensis*.
4. To study modulation of coelomic constituents following *B. thuringiensis* challenge.
5. To study wound healing including regeneration (after amputation) with concurrent monitoring of innate immune status and indigenous microflora of the coelomic fluid.