GENERAL DISCUSSION AND SUMMARY

Eisenia fetida is a rich source of novel microorganisms. Altogether 25 unique colonies (8 from the cast, 3 from the posterior gut and 5 from the anterior gut, 5 from coelomic fluid and 4from the whole gut in anaerobic condition) were isolated. Phylogenetic trees constructed using MEGA ver 7.0 for all the 20 gut-strains depict their unique phylogenetic positions in three major phyla- *Proteobacteria*, *Firmicutes* and *Actinobacteria* (Fig. 1.13). Whole genome sequencing has been carried out for four unique strains. These are ET03^T isolated from the cast (Saha *et al.,* 2018) ; EPG1 from the posterior gut; EAG2 and EAG3 from anterior gut of *E. fetida*. Earthworms, the so-called 'ecosystem engineers', play a key role in nutrient cycling by interacting with microorganisms particularly responsible to the turnover of organic matter in soil systems (Cao *et al.,* 2015). As detritivores, earthworms ingest and digest a mixture of dead organic matter and microorganisms, like animal manures. Manure type though does not significantly influence the taxonomic and phylogenetic composition of the cast, the earthworm produces. Manures strongly differed in their taxonomic and phylogenetic composition, but these differences were markedly reduced by the earthworm gut. The core earthworm cast microbiome comprised of the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* as found in our metagenomic data. Our results suggest that earthworms build up their cast microbiome by selecting from the pool of ingested bacteria.

The biological process of the nitrogen cycle is a complex interplay among many microorganisms catalyzing different reactions, where nitrogen is found in various oxidation states ranging from +5 in nitrate to -3 in ammonia. Reduction pathways are assimilatory nitrate reduction (MD:M00531) and dissimilatory nitrate reduction (MD:M00530) both for conversion to ammonia, and denitrification (MD:M00529). In denitrification nitrate or nitrite is reduced to gaseous nitrogen compounds (N_2, NO and N_2O) as a terminal electron acceptor at low oxygen or anoxic conditions and liberated to the atmosphere. Genes of nitrogen metabolism with significant hit were nitrite reductase (NO-forming), nitrate reductase catalytic subunit, *cyn*T, can; carbonic anhydrase , *nap*A; periplasmic nitrate reductase NapA , nitrate reductase (NADH) , *nir*A; ferredoxin-nitrite reductase , *nir*B; nitrite reductase (NAD(P)H) large subunit , *nor*B; nitric oxide reductase

subunit B , *nor*F; nitric-oxide reductase NorF protein and *nos*Z; nitrous-oxide reductase. Their effects on the nitrogen cycle are highlighted (Fig. DS-1).

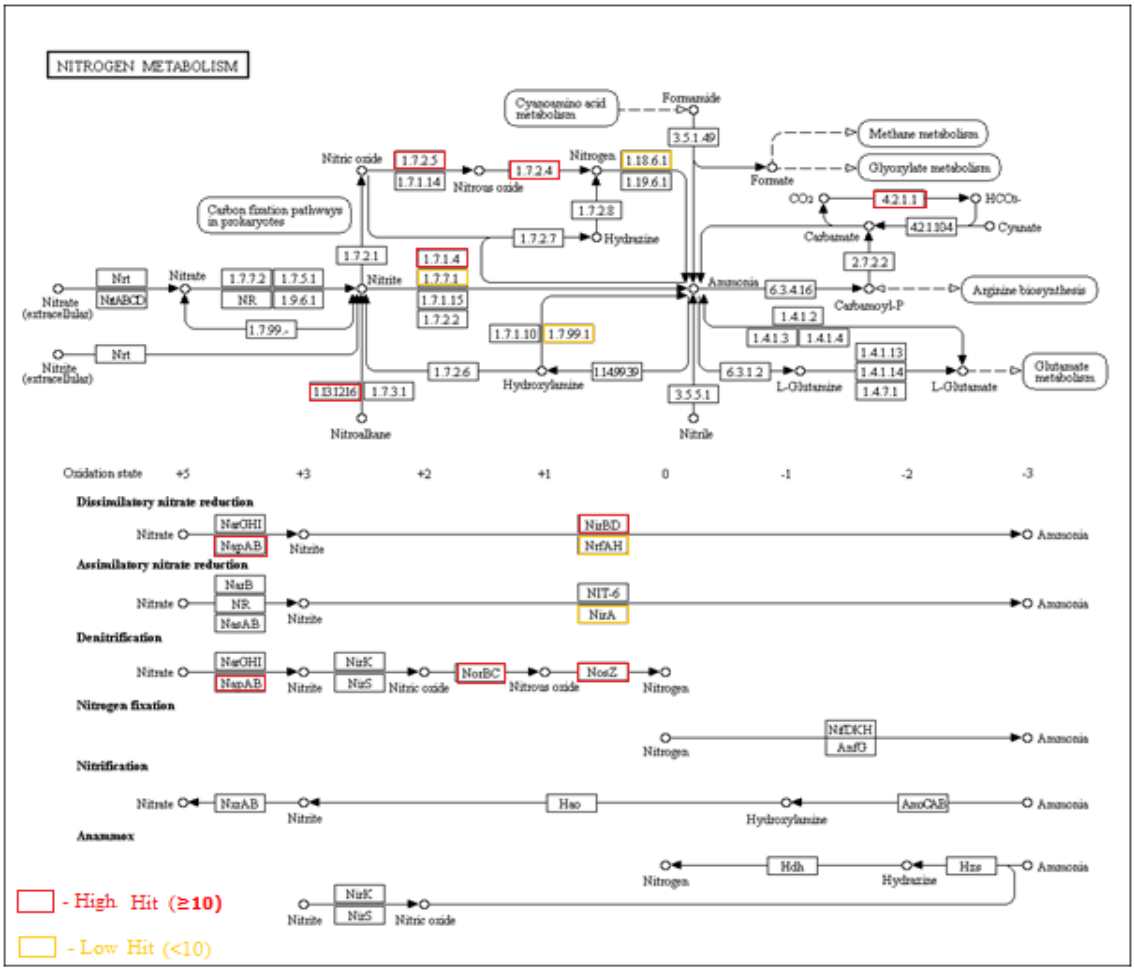

Fig. DS-1: Contribution of gut microbes of *E. fetida* on Nitrogen metabolism pathway/ Nitrogen cycle (The KEGG pathway was adopted from Kanehisa Laboratories, Japan).

Sulfur is an essential element for life and the metabolism. Sulfur occurs in various oxidation states ranging from +6 in sulfate to -2 in sulfide (H_2S). Sulfate reduction can occur in both an energy consuming assimilatory pathway and an energy producing dissimilatory pathway. In the assimilatory pathway, found in a wide range of organisms, sulfur compounds are reduced to compounds which ultimately are used for the biosynthesis of S-containing amino acids. In the dissimilatory pathway, which is restricted to obligatory anaerobic bacterial and archaeal lineages, sulfate (or sulfur) is the terminal electron acceptor of the respiratory chain producing large quantities of inorganic sulfide. The SOX (sulfur-oxidation) system (MD:M00595) is found in both

photosynthetic and non-photosynthetic sulfur-oxidizing bacteria. Genes of sulfur metabolism with significant hit were sulfate adenylyltransferase (*cys*D), adenylylsulfate kinase (*cys*C), phosphoadenosine phosphosulfate reductase (*cys*H), sulfite reductase (NADPH) hemoprotein beta-component (*cys*I), sulfite reductase (NADPH) flavoprotein alpha-component (*cys*J), sulfate adenylyltransferase subunit 1 (*cys*N) and bifunctional enzyme CysN/CysC (*cys*NC). Some other genes of sulfur metabolism with less hit (<10) were sulfite oxidase (SUOX), adenylylsulfate reductase, subunit A (*apr*A), adenylylsulfate kinase (*cys*C) and sulfite reductase (ferredoxin) (Fig. DS-2)

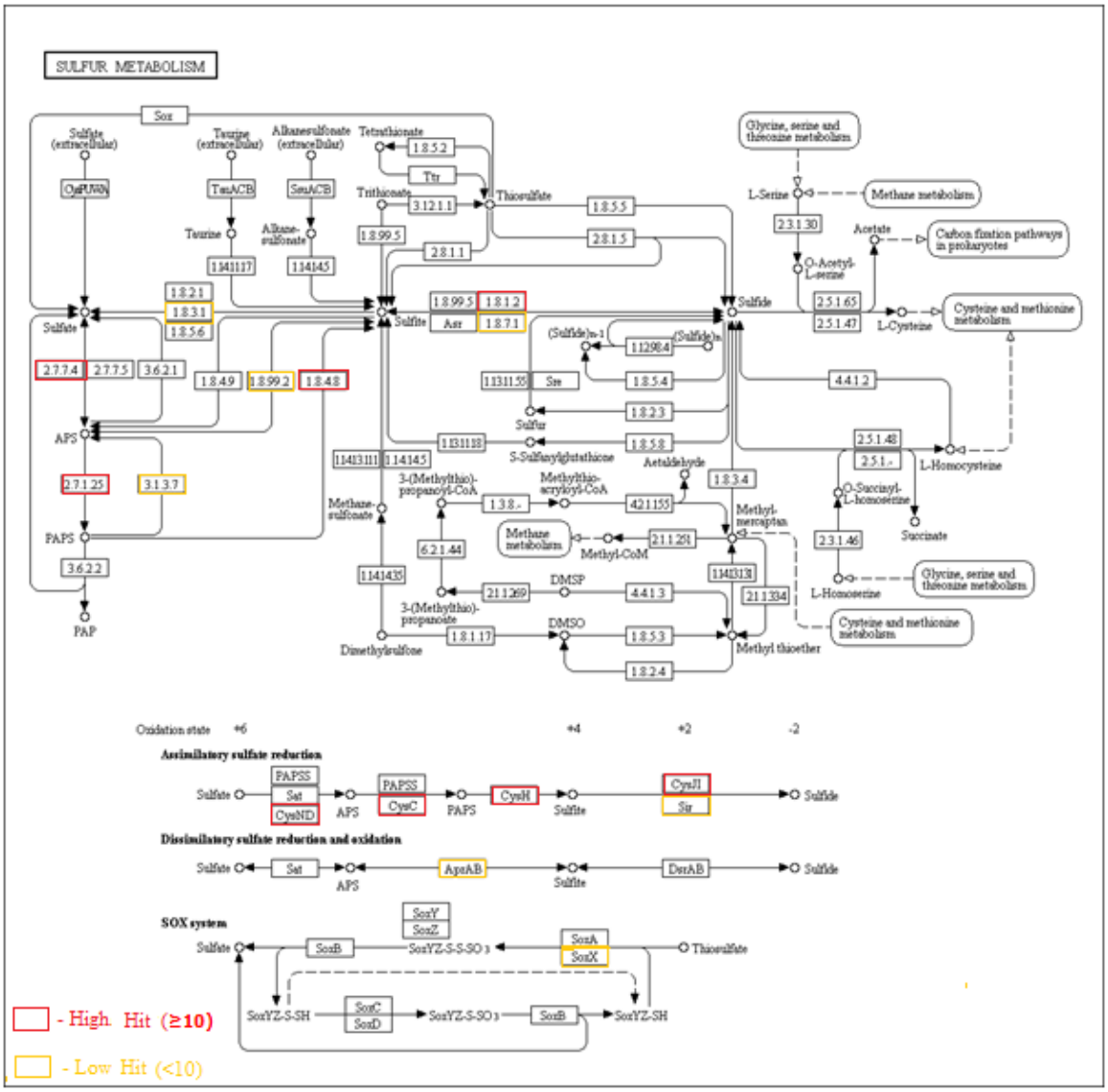

Fig. DS-2: Participation of gut microbes of *E. fetida* in sulfur metabolic pathway (The KEGG pathway was adopted from Kanehisa Laboratories, Japan).

Methane is metabolized principally by methanotrophs and methanogens. Methanotrophs consume methane as the only source of carbon, while methanogens produce methane as a metabolic byproduct. Methylotrophs can obtain energy for growth by oxidizing one-carbon compounds, such as methanol and methane. Genes of methane metabolism with significant hit were tetrahydromethanopterin S-methyltransferase subunit A (*mtr*A),

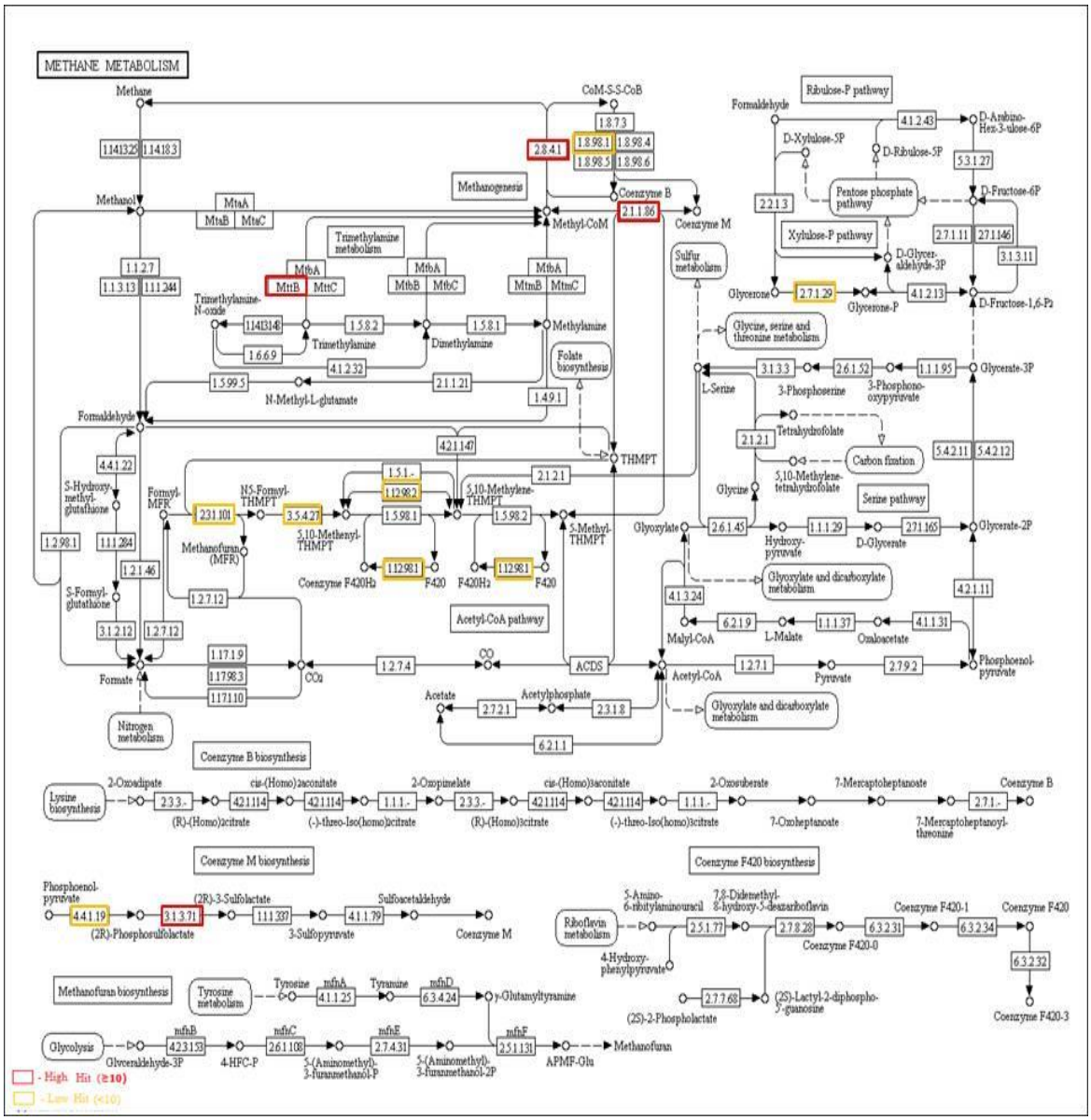

Fig. DS-3: Influence of gut microbes of *E. fetida* on methane metabolic pathway (The KEGG pathway was adopted from Kanehisa Laboratories, Japan).

2-phosphosulfolactate phosphatase (*com*B), methyl-coenzyme M reductase alpha subunit beta subunit (*mcr*A and *mcr*B) and tetrahydromethanopterin S-methyltransferase subunit

C, D, E and B (*mtr*C, *mtr*D, *mtr*E and *mtt*B). Some other genes of methane metabolism with less hit (<10) were formylmethanofuran-tetrahydromethanopterin N-formyltransferase (*ftr*), dihydroxyacetone kinase (DAK1, DAK2), phosphosulfolactate synthase (*com*A), coenzyme F420 hydrogenase beta subunit and gamma subunit (*frh*B, *frh*G), heterodisulfide reductase subunit A (*hdr*A), 5,10 methenyl-tetrahydro-methanopterin hydrogenase (*hmd*) and methenyltetrahydromethanopterin cyclohydrolase (*mch*)(Fig. DS-3).

Earthworms are constantly exposed to pathogens due to their detritivorous mode of life. Consequently, they have evolved various immuno-defense mechanisms which are assigned to coelomocytes, localized in the coelomic cavity. Light microscopic studies of coelomocytes in the *Eisenia fetida* coelom revealed the following morphologically distinct groups - amoebocytes (23±9%), granulocytes(18±7%) eleocytes(6±3%). Other than that small spherical cells (51±8%) has also been observed. Amoebocytes with phagocytotic activity can be identified from the size and shape of the nucleus. The cells tend to be smaller than, as little as 8 µm, but occasionally may be as large as 15 µm with fewer granules. Granulocytes have cytoplasm completely filled with small granules and have diameter of 15-22 µm. The eleocytes are large cells with 30-60 µm diameter. These cells possess a natural fluorescence like activity. The fourth group of cells in the coelomic fluid may be the transitory cells which are progenitors of the distinct cell lineages at different stages of maturation. We have differentially interrogated the growth patterns of *B. megaterium* and *B. thuringiensis* in Luria Broth and formulated broth that mimics the coelomic fluid of *Eisenia* in composition. It has been observed that both the species have similar growth kinetics in the Luria broth, but *B. megaterium* has distinctly faster growth rates in the coelom mimicking broth. Thus, the symbiosis like co-relation of *B. megaterium* and *Eisenia fetida* is evident. Further, closely related bacteria can secrete a wide array of antibacterial compounds such as bacteriocins when competing with other bacteria for the same resources. This may be the sources of future research to elucidate this bacterial competition. The bacterial growth in the coelomic fluid is controlled by the phagocytic and antimicrobial activity of coelomocytes. Expression of defence molecules like coelomic cytolytic factor (CCF) in the gut epithelial tissue is high due to continuous flow of immune response to an incessant flow of microbes with

ingested food. The coelomocyte population in response to forced introduction of a pathogen (*B. thuringiensis*) in the test and control *E. fetida* was compared. When there is a possibility of huge upsurge due to mobilization of pathogenic bacteria, pathogens in mass are captured or in other words encapsulated by multicellular entities produced by amoebocytes and eleocytes. The plausible immunomodulatory function of riboflavin (stored in chloragosomes) was demonstrated by establishing it as chemoattractant for coelomocyte-taxis. Riboflavin (vitamin B2) synthesis efficiency of the coelomic bacterial isolates was measured in a time-dependent manner during growth in a medium formulated on the basis of composition of the coelomic fluid. Chemotaxis of the environment bacteria towards coelomic fluid was discovered. Chemotaxis was studied by an improvised technique where bacterial mobilization to the CF contained in a capillary tube was quantified in a time-dependent manner. The notable findings of the present study are: (i) *Bacillus* population in the CF is up to the magnitude of 10^6 cells/ml (~68% of total bacterial load). (ii) The three major species recognized are- *Bacillus megaterium* (representative strain Ah4), *B. cereus* (representative strain BCR) and *B. pumilus* (representative strain BP). (iii) The number of coelomocytes particularly chloragocytes (or eleocytes) was found to increase from 12h of pathogen challenge. Since *E. fetida* is unable to produce riboflavin but compulsorily required for immune functions, chloragocytes store considerable amount of riboflavin in their chloragosomes (possibly derived from the symbionts). Strain Ah4, isolated from CF, has shown the fastest chemotactic mobility towards CF and at the same time its riboflavin production rate was highest among the *Bacillus* species that survive in the coelome. The basis of host-bacterial symbiosis (give-n-take phenomenon) was thus revealed.

Regeneration or re-growth of lost body parts after amputation,is restricted and sparsely found in few discrete groups of kingdom Animalia. *Eisenia fetida*, an annelid, besides being well-known for its usage in composting, has drawn special interest to the biologists because of its regenerative property. The present study is undertaken to describe tissue reorganization after amputation in *E. fetida.* Transverse amputation of adult *Eisenia fetida* at different regions of body followed by survival and development studies revealed that anterior fragments can regenerate missing posterior tail regions when amputations are done at least beyond the clitella. Tail regeneration from the 66th

metameric ring occurs with highest survival rate (89%). Normal nutritional activity and defecation restart from 9th day post amputation (dpa). Internal tissue reorganization and formation of the major tissues are complete on 11th dpa. The amputated segment's growth in respect to body weight gain was noted from 42 dpa. Histological studies reveal that neoblast cells originate not only from dedifferentiation of the longitudinal muscle cells, but also by multiplication of basal epithelial cells and visceral peritoneal cells. The blastemal mass comprising of chloragogue tissue has also been observed which might serve as reservoir for nutrition during regeneration. This histological study attempts to reveal the origin of blastemal cells during earthworm's wound healing and posterior regeneration. From 1 day post-amputation, neoblast cells could be observed to riginate from the de-differentiating longitudinal muscle layer of the body wall facing the coelomic side (Fig. 4.5 Ic). Similar observations were also reported by Park *et al.* 2013. These de-differentiating cells after proliferation and re-differentiation can rebuild the longitudinal muscle layer (Fig. 4.5 Vc). There was no existing report on de-differentiating cells from tissues other than longitudinal muscle cells. Here, it has been reported for the first time that dedifferentiating cells have also originated from- (i) the chloragogue tissue (Fig. 4.5 Ic), and (ii) intestinal tissue especially the basal cells of the typhlosole region (Fig. 4.5 IIe). The dedifferentiated chloragouge cells formed the blastema mass on the visceral side of the coelom which was not observed in the de-differentiated longitudinal muscle cells. From 5dpa onwards, the neoblast chloragogue cells could be found to accumulate in the coelomic space and by 11dpa it occupied most of it (Fig. 4.5 VIIa). This blastema might act as a nutrition-reservoir for the regenerating tissue. Most of the segmental structures viz. epithelium, circular and longitudinal muscle layers, wall of intestine were reformed by 11dpa. The release of cast through the regenerated pygidium by A4 amputee at 9dpa was also observed. Muscle-cells were previously thought to regenerate from cells embedded in the muscle layer itself as precursor cells (Bely, 1999). In contrst, we have observed during this histological study that, longitudinal muscle-cells loose-offfrom each other possibly by losing intra cellular adhesion molecules and divide resulting into the separation of nucleated part and contractile part, thus forming the de-differentiated neoblast cells. Cell-tracking studies by Tweeten & Reiner, 2012 conducted on earthworm *Lumbriculus,* also confirm that old gut tissue contribute to regenerate new gut tissue. In

Planarian regeneration, stem cells distributed in different regions of the body help to regenerate lost tissue structures (Tanaka & Reddien, 2011). Whether or not the earthworms follow the same rule has long been a debated issue. Blastema which is reported to be a homogeneous mass of neoblast (undifferentiated) cells is formed during regeneration of the fish fin and amphibian limb. Body part or limb regeneration experiments which were conducted in some vertebrates support lineage specific origin of blastema cells with unipotent functional attributes. Actually, the blastema in such cases was argued to be a heterogeneous mass of lineage-restricted cells (King and Newmark, 2012). The present findings suggest that during regeneration in *E. fetida* the origin of neoblast cells are tissue restricted. The neoblast cells originated through proliferation of the de-differentiated muscle cells and the chloragogue cells in respective tissue layers. These neoblast cells re-differentiated into tissues in the regenerating region of the amputated region of the earthworm (Fig. 4.5 IIIb, Vb, VIc). There could be cellular regulatory mechanisms which control the process of regeneration. Deep investigations at molecular level on model organisms may identify key molecules required to initiate and modulate the regenerative events. Some reports suggest that nerve cord may have cardinal role during annelid regeneration (Bely, 2014). Absence of the nerve cord at the amputed region of *Eisenia* inhibits ectodermal and mesodermal growth, but, endodermal growth remains undisturbed. The deep cellular events of de-differentiation are still not well understood. So, it is important to characterize how the extracellular environment is altered during regeneration, and how different extracellular factors may shape cellular activity in the blastema. The present histological guide is a preliminary advancement which offers an opportunity to the molecular biologists to use the anatomical events following amputation to link gene function during regeneration.

The molecular level characterization vows to our hypothesis that the gut of *E. fetida* houses novel bacteria and these unique bacteria might have an important biotechnological application in the field of complex sugar digestion or as the source of novel antibiotic or antifungal agents. Study of the *Eisenia fetida* coelomocytes' gene expression under bacterial challenge at genome-wide-transcriptomic level also has produced significant data having implications in both academic and potential biotechnological applications.