CHAPTER 4

WOUND HEALING & REGENERATION IN *EISENIA FETIDA*

4.1.Introduction

The process of wound healing and regeneration in the amputated body parts is studied in few isolated groups of animals. This bio-medically relevant field of scientific research still have the potential in revealing knowledge about the differential regeneration capability of different animals and their cellular and molecular basis. Some diploblastic groups, acoelomate triploblasts, annelids, insects, asteroid echinoderms, fishes, amphibians and few lizards has the ability to regenerate amputated body parts (Sa´nchez Alvarado, 2000; Myohara, 2004; Bely & Nyberg, 2010). Some lower group of animals like hydra, planarians can regenerate complete individuals from small pieces of the body while in the complex forms like fish, amphibians and lizards replacement of lost fins appendages or tail respectively is observed. In between these lower and higher groups of animals, annelids occupy an intermediate position being the simplest eucoelomate triploblast. Hence, reasonably annelids particularly earthworms have long been subjects of regeneration research. Regeneration in annelid groups like nereids (errant polychaetes), enchytraeids (Sedentaria), naidids (Sedentaria), capitellids (Sendentaria), and Lumbricids (oligochaetes) have been studied (Berrill, 1952). Among these different groups of annelids successful regeneration of posterior segments have been reported, but, the ability to regenerate anterior segments were found to be futile in most cases (Bely, 2014). Because of the easy availability, convenience in culture and handling in the laboratory and rapid regenerative power, the earthworm *E. fetida* has been preferred in regeneration research (Edwards & Bohlen, 1996). Earlier reports regarding the correlation of the survival rate and regeneration capacity of immature and adult *E. fetida* are available which indicate that survival and regeneration proportionally depend on the number of annules retained in the amputated earthworm (Xiao *et al.*, 2011). The processes of wound healing and cell proliferation at the wound site after amputation were described by Hill (1970). The nature of tissue interactions between the epidermis, nerve cord, and gut endoderm in regenerating earthworms was reported by Fitzharris & Lesh (1972). Few histological studies have postulated that all of ectoderm, mesoderm and endoderm proliferate near the wound site (Clark, 1972; Cornec *et al.*, 1987). Neoblast cells, observed near the severed region in many clitellate annelids, has been in focus for their origin and function (Cornec *et al.*, 1987; Tadokoro *et al.*, 2006; Myohara, 2012;

Sugio *et al.*, 2012). These neoblast cells have been postulated to form 'regeneration blastema' in the regenerating region. The blastema is said to form most of the missing structures (Müller *et al.*, 2003; Müller, 2004; Müller & Henning, 2004; Zattara & Bely, 2011). Tweeten and Reiner (2012) pointed out that gut endoderm has an important role in the regeneration of gut in the amputated animals. Park *et al.*, (2013) described circular muscle layer to be the major origin of blastemal cells during regeneration in *Eisenia andrei* with histological evidences of de-differentiation restricted to 1 and 3 day(s) after amputation. There was no available report on the origin of vital tissues like epithelium, circular muscle layer or gut wall in the regenerating region of *E. fetida*. Under this scenario, further research attempting revelation of mechanism of tissue reorganization and regeneration of body wall, muscle layers and gut lining is needed.

Some authors have recently speculated about the possible contribution of other tissues in the blastema formation (Park *et al.*, 2013). In this study, a histological investigation of de-differentiation followed by re-differentiation is carried out from day 1 to day 11 following amputation of posterior one-third of the *E. fetida*'s body. In particular we investigated the importance of the vital organs in relation to the survival and regeneration of the amputated fragments in *Eisenia fetida* focussing mainly into the process of regeneration with the classical histology tool.

4.2. Materials and methods

4.2.1. Amputation experiment of *E. fetida*

Earthworms *(E. fetida)* were reared in cow manure at 22 ± 1◦C, in a laboratory climate chamber for several generations. Cocoons were collected from the culture beds and reared in small aerated plastic chambers with adequate cow dung manure, humidity, and temperature maintenance. Newly hatched earthworms took about 8 weeks to develope clitella until which they were regarded as immature. Amputation experiments were done on the adult *E. fetida* by severing the worm on four different amputation sites (Fig. 4.1). Adult *E. fetida* has approximately 97 segments (Halder, 1999) including a well-developed clitellum on annule number 25–31 (Fig. 4.2a) and generally weighs 0.35±0.03g.

4.2.1.1 Amputation, rearing and estimation of survivability of amputated parts

Four types of amputations (in between segments 10-11 [A1 (Anterior); P1, (Posterior)], 24-25[A2 (Anterior); P2, (Posterior)], 31-32 [A3 (Anterior); P3, (Posterior)] and 66-67[A4 (Anterior); P4, (Posterior)] were done. Sets of amputated segments {A1, P1}, {A2, P2}, {A3, P3}, and {A4, P4}, were selected as amputation sites to represent anterior segments without esophagus and crop (A1), without clitellum (A2), with clitellum (A3), and body part (2/3rd) with most known vital organs (A4); on the other hand, the posterior segments with esophagus and crop (P1), with clitellum (P2), without clitellum (P3)and body part (1/3rd) without most known vital organs (P4).

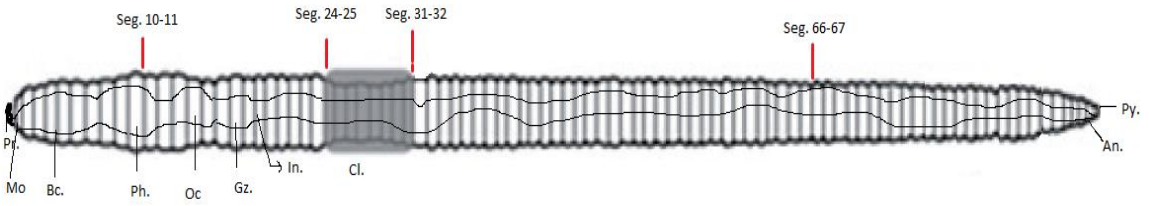


Fig. 4.1: Schematic drawing of an adult *E. fetida* depicting the four selected regions of amputation (An-Anus, Bc- Buccal cavity, Cl- Clitellum, Gz- Gizzard, In- Intestine, Mo-Mouth, Esophagus, Ph- Pharynx, Pr- Prostomium, Py- Pygidium).

The above-mentioned scheme of amputation was designed to observe the effects of position of amputation on survival and recovery rate (weight gain). Controls were *E. fetida* with no amputations. All amputations (as per scheme) were replicated three times with 20 earthworms per treatment; each kept in an aerated plastic container with cow manure and hydrated tissue paper (Fig.4.2c). Amputation was done following standard method (Xiao *et al.*, 2011).

4.2.1.2 Estimation of body weight alteration of amputated parts

The survival rates, growth in terms of body weight (including gut content) were checked every 2 days for the first week and then weekly up to 10 weeks.

4.2.1.3 Periodic histological observations

For histological studies, surviving representatives of A4 (as A4 regenerated most successfully) were selected and sacrificed. The nearby region of amputation of each A4 was severed out with a sterile sharp scalpel and processed for histological observation

(Cui, 2011). Briefly, the whole tissue was fixed in Bouin's fixative for 18hours. After ethanol washings at different increasing grades upto absolute for dehydration, xylol was used for clearing. The tissues were then infiltrated with paraffin (MT56-58◦C) by warming in oven. Microsections (6 µm) were cut (using Lipshaw type rotary microtome, York Scientific Industries Pvt. Ltd.) and mounted on slides. Microsections were stained with haematoxylin and eosin and observed under a light microscope (Dewinter, Model DEW/002, Dewinter Optical Inc., INDIA) and photographed with DIGIEYE powered through USB 2.0 equipped desktop computer.

4.2.2. Dynamics of indigenous *Bacillus* species in coelomic fluid of the amputated body parts of *E. fetida*

4.2.2.1 Periodic withdrawal of coelomic fluid of E. fetida

Coelomic fluid from the survived body part (A4) of the earthworms was periodically (at day1, 3, 7 and 11) were pooled out in separate siliconized centrifuge tubes. Approximately 100µl from ~ 20 A4 animal (part) was collected at each study day. Sterile, sharpened capillary tubes were used to withdraw the coelomic fluid from surface cleaned earthworms (Bilej *et al.,* 1990). Sterile 1x PBS, pH7.4, prepared by using PBS powder (Himedia M1866) and MiliQ water was used to wash the coelomocytes. Total process was done on ice.

Coelomocytes were separated by centrifugation (100xg for 10 min) and cell-free coelomic fluid was collected and kept at -20 °C. Isolated cells were resuspended in PBS

4.2.2.2 Serial dilution, plating and incubation

The supernatant collected in the previous step was serially diluted in sterile PBS. Multiple dilutions and plating on HBA plates were done to enumerate the number of indigenous *Bacillus* spp. content (CFUs) in the coelomic fluid during different stages of regeneration.

4.2.3. Compositional changes of coelomic fluid relating to coelomocytes of selected regenerating *E. fetida*

4.2.3.1 Isolation of coelomocytes

Coelomocytes separated on the previously mentioned step on day 1,3,7 and 11 from the A4 body part of *E. fetida* were isolated, identified, counted (DC) and studied for their phagocytic nature.

4.2.3.2 Differential count (DC) of coelomocytes

Differential counts were made for each group of regenerating worms by placing a drop of coelomocyte suspension in PBS onto a clear glass slide and making a smear. After air-drying, coelomocytes were fixed in methanol for 20 s and stained with modified Wright's stain (Sigma WS16) for 20s. One hundred leukocytes were classified under oil immersion using 100 x objective lens.

4.2.3.3 *Study of phagocytic behaviour of coelomocytes*

Time-lapse study of phagocytosis percentage at 30min, 1h and 1h 30min from the coelomocytes collected from A4 regenerating segment of *E. fetida* was performed following the same process described in section 3.2.2.5.

4.3. **Results**

4.3.1 Amputation of *E. fetida*

4.3.1.1 *Survivability of amputated parts*

The survival rates of the anterior and posterior segments of the amputated *E. fetida* (as per scheme) are calculated as survival percentage for each amputated part by using the formula, (surviving amputated parts / total initial number of that amputated part i.e.20) X 100. A2, A3, and A4, have shown the higher rate of survival (78%, 78% and 89% respectively after 10 weeks) during the process of regeneration, while A1 failed to survive and 89 % died within day 1 and the remaining (11%) in day2 (Fig 4.3a).

All the posterior amputated parts P1, P2, P3, P4, have strikingly low survival rates during the process of regeneration (Fig. 4.3b). All the P1 parts died within 2 weeks of amputation. P2, P3 and P4 have 22%, 44% and 33% survival rate respectively up to 10 weeks of the experiment period.

4.3.1.2 *Body weight alteration of amputated parts*

The mean body weights of the survived amputated parts were taken into account for calculating the alteration of body weight. The reduction in weight with time was noticed among the survived anterior amputated parts, A2, A3, A4, up to 6 weeks (Fig. 4.4a). A3 and A4 gained weight from 7th week which even surpassed their initial (Day 0) weight. The weight of amputated amputated part, A2 remained unchanged. All the posterior amputated parts (P1, P2, P3 and P4) of the amputated *E. fetida* have shown progressive decline in body weight (Fig.4.4b). The decrease in body weight of the posterior amputated parts was found to be much faster than the decrease shown by the anterior parts in the initial days of observation.

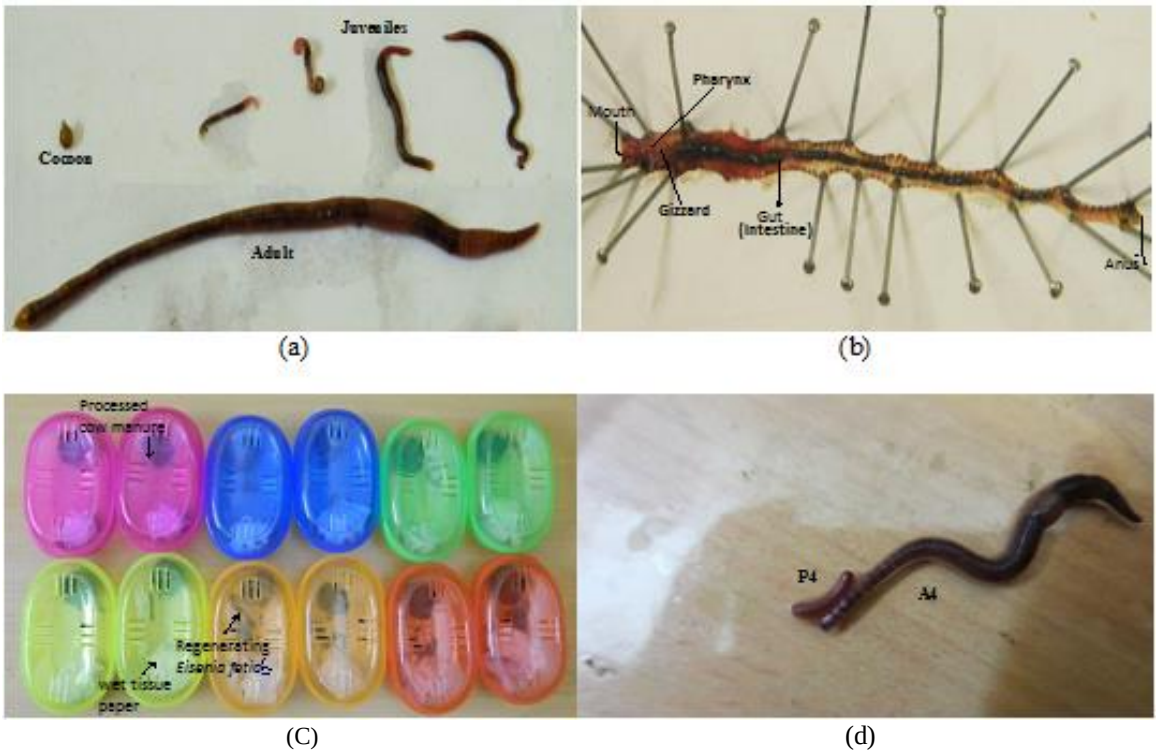
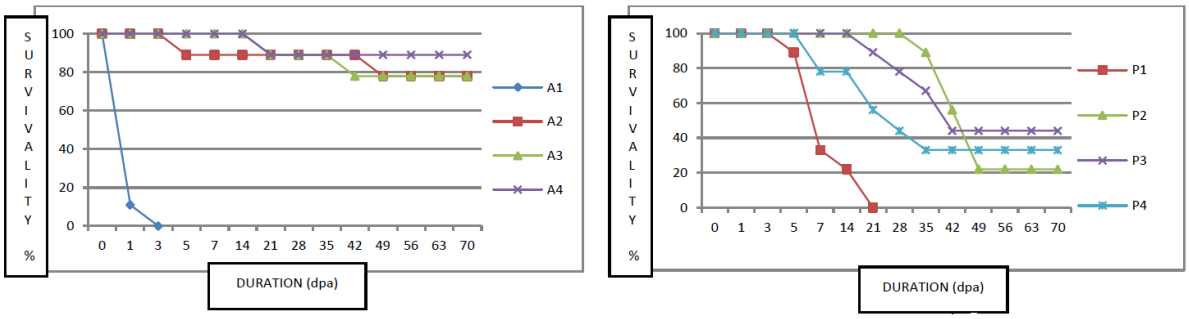
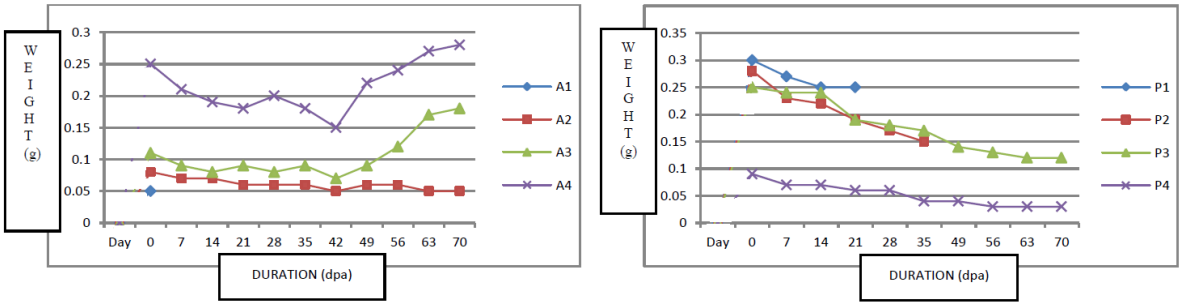


Fig.4.2: Photographs taken during different phases of *E. fetida* regeneration experiment. (a) Adult *E. fetida* compared with juveniles (b) Dissected adult *E. fetida* with intact gut (c) Rearing containers of individual amputated *E. fetida* (d)11 dpa A4 amputee with regenerated posterior part ; survived P4 amputee without regeneration



(3a) (3b)

Fig. 4.3 Survival (%) of the amputated *E. fetida* in respect to days post amputation. (a)Anterior parts (b)Posterior parts



(4a) (4b)

Fig. 4.4 Body weight (g) of the amputated *E. fetida* in respect to days post amputation. (a) anterior parts (b) posterior parts.

4.3.1.3 *Histological observations*

Since A4 demonstrated the most successful development process in terms of gaining net body weight following amputation, the tissue level dynamics during regeneration of A4 amputated part with the aid of histological techniques was studied. It was revealed from the histological section of one-day- post- amputation (1dpa) A4 part that, all the major tissues like epidermal layer, circular muscle layer, longitudinal muscle layer, gut wall epithelium show anatomical variation than the normal tissue organization. Proliferating cells appeared in all the above mentioned tissues (Fig. 4.5 IIa). Neoblast cells were clearly observed to originate from de-differentiating longitudinal muscle layer and the chloragogue tissue in the coelomic face of those tissues (Fig. 4.5 IIc). The longitudinal muscle cells delaminated in the coelomic space, proliferated and formed neoblast cells.

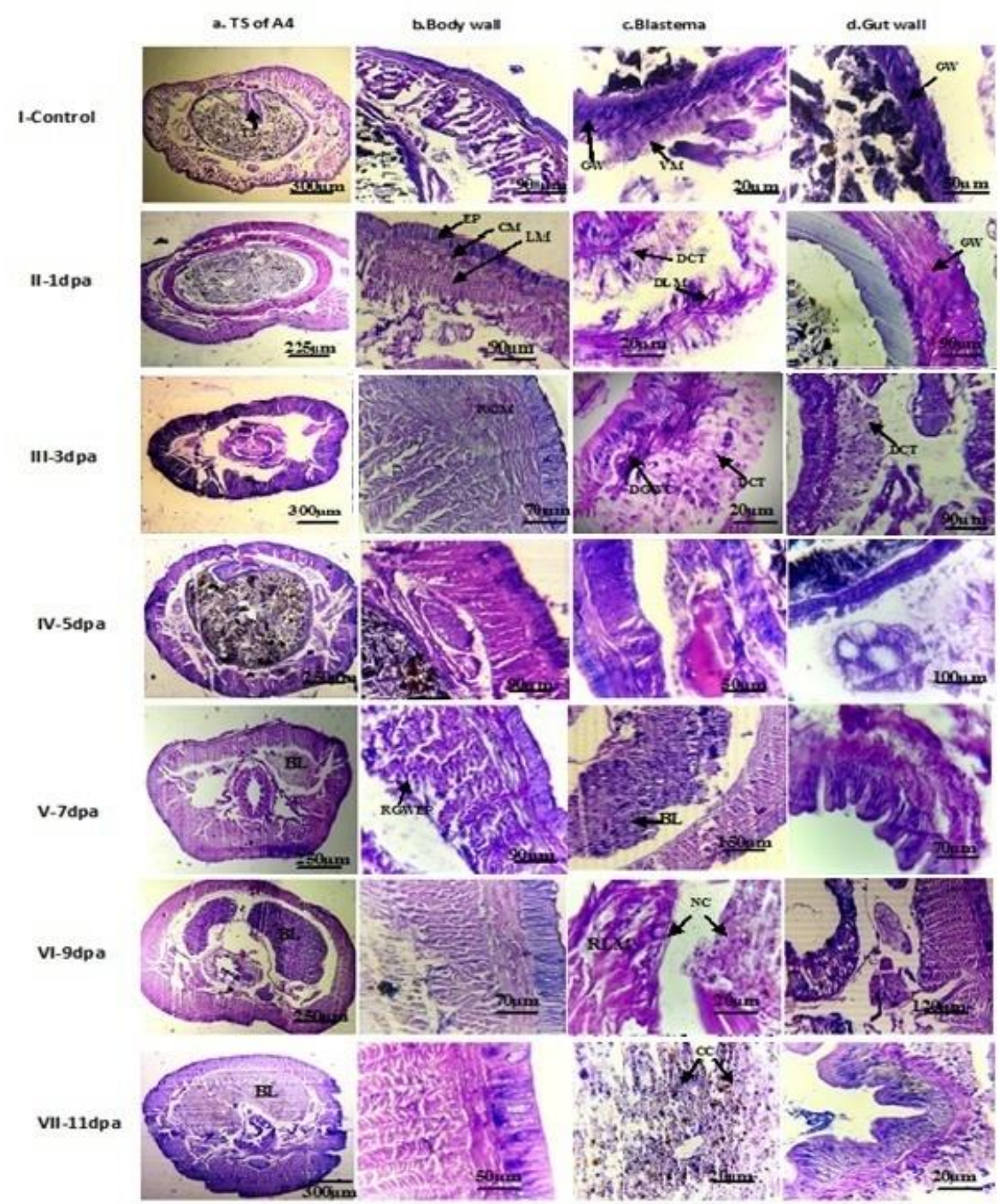


Fig.4.5: Photographs of histological preparations of tissues in regenerating region of A4 amputee up to 11 days post amputation and corresponding regions from control *E. fetida*.
(BL-Blastema, CC-Chloragogenous Cells, CM- Circular muscle, CT-Visceral mesoderm, DCT-De-differentiating chloragogue tissue, DGWC- De-differentiating gut wall cells, DLM- De-differentiating longitudinal muscle, EP- Epithelium, GW- Gut wall, LM- Longitudinal muscle, NC- Neoblast Cells, RCM- Re-differentiating circular muscle, RGWEP- Re-differentiating gut wall epithelium, RLM- Re-differentiating longitudinal muscle, TS-Typhlosole)

The chloragogue tissue layer grew thicker than the normal (Fig. 4.5 Ic, IIc & IIIc) and multiple layers of proliferating neoblast cells could be found. By day 3 (3dpa), all the major tissues were observed to develop growth by the neoblast cells. Regeneration blastema (cells in mass) was developed in the coelomic space (Va, VIa, VIIa). The muscle layers of the body wall were found to get re-established by 7dpa. The gut wall epithelium reformed by 7-9dpa. The blastema cells were found to contribute the re-differentiating longitudinal muscle layer (Fig. 4.5 VIc). (iv) Since histological surveillance was continued even after 10dpa, the complete differentiation of the mid dorsal groove of intestine (typhlosole) was found to be taking place by 11dpa. (v) Interestingly, blastema tissue was located and photographed to be formed not only from de-differentiating longitudinal muscle cells (Fig5 IIc) but also from chloragogen cell mass (Fig. 4.5 IVc). The other organs viz. excretory segmental nephridia were also found to develop fully by day 11 (Fig 4.5 VIIa).

Eisenia is the simplest eucoelomate resembling basic body plan of the higher groups of animals. The digestive system in *Eisenia* resembles a straight tube with various degrees of regional specialization at the anterior end (Fig. 4.2b). Below the epidermis there are thin circular and thick longitudinal muscle layers. The longitudinal muscle layer is bounded internally by the parietal peritoneum (Fig. 4.5 Ia). Mouth leads to a short buccal cavity, muscular pharynx, and esophagus. The posterior esophagus bears a crop, and one muscular gizzard. The rest of the digestive tube is a straight intestine leading to a short proctodeal hindgut and anus located on the pygidium (Brusca & Brusca, 2003). In the present study, it was observed that, presence of the anterior organs of the digestive system was vital for the survival of the severed worms. Prior to the present study, linear correlations between the survival frequencies of amputated earthworms with the differential length of the amputated segments were observed. Xiao *et al.*, (2011) reported that greater are the chances of survival when lesser segments were amputated in *E. fetida*. In our study, we have demonstrated that absence of vital organs in the amputated segment (A1) did not support survival vis-a-vis regeneration of the amputee *E. fetida* worms. Hence, anterior segments except the A1 amputee survived (survival rate >70%). This might be due to intactness of the anterior gut portions (vital for feeding and uptake of nutrition from the ingested food which the amputees draw after recovery from the severe

shock). It was observed that all the live anterior amputees lost their body weight to some extent during the initial period (1st to 6th week), but from 7th week onward gradual increase in body weights were noted in case of A3 and A4 amputees. The A2 amputee survived (for > 8 weeks) but failed to grow and regenerate. This may be because of the fact that they did not have sufficient intestine and associated tissue like typhlosole that increases the surface area of the intestine in *Eisenia* (Fig. 4.5 Ia). Many layers of chloragogen cells remain associated with the intestinal wall and typhlosole. These chloragocytes contain greenish, yellowish, or brownish globules and are reported to serve as producer and store-house of glycogen and lipids along with other important functions. The blastema tissue from 9 dpa was observed to be mainly occupied by the chloragogue tissue (Fig. 4.5 VIIc). Hence, the function of this tissue in the later stage of development seems to serve nutrition to cellular growth and proliferation of the regenerating part. All the severed parts containing the posterior region survived the amputation shock better than the counterpart A1 amputee but failed to gain weight. Few posterior fragments survived for as long as 60 days, presumably by slow exhaustion of nutrition which was stored in the chloragogenous tissue before amputation.

4.3.2. Dynamics of indigenous *Bacillus* species in coelomic fluid of the amputated body parts of *E. fetida*

Study of coelomic fluid at different intervals (at day1, 3, 7 and 11), from the survived body part (A4) of *E. fetida*, reveals a population fluctuation of the major Bacillus spp. An adventitious pathogen, *B. thuringiensis* is observed at very small number compared to other *Bacillus* spp. in the amputated A4 segment from day up to Day7. This pathogen is completely out populated by other indigenous *Bacillus* species by day11. The population of different indigenous *Bacillus* species is considerably lowered than the normal bacilli count in control animals. The number of *B. cereus* is significantly reduced on Day 7 ($p < 0.01$), which is slightly recovered on Day 11. Population of *B. megaterium* seems to have the least reduction ($p < 0.05$) compared to other bacilli. This outcome indicates that *B. megaterium* is favoured in the coelomic fluid than other bacilli (Fig. 4.6).

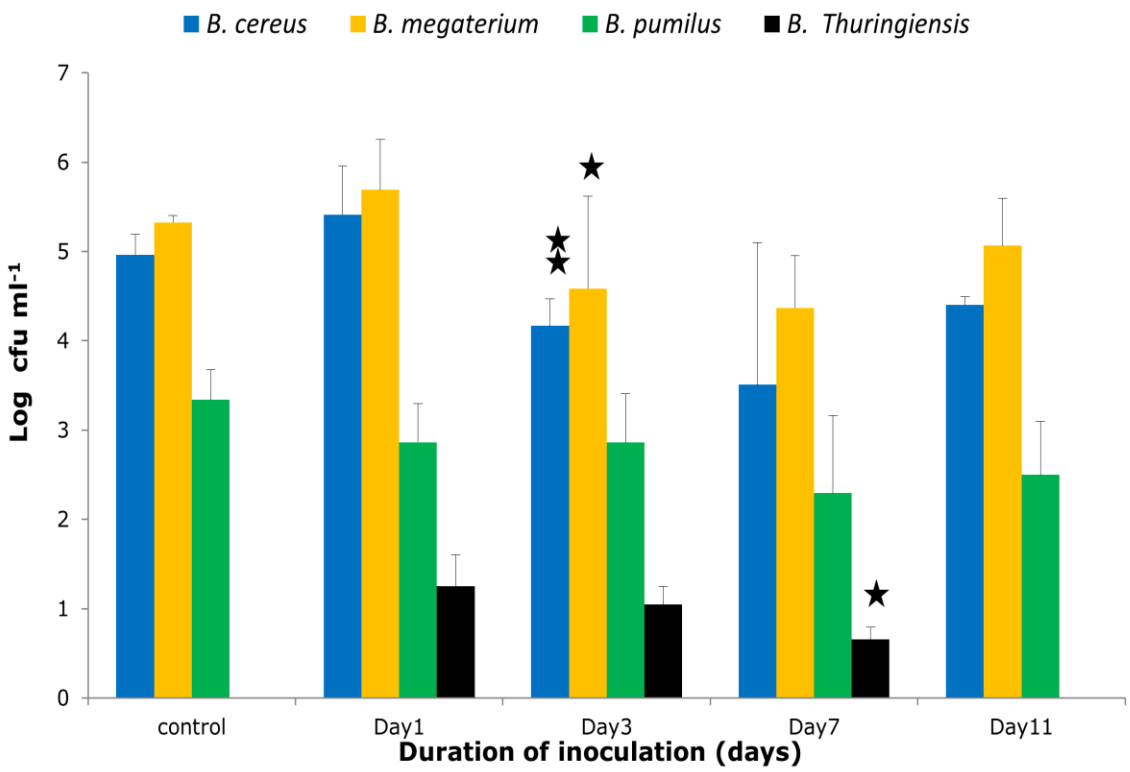


Fig 4.6: Dynamics of indigenous *Bacillus* species in coelomic fluid at different intervals (at day1, 3, 7 and 11), from the survived body part (A4) of *E. fetida*

4.3.3. Compositional changes of coelomic fluid relating to coelomocytes

4.3.3.1 Differential count (DC) of coelomocytes

Light microscopic studies of coelomocytes at different intervals (at day1, 3, 7 and 11), from the survived body part (A4) of *E. fetida* revealed modulation of the four group of coelomocytes, the normal count of which are- amoebocytes (or hyaline amoebocytes)(23±9%), large granulocytes (or basophils)(18±7%), eleocytes (or chloragocytes) (6±3%) and small granulocytes (or granular amoebocytes) (51±8%).

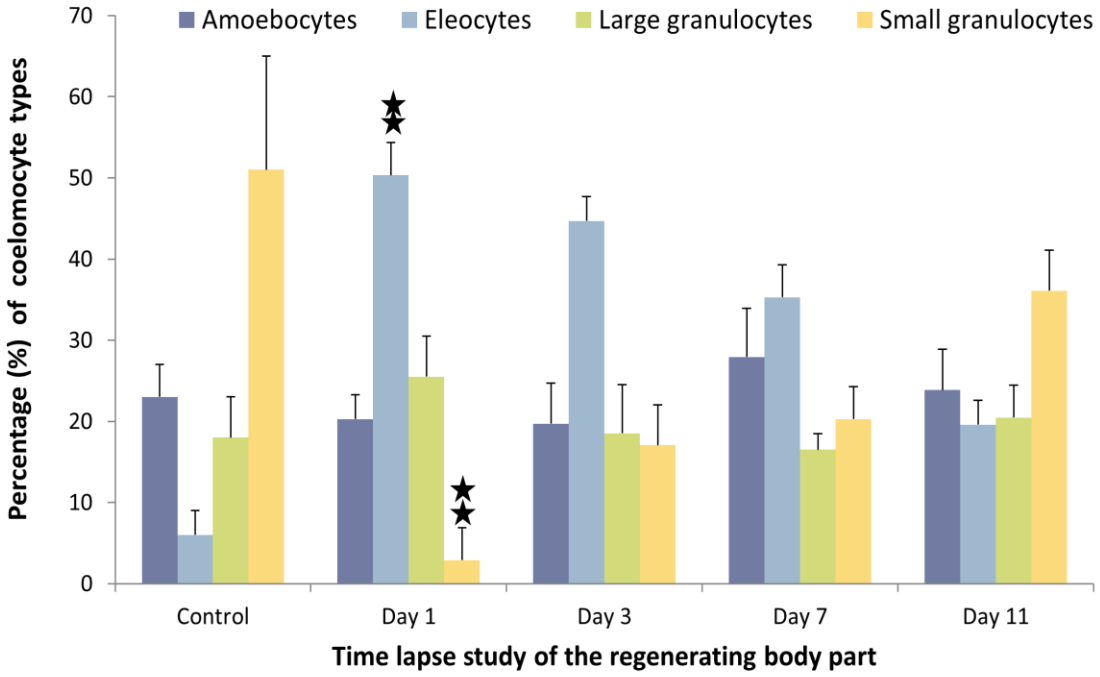


Fig 4.7: Differential count (DC) of coelomocytes at different intervals (at day1, 3, 7 and 11), from the survived body part (A4) of *E. fetida*

The population of eleocytes was significantly increased and the population of small granulocytes was significantly decreased on day 1 after amputation of the A4 fragment in comparison to the control animals. This deviation of the differential count of coelomocytes is minimized to some extent by day 11 (Fig. 4.7).

4.3.3.2 Study of the phagocytic behaviour of coelomocytes

Both amoebocytes and granulocytes were frequently found with inclusion bodies including nano-carbon particles. These coelomocytes were frequently observed to accumulate towards the activated carbon particles, which indicate their increased efficiency of chemotaxis and phagocytosis. Active phagocytic cell increased significantly ($p < 0.001$) on day 1 after amputation of the A4 fragment in comparison to the control animals. This difference in the number of active phagocytic cells remained significant (p

< 0.05) till day11 (Fig. 4.6). The fraction of amoebocytes and granulocytes with visible inclusion bodies in respect to the total number of coelomocytes was considered to be the phagocytic percentage.

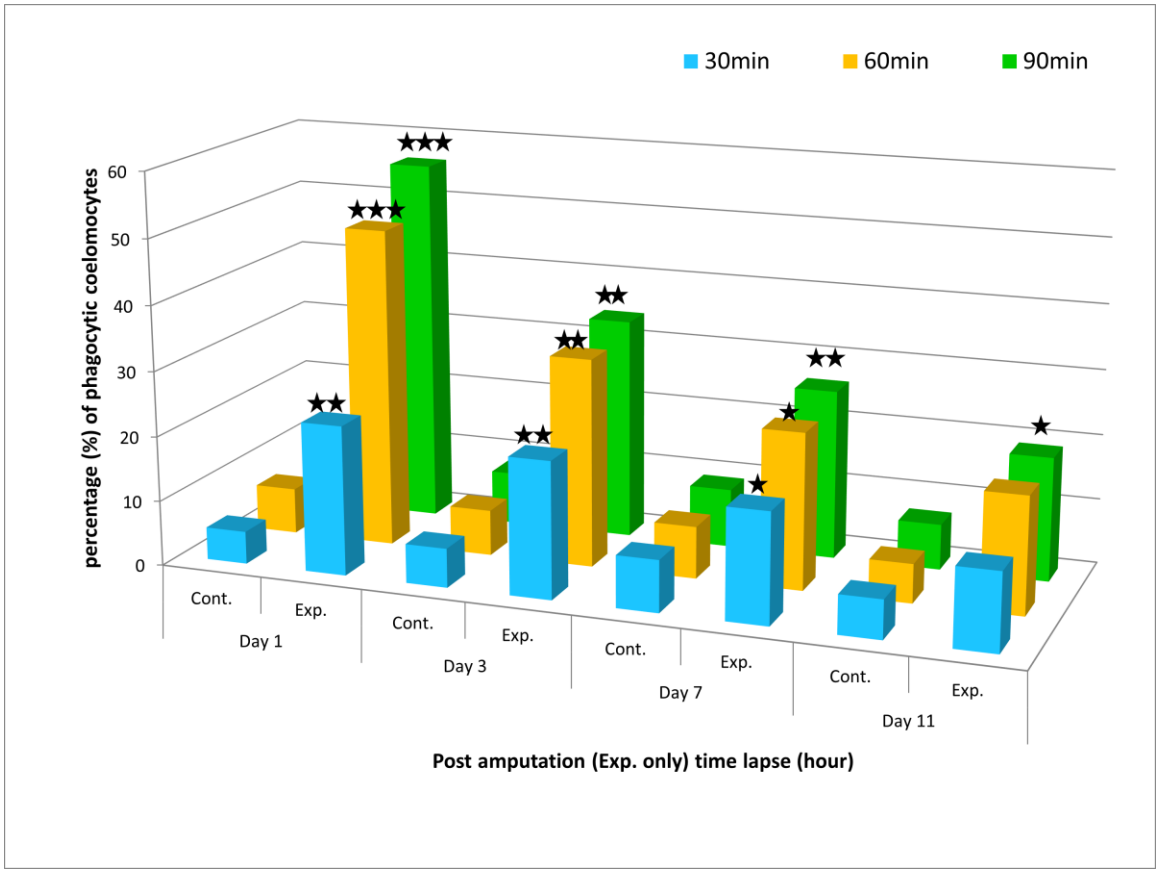


Fig.4.8: Bar graph showing percentage of coelomocytes with phagocytic inclusion bodies in the experimental (A4) and control (without previous amputation) *E. fetida* at different time intervals.