# CHAPTER - 3

# MODULATION OF COELOMIC FLUID CONSTITUENTS UNDER BACTERIAL CHALLENGE

### 3.1 Introduction

*Eisenia fetida* (Savigny, 1826), the clitellate annelid is a universally accepted vermicomposting earthworm living in and on the decomposing organic matter. Soil and organic debris contain different groups of bacteria along with other microorganisms. *Eisenia,* in context to its living zone certainly has to face the bacterial challenge (Aira *et al.,* 2007). *E. fetida* hosts $6X10^5$ /ml ($0.9X10^5$ /adult individual) bacteria in its coelom (Edwards and Lofty, 1977). The Superior numbers of phagocytes along with various humoral factors prevent the microorganisms from outgrowth (Bilej *et al.,* 2000; Ko¨hlerova´, 2004). If the bacterial burden surpasses the optimum limit it may cause disease in the 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. So, logically *E. fetida* seems to have certain defence mechanisms that would be able to protect it against invading pathogens. Coelomocytes in the coelomic cavity are reported to be the major players of the immune system (Dhainaut and Scaps, 2001; Dvořák *et al.,* 2016). Semi-quantitative Expressed sequence tag (EST) based genomic study on a closely related species *E. andrei* following bacterial challenge reported 24 immune-related and cell defense genes (Bilej 2014). However, there is limited genetic information available for immunological pathways in *E. fetida* under bacterial challenge. This study compared transcriptomic profiles of coelomocytes from normal and bacteria infected *E. fetida* by Illumina-based paired-end sequencing to explore the molecular immune mechanism of *E. fetida* against bacterial infection. Adult *E. fetida* earthworms maintained at 22 ℃ in compost were transferred 2 days before experiments on filter paper soaked with an isotonic balanced salt solution (BSS) (Stein and Cooper, 1981). Bacterial inoculation in the coelomic fluid was done following the protocol described by Kauschke and Mohrig in 1987 with minor modifications.

Percoll (Pharmacia) was used as a cell separation media by Hamed *et al.,* 2005. Coelomocytes thus isolated was evaluated for living cell numbers by trypan-blue exclusion method; and Wright-Giemsa staining was performed for cytological study. Cell

size as well as nuclear diameter are measured with a calibrated ocular scale (10x), using a Zeiss microscope (40x) objective and a microcytometer.

Modern medical research has indicated that the coelomic fluid (CF) of earthworms contains an abundance of bioactive substances including lectin (Suzuki *et al.,* 2009), polysaccharide (Wang *et al.,* 2007), protease (Sugimoto *et al*., 2003), antibacterial peptide (Wang *et al.,* 2003), metalloenzyme (Sturzenbaum *et al.,* 2001), fibrinolytic enzyme (Wang *et al.,* 2005), and so on. Earthworm proteins and peptides have exhibited various biological activities (Liu *et al.,* 2004; Wang *et al.,* 2007). Fetidin, with apparent molecular weight of 40 kDa, was purified from earthworm coelomic fluid and its bioactivities of antibacterial action, hemolysis and hemocoagulation were estimated (Milochau *et al.,* 1997). A 42 kDa protein, named coelomic cytolytic factor 1 (CCF-1), was reported to have cytolytic, opsonizing and hemolytic properties (Beschin *et al.,* 1998). Lumbricin I which was isolated and characterized from the earthworm showed antimicrobial activity *in vitro* against a broad spectrum of microorganisms without hemolytic activity (Cho *et al.,* 1998).

ECFP, 38.6 kDa protein, was isolated from *E. fetida* and shown to possess significant hemolytic activity to chicken red blood cells (CRBC) (minimal hemolytic concentration 0.39 µg/mL) and antibacterial effect against *Escherichia coli* (minimal bactericidal concentration, MBC 180 µg/mL) and *Staphylococcus aureus* (MBC 90 µg/mL) (Hua *et al.,* 2011).

Only fragmented data regarding the immune signaling and function of immune system in lumbricids are available, so, there is ample scope to undertake through investigation to unveil the immune pathways in these group.

Whole transcriptome analysis is a powerful tool for understanding different cellular, metabolic and developmental pathways that continue in an organism (or in any particular tissue or cell type that is sampled) under a specific condition like bacterial challenge (Khoo *et al*., 2012). Recently, NGS transcriptome sequencing technology has gradually replaced the gene chip technology and becomes a major tool for studying gene expression (Mu *et al*., 2014; Zhou *et al*., 2015; Dheilly *et al*., 2014). The high-throughput transcriptome sequencing is particularly important to provided insights into the immunogenetics of nonmodel

organisms, lacking reference genomes (Hanelt *et al*., 2008).

In earthworms, coelomocytes are involved in immune responses involving phagocytosis, encapsulation, coagulation etc. (Li *et al*., 2013; Gliński *et al*., 2000; Eliseikina *et al*., 2002; Dolmatova *et al*., 2004; Mutz *et al*., 2013). In this study, we focused on coelomocytes for the transcriptome analysis to have a deeper look into the immune mechanism of *E. fetida* against bacterial infection.

Zhang *et al.,* (2018) studied transcriptome of coelomocytes of Onchidium struma after bacterial challenge. Hanelt *et al.,* (Adema *et al*., 2010) conducted a similar transcriptome study of *Biomphalaria glabrata* snails challenged with *E. coli*. No studies have been reported on the coelomocytes transcriptome of *E. fetida* in response to bacterial infection. There is limited genetic information on *E. fetida* and further study is needed for understanding its disease resistance mechanism.

To gain deeper insight into the molecular immune mechanism of *E. fetida* against bacterial infection, *de novo* transcriptome sequencing of its coelomocytes after infection with *Bacillus thuringiensis* was performed on Illumina NextSeq 500 using 2 x 75 bp chemistry, and performed a global survey of immune-related genes, annotation of immune signaling pathways and determination of gene expression.

## 3.2. Materials and methods

### 3.2.1 Microbial challenge to *E. fetida*

Adult *E. fetida* worms were acclimatized to the laboratory conditions and were then distributed in experimental and control groups. The experimental animals were challenged by *Bacillus thuringiensis*

#### *3.2.1.1 Dose determination for microbial challenge experiment*

The $LD_{50}$ value for the number of live *B. thuringinsis* cell injected in adult *E. fetida* worm (Body wt. ~ 0.3g) was calculated using arithmetic method of Karber as adapted by Alui and Nwude (1982). Log phase culture (~1 OD) of *B. thuringiensis*, supposed to carry 5.0E+8 cfu/ml (Fisheret *et al., 2001)* was taken in microfuge tubes, centrifuged at 2000g for 5 minute at 4°C, washed twice with sterile MiliQ water and finally re-suspended to the concentrations $10^7$ cfu/ml, $10^8$ cfu/ml, $10^9$ cfu/ml and $10^{10}$ cfu/ml. Approximately 10 µl of this bacterial suspension was injected in each worm of the four

dose-groups (each group contained 10 animals) using insulin syringe (Dispo-van, India) with 31 gauze fine needle. The lethal effects of various doses of bacterial injection are shown in Table 3.1.

$1/10^{th}$ of $LD_{50}$ was taken as the dose for injecting each individual of the experimental groups of the earthworm.

*3.2.1.2 Injecting microbes into coelomic space*

$1/10^{th}$ of $LD_{50}$ dose of live *B. thuringiensis* is administered as the sub-lethal bacterial challenge per adult *E. fetida.* For this, 400 µl of Log phase culture (~1 OD) of *B. thuringiensis* was taken in the microfuge tube, centrifuged at 2000g for 5 minutes at 4°C, washed twice with sterile MiliQ water and finally resuspended in 1 ml. Approximately 10 µl of this bacterial suspension (or ~2 x $10^6$ live *B. thuringiensis*) was injected in each worm of the three treated groups using insulin syringe (Dispo-van, India) with 31 gauze fine needle. All *E. fetida* of other three Petri-plates were injected with 10 µl of sterile water.

The experiments were carried out in three (03) different sets. In each set the worms were kept in Eight (08) Petri-plates distributed equally in experimental and control groups (each plate with twenty-five adult earthworms) and then incubated at 25°C and fed on rehydrated (moisture, 80%) cow dung chips.

3.2.2. Comparative study of coelomocytes in challenged and control groups of *E. fetida*

Coelomic fluid was periodically withdrawn from the body of *E. fetida* of the experimental and control groups. Coelomocytes were isolated, identified, counted and studied for their phagocytic nature.

*3.2.2.1 Periodic withdrawal of coelomic fluid of E. fetida*

Coelomic fluid from the treated and control groups of earthworm was periodically (at 12h, 24h, 36h and 48h) pooled out in separate siliconized centrifuge tubes. 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.

#### *3.2.2.2 Separation of coelomocytes*

A portion from each group of the Isolated cells was separated on a Percoll gradient (Pharmacia, Sweden) created by mixing with 0.15 M NaCl to prepare six step concentration gradients, 5-15%, 25-35% and 45-55% from top to bottom of the glass centrifuge tube (Hamed *et al.,* 2005). Coelomocyte suspension was put on top of the concentration-gradient and centrifuged at 1000g for 10 minutes. Coelomocyte bands from different layers were collected carefully by Pasteur pipette. Separated coelomocytes were washed twice in PBS.

#### *3.2.2.3 Total count (TC) and Differential count (DC) of coelomocytes*

Coelomocytes collected from each group of earthworm were counted using an improved Neubauer haemocytometer using standard methods (Kirk *et al.,* 1975) and reported as the mean ± standard deviation (SD) per ml. Cell viability was determined by staining with 0'4% trypan blue (Sigma T0887). Viability was expressed as the percentage of live-cells at counting.

Differential counts were made for each group of 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 20 s. One hundred leukocytes were classified under oil immersion using 100 x objective lens.

#### *3.2.2.4 Micrometry of coelomocytes*

The diameter of the stained coelomocytes and their nuclei were measured using an ocular micrometer which was calibrated using a stage micrometer. Three readings were taken to determine the average diameter in each case.

*3.2.2.5 Study of phagocytic behaviour of coelomocytes*

A carbon particle suspension in PBS (pH, 7.4) solution was prepared by centrifuging Higgins India ink at 1,300g for ten minutes, removing the supernatant, and washing the pellet four times in PBS (Stein *et al.,* 1975). After the final wash, PBS amount was adjusted such that the carbon pellet was 25% (V/V). 200µl of live coelomocyte suspension in PBS (adjusted to $10^6$ cells/ml) was taken on a clear glass slide with 20µl of the carbon suspension and incubated at 28 °C in clean moist Petri plate chambers. Three such slides were prepared from each experimental and control groups of *E. fetida* for time-lapse study of phagocytosis percentage at 30min, 1h and 1h 30min. After incubation at different time intervals the slides were taken out, gently smeared, fixed and stained following procedure mentioned in section 3.2.2.3 and observed under the microscope.

*3.2.2.6 Scanning Electron Microscopy (SEM) of the* coelomocytes.

Coelomocytes from different experimental and control groups of *E. fetida* were spread on coverslips, semi-dried and were then fixed in the mixture of 1% paraformaldehyde and 2,5% glutaraldehyde, then postfixed in OsO4. The coverslips containing coelomocytes were completely dehydrated by passing through increasing grade of alcohol and drying in a critical point dryer. The cells were gold metal coated and observed in an scanning electron microscope (JS MIT 100, JEOL Ltd., Tokyo, Japan) at USIC, NBU.

3.2.3. Comparative study of coelomic fluid in challenged and control groups of *E. fetida*

Coelomic fluid and coelomocyte lysate (obtained by five cycles of freezing and thawing and treated with a proteinase inhibitor cocktail; Sigma P2714) from the treated and control groups of earthworms at different time intervals were analyzed for total protein estimation and SDS-PAGE.

*3.2.3.1 Estimation of total protein content*

Protein concentration was determined by the Bradford method (1976), using bovine serum albumin (BSA) to construct the standard curve. The standard curve was established as follows: y = 0.018x + 0.175 (R2=0.967) [x-axis represented the protein concentration and y-axis represented optical density (OD)].

*3.2.3.2 SDS-PAGE analysis*

The crude coelomic fluid and coelomocyte lysate after making bacteria free by filtering through 0.2 um membrane were separated in SDS-PAGE (chamber 0.1×10×10cm) using 10% polyacrylamide resolving gel and a 4% stacking gel in the presence of 1% SDS. The migration buffer consisted of 25mM Tris and 192mM glycine (pH 8.6). After migration, the gel was stained with Coomassie brilliant blue R-250, and decolored with destaining solution (10% methanol:20% ice-cool acetic acid:70% $dH_2O$).

3.2.4. Dynamics of indigenous *Bacillus* spp. in coelomic fluid of *E. fetida* under challenge by *B. thuringiensis*

Five earthworms (*E. fetida*), at different intervals (12h, 24h, 36h and 48h), were taken from each of the three experimental plates and washed several times with sterile distilled water. Thoroughly washed individual earthworm was held firmly in a sterile tissue paper and coelomic fluid (~10 µl) was collected in a fine- sharp sterile capillary tube (diameter ≤ 1.0 mm) by puncturing the coelomic cavity. The coelomic fluid contents of the capillary tubes were pooled in a PCR microfuge tube, centrifuged at 150g for 5min to pellet out the coelomocytes. The supernatent was serially diluted in sterile PBS. Multiple dilutions and plating on HBA plates were done to enumerate the number of *B. thuringiensis* content (CFUs) in the coelomic fluid and differentiation between various species of *Bacillus* along with viable count respectively.

3.2.5. Comparative gene expression studies via whole transcriptomics in coelomocytes of *'B. thuringiensis* challenged' and 'control' *E. fetida*

*3.2.5.1 Total RNA Isolation and cDNA library construction*

Total RNA was isolated from both the treated and control coelomocyte samples of *Eisenia fetida* using commercially available Quick-RNA Miniprep Plus kit (ZYMO Research) as per the manufacturer's instruction. The quality and quantity of the isolated RNA was checked on 1% denaturing RNA agarose gel and NanoDrop, respectively.

The RNA-Seq paired end sequencing libraries were prepared from the QC passed RNA samples using illumina TruSeq Stranded mRNA sample Prep kit. Briefly, mRNA was enriched from the total RNA using poly-T attached magnetic beads, followed by

enzymatic fragmentation, 1st strand cDNA conversion using SuperScript II and Act-D mix to facilitate RNA dependent synthesis. The 1st strand cDNA was then synthesized to second strand using second strand mix. The ds-cDNA was then purified using AMPure XP beads followed by A-tailing, adapter ligation and then enriched by limited no of PCR cycles. The PCR enriched libraries were analyzed in 4200 Tape Station system (Agilent Technologies) using High Sensitivity D1000 Screen Tape as per manufacturer instructions.

#### *3.2.5.2 Sequencing and assembly*

After obtaining the Qubit concentration for the libraries and the mean peak sizes from Agilent Tape Station profile, the PE illumina libraries were loaded onto NextSeq 500 for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions on NextSeq 500 using 2 x 75 bp chemistry. The kit reagents were used in binding of the samples to complementary adapter oligos on the paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

The filtered high-quality reads of the 2 samples were pooled together and assembled into transcripts using Trinity de novo assembler (V2.5) with a kmer of 25 and minimum contig length of 200. The assembled transcripts were then further clustered together using CD-HIT-EST-4.5.4 to remove the isoforms produced during assembly. This resulted in sequences that can no longer be extended. Such sequences are defined as Unigenes. Only those unigenes which were found to have >90% coverage at 3X read depth were considered for downstream analysis.

#### *3.2.5.3 Coding sequence (CDS) Prediction*

TransDecoder-v2.0 was used to predict coding sequences from the above-mentioned unigenes. TransDecoder identifies candidate coding regions within unigene sequences. TransDecoder identifies likely coding sequences based on the following criteria:

# A minimum length open reading frame (ORF) is found in a unigene sequence

# A log-likelihood score similar to what is computed by the GeneID software is > 0.

The above coding score is greatest when the ORF is scored in the 1st reading frame as compared to scores in the other 5 reading frames. If a candidate ORF is found fully encapsulated by the coordinates of another candidate ORF, the longer one is reported. However, a single unigene can report multiple ORFs (allowing for operons, chimeras, etc.).

### *3.2.5.4 Functional unigene annotation and classification*

Functional annotation of the genes was performed using DIAMOND program, which is a BLAST-compatible local aligner for mapping translated DNA query sequences against a protein reference database. DIAMOND (BLASTX alignment mode) finds the homologous sequences for the genes against NR (non-redundant protein database) from NCBI. Majority of the blast hits were found to be against *Capitella teleta* which also belongs to the phylum Annelida.

To identify sample wise CDS from above mentioned pooled set of [control(Cn) and treated (Ex)] CDS, reads from each of the samples were mapped on the final set of pooled CDS using bwa (-mem) toolkit. The read count (RC) values were calculated from the resulting mapping and those CDS having 90% coverage and 3X read depth were considered for downstream analysis for each of the samples. Sample-wise CDS statistics have been summarized in the following table.

### *3.2.5.5 Gene Ontology Analysis*

Gene ontology (GO) analyses of the CDS identified for each of the 2 samples were carried out using Blast2GO program. GO assignments were used to classify the functions of the predicted CDS. The GO mapping also provides the ontology of defined terms representing gene product properties which are grouped into three main domains: Biological Process (BP), Molecular Function (MF) and Cellular Component (CC).

GO mapping was carried out in order to retrieve GO terms for all the functionally annotated CDS. The GO mapping uses following criteria to retrieve GO terms for the functionally annotated CDS:

# BlastX result accession IDs are used to retrieve gene names or symbols, identified gene names or symbols are then searched in the species-specific entries of the gene- product tables of GO database.

# BlastX result accession IDs are used to retrieve UniProt IDs making use of PIR which includes PSD, UniProt, SwissProt, TrEMBL, RefSeq, GenPept and PDB databases.

# Accession IDs are searched directly in the dbxref table of GO database.

# BlastX result accession IDs are searched directly in the gene product table of GO database.

*3.2.5.6 Functional Annotation of KEGG Pathway*

To identify the potential involvement of the predicted CDS in biological pathways, 21,722 and 22,143 CDS of Cn and Ex samples respectively were mapped to reference canonical pathways in KEGG.

*3.2.5.7 Differential Gene Expression Analysis*

To perform differential expression analysis for Cn andEx samples, reads from the individual samples were mapped on the final set of CDS using bwa (-mem) toolkit. The read count (RC) values were calculated from the resulting mapping and only those CDS were considered for differential expression analysis which passed the 90% coverage at 3X depth criteria. Then by employing a negative binomial distribution model in DESeq package (version1.22.1 http://www.huber.embl.de/users/anders/DESeq/) differential gene expressions were calculated. Dispersion values were estimated with the following parameters: method = blind, sharingMode = fit-only and fittype = local. Finally Log2 fold change (FC) value was calculated from the aforementioned RC values.

$$FC = Log2\,(treated/control)$$

The CDS with FC value greater than zero were considered as up-regulated whereas less than zero as down-regulated. P-value threshold of 0.05 was used to filter statistically significant results. The CDS with FC value equal to zero are considered as "not regulated".

The combination used to identify the differentially expressed genes was 'Cn' for control and 'Ex' for experiment or treated groups respectively.

## 3.3. Results and discussion

3.3.1 Microbial challenge to *E. fetida*

*3.3.1.1 Dose determination for microbial challenge experiment*

| **Group** | **Dose (cfu/ml)** | **Dose Difference (cfu/ml)** | **No. o f Animal per group.** | **No. Dead** | **Mean Dead** | **Dose difference X mean Dead** |
|---|---|---|---|---|---|---|
| 1 | control | - | 10 | 0 | 0 | 0 |
| 2 | 10,000,000 | 10,000,000 | 10 | 0 | 0 | 0 |
| 3 | 100,000,000 | 90,000,000 | 10 | 4 | 2 | 180,000,000 |
| 4 | 1000,000,000 | 900,000,000 | 10 | 7 | 5.5 | 4950,000,000 |
| 5 | 10000,000,000 | 9000,000,000 | 10 | 10 | 8.5 | 76500,000,000 |
| $LD_{50}$ (*B.thuringiensis)* =10000,000,000-(81630,000,000/10)= 1837,000,000 cfu/ml | | | | | | Sum=81630,000,000 |

The $LD_{50}$ of live *B. thuringiensis,* injection was found to be 1.8 x $10^9$ cfu/ml or ~2 x $10^7$ live *B. thuringiensis* in 10 µl dose per adult *E. fetida*. 1/$10^{th}$ of $LD_{50}$ was taken as the dose for injecting each individual of the experimental groups of the earthworm.

3.3.2. Coelomocytes in challenged and control groups of *E. fetida*

*3.3.2. 1* W*ithdrawal volume of coelomic fluid of E. fetida*

Coelomic fluid was periodically withdrawn from the body of *E. fetida* of the experimental and control groups. 200 µl of coelomic fluid (from~20 earthworms) was extracted from each of the experimental and control groups of the different sets.

*3.3.2.2 Separation of coelomocytes*

Four semi-separated cell bands were observed at percoll concentrations of 10, 25, 40 and 50% contining amoebocytes, large granulocytes, eleocytes and small granulocytes as the

main components correspondingly. This observation is comparable to the similar report by Hamed *et al.*, (2005), where the authors found bands of acidophils, basophils, chloragocytes and nutrophils at more or less similar percoll gradients.

### *3.3.2. 3 Total count (TC) and Differential count (DC) of coelomocytes*

Light microscopic studies of coelomocytes in the untreated adult (Bd wt. 0.3±0.05g ) *Eisenia fetida* coelom contains 1.2 ± 0.7 x $10^7$ coelomocytes (TC) /ml, with the following morphologically distinct groups - 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%).

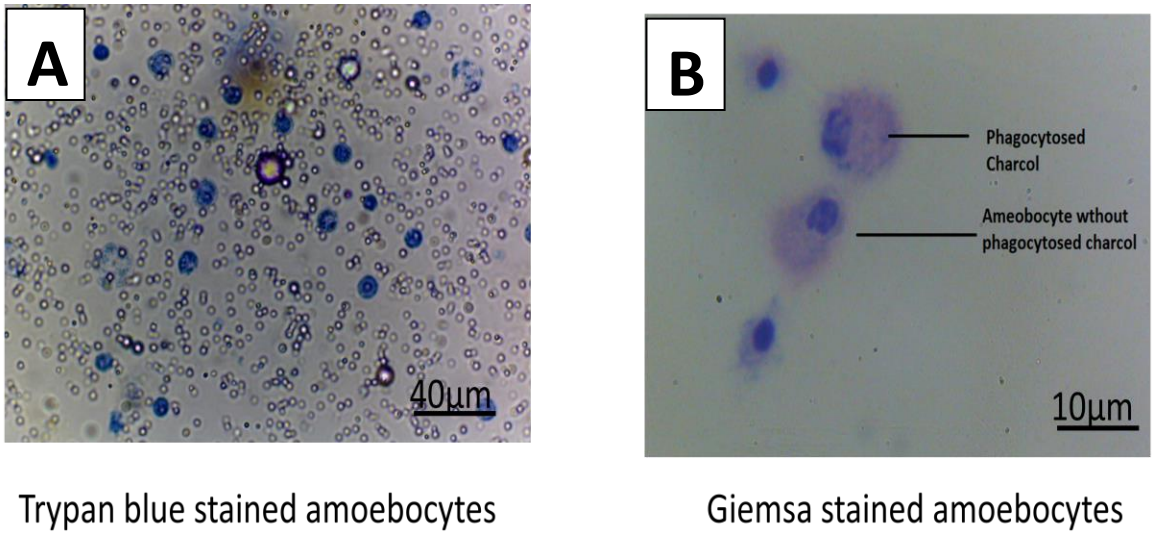


Fig. 3.2: Microscopic observations during Total count (A) and Differential count (B) of coelomocytes of *E. fetida*

Amoebocytes with phagocytotic activity can be identified from the size and shape of the nucleus. The cells tend to be smaller, as little as 8 µm, but occasionally may be as large as 15 µm with fewer granules, eccentric nucleus and large pseudopodia. Granulocytes have cytoplasm completely filled with small granules (basophilic) and have the diameter of 15-22 µm. The eleocytes are large cells with 30-60 µm diameter (with fluorescent yellow pigmentation). The fourth group of cells, small granulocytes are generally, <6 µm in diameter and are densely packed with granules (acidophilic).

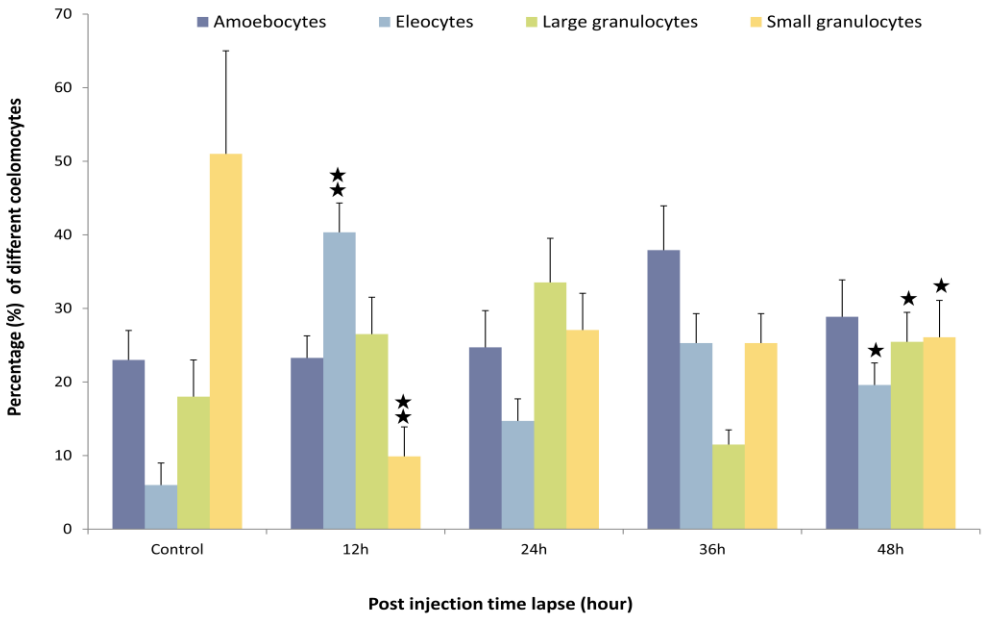


Fig. 3.2: Differential count of coelomocytes in *B. thuringiensis* challenged *E. fetida* at different time intervals

### *3.3.2. 4 Microscopic attributes of the coelomocytes*

Micrometric reports of the different coelomocytes are enlisted below-

Table3-2: Cell and nuclear size of different coelomocytes

| Cell Type | Size of the Cell | Size of Nucleus |
|---|---|---|
| Amoebocytes | 8-15 µm | 3-5 µm |
| Large granulocytes | 15-22 µm | 4-6 µm |
| Eleocytes | 30-60 µm | 5-8 µm |
| Small granulocytes | <6 µm | <4 µm |

### *3.3.2.5 Phagocytic behavior of coelomocytes*

Both amoebocytes and granulocytes were frequently found with inclusion bodies including nano-carbon particles. In the treated groups, these cells were often found to interact with bacilli especially at 24 and 36 h post inoculation groups (Fig. 3.3). These

coelomocytes were frequently observed to accumulate towards the activated carbon particles or formalin fixed *B. thuringiensis* cells (Fig. 3.4), which indicate their increased efficiency of chemotaxis and phagocytosis.

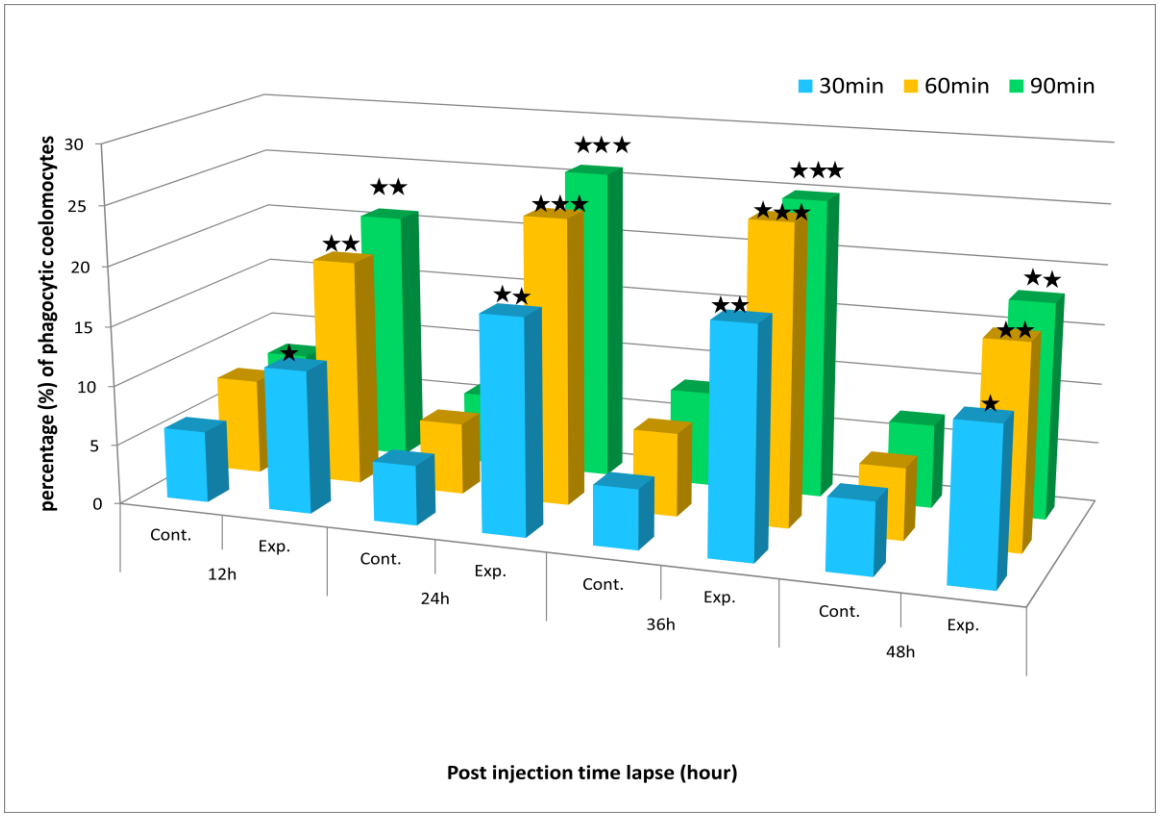


Fig. 3.3: Bar graph showing percentage of coelomocytes with phagocytic inclusion bodies in the experimental and control *E. fetida* at different time intervals after challenge.

### *3.3.2.6 Scanning Electron Microscopy (SEM) of the* coelomocytes

Outer morphology of the four types of coelomocytes were revealed by SEM. Fig 3.5 depicts the SEM plates of glutaraldehyde fixed coelomocytes from *E. fetida*. Amoebocytes (Fig. 3.5-A) are seen to throw extensive pseudopods in all directions. Large granulocte (Fig. 3.5-B) can be easily identified with its numerous small pseudopodia. Eleocyte (Fig.3.5-C) are large spherical cells. Small granulocyte (Fig. 3.5-D) are the smallest spherical cells. Different types of coelomocytes present in coelomic fluid of *E. fetida* interact with bacteria and possibly keep the bacterial population under control by regularly engulfing them.

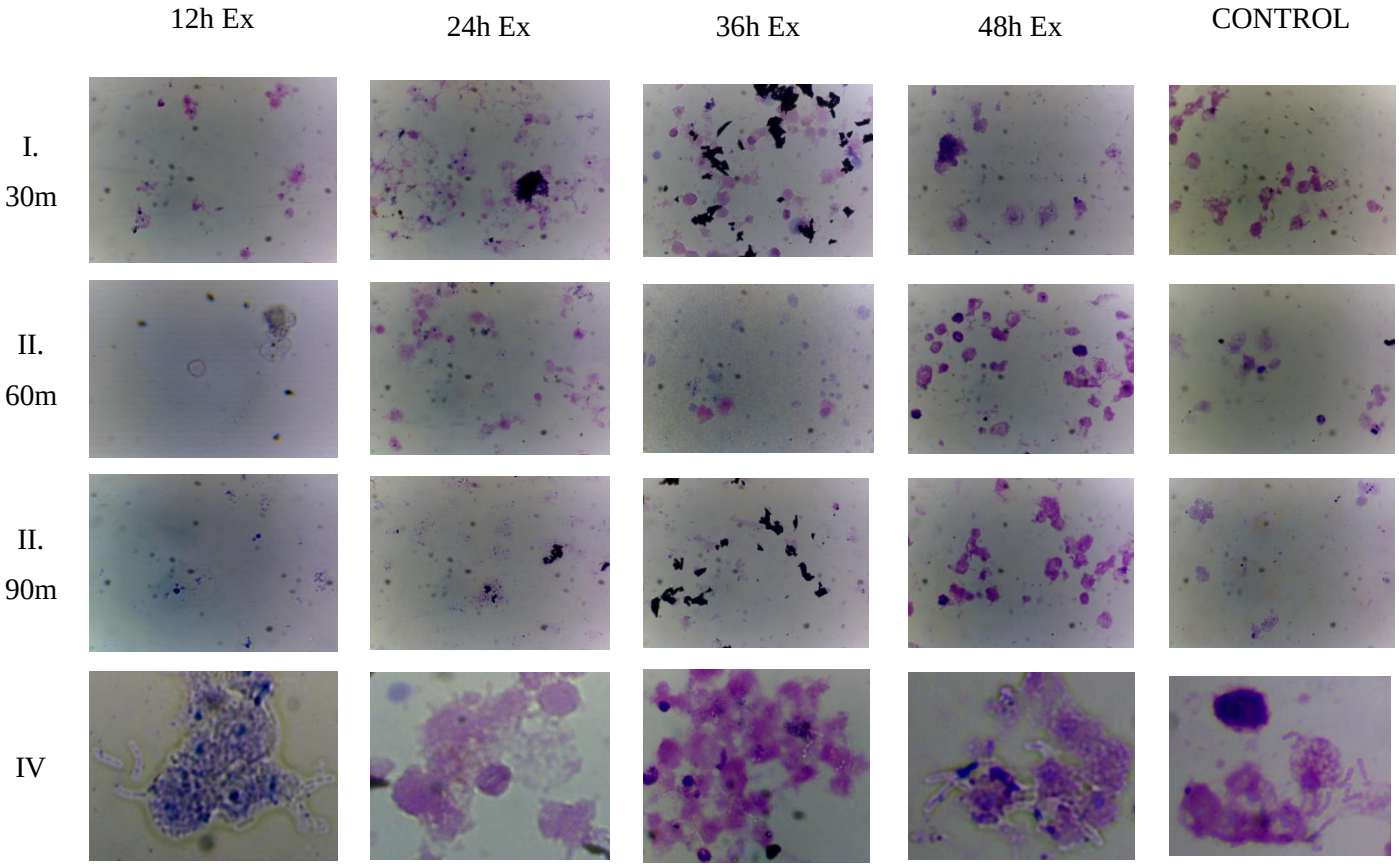


Fig.3.4: Photographic plates of giemsa stained coelomocytes from *B. thuringiensis* challenged *E. fetida* at different time intervals with activated carbon (Row I, II and III; 40x objective) or formalin fixed *B. thuringiensis* cells (Row IV; 100x objective).

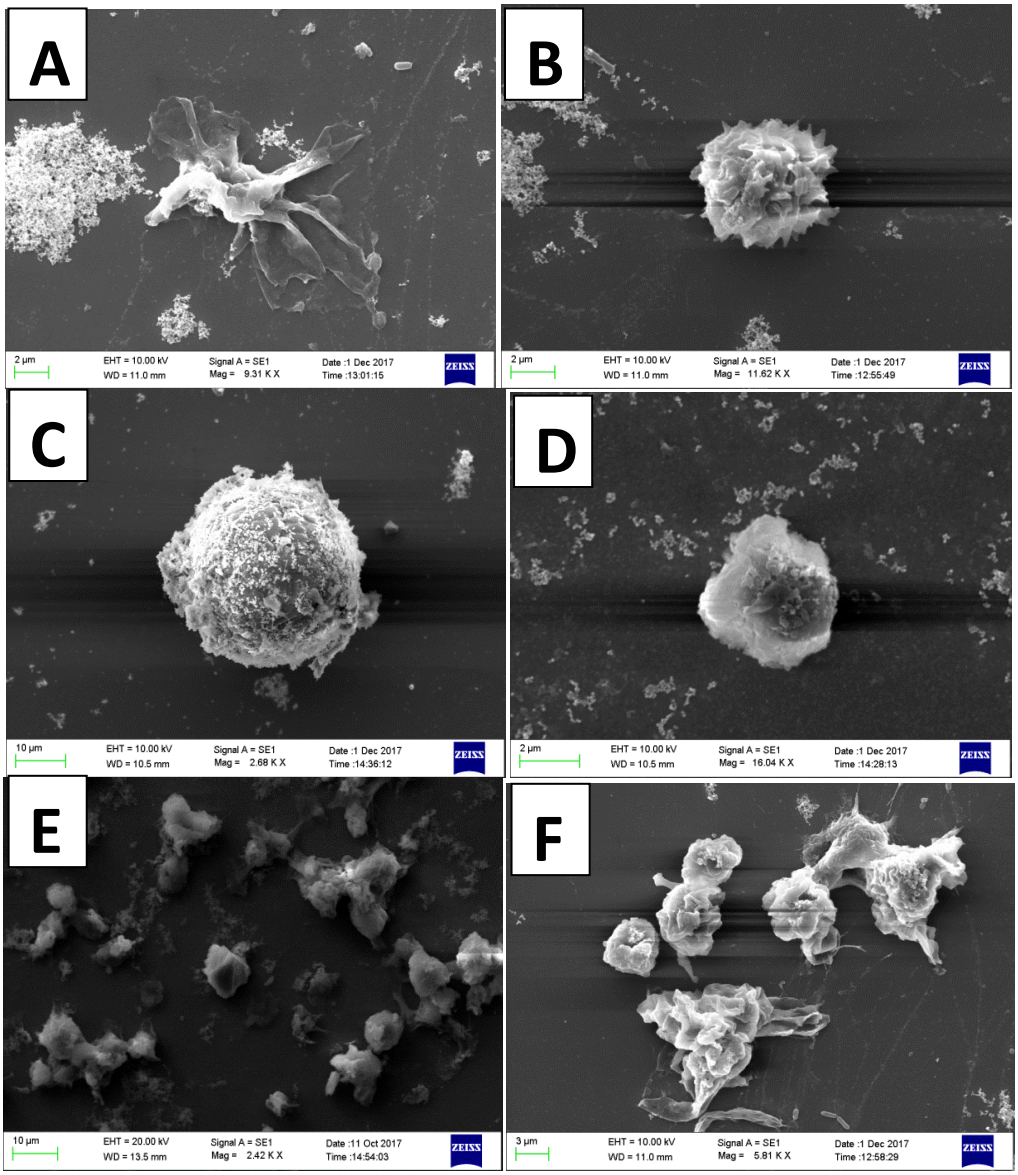


Fig.3.5**:** SEM plates of glutaraldehyde fixed coelomocytes from *E. fetida*. A- Amebocyte, B- Large granulocte, C- Eleocyte, D- Small granulocyte, E- Different types of coelomocytes present in coelomic fluid of *E. fetida* and F- Coelomocytes interacting with bacteria.

3.3.3. Protein analysis of coelomic fluid in challenged and control groups of *E. fetida*

Coelomic fluid protein content was analysed in the following two steps-

*3.3.3.1 Total protein content*

Total protein content estimated in the untreated animals was 1.2±0.2 mg/ml in coelomic fluid and 6.9±0.6 mg/ml in the coelomocytes. Time lapse analysis of the total protein content is observed to increase in both coelomic fluid and coelomocytes after inoculation

of live *Bacillus thuringiensis* after 24 and 36h lapse to upto 10.2±0.4 mg/ml in coelomic fluid and 12.5±0.8 mg/ml in the coelomocytes (Fig. 3.6). This trend is observed to decrease at 48h onwards.

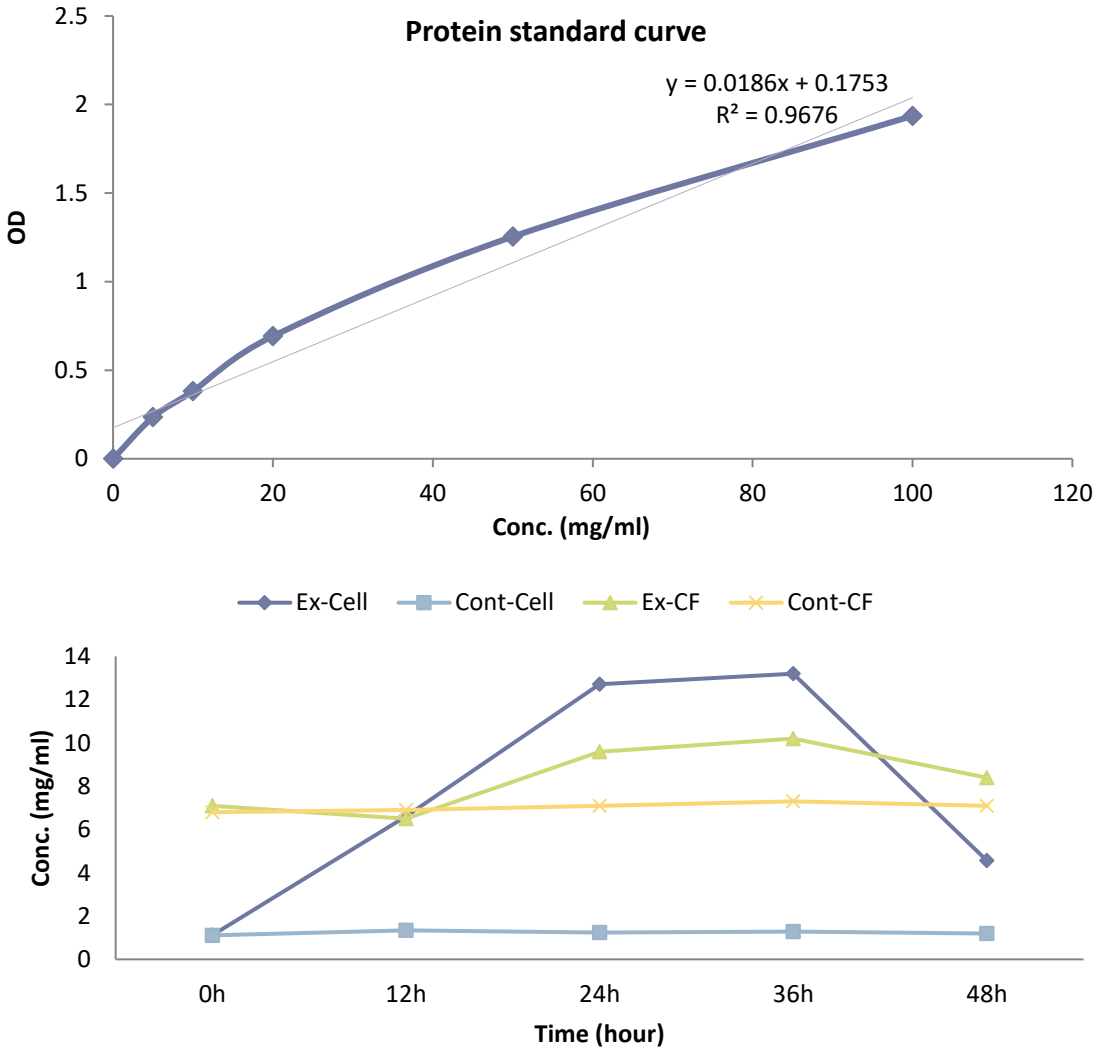


Fig.3.6: Time lapse analysis of total protein contents in coelomic fluid and in the coelomocytes of *B. thuringiensis* challenged and control *E. fetida*

### *3.3.3.2 SDS-PAGE analysis*

Total protein content estimated in the coelomocyte lysate was approximately 5-6 times higher.

Thin bands of ~ 38 kDA and ~ 42kDA were found in the coelomic fluid lanes, which may be corresponding to the ECFP and CCF1 proteins described earlier. Some other smaller protein bands of unknown function were also found to be expressed. The cell lysate lanes could not be separated well may be because of different types of proteins expressed in the cells.

3.3.4. Dynamics of indigenous *Bacillus* species in coelomic fluid of *E. fetida* under *B. thuringiensis* challenge

Study of coelomic fluid at different intervals (12h, 24h, 36h and 48h), from each of the experimental and control plates, clearly reveals that the population of introduced *B. thuringiensis* started declining from the day 2 onwards and at day6 there was significant reduction in pathogen count (Fig. 3.7).

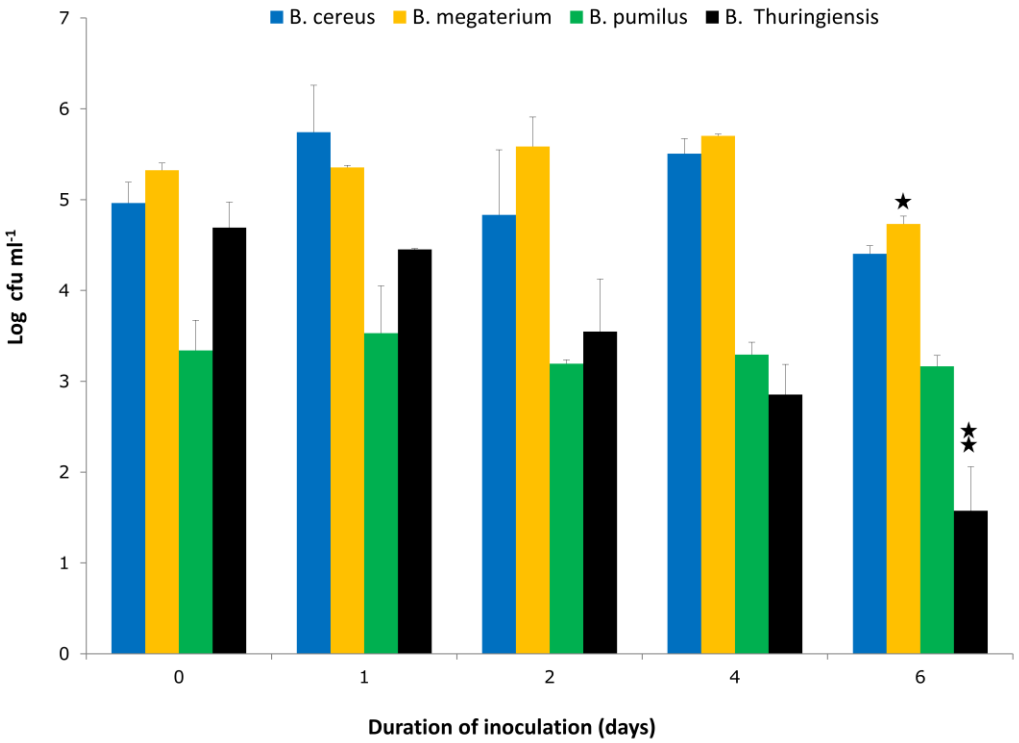


Fig.3.7: Dynamics of indigenous *Bacillus* species in coelomic fluid of *E. fetida* under challenge by *B. thuringiensis*

3.3.5. Comparative gene expression studies via whole transcriptomics in coelomocytes of *'B. thuringiensis* challenged' and 'control' *E. fetida*

*3.3.5.1 Total RNA Isolation and cDNA library construction*

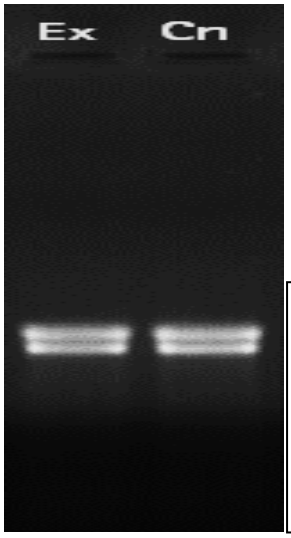


Fig.3.9: Isolated RNA from the Experimental (Ex) and Control (Cn) samples, run on on 1% denatured Agarose gel shows mRNA smears below the 23S and 16S bands.

The total RNA isolated from the two samples (Experimental and control) were Qualified using NanoDrop and Qubit. The results (Table- 3.1) indicated good quality RNA isolation sufficient for the transcriptomic pipeline.

Table 3.1: Quantification of receivedRNAsamples using NanoDrop/Qubit

| **Sr. No.** | **Sample ID** | **NanoDrop Readings** (ng/µl) | **Qubit Readings** (ng/µl) | **NanoDrop** OD $A_{260/280}$ | **NanoDrop** OD $A_{260/230}$ |
|---|---|---|---|---|---|
| *1* | *Ex* | *247.8* | *124.0* | *2.13* | *1.64* |
| *2* | *Cn* | *266.5* | *144.0* | *2.13* | *2.27* |

*3.3.5.2 Sequencing and assembly*

The paired-end (PE) libraries that were prepared from total RNA had mean fragment size distributions of 442bp and 457bp for the samples Ex and Cn, respectively. After the transcriptome was conducted, 34.67 Mb and 36.07 Mb high quality reads (QV>20) were obtained from coelomocyte samples of unchallenged and *B. thuringiensis* challenged *E. fetida*, respectively (Table 1). Total clean data generated were 5.5 Gb and 5.7 Gb from two groups, respectively (Table 1). The clean reads from the two libraries were assembled into 83,192 unigenes. The total length of all unigenes was 88,326,196 bp and average length was 1062 bp.

$N_{50}$ was 1,799 bp.

The length distribution of assembled unigenes obtained from coelomocytes in unchallenged and *B. thuringiensis* challenged *E. fetida* revealed that most of unigenes ranged from 200 bp to 2000 bp, and approximately 6.5% of unigenes were over 3000 bp in length (Figs. 1–3). The sequence data of unigenes were deposited in the Sequence Read Archives (SRA) at NCBI under accession number SRP6138677.

### *3.3.5.3 Coding sequence (CDS) Prediction*

TransDecoder predicted 28,770 CDS. Functional annotation of the CDS using DIAMOND (BlastX mode) against the NCBI 'Nr" database cloud classify 18,602 CDS most of which annotated against Capitella telata, a polychaete worm. 10,168 CDS were without Blast hit.

### *3.3.5.4 Functional unigenes annotation and classification*

Majority of the blast hits were found to be against *Capitella teleta* which also belongs to the phylum Annelida.

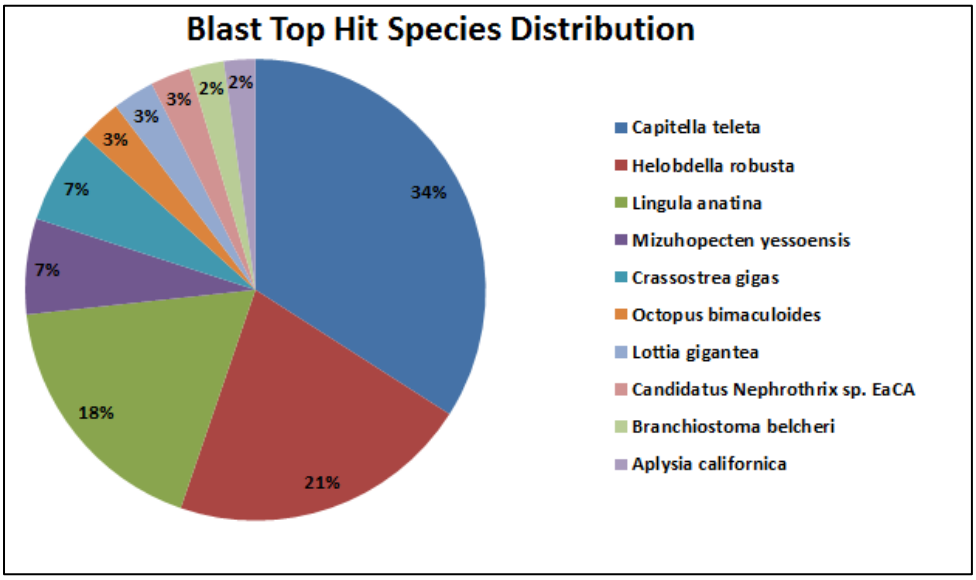


Figure 3.9: Top blast hit species distribution of pooled CDS

### *3.3.5.5 Gene Ontology Analysis*

Gene Ontology (GO) analysis using B2G framework, categorized 4,138 and 4,206 CDS from the control and treated samples respectively to three main domains: Biological process (BP) Molecular function (MF) and Cellular

component(CC) (Table 2).

### *3.3.5.6 Functional Annotation of KEGG Pathway*

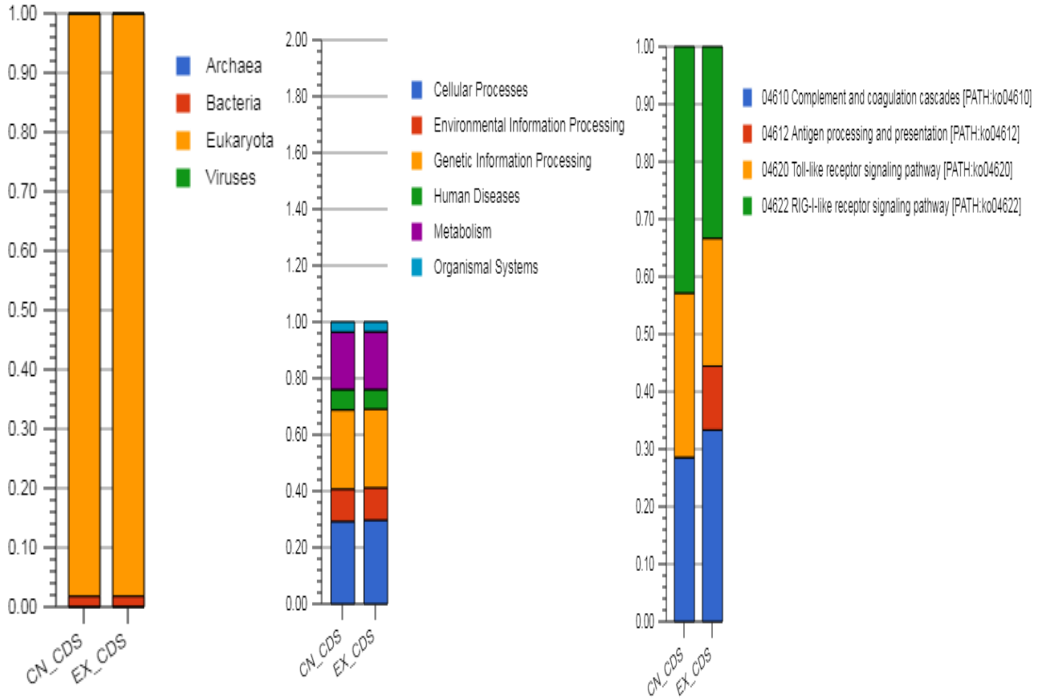


Fig.3.10: KO analysis of the Control (CN_CDS) and experimental (EX_CDS) CDS in MG-RAST

KEGG pathway analysis was carried out for all the individual set of CDS. A total of 5,343 and 5,425 CDS of the control and treated samples respectively could be categorized into 29 different pathways, the majority of which were signal transduction, transport and catabolism, translation, endocrine and immune system etc. The output of KEGG analysis includes KEGG Orthology (KO) assignments and corresponding Enzyme Commission (EC) numbers and metabolic pathways of predicted CDS using KEGG automated annotation server, KAAS. The results are briefed in the table S2. KO analysis of the Control (CN_CDS) and experimental (EX_CDS) CDS in the MG-RAST website also produces similar results (Fig: 3.10)

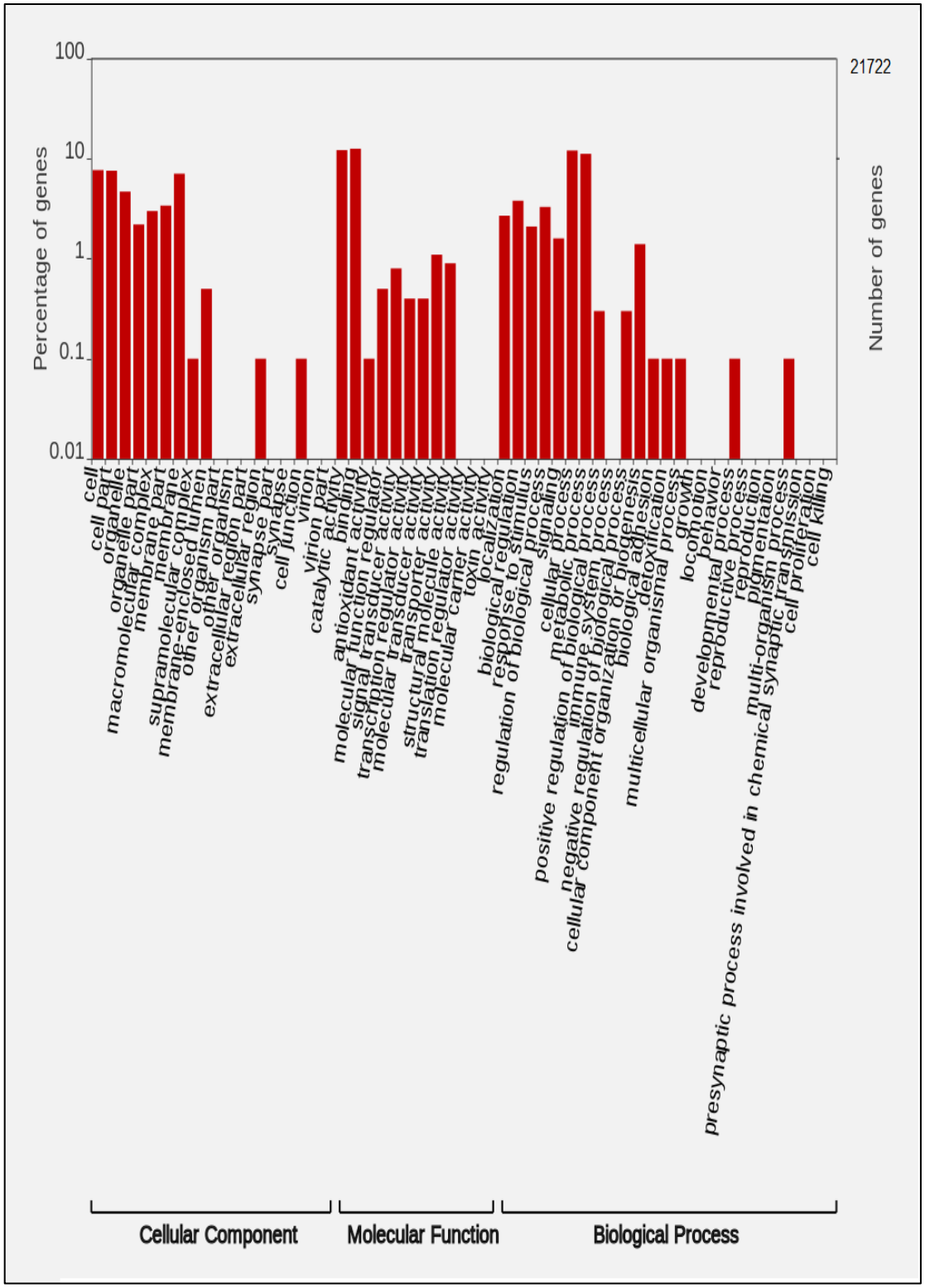


Fig. 3.11: WEGO Plot for Cn Sample

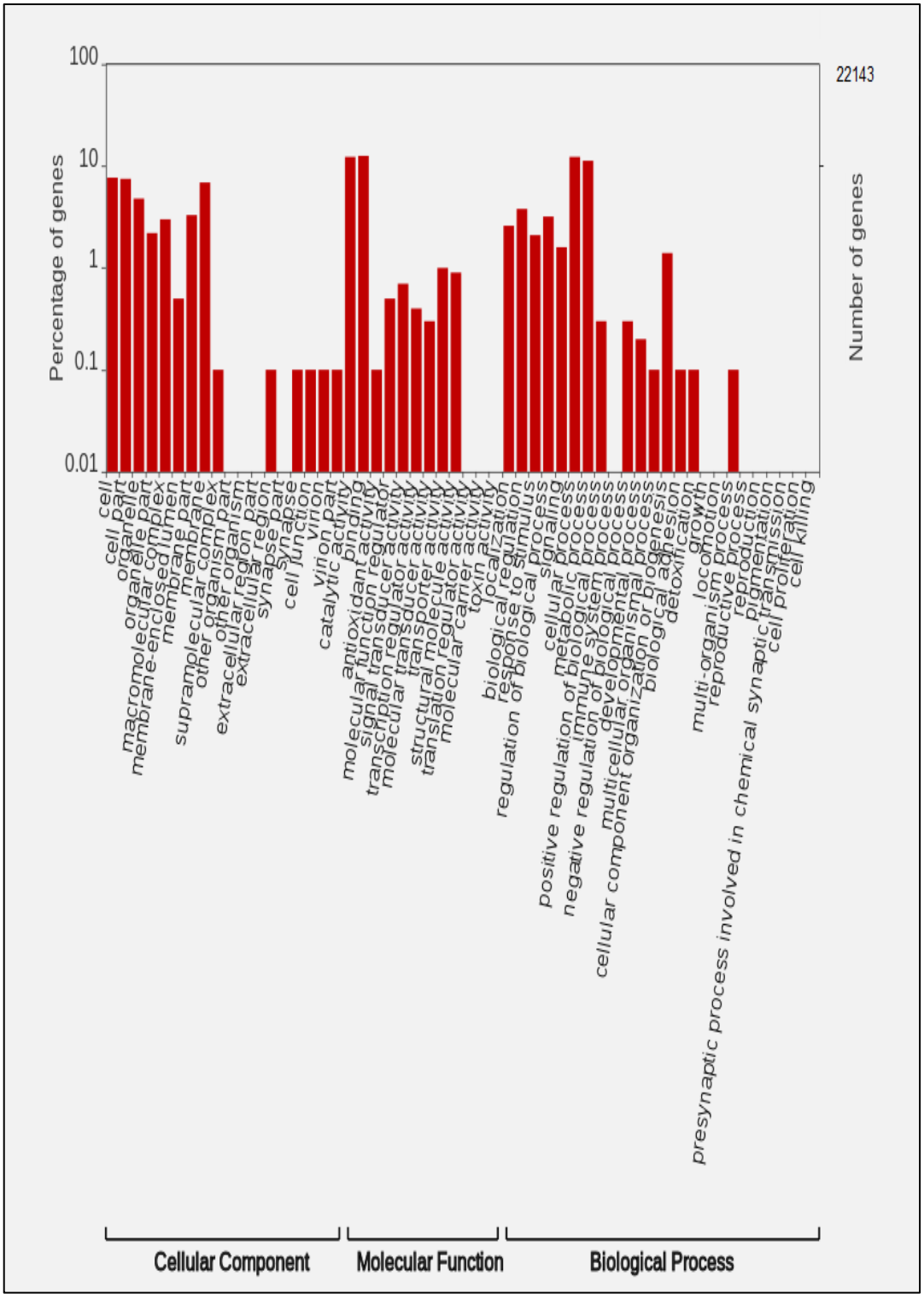


Fig. 3.12: WEGO Plot for Ex Sample

*3.3.5.7 Differential Gene Expression Analysis*

Differential gene expression analysis revealed that the expression of 646 genes was significantly up-regulated and 512 genes were significantly down-regulated in the treated group. 19,616 genes commonly expressed in both the samples. 98 unigenes were up-regulated more than two folds, and 15 unigenes were down regulated more than 2 folds, respectively (Table S1). Volcano plot (Fig 3.14) and Scatterplot (Fig 3.15) of differentially expressed genes have been created. Green dots represent the down-regulated (significant) and red dots represent the up-regulated (significant) genes. Fig 3.13 depicts the heat map of the top 50 differentially expressed genes (significant). COG analysis of DEGs showed that 621 DEGs (11.4%) were related with the immune system and diseases.

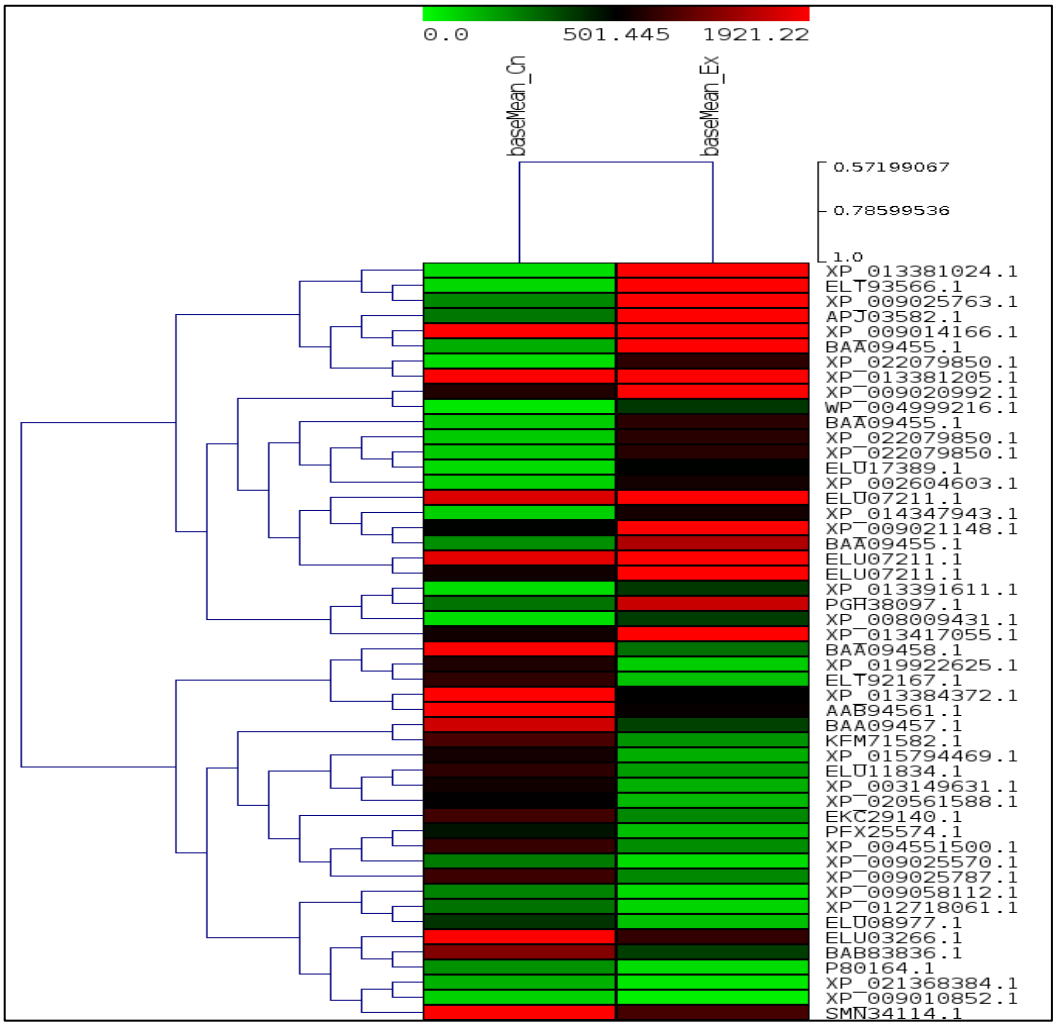


Fig 3.13: Heat map **of** the top 50 differentially expressed genes (significant); baseMean_Cn represents the normalized expression values for Cn sample **&** baseMean_Ex represents the normalized expression values for Ex sample.

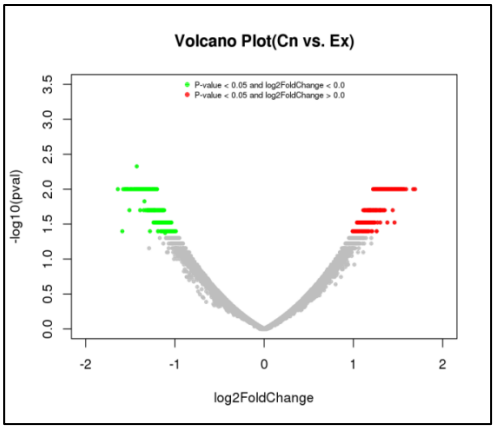


Fig. 3.14: Scatterplot of differentially expressed genes; green dots represent the downregulated (significant) and red dots represent the upregulated (significant) genes; baseMean_Cn represents the normalized expression values for Cn sample and baseMean_Ex represents the normalized expression values for Ex sample.

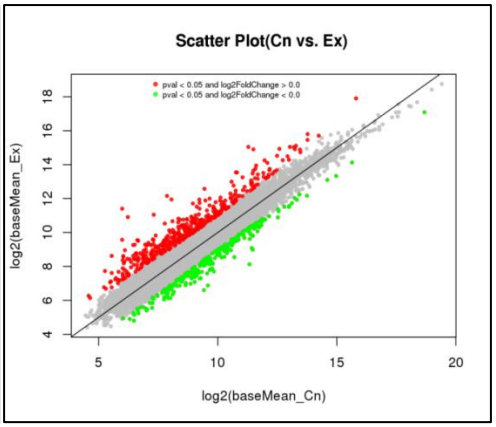


Fig.3.15: Volcano plot of differentially expressed genes; green dots represent the downregulated (significant) and red dots represent the upregulated (significant) genes.

Identification of immune-related genes and pathways

The immune related pathways significantly up-regulated were Toll-like receptor signaling pathway (PATH:ko04620), NOD-like receptor signaling pathway (PATH:ko04621), RIG-I-like receptor signaling pathway (PATH:ko04622), FcyR-mediated phagocytosis (PATH:ko04666), leukocyte trans-endothelial migration (PATH:ko04670).

*Toll-like receptor (TLR) signaling pathway*

Fifteen genes involved in the TLR pathway were found to be regulated in *B. thuringiensis* infected *E. fetida* compared to non-infected control. The DEGs, Lipopolysaccharide binding protein (LBP), TLR2, TLR13, FADD and TRAF3 were up-regulated (Fig. 3.16). The LBP expression was found to be proliferated by 2 fold suggesting an accurate and rapid response of *E. fetida* to bacterial invasion (Table 3). The TLR4 located adjacently down-stream of LBP was not found in *E. fetida,* compared to other animals, suggesting an alternative LBP sub-pathway.

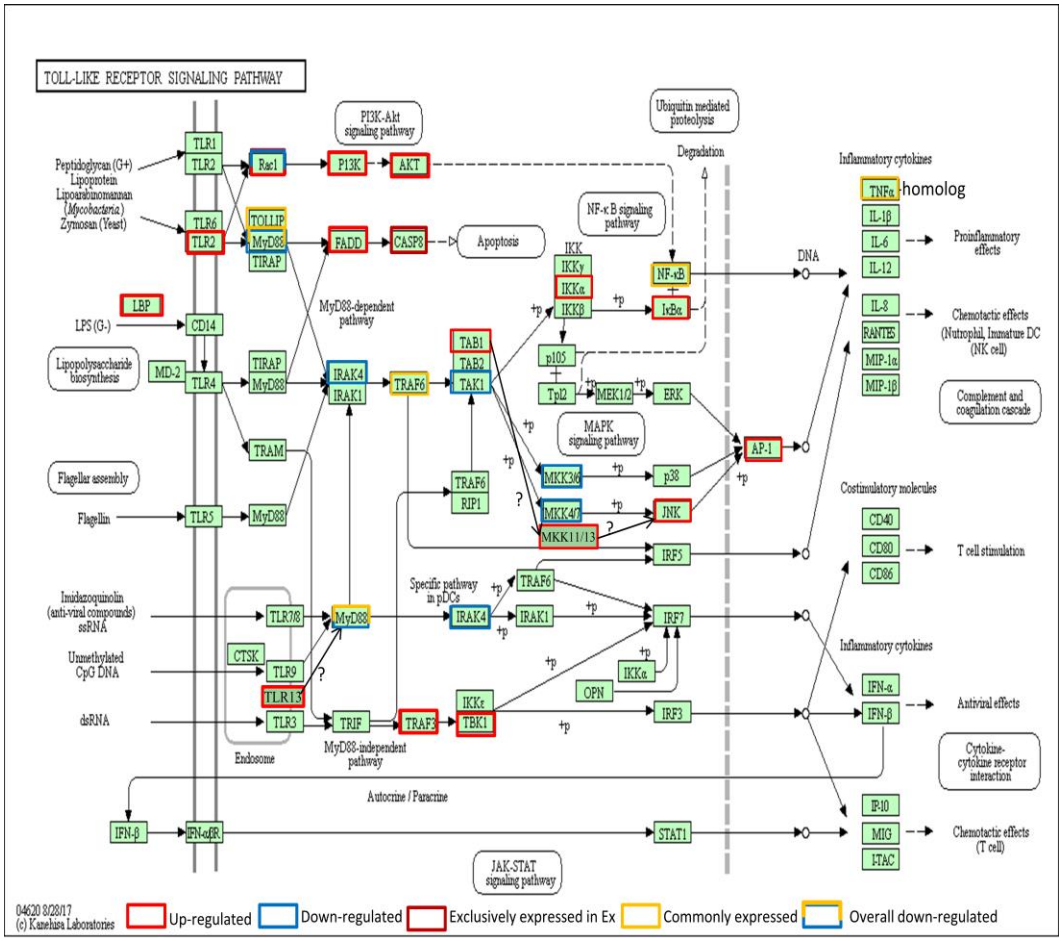


Fig. 3.16: Mediators of Toll-like receptor (TLR) signaling pathway that are differentially modulated in the experimental groups compared to the control (The KEGG pathway was adopted from Kanehisa Laboratories, Japan).

A specific study on TLR immunity pathway in a marine mollusk (*Crassostrea gigas*) showed that time-dependent patterned expression regulation of MyD88 along with TLR4 was observed while incubation with LPS. The expression of TLR4 in *C. gigas* was initially depressed and followed by a gradual proliferation. MyD88 is the first molecule acti- vated in the TLR signaling pathway (Muzio *et al.*, 1997; van dar Sar *et al.*, 2006), and has been found in vertebrates (Bonnert *et al.*, 1997; Wheaton *et al.*, 2007; Zhang *et al.*, 2012[a]) and invertebrates (Zhang *et al.*, 2012[b]; Ren *et al.*, 2014; Chu *et al.*, 2013) Adema *et al.* (Morozova *et al.*, 2009) found that snails challenged with *B. thuringiensis* showed more than 2 fold upregulation on JNK-interacting protein 3 which involved in signal transduction after TLR 4 activation. In our study, the MyD88 gene's expression was common

in both *B. thuringiensis* challenged and control animal with an overall down regulation, implying a similar regulation pattern (Fig. 3.16). The TLR2 and TLR13 expressions are up-regulated. Although the intermediate proteins of the TLR2 pathway are observed to be up-regulated leading to caspase 8 mediated apoptosis which is only seen in experimental worms along with activation of AP-1 complex leading to the expression of pro-inflammatory cytokines.

*RIG-I-like receptor signaling pathway*

RIG-I is one of the three RLR signaling pathway reported to be mainly involved in intracellular virus sensing. Non-self RNA appearing in a cell as a result of intracellular

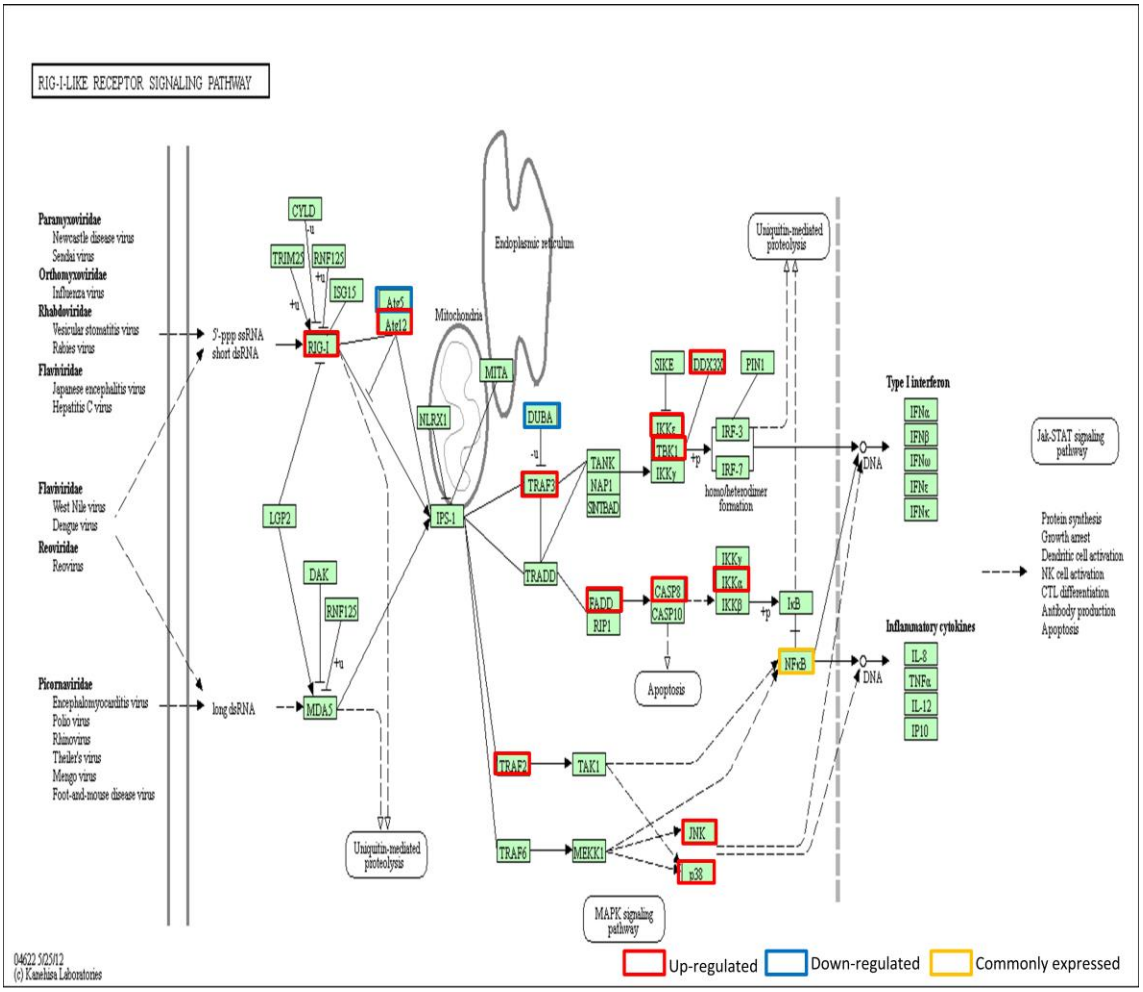


Fig. 3.17: Mediators of RIG-I-like receptor signaling pathway that are differentially modulated in the experimental groups compared to the control (The KEGG pathway was adopted from Kanehisa Laboratories, Japan).

viral replication is recognized by a family of cytosolic RNA helicases termed RIG-I-like receptors (RLRs). Fourteen genes involved in the RIG-I pathway were found to be regulated in *B. thuringiensis* infected *E. fetida* compared to non-infected control. The DEGs, RIG-I, Ate12, TRAF2, TRAF3, FADD, CASP8, IKK, p38, JNK, TBK1 and DDX3X were up-regulated (Fig. 3.17). Inhibitors in the pathway such as Ate5 and DUBA were down regulated in the *B. thuringiensis* injected worms compared to the control ones. The IPS-1 located adjacently down-stream of RIG-I was not found in *E. fetida*, compared to other animals, suggesting an alternative RIG-I sub-pathway. Upon recognition of viral nucleic acids, RLRs recruit specific intracellular adaptor proteins to initiate signaling pathways that lead to the synthesis of type I interferon and other inflammatory cytokines, which are important for eliminating viruses.

*C-type lectin pathway*

C-type lectin receptors (CLRs) are a large super-family of proteins characterized by the presence of one or more C-type lectin-like domains (CTLDs). CLRs function as pattern-recognition receptors (PRRs) for pathogen-derived ligands in dendritic cells, macrophages, neutrophils, etc., Ten genes involved in the C-type lectin pathway were found to be regulated in *B. thuringiensis* infected *E. fetida* compared to non-infected control. The DEGs, p38, JNK, AP-1, CYLD and CALM were up-regulated (Fig. 3.18). Two novel lectin receptors Galectin and Rhamnose binding lectins were found to highly up-regulated in the experimental worms after bacterial challenge. Many other factors involved in this pathway viz. Syk, Pak-1, Bcl-3, Bcl-10, PLC$_{\gamma}$2 and NEMO were highly expressed in both experimental and control group of animals. Upon ligand binding, CLRs stimulate intracellular signaling cascades that induce the production of inflammatory cytokines and chemokines, consequently triggering innate and adaptive immunity to pathogens. *B. thuringiensis* is a potential pathogen for many invertebrate groups (WHO, 2003). In this study, the differently expressed unigenes (DEGs) were analyzed to enrich our knowledge and the DEGs information obtained will help to understand molecular immune mechanism of

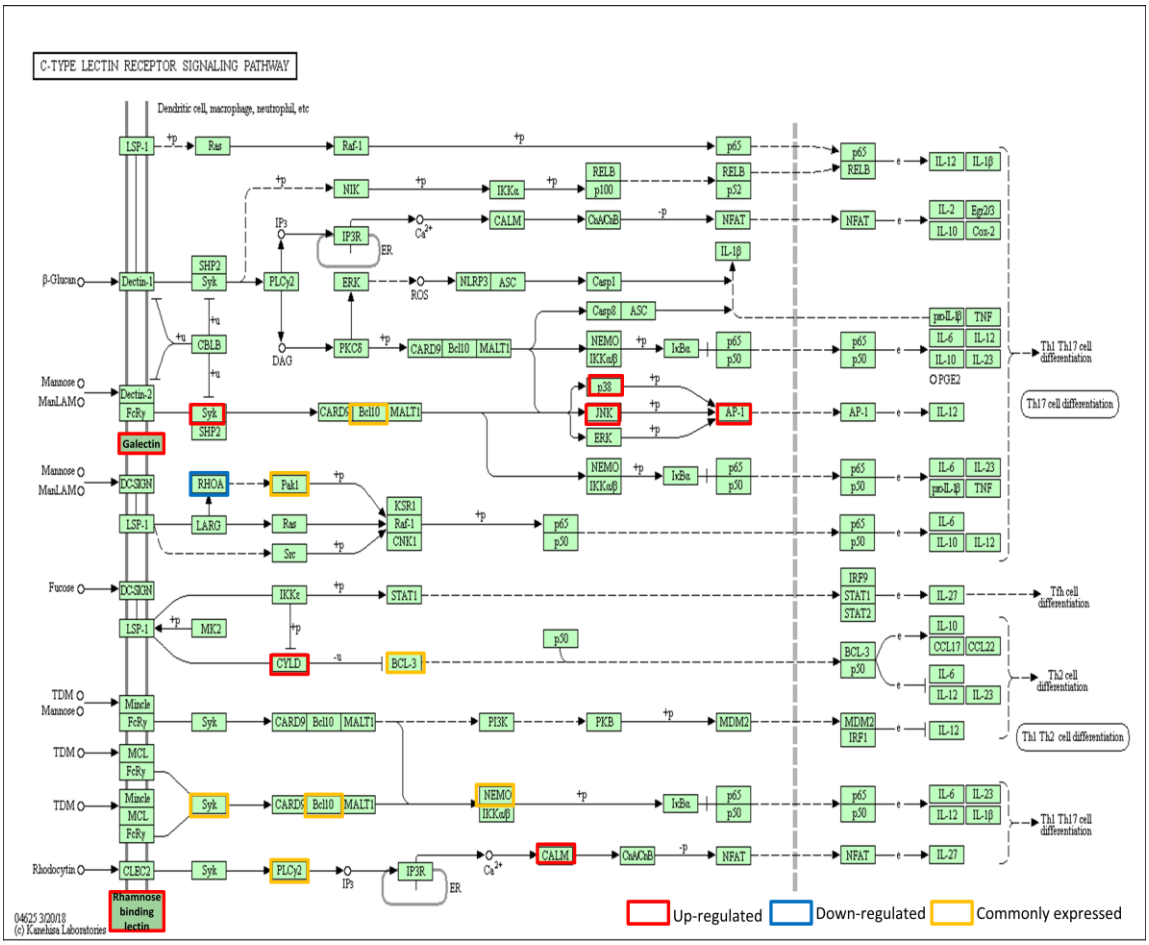


Fig. 3.18: Mediators of RIG-I-like receptor signaling pathway that are differentially modulated in the experimental groups compared to the control (The KEGG pathway was adopted from Kanehisa Laboratories, Japan).

*E. fetida* in response to bacterial infection. *E. fetida* is a cosmopolitan representative of the Phylum Annelida which is the first triploblastic eucoelomate group, thus to be a potentially valuable representative species for providing information on evolution of immune system in the eucoelomates (Qlu, 1991; Zhang *et al.*, 2017; Sun *et al.*, 2016).

However, studies on the generation, development, differentiation and immune system of *E. fetida* are limited due to the lack of transcriptomic and genomic information. The transcriptomic and genomic information obtained in the present study will be helpful for future studies of *E. fetida*.