CHAPTER 5

DISCUSSION

Discussion

Microorganisms have evolved to adapt the changing environmental conditions and have developed various molecular and biochemical processes to overcome adverse environmental conditions. Heavy metal contamination of the environment due to increased industrial activity is considered as a threat not only to the flora and fauna of the ecosystem but also for the human beings. The release of heavy metals into the environment causes an environmental pollution as they are non-biodegradable and accumulates in the living organisms [223]. Microbes, particularly the bacteria representing the largest domain of prokaryotes have adapted to tolerate the metal stress and can grow easily in their presence. The industrial areas with major mining activities are rich source of heavy metal resistant microbes [57]. These microbes are of practical interest to microbial ecologist not only to understand the emerging concept of metal resistance but also to know the evolutionary aspect and significance of metal resistance in microbes. In the present study the soil samples were collected from the nearby areas adjoining the mining sites of East Singbhum district of Jharkhand (India) and screened for isolation of metal resistant bacteria by serial dilution. Forty two (42) bacterial isolates representing different bacterial colonies were found resistant to different concentrations (0.5, 1 & 2 mM) of Cd, Cr and Ni (Table 1) and among them eight (8) strains were resistant to all the metals used in the study. All the identified bacterial strains were further subjected for morphological, biochemical and phylogenetic analysis.

The morphological characterization and gram staining are the first step followed by biochemical and molecular identification. The identified metal resistant bacterial strains were tested for morphology (shapes: rod/cocci), gram staining and biochemical tests such as cellulose, amylase, hydrolysis, triple sugar etc. (Table 2). Based on the morphological and biochemical analysis five (05) strains (highlighted in Table 2) with distinct biochemical characteristics were selected for Biolog analysis.

Biolog microbial identification system is based on the principle of numerical taxonomy and uses easy determinable color reactions indicating metabolic activities such as the oxidation of wide range of carbon compounds [224, 225]. With this system five (05) selected strains were identified as *Bacillus* (two strains), *Pseudomonas, Lactobacillus* and *Staphylococcus* (Figure 1 a-e). However, for the species level identification genotypic studies using 16S rDNA sequencing is needed for the confirmation of the selected *Pseudomonas* and *Bacillus* strains.

Based on Biolog analysis and ability of the bacterial strains to grow in presence of all the metals (Cd, Cr & Ni), 16S rDNA sequence of two strains *Pseudomonas* sp. (JHCO2018) and *Bacillus* sp. (JHNI40215) were analyzed. The two strains were identified as *Pseudomonas aeruginosa* RV3 (accession no: JX13019) and *Bacillus cereus* NI40215 (accession no: awaited) showed 99% identity of 16S rDNA sequences similarity with the corresponding sequences of *Pseudomonas* and *Bacillus* species respectively (Figure 3 & 4).

The optimization of the growth conditions showed that the optimal temperature and pH for both the strains *Pseudomonas aeruginosa* RV3 and for *Bacillus cereus* NI40215 are pH 7 and 37 °C (Figure 5 & 6). Both the bacterial strains were found resistant to multiple antibiotics (Figure 7 & Table 3) and such resistance might be associated with a high degree of tolerance to different metals [59, 226, 227]. The resistance to antibiotics and tolerance to metals might be due to exposure to metal contaminated environment that could have caused the selection for resistance factors in the bacterial strains [59]. Interestingly, both the bacterial strains were resistant to Cd, Cr and Ni with average MIC value for all the metals were found as 2.46 and 2.16 for the strain RV3 and NI40215 respectively. The order of toxicity of the metals to the bacterial strains were found to be Cd > Cr > Ni (Figure 8). Also it was observed that inhibitory concentrations of metal were higher in solid medium as compared to liquid

medium. This difference might be due to the difference in the diffusion, complexation and availability of metals in the medium [228].

Both the bacterial strains exhibited slower growth in the presence of metals (Cd, Cr & Ni) in comparison to the untreated control (Figure 9). Heavy metals have been reported to inhibit bacterial growth indicated by an extension of the growth rate and generation time [228]. In the present study significant decrease in the growth rate were observed for both the bacterial strains (Table 4)

The decrease in the growth rates of bacterial strains (RV3 & NI40215) might be due to heavy metal induced oxidative stress. The heavy metals are known to generate reactive oxygen species (ROS) leading to oxidative damage [229]. Reduced glutathione and antioxidant enzymes (i.e. SOD, CAT, GPx, GR) plays important role in neutralizing ROS and protects cellular system from metal toxicity. Although such studies are comparatively more in plants and animals [230, 231] and to some extent in cyanobacteria, information on this aspect is limited in the soil bacteria.

GSH is considered as one of the important non-enzymatic antioxidant and reported to protect *Saccharomyces cerevisiae* [232, 233] and Gram-negative bacteria [234] from oxidative stress. In the present study dose dependent decrease in GSH levels were observed in both the bacterial strains (RV3 & NI40215) in response to heavy metals (Cd, Cr & Ni) (Figure 10). The decrease or depletion of GSH in response to metals might be associated with heavy metal detoxification mechanism. Recent reports on the depletion of GSH in response to metals [231], supports the present findings. The depletion of GSH in the present situation might be due to significant increase in the lipid peroxide levels on exposure to the metals in both the strains (Figure 11) and could be responsible for the decreased growth rates. The decreased growth rates with decrease in the GSH levels in response to metal stress, suggests possible role of GSH in the proliferation of the bacterial cells. Recently, Perez *et al.* [233] reported that *S.*

cerevisiae (yeast) when exposed to lead (Pb) decreased its proliferation rate with depletion of intracellular thiols.

Even though it is not clearly understood how the antioxidant enzymes in bacteria are involved in providing protection from the metal stress, it is presumed that high antioxidant capacity in the bacterium might be associated with metal resistance. SOD and CAT are front line antioxidant enzymes which neutralizes the superoxide radicals and H_2O_2 respectively [235]. In the present study the metal resistant bacterial strains (RV3 & NI40215) showed significant increase in the SOD and CAT activity in response to the metals (Cd, Cr & Ni) at 6h and 12h (Figure 12 & 13). Behera *et al.* [231] and Corticeiro *et al.* [115] in their independent studies reported similar increase in SOD and CAT activity in response to metals in *B. cereus* and *R. leguminosarum*. Pandey *et al.* [39] observed similar increase in the activity of SOD and CAT in response to cadmium, lead and arsenic in the metal resistant strains of *Ochrobactrum* and *Bacillus* sp. The increase in SOD and CAT activity in response to metal stress in both the metal resistant strains is possibly an adaptive response for the removal of superoxide radical and H_2O_2 from the bacterial cells.

GPx and GR are important GSH-related enzymes and have an important role in GSH/GSSG maintenance which is crucial for cellular redox status [116]. In the metal resistant bacterial strains(RV3 & NI40215) significant dose dependent increase in the GPx and GR activity were observed in comparison to the untreated conditions (Figure 14 and 15). Bianucci *et al.* [236] reported enhanced GPx and GR activities in *Bradyrhizobium* sp. NLH25 exposed to cadmium (Cd). Lenartova *et al.* [35] showed increased activities of GPx and GR in *Streptococcus bovis* in the presence of mercury (Hg). Similar increase in the GPx and GR were observed in *Pseudomonas putida* [35, 42]. The increase in the activity of GPx and GR in response to metals (Cd, Cr & Ni) might be probably due to the alterations in the cellular thiol redox balance as a result of decrease in the GSH levels in the bacterial strains.

The present data on the modulation of GSH and antioxidant enzymes in response to the heavy metals (Cd, Cr & Ni) suggests their possible role in conferring resistance in both the bacterial strains (RV3 & NI40215). However, apart from modulation of GSH and antioxidant enzymes metal resistant bacteria are known to adopt several mechanisms including regulation of uptake, transformation into less toxic species and intracellular mobilization etc. to detoxify the metal ions.

SEM image data exhibited morphological changes in the both the bacterial strains (RV3 & NI40215). In both the strains increase in the volume but decrease in surface/volume ratio were observed particularly at the highest dose of the metal treatment (Figure 16 & Table 5; Figure 20 & Table 7). The relative reduction in cell surface/volume ratio is considered as an effective mechanism adopted by the cells to lower the toxic effects of environmental stress by decreasing the attachable/exposed surface [214]. Similar morphological changes have been reported in the phototropic bacteria after exposure to environmental stress [237]. The changes in cell morphology in strains RV3 and NI40215 may be a part of protective mechanism in response to metal stress. Chakravarty *et al.* [238] also reported such changes in heterotrophic acidophiles. EDX analysis showed the presence of metals (Cd, Cr & Ni) on the surface of both the bacterial strains (RV3 & NI40215). The cell size and morphometric properties is important parameter for bio-sorption process of heavy metals by the bacteria and is directly proportional to the metabolic activity and growth rates [239]. The present data suggests that metal induced morphological changes might have important role to keep up the metabolic activity and for survival in the metal resistant bacterial strains.

The functional groups such as carboxyl, amine, hydroxyl, phosphate and sulfhydryl etc. located on the bacterial cell surface are known to play important role in metal interaction and bio-sorption. FTIR spectra were recorded to investigate the role of cellular functional groups involved in metal biosorption by the bacterial biomass of the strains RV3 and NI40215. Analysis of complex IR

spectra, revealed shift in the major absorption peak in the metal (Cd, Cr & Ni) treated bacterial strains as compared to metal free conditions (Figure 18 & Table 6; Figure 22 & Table 8). The untreated biomass displayed number of absorption peaks in both the strains, reflecting the complex nature of the biomass. The spectra of the untreated biomass of both the strains (RV3 and NI40215) showed characteristic peaks in 3600 – 3200 cm^{-1} region due to stretching of the N-H bond of amino groups along with γ O-H of the hydroxyl groups [240]. A change in this region for all the metals loaded biomass suggests the involvement of amino and hydroxyl groups in metal binding to the bacterial surface [57]. Previous studies suggest hydroxyl groups to have high affinity for divalent cations [241]. These hydroxyl groups are present in all polysaccharides can become negatively charged contributing to metal adsorption [57]. The region 3000 – 2700 cm^{-1} is dominated by the C-H stretching vibrations of CH_3, $>CH_2$, CH and CHO functional groups respectively [242]. All the bands in this region shifted to lower wavenumber in metal (Cd, Cr & Ni) treated bacterial strains (RV3 and NI40215) (Table 6 & 8). The region between 1800 – 1500 cm^{-1} show characteristic bands for proteins while the region 1700 – 1600 cm^{-1} is specific for amide-I bands [243] that is mainly due to C=O stretching vibrations of peptide bonds [244]. The bands in amide region provide insight into the protein secondary structures [244]. On the other hand the region from 1600 – 1500 cm^{-1} is specific for amide-II bands, which is due to N-H bending vibrations [57]. IR spectrum of bacterial strains RV3 and NI40215 showing band at 1653 and 1652 cm^{-1} respectively is due to the C=O stretching of ester carbonyl group [244, 245] and was shifted to lower wavenumber in the metal treated strains.

The change in the bands 1547 and 1556 cm^{-1} observed in metal free biomass of bacterial strains RV3 and NI40215 respectively also shifted which might be due to N-H bending vibrations [57]. The bands in the 1500 – 1200 cm^{-1} region arise mainly form the C-H bending vibrations of CH_3, CH_2 and CH functional groups [42, 46]. The information on the phosphodiester functional groups can be

obtained in the region between 1250 – 1200 cm^{-1} which corresponds to >P=O asymmetric stretching frequencies [243]. In the present study bacterial strain RV3 showed absorption band at 1459 and 1407 cm^{-1} in the untreated biomass which were shifted to the lower frequencies in the metal treated conditions but in the case of bacterial strain NI40215, no change in the frequencies were observed in Cd treated conditions while in the Cr treated band 1455 cm^{-1} disappears and in the Ni treated biomass shift was observed at the lower dose, which disappears with the higher dose. The biomass of both the bacterial strains RV3 and NI40215 showed absorption band at 1230 and 1238 cm^{-1} respectively, that were shifted to the higher frequencies in the metal treated conditions which might be due to P=O asymmetric stretching vibrations of PO_2^-phosphodiesters. Phosphate moieties such as phosphate containing metabolites, sugar phosphate esters etc. are known to play crucial role in metal chelation [248].

The region from 1200 – 900 cm^{-1} are mainly dominated by the sequence of bands due to C-O, C-C, C-O-C and C-O-P stretching vibrations [246, 247] and also CH_3, CH_2 rocking modes [249]. These functional groups are present in the carbohydrates and polysaccharides. The polysaccharide absorption bands were observed at 1076 cm^{-1} in strain RV3 and at 1079 cm^{-1} in strain NI40215 that shifted to the lower frequencies in the metal treated conditions. There was an additional band at 1167 cm^{-1} in the strain NI40215 which shifted to higher frequencies in the metal loaded biomass. The changes in the absorption bands in the region 1200 – 900 cm^{-1} might be due to the chelation of metals by the polysaccharides but due to complexity of the absorption of various cellular polysaccharides specific assignment will be rather difficult. FTIR studies showed complex interactions of metals (Cd, Cr & Ni) within wide range of functional groups such as –OH, aliphatic C-H, secondary amines, carboxyl, carbonyl, phosphate and phosphate diester present on the bacterial biomass of the strains RV3 and NI40215.

FTIR analysis of metal loaded biomass of bacterial strain RV3 and NI40215 suggests the possible interaction with the functional groups present on the bacterial cell surface. TEM analysis was carried out to further investigate the intracellular localization of absorbed metals within the bacteria *Pseudomonas aeruginosa* RV3. The electron micrograph of metal free control cells exhibited relatively diffused boundary and homogenous cytoplasm with few electron dense granules. The metal loaded bacterial cells showed metal deposition in both membrane and cytoplasm but preferentially in the periplasmic and cell wall which represented relatively darker and opaque region in the electron micrograph [Figure 19]. Earlier reports on the *Pseudomonas* strains also showed preferential localization of metals in the cell periphery and relatively lesser fraction in the cytoplasm [57]. The peripheral distribution of metals in the Gram-negative cell envelop might be due to its anionic character, which is contributed by the phosphoryl and carboxyl moieties in the cell wall and membrane components [250]. But there are reports on cytoplasmic deposition of heavy metals such as U, Th and La by different *Pseudomonas* strains [251]. The present study showed Cd, Cr and Ni accumulation both in the region of cell wall and cytoplasm but preferentially more in the peripheral region.

TEM analysis also exhibited bioaccumulation of metals (Cd, Cr & Ni) in the metal resistant bacterial strain RV3. Bacteria can adopt two processes, one is active metabolism in which metals accumulated inside the cell and another is passive metabolism in which metal adhere to the surface molecules such S-layer proteins [252]. Such processes have been reported in *Pseudomonas aeruginosa* and *Cupriavidus metallidurans* [253]. The present investigation showed increased levels of metals in dose dependent manner in the dry biomass of both the bacterial strains (RV3 and NI40215). Biosorption/accumulation of metal in the dry biomass were found to be greater for Cd followed by Ni and Cr. *Bacillus* strain NI40215 exhibited greater bio-sorption/accumulation in comparison to the *Pseudomonas* strain RV3. The higher absorptive capacity in Gram positive

bacteria particularly *Bacillus* sp. is might be due to the presence of higher peptidoglycan and teichoic acids in their cell wall [254]. ICP-OES analysis of metal loaded bacterial dry biomass showed similar trend of dose dependent increase in the metal biosorption/accumulation in both the metal resistant bacterial strains (RV3 and NI40215) even though the amount of metal detected were much higher as compared to AAS which might be due to difference in the sensitivity of the instruments.

The SEM, FTIR and TEM analysis confirms the interaction and localization of metals within the bacterial strains. Though significant increase in the lipid peroxidation were observed in response to metals (Cd, Cr & Ni), membrane damage were not observed in the SEM images of the metal resistant bacterial strains (RV3 and NI40215) instead increased level of bisorption/accumulation were seen with increase in dose of the metals. It has been suggested that metal resistant bacteria adopts multiple mechanism to overcome toxic effects of the metals. To study the molecular level interaction proteomic approach was used to identify differentially expressed proteins and their role in metal detoxification in *Pseudomonas aeruginosa* RV3. The bacterial strain RV3 responded to metals (Cd & Cr) by up-regulation involved in protein repair, ATP synthesis, protein biosynthesis, maintenance of cell shape/cell envelop, transportation, Cd-Zn-Co resistance and arginine synthesis. Major proteins identified from 2D spots in RV3 strain in response to Cd were identified as groEL, prolyl-tRNAsynthetase, outer membrane porin F precursor, ATP synthase subunit beta, hypothetical protein PaerPA_01001816, binding protein of ABC transporter, lipoyl synthase, tRNA pseudouridine synthase A, 50S ribosomal protein L3, arginine repressor and putative heavy metal chaperone/transporter. In the Cr treated condition major spots identified were for proteins chaperonin groEL, atpD gene product, L-isoaspartate O-methytransferase, F0F1 ATP synthase, 50S ribosomal protein L6, putative outer membrane protein, czcC gene product and hypothetical protein HMPREF9505_02475.

The chaperonin proteins such as groEL are known stress proteins conserved in almost all organisms from prokaryotes to eukaryotes. The previous reports indicate the overexpression of the chaperonins in *Euglena gracilis* exposed to Cr ions [255] and protect the cells from the oxidative damage induced by the metal ions. Not only chaperonin system such as DnaK/DnaJ/GrpE protects the cell against oxidative stress but also helps in synthesizing new proteins and protein translocation resulting in translocation of proteins across the membrane as unfolded peptides [256]. The up-regulation of groEL in the present study suggests for the similar role on exposure of the cells to metals (Cd &Cr).

Reports have suggested the overexpression of ribosomal proteins in response to the high concentration of metal [257]. Similar observations were also recorded in Cd and Cr treated *Pseudomonas aeruginosa* RV3 suggesting the increased level of protein biosynthesis. This increase in the protein biosynthesis is well supported by the induction in tRNA synthase/synthetase. Ferreira *et al*. [257] also showed that under metal stress *E. gracilis* and *P. aeruginosa* produced many more ribosomal proteins. All these proteins are intimately involved in protein biosynthesis which reflects vigorous synthesis of protective proteins in response to metal exposure. The increased level of synthetic activity could require more ATP to counteract the metal stress supports the present findings.

Several studies have shown that heavy metals might be adsorbed on cell surface, cell wall or cell envelops [258, 259]. The Gram-negative cell envelop, consisting of the outer membrane together with the peptidoglycan layer, plays key role in heavy metal binding. An increase in outer membrane component will lead to increase in heavy metal binding [259], which supports the present observations.

Even though heavy metals are known to play important role as trace elements in many biochemical reactions, higher concentrations forms unspecific complex compounds in the cells leading to toxic effects. Some metals ions e.g. Cd^{2+}, Cr^{2+}, Hg^{2+} and Ag^{+} forms strong toxic complexes that have adverse effects on the

physiological functions. The cells regulates uptake of heavy metals either by the chemiosmotic gradient across the bacterial membrane or through ATP hydrolysis. There are inducible P-type ATPase for magnesium uptake, ATP binding cassette (ABC) transporters for Mn^{2+}, Zn^{2+} and Ni^{2+} and some specific chemi-osmotic transporters of HoxN family for Ni^{2+} and Co^{2+}, and also ABC transporters for sulfate and phosphate in the bacteria.

In the Gram-negative bacteria resistance to Cd is known to be conferred by efflux mechanism, utilizing RND-drive systems like czc which is mainly a zinc exporter [23]. The present investigation exhibited differential expression of ABC transporter and induction of outer membrane porin F precursors and putative heavy metal chaperone/transporter in response to Cd in the bacterial strain RV3 suggesting possible role in Cd efflux conferring resistance to the bacteria. The role of ABC transporter in Cd resistance to the Gram-negative bacteria *Pseudomonas aeruginosa* RV3 has not been reported. Studies on *S. cerevisiae* showed that Cd binds with the glutathione forming cadmium-bisglutathionato complex and transported by the ABC transporters. The present data suggests similar mechanism of metal detoxification might exist in the RV3 strain. The 2DGE analysis also showed differential expression of czc transporter protein and induction of putative outer membrane protein in response to the Cr, which might also be involved in the efflux of the metals. Czc belongs to the family of CDF transporters responsible for efflux of Zn^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+} etc. [260] might also be responsible for the efflux of Cr, imparting resistance to the bacterium.

The present protein expressions in response to Cd also showed induction of lipoyl synthase in metal resistant RV3 strain. Lipoyl synthase (EC 2.8.1.8) is a key enzyme that catalyzes the synthesis of lipoic acid through lipoylation which is essential for the functioning of several important enzymes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched chain ketoacid dehydrogenases and the glycine cleavage enzymes etc. involved in oxidative metabolism. Recently lipoic acid has received attention for its role as a biological

antioxidant and has protective function in both its reduced (dihydrolipoic acid: DHLA) and oxidized form (Lipoic acid: LA). Both LA and DHLA forms stable complex with transition metals and may bind and eliminate heavy metals from the biological system. It has been reported that the LA chelates Mn^{2+}, Cu^{2+}, Zn^{2+} and Pb^{2+} whereas DHLA chelates Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+} and Hg^{2+} [261, 262, 263]. The chelating ability of DHLA and LA, due to the presence of vivinal sulphur atoms and carboxylic group, appears to be important in the biological system as they can participate in chain reactions leading to O_2 reduction by catalyzing transfer of electron from one oxygen to another in Fenton like reaction [264].

Even though the role of lipoyl prosthetic group in the functioning of the different multi enzyme complexes is well established, the biosynthesis of LA is poorly understood [264]. In prokaryotic cells the eight carbon atoms of LA is derived from octanoic acid: 8-thioctanoic acid and 6-thioctanoic acids are intermediates in LA biosynthesis as shown in *Escherichia coli* using labeling experiments [265]. Three genes have been linked to the formation of the two carbon sulfur bonds at the inactivated carbon atoms through radical based mechanism [265]. For insertion of the first sulfur atom into the octanoate backbone, the *lipA* gene encodes a LA synthase similar to biotin synthase. The other two genes *lipB* and *lplA* have been identified as lipoyl ligase that transfers the lipoyl groups to the E_2 subunit of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes. Genetic studies on *E.coli* showed the cell with mutation in *lipA* does not produce LA [266, 267] and that the only gene of the three mentioned involved in the biosynthesis of LA is *lipA* [268]. However, till now it is not clear whether *lipA* gene product LipA catalyzes the *in vivo* formation of one or both of the carbon-sulfur bonds [269]. In the present study the induction of lipoyl synthase in response to Cd in the metal resistant RV3 strain, suggests the possible role of lipoic acid to overcome metal stress. The information on the lipoyl synthase and its possible role in heavy metal detoxification in metal

resistant *Pseudomonas aeruginosa* RV3 is not available. In the present investigation *lipA* gene was PCR amplified and cloned in InsTA cloning vector. The cloned *lipA* gene was sequenced and the length of gene was 972 base pairs.

The phylogenetic analysis revealed that the *lipA* gene belonging to *Pseudomonas aeruginosa* species having identity of 99% with a bootstrap value of 58 out of 100 replicates (Figure 33 &Table 17). Further the PDB BLAST result showed the translated LipA enzyme (Table 16) having a 47% structural similarity with Chain B, crystal Structure of *Thermosynechococcus elongatus* Lipoyl Synthase 2 Complexed with Mta and Dtt [*Thermosynechococcus elongatus* BP-1 (Table 18). Hence the 3D structure was predicted using I- TASSER [218] server, an on-online platform for protein structure predictions as well as function. The server provides the most accurate structural and function prediction using state –of –the art algorithms [219]. On the basis of the result of I - TASSER server and the predicted secondary structure, the 5 best models were taken for model refinement using ModRefiner to refine the geometrical errors of the predicted structures. ModRefiner is an algorithm for atomic level, high resolution protein structure refinement [220]. The result of this study is shown in the Table 6. The energy calculations of the 5 models were assessed using ANOLEA (Table 21).

For the better structural assessment of protein, Ramachandran plot analysis is necessary. Ramachandran plot is a way to visualize backbone dihedral angles ψ against φ of amino acid residues in protein structure. A Ramachandran plot can be used in two different ways. One is to show in theory which values or conformations, of the ψ and φ angles are possible for amino acid residues in a protein and second is to show the empirical distribution of data point observed in a single structure in usage for structure validation [221]. The analysis of Ramachandran plot has been given in Table 22.

From Table 21 and Table 22, considering the ANOLEA assessment analysis and the Ramachandran plot, having an energy value of -1204 and 299 (93.1%)

residues present in favored region with only 5 (1.6%) residues in the outlier region (Figure 36). Considering all these facts the final refined structure of **model-4** (Figure 35) is proposed as the most stable structure.