Chapter 5

Discussions and Conclusion

5.1. Environmental stimulants behind chromium bioremediating microbial strain generation:

In order to understand the role of rhizospheric soil microbes in environmental well-being, their community composition, the mechanism in environmental chromium removal, and influence on the plants growing in the polluted soil were inspected. The community composition study revealed that a small number of aerobic bacteria (lower soil dehydrogenase enzyme activity exhibiting) had grown at the different polluted sewage sites of Kolkata and the lower DO and higher COD values determined that the sewage sites studied herein are highly polluted with organic materials, but have not yet crossed the borderline, which is why different pollutant tolerant plants, animals, and microbes are still able to grow and survive. As, COD represent the consumed oxygen mass per unit volume [Li and Liu, 2019], high COD values of different sewage sites indicated that the sewage bodies have experienced an ongoing competition for bio-available oxygen because of the presence of a huge amount of oxidizable organic matter. Consequently, most of the sewage bacteria can survive in a diminished or no oxygen accumulated environment.

The presence of soil microorganisms can alter the waste constituents through organic matter decomposition, inorganic transformations, and nutrient assimilation [Buerkert et al. 2012]. These processes are largely restricted to the surface soil which represents several upper meters. The ability of soil microorganisms to decompose organic matter is a function of their population complexity [Swift et al. 1979]. The presence of stress resisting microorganisms enhances the capability of the soil to degrade a wide variety of organic substances, pathogenic bacteria and viruses [Enebe and Babalola, 2019]. The rate at which organic matter decomposition occurs and the exact nature of the intermediate and end products depend on the composition of the added organic matter in the wastewater [Dlugokencky and Houweling, 2003]. Soil factors, however, exert considerable control as well. The presence or absence of oxygen, more than any other single factor, determines the rate and end products. The oxygen status of the soil is a function of soil porosity, and properties that favour rapid transmission of water also favor oxygen movement unless the soil is completely saturated [Indoria et al. 2020]. Naturally, the clay containing sewage soil contains a lesser amount of oxygen and decomposition happened in the anoxic or less oxygenic environments [Jiang and Li, 2020]. Decomposition generally proceeds most rapidly under aerobic conditions. But, under anaerobic conditions, up to 60% of the organic carbon may be respired by organisms as carbon dioxide in the initial stage of decomposition. Much of the rest is incorporated into microbial cells, and some of this is subsequently respired when the population declines [Gougoulias et al. 2014]. Through organic matter decomposition, minerals like phosphorus, sulfur, and many other trace elements are converted from organic to inorganic forms. This kind of conversion has called mineralization. Many of the elements that are mineralized during organic matter decomposition are then subject to inorganic transformation in the soil and are assimilated by plants in overland-flow and irrigation-treatment systems [Prescott, 2005]. Vegetations thus function as a sink where many waste nutrients, most notably phosphorus, can be effectively immobilized. In this manner, sewage harvested vegetation and sewage dwelling microbes play an important role in the renovation or 'treatment' of the wastewater.

The chromium resistance strains *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018 were isolated and identified from slurry sludge of three highly chromium contaminated sewage regions of Kolkata namely, Circular canal, East Kolkata Wetland and Kestopur Khal. The reason behind taking slurry soil for microbial isolation was, slurry phase of soil is a mixture of water, soil and many other living and dead bioreactors on which the microbial population depends for survival [Fomina and Gadd, 2014]. We had described in chapter 2 and chapter 3 that, the isolated chromium resistant, thickened cell wall containing (Gram-positive) and endospore-forming sewage microbes could withstand the hazardous effects of both hexavalent and trivalent chromium by implementing several strategies. Most of the isolated soil bacteria could also resist Co and Cd in addition to chromium. The microbes showed the similar kinds of mode in chromium accumulation, chromium removal and soil-plant-chromium interaction.

5.2. Sewage microbe implemented mode of action in soil chromium removal and role of sewage microbe in plant well-being:

Generally, bioremediating, heavy metal resistant bacteria reduce environmental metal pollution by five main mechanisms [Inavena, 2009] as surface absorption, efflux, extracellular sequestration, intracellular sequestration and precipitation by oxidation or reduction of the metal. The significance of bioremediation is in light because of its eco-friendly nature and minimum health hazards than the other physio-chemical strategies, which are less eco-friendly and dangerous to organisms. [Verma and Kuila, 2019]. Being the transition element, chromium (atomic number 24) possesses different oxidation states such as trivalent-Cr(III), pentavalent-Cr(V), hexavalent-Cr(VI). Among the different oxidation states, the less mobile trivalent form is abundant in the earth. The trivalent chromium generally forms a different crust with other soil minerals like calcium or silicon and remain present as ore within soil or rock. But, the second stable form i.e., the hexavalent chromium, is not the usual component of the environment and it does not take part in any biological well-being rather leave many deleterious effects on various life forms. The unusual oxidation state of chromium, i.e. hexavalent chromium [Cr(VI)], come to nature through several anthropogenic activities like random industrialization and the quick and easy spreading of hexavalent chromium has occurred because of its high water solubility and soil mobility. The microbes which are inhabitants of different metal-containing anaerobic sites, mainly, the wastewater reservoirs, have developed their own mode of action through which they convert the hexavalent chromium to less mobile and less hazardous trivalent one to fulfil their metabolic welfare [Kanmani et al. 2012]. As recently Novotnik et al. (2019) had reported the microbial reduction of transition element manganese. They [Novotnik et al. 2019] had also described that manganese tolerant microbe utilized the metal reduction potential in their cellular metabolic reaction at the anoxic situations even more efficiently than fermentation. In case of the anaerobic condition, when, the oxidative state of chromium (Cr^{6+}) remain absent, the bacteria have taken part in fermentation [Breda et al. 2019]. Normally, the microbes undergo bio-absorption, bio-accumulation and enzymatic redox reaction process to interact with the environmental hexavalent chromium [Mishra et al. 2012]. The chromate reductase enzyme present within the microbial cell membrane have taken an important part in chromium reduction under anaerobic condition and the activity of this enzyme induced by co-factor NADH or glutathione [Elangovan et al. 2006] and hexavalent chromium behave as terminal electron acceptor of this pathway [Camargo et al. 2004]. While oxygen competes with chromium reduction as an electron acceptor, the presence of oxygen vigorously affects the chromium reduction pathway [Han et al. 2016]. One of the key blockades of microbial chromium reduction at neutral soil is, the oxides of both hexavalent and trivalent chromium remain poorly soluble at neutral pH. The hexavalent chromium oxides are soluble in acidic environments and trivalent chromium oxides are soluble at alkaline environment. This phenomenon creates a serious problem for microbe as the microbial cell membrane becomes impermeable to chromium. The microbes overcome the situation by secreting extracellular siderophore which can solubilize the chromium ions and make them available for microbial reduction. Soil microbes can bind heavy metals by producing siderophores [Glick and Bashan, 1997; Braud et al. 2009a; Rajkumar et al. 2010] and low molecular weight organic acids [Rózycki and Strzelczyk, 1986; Renella et al. 2004] which make chromium available to the microbes by enhancing cation mobility. Most of the soil microbes investigated in the present work, produced considerable quantities of hydroxamate siderophores and organic acids (evaluated by the positive results in the MRVP test) which have a significant impact on soil cation mobility, iron molecule gathering and chromium reduction. The soil microbe secreted siderophore also have taken an important part in the extraction of iron from soil organic substances and made it available for the microbes [Dale et al. 2004]. When the iron become solubilized and entered within the microbial cell, it potentially enhanced the chromium reduction and stability within the microbial cell by performing a redox reaction. Recently, Brumovský et al. (2021) stated that sulfur-containing zero-valent iron had reduced hexavalent chromium (Cr^{6+}) to trivalent chromium (Cr^{3+}) and made the formed trivalent chromium immobile by forming complex oxide or hydroxide salts of chromium and iron. Earlier Emily et al. (2017) had also examined the stability of microbial chromium bioremediation product Cr(III)-Fe(III)-(oxy)hydroxides. Thus, the microbial siderophore had made iron available within the microbial cell and the cellular iron had helped in chromium removal from the environment for the long term by taking part in the redox reaction. The compound, which had formed by the reaction among trivalent chromium and ferric ion, converted to green rust of carbonate and iron oxide by slow turning. The same trend was followed by our microbial isolates. We described in chapter 3 that how the reduced trivalent chromium remained persisted within the microbial cell when we investigated the microbial batch cultures. This finding indicated that the hexavalent chromium had converted to persistent trivalent chromium. Our isolated soil bacteria also produced an ample amount of hydroxamate siderophore which is specifically important for the microbial chelate compound formation and ferric iron uptake mechanism through iron mobility [Powell et al. 1980; Mawji et al. 2008]. Thus, we can affirm from the previous literature reviews and present investigation, that our isolated microbe may employ the siderophore gathered iron in chromium removal.

The hexavalent chromium entered within the microbial cell with the help of organic acid and siderophore had taken part in cellular energy production by breaking down organic macro-molecules. The organic macro-molecules were come from surrounding soil within the microbial cell by different starch or protein degrading enzymes like amylase, gelatinase, and urease. The lesser soil dehydrogenase activity of the presently investigated sewage soil samples revealed that the soil microbes generally did not take part in aerobic respiration or fermentation in presence of hexavalent chromium; instead, they produced their required metabolic energy through the reduction of hexavalent chromium to trivalent chromium. The extracellular lytic enzymes produced by the microbes hydrolysed the large carbohydrate or protein macro-molecules like starch or collagen and brought them within the cell directly as they could further be used in energy production. Previous literature revealed that the anaerobic bacteria normally do not preserve the energy which they gained by metallic reduction instead, utilized the metal reduction procedure as electron-donating step which enhances fermentation [Novotnik et al. 2019]. Many sulfur-reducing and fermentative bacteria have taken part in this kind of metabolic reaction. Brumovský et al. (2021) also stated the role of reduced sulphur in trivalent chromium immobilization within the microbial cells. But in the case of chromium reduction, the microbe generally preserves the obtained metabolic energy for future use. Some chromium reducing *Shewanella* (Hunt et al. 2010) and *Geobacter* (Esther et al. 2015) species were reported which conserved the energy gained through chromium reduction for further cellular metabolic purposes. Naz et al. (2021) had also revealed the role of chromium in anaerobic energy formation. Our investigated microbes have also taken part in chromium reduction and formation of energy by bound environmental inorganic phosphate. We have discussed in chapter 4, the isolated and investigated microbes acquired a huge amount of stable phosphorus from soil and had accumulated it within their cell as organic phosphate.

According to our observation, the isolated *Microbacterium radiodurans* K12016 showed an accelerated chromium reduction rate in presence of 2,4 dinitrophenol (2,4 DNP). The previous study had also revealed that protonophore uncoupler DNP increased the rate of respiration by reducing the ATP/ADP and NADH/NAD ratio respectively [Leverve et al. 1998]. And, because of that, in anaerobic conditions, the glucose degradation rates remain just as in aerobic conditions when, uncoupler like DNP remain present [Mickelson, 1974]. *Microbacterium radiodurans* K12016 accelerated the rate of chromium reduction initially in presence of DNP. The same trend was reported in *Arthrobacter* strain SUK 1201 where almost 80% hexavalent chromium got reduced compared to control and after prolonged incubation tenure, both control and DNP treated cells get reduced chromium completely [Dey et al. 2014]. The same kind of metal reduction enhancing effect of DNP was found at chromate reducing *Burkholderia cepacia* MCMB-821 strain which utilized lactose and salt in respiration as electron doner in the anaerobic and aerobic environment as well. Presently investigated *Microbacterium radiodurans* K12016 used glucose and sodium chloride from surrounding LB media for the production of energy and the chromium reduction happened simultaneously along with bacterial growth. Alam and Ahmad (2012) had reported that in chromate reducing strain of *Staphylococcus gallinarum* W-61, *Stenotrophomonas maltophilia* ZA-6, *Pantoea* KS-2 and *Aeromonas* KS-14, sodium azide and sodium cyanide depleted the chromium reduction rate whereas, uncoupling agent dinitrophenol (DNP) triggered it. The same trend was found in our isolated *Microbacterium radiodurans* K12016 strain. The chromate reduction stimulating capability of uncoupler dinitrophenol (DNP) suggested a strong association-ship among electron transport chain (ETC) and chromium bio-reduction. As Han et al (2016) stated previously that oxygen competes with hexavalent chromium for an electron within the microbial cell, it could be inferred that, like oxygen, the hexavalent chromium can be attached at the terminal complex (generally complex IV) of electron transport chain and react with a proton to form water. The significance of chromate reduction in the anaerobic conditions is that, if it does not potentially collect electrons in absence of oxygen, the electron transport chain (ETC) cannot work properly, and the cell does not produce ATP by implementing proton motive force (PMF). Naturally, without ATP the normal cellular physiological occurrence should be stopped and the cell may even die. So, in the present investigation, the *Microbacterium radiodurans* K12016 had reduced the accumulated chromium with their concurrent growth and the rate of chromium reduction acceleration in presence of DNP revealed that chromium reduction might take a significant part in the respiratory electron transport chain (ETC). The deceleration of chromium reduction rate in presence of sodium azide also strengthens the previous finding because sodium azide plays a notable role in preventing cellular respiration by inhibiting the electron acceptors. The newly acquired chromium removing *Microbacterium radiodurans* K12016 was used to carry out megablast alignment with chromium reducing *Microbacterium oleivorans*, *Microbacterium liquefaciens* and get respectively 90.72% and 87.08% 16S rRNA based sequence identity. *Microbacterium oleivorans* was reported as chromium reducing strain and expressing chromate reductase gene chrA (Sarkar et al. 2016). As, in prokaryotes, 16S rRNA control the function of mRNA through small ribosomal sub-unit (Rinke-Appel et al, 1994) and contribute to mRNA stabilization (Abdi and Fedrick, 2005), results had indicated that the bacteria which showed 16S rDNA-based similarity with our identified strain of *Microbacterium radiodurans*, should also exhibit some functional similarity. 16S rRNA based analysis revealed that our isolated microbial strains showed nucleotide sequence similarity with some *Microbacterium* and *Bacillus* species which were highly abundant in chromium contaminated regions [Garbisu et al. 1998; Liu et al. 2006; Sau et al. 2010; Zheng et al. 2014; Sarkar et al. 2016; Xiao et al. 2017]. One UV-resistant strain of *Microbacterium radiodurans* was also showed nucleotide resemblance with *Microbacterium radiodurans* K12016 [Chatterjee et al. 2018]. Sequence comparison study displayed that different geochemically important chromium tolerant and chromium resistant genes which translate numerous stress-induced proteins like ATP-dependent chaperon, ABC transporter, GTP binding protein, siderophore biosynthesis protein, chromium transporter protein, chromium reducing protein were native in *Microbacterium* [Learman et al, 2019] and some species of *Bacillus* [Wang et al. 2015, He et al. 2010]. Hemme et al (2016) reported that by lateral gene transfer, different stress resisting sewage microbes become metal tolerant permanently. Some *Bacillus* sp. including *Bacillus subtilis* (express chromate reducing nfrA gene), *Bacillus cereus*, *Bacillus firmus* revealed more than 90% sequence similarity with our newly identified strains of *Bacillus xiamenensis* predicted that, our isolated strains might play some potential role in the chromium reduction (Garbisu et al. 1998; Liu et al. 2006; Xiao et al. 2017; Sau et al. 2010; Zheng et al. 2014). Though, as we stated in chapter 3 that, our isolated *Bacillus xiamenensis* strains showed relatively lower hexavalent chromium resistance as compared to *Microbacterium radiodurans* K12016, and in the presence of microbial growth stimulator DNP, they didn't manifest any considerable change in chromium reduction rate, chromium had accordingly accumulated and simultaneously reduced with microbial growth within the *Bacillus xiamenensis* cells. But, the chromium resistance and accumulation efficiency of *Microbacterium radiodurans* K12016 was comparatively greater than strains of *Bacillus xiamenensis.* As the chromium reduction mechanism varies microorganism to microorganism, the *Bacillus xiamenensis* might not directly employ chromium reduction procedure as terminal electron acceptor of respiratory ETC rather, they implemented another detour mechanism to avail the advantage of chromium reduction. While the strains of *Bacillus xiamenensis* produced more concentrated hydroxamate siderophore as compared to *Microbacterium radiodurans* K12016, the siderophore should assemble the soil iron molecule which had taken part in chromium reduction. Iron mediated chromium reduction in *Bacillus* had been reported in many previous pieces of literatures [Su et al. 2019; Xu et al. 2020]. Following the results we had got throughout our experiments, and, the postulates [Ahemad, 2015] obeyed in many previous investigations, it can be deduced that in *Microbacterium radiodurans* K12016 might serve as a potent terminal electron acceptor to gain energy and the strains of *Bacillus xiamenensis* serve as potent chromium reducing agent by forming adequate amount of enzyme catalase at the time of chromium intoxication which had significantly taken part in simultaneous redox reaction of hydrogen peroxide and chromium. Strains of *Bacillus xiamenensis* as well produced chelating agents like exopolysaccharides (EPS) and siderophores which could react with hexavalent chromium in several ways. The different enzymes secreted by chromium reducing microbes could take an active part in chromium reduction. The chromium resistant *Bacillus xiamenensis* secreted exoenzyme catalase help in microbial chromium reduction by means of hydrogen peroxide break-down [Pettine et al. 2002] along with simultaneous iron reduction. As sodium azide inhibits the activity of enzyme catalase in presence of hexavalent chromium [Itoh et al. 1995], it should affect the hydrogen peroxide mediated chromium reduction. The initial deceleration of chromium reduction in *Microbacterium radiodurans* K12016, *Bacillus xiamenensis* 1E0018 and *Bacillus xiamenensis* 26K018, at the presence of sodium azide also affirmed the active role of catalase in microbial chromium reduction within our presently investigated microbial strains. The oxygen molecule which had formed by hydrogen peroxide depletion at the time of chromium reduction might take part in microbial respiration as an electron acceptor instead, directly using hexavalent chromium as a terminal electron acceptor. So, chromium reduction helps in microbial respiration in a direct or indirect manner by supplying terminal electron acceptors to the respiratory electron transport chain (ETC). Dang and Jiao (2014) had also stated previously that facultative anaerobic microbes engaged different soil elements like sulfur, iron, manganese in anoxic or hypoxic conditions to continue their cellular respiration.

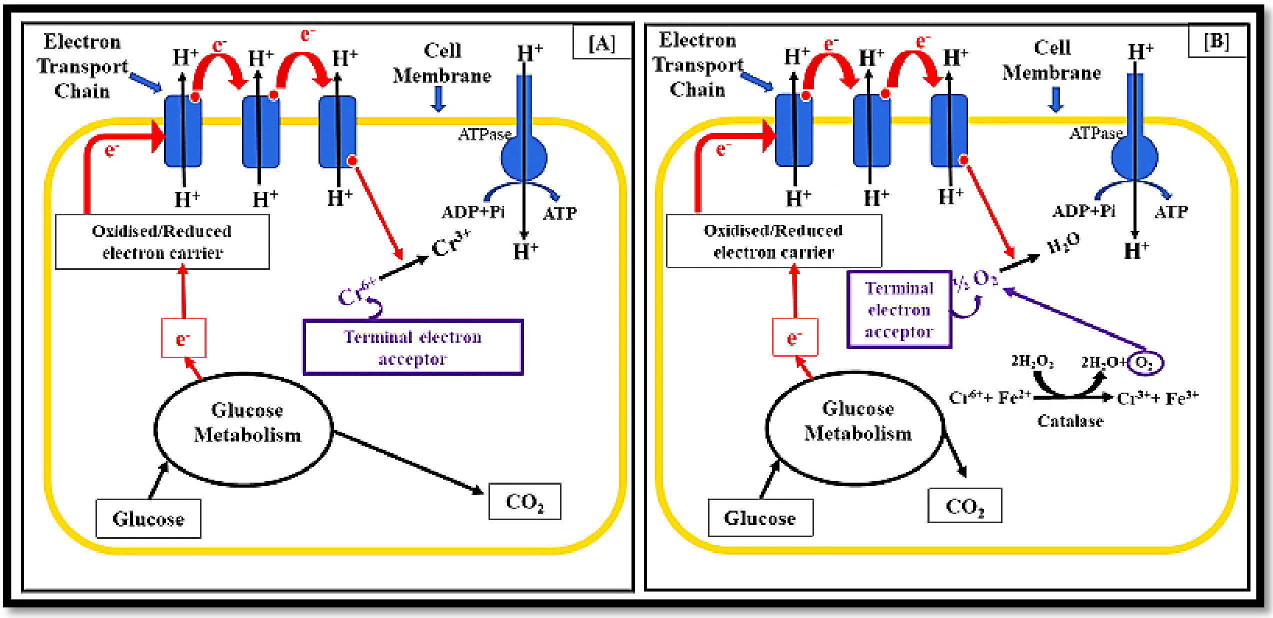

Figure 5.2.1: The schematic representation of predicted anaerobic respiration techniques implied by chromium resistant sewage dwelling microbes. [A] In

***Microbacterium radiodurans* K12016, the hexavalent chromium directly behaving as terminal electron acceptor of respiratory electron transport chain (ETC). [B] In *Bacillus xiamenensis* 1E0018, *Bacillus xiamenensis* 26K018, as well *Microbacterium radiodurans* K12016, the chromium reduction can control respiratory electron transport in a bypass strategy; here the chromium reduced by means of simultaneous redox reaction of hydrogen peroxide and the oxygen molecule produced, behaving as terminal electron acceptor of respiratory electron transport chain (ETC)**

Chromium reduction and bioremediation in different species of *Bacillus* also depend on the other mineral (like sulfur, phosphorus) constituent of the soil. Many previous works reported that the trivalent chromium, which is the end product of chromium reduction, can form an immovable complex with extracellular EPS or intracellular minerals like sulfur or iron [Zhu et al. 2019; Xu et al. 2020]. Chromium has also competed with other elements like phosphorus, sulfur or iron [Shanker, 2005] to enter within the plant or microbial cells and finally entered by forming chelate compounds or by means of active transport. The presence of hexavalent chromium may also increase the plant's soil mineral consumption [Wyszkowski and Radziemska, 2009] which is not a good sign for plant health. Many soil microbores including *Bacillus*, has competed for nutrient minerals like sulfur, phosphorus and even chromium with plants. Different *Bacillus* species played a significant role in soil mineral mobility, mineral accumulation and their absorption at microbial vicinity. We had described in chapter 4 that, soil *Bacillus* and other chromium resistant microbes reduced plant chromium absorption and accumulation singly and also in consortia. The soil microbe mediated plant chromium absorption declination has a good impact on human health because it lowers the chances of chromium bio-magnification. So, it could be predicted that the soil-dwelling *Bacillus* assist plant growth by lowering plant mineral absorption. The presently isolated strains of *Bacillus xiamenensis* possess quite a potent role in plant growth promotion compared to *Microbacterium radiodurans* K12016. The chromium resistant soil microbe enhanced the growth and metabolism of plants by forming different chelating compounds and phytohormones [Sarathambal et al. 2017]. Chelating compound siderophore has enhance plant growth-promoting capacity and also taken part in governing plant-soil-microbe biocontrol mechanisms [Saharan and Nehra, 2011]. One of the major causes of plant growth induction by metal resistant bacteria is their metal assimilation and metal translocation enhancement efficiency [Hansda et al. 2014]. The metal migration procedure has a good impact on plant growth and well-being. Most of the soil microbes investigated here produced considerable quantities of hydroxamate siderophores and organic acids (evaluated by the positive results in the MRVP test) which have a significant impact on soil cation mobility and the regulation of plant's nutrient uptake. Hence, the presently investigated microbes might reduce the plant absorption of heavy metals by producing siderophores and extracellular acids. Additionally, the microbial isolates were found to produce the plant growth-promoting hormone, IBA and IAA. Previous reports have indicated that rhizospheric microbes secreted IAA can alter the endogenous IAA pool in plants [Arshad et al. 2007; Glick 2012] and increase the length and surface area of plant roots [Glick 2012]. Many previous works of literatures also specifically described the role of *Bacillus* species in plant growth promotion by forming phytohormones like IAA or IBA [Saharan and Nehra, 2011; Shukla et al. 2012; Cherif-Silini et al. 2013; Ndeddy Aka and Babalola, 2016]. The present study also follows the trend described in previous literature. The isolated soil *Bacillus* help in plant growth induction by producing siderophore, organic acid and indole compounds like IBA and IAA. The subsequent morphological and physiological features of the chromium resistant microbes were also studied to investigate their bioremediation impact on the environment. Our isolated chromium tolerant sewage microbe showed 16S nucleotide-based similarity with the previously identified chromium resistant strains and also implemented several mechanisms to bind soil chromium. They synthesized siderophores and exopolysaccharides which had helped in the aggregation of chromium and their entrance within the microbial cell. Then the microbial isolates reduced the cell-incorporated hexavalent chromium to less hazardous trivalent chromium and utilized the chromium reduction potential in their cellular profit and welfare. The morphologically and biochemically identical, organic acid, siderophore and catalase producing chromium resistant soil microbes were found in abundance to reside in the different chromium polluted sewage regions of Kolkata. *Microbacterium radiodurans* strain K12016 isolated from Circular Canal and *Bacillus xiamenensis* (strain 1E0018 and 26K018) isolated from East Kolkata Wetland and Kestopur Khal, exhibited a similar trend in chromium accumulation and chromium reduction. But, the chromium reduction mechanism of *Microbacterium radiodurans* strain K12016 had been varied with the strains of *Bacillus xiamenensis* (1E0018 and 26K018) to some extent.

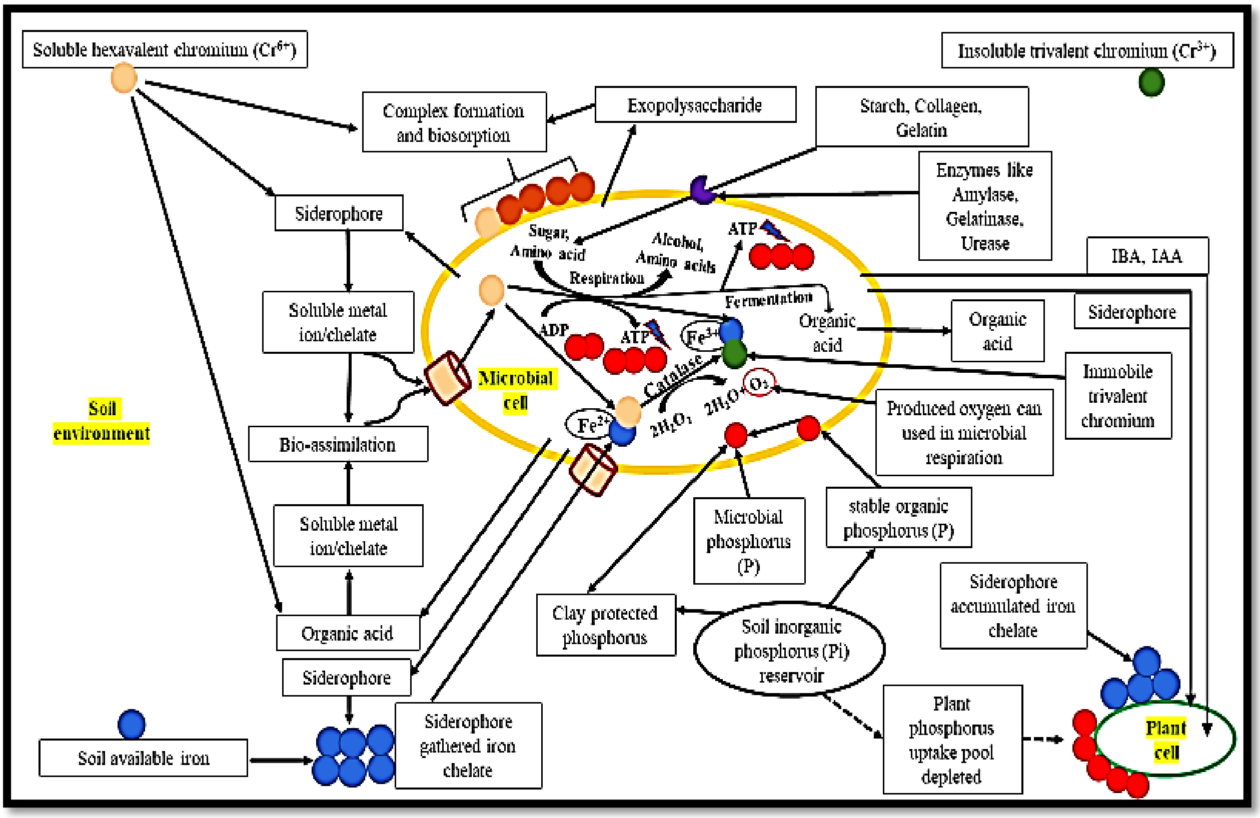

Figure 5.2.2: Diagrammatic representation of microbial interrelationships with soil and plants within the hexavalent chromium contaminated sewage regions of Kolkata with the special emphasis on metabolic pathways involved in microbial chromium reduction

5.3 Conclusion of thesis:

It is possible to conclude that isolated strains can serve as potent bioremediating bacteria, because of their significantly higher chromium tolerance, reduction and accumulation ability. Heavy metal profiling of the soil samples and the plants growing in the sewage soil indicated that the resident soil microbes greatly influence heavy metal absorption by the plants. From these findings, it may be concluded that the isolated chromium removing, eco-friendly strains have a great impact on agro-economic society as they could be applied to eliminate environmental chromium, as well as improve plant growth in heavy metal contaminated, poorly cultivable soil and decrease the risk of metal accumulation and biomagnification by the edible plants that are consumed regularly. Further field application should be required to clarify their bioremediation and plant interaction strategies.

5.4 Future directions:

After inspecting their in-situ chromium removal efficiency, the isolated chromium reducing strains can be used as potent agro-industrial agents, whereas, their capability in decreasing plant chromium accumulation was already been inspected in field and experimental conditions as well. In future, more investigation has to be put into the expression of genes responsible for conducting the respiratory electron transport chain (ETC) and chromium reduction pathway. The genes responsible for microbial chromium reduction and plant chromium accumulation, and their cross-talk mechanisms possess a considerable impact on human and plant well-being because we found that the pollutant tolerating microbe-plant consortia had developed a certain mechanisms of actions that should help to overcome chromium toxicity.