CHAPTER 4

DISCUSSION

4.1 FERRON ASSAY

The acidophilic iron oxidizing bacteria represent a group of, in the main, obligately autotrophic chemolithotrophs. The acidophilic, iron (II) ion-oxidising bacteria *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* are the most important mesophiles for the extraction of metals from sulfidic ores by bioleaching (Sand et al., 1992; Colmer et al., 1950). Acidophilic microorganisms show sulfur or iron oxidizing abilities which serves as their energy source. These bacteria cannot thrive in the presence of organic salts. Owing to the low biomass and specific growth rates of *Thiobacillus ferrooxidans* measurement in continuous cultures is very time consuming. Comparison of the published values of kinetics and energetic constants showed that large variations occurred between data from different authors who used similar systems (Boon et al., 1999). As already published by authors an analytical assay technique is the only possible way to measure the growth of such acidophiles. Immunological methods can detect the presence or the absence of the iron oxidizing bacteria, but will not prove effective to monitor the growth of the organism over a period of time. Shortcomings did exist in some of the standard assay methods like titrametric methods, orthophenanthroline method (at low ferrous iron concentrations the background color of the ferric iron in the sample caused an error in the colorimetric measurement) (Boon et al., 1999), electrochemical method, Sagaidachny’s method and potassium thiocyanate method (color fades on standing) (John, 1932).
The purpose of this study was to develop a simple assay to detect the iron oxidizing bacteria from isolates and use it as an indirect measurement of growth for pure culture. In case of chemolithotrophic microorganisms such as *Thiobacillus ferrooxidans*, for which plating is not easy and the results are obtained after few days, the ferron assay was found to be efficient, accurate and robust method. This study reports an efficient and stable method to assay the oxidation of ferrous to ferric, which indirectly relates to the growth pattern of acidophilic bacteria i.e (*Thiobacillus ferrooxidans*, *Leptospirilum ferrooxidans* & *Acidothiobacillus ferrooxidans*). The complex formed from the reaction of ferric and ferron is stable over a period of time and ferron does not act as an environmental hazardous chemical as compared to the chemicals used in other methods. 7-Iodo-8-hydroxyquinoline-5-sulfonic acid dissolves in water to give a bright yellow solution which reacts with ferric iron to give a green colored solution and intensity varying with the ferric ion concentration. The reaction is very sensitive and is best carried out in an acidic solution (John, 1932). Ferron assay is a direct and easy method. Ferron forms a complex with ferric which is stable irrespective of the pH or the media composition. The chemicals used in the media were added separately to ferron solution and no green color formation was observed. This clearly indicates that the media components do not interfere with the color formation and it is clearly a reaction between the ferric and ferron. Considerable progress has been made in studying the biochemistry and molecular biology of the acidophilic species in the recent years. It therefore becomes necessary to design analytical and molecular techniques to ensure work with pure cultures and also to study their respective growth patterns.

Ferron assay has also proved to be useful to do initial characterization of any isolate since the green color formed clearly indicated the mine samples had microorganisms capable of oxidizing ferrous. The growth of the isolates from Kudremukh and Kolar Gold Waste samples in ferrous sulphate medium followed by its assay for growth by indirectly measuring the oxidation of ferrous to ferric by the iron eating acidophiles. This clearly showed that ferron assay can be used as the first
step in characterizing an isolate from natural environment. This assay technique is the first of its kind, in using ferron to detect iron oxidizing bacteria.

4.2 PLATE ASSAY FOR THE DETECTION OF IRON OXIDISING ACIDOPHiles

Microbial isolation and purification of colonies grown on solid media is always the best way to check for the purity of the strains. Growth on solid media precedes any kind of cultural and phylogenetic analysis like the immunological detection (Jerez and Arredondo, 1991; Muyzer et al., 1987) or the use of genetic probes (Yates et al., 1986). Acidophilic organisms have been repeatedly tried to be grown on solid media during the last years by several scientists. Limited success and non-reproducible results on solid media may be due to a number of factors. Agar when added from a concentration of 2% to 7% directly into the minimal media with ferrous sulphate has not been successful in solidification. One of the reason could be that agar being a polysaccharide hydrolyses under acidic conditions of the media. Accumulation of oligo and monosaccharides could be inhibitory on the iron oxidizers. Different gelling agents have been used like silica gel (Kawarazaki et al., 1986), agarose and gelrite (Lindstrom and Sehlin, 1989). Silica gel and gelrite have not been very successful as gelling agents and also different strains could not be cultivated on these solid plates.. A drawback with agarose is that of it being expensive. Based on the problems experienced in culturing varied strains like *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans* & *Acidothiobacillus ferrooxidans* for the first time solidification at pH 2 with 2% agar has been made successful. With this plating technique, it has been successful to grow cultures on solid media for the above mentioned strains and the isolates from natural environments which have been photographed. The colony color ranges from light yellow for some strains to brown for the other.
A novel concept of detecting iron eating acidophiles has been introduced in this work. The ferro ferric iron complex formation yields a blue color which is a known concept. We have used this concept to detect iron eating acidophiles from a mixed population or microbial isolates of natural environments is a preliminary step for the characterization and has been discussed for the first time in this work. The blue colored formed on flooding the plates with potassium ferroocyanide is due to the complex formation between the ferric formed due to the oxidation of ferrous by the bacteria, reacting with the ferro of the complex to form the ferri-ferro ion complex. The potassium ferrocyanide solution dissolved in acidic water is light yellow in color. Keeping this novel concept of introducing a color based detection of iron oxidizing acidophiles, a medium was further designed which would allow only the growth of iron oxidizing bacteria.

Photoactivation of ammonium ferrioxalate plate yields ferrous, nitrogen and carbon dioxide which are the essential nutrients for iron bacteria as they are chemolithoautotrophs. In this plate we have excluded the use of any kind of sulphur source so bacteria which essentially grows utilizing sulphur will not grow on these plates. This plate can be used to differentiate and isolate only iron eating bacteria from mixed population of isolates from natural environments. We have applied this concept to isolate iron acidophiles from Kudremukh samples since the Kudremeukh is mainly an iron ore area. *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans* and *Ferroplasma acidiphilum* have been short listed from a list of microorganisms known to mediate bioleaching reactions in acidic environments utilisin mainly iron as their source of energy as discussed by (Eleanora, 2000). There are definitely some organisms which utilize iron as their source of energy, but these organisms differ from the above mentioned three organisms in their survival temperature and pH conditions. Thereby the plate assay designed based on the concept of ferro-feric complex formation on ammonium ferrioxalate plates definitely would support only the growth of *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans* and *Ferroplasma acidiphilum*. This method therefore becomes the first step to isolate on plates only iron...
eating bacteria from mixed population of microorganisms present in natural environments like the Kudremukh iron ore soil or the Kolar gold soil or for that matter from any mining or ore site. *Thiobacillus ferrooxidans* inspite of its efficiency to utilize both iron and sulphur, has been reported to effectively utilize iron and in the absence of iron can shift its metabolism to utilize sulphur. This property would not be drawback in utilizing the ammonium ferrioxalate plates.

4.3 EFFICIENCY OF PYRITIC LEACHING BY THE ISOLATES FROM KUDRE MukH IRON ORE SOIL, KOLAR GOLD MINE SOIL AND NEYVELI LIGNITE MINE SOIL

Microbiologically accelerated oxidation of pyrite is important in both environmental and applied microbiology. Two mesophilic bacteria are known to cause the pyritic oxidation namely *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*. Thiosulfate and iron(II) ions are formed as the first intermediates during the oxidation of the pyrite. The leaching bacteria *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* has been found to carry a considerable load of iron (III) ions complexed in their exopolymer layer (Gehrke et al., 1995). Ferric iron bound to exopolymers produced by iron oxidising bacteria acts as an electron shuttle; it is reduced when it reacts with the sulfide and is reoxidised in an energy generating reaction by the bacteria. The exopolymer layer provides a reaction compartment allowing the above mentioned processes to occur. The reaction is as follows

\[
\text{FeS}_2 + 2\text{Fe}^{3+} \rightarrow 3\text{Fe}^{2+} + 2\text{S}^0
\]

The reaction series described supplies the bacteria with iron (II) ions (Axel et al., 1996).

Helle and Onken (1988) have found that the pyrite leaching in continuous cultures was significantly accelerated when using mixed culture with *Thiobacillus*
*ferrooxidans* and *Leptospirillum ferrooxidans* bacteria, compared to pyrite leaching in pure cultures of *Thiobacillus ferrooxidans*. Iron oxidizing bacteria may have evolved to optimize electron extraction from pyrite to obtain ferrous. When grown in batch culture in a liquid ferrous iron or pyrite medium, *Thiobacillus ferrooxidans* will initially outgrow its iron oxidizing competitors and dominate the population. This is largely because during the initial stages of batch culture the redox potential is low and *Thiobacillus ferrooxidans* has a faster growth rate than *Leptospirillum*. Under such conditions *Thiobacillus ferrooxidans* is able to build up large number of cells before conditions become more favorable for *Leptospirillum ferrooxidans*. However because the *Leptospirilli* have a greater affinity for ferrous iron and are less sensitive to inhibition by ferric iron on prolonged aeration *Leptospirillum ferrooxidans* is likely to dominate (Norris et al., 1988).

The isolates from the Kudremukh iron ore was found to be the most efficient among the other isolates from Neyveli mine or Kolar gold mine. The isolates were identified as acidophilic iron oxidizing bacteria based on their growth on specialized iron plates with no sulfur in it as mentioned above. The probability of the isolates could be *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*. The isolates from the Kudremukh iron ore site took more than five days for maximum leaching of ferric as assayed by ferron. Experiments have been reported to show the mixed culture population of acidophiles favouring higher efficiency in leaching pyrite. The main idea behind this work was to identify isolates from natural environments that are capable of leaching and the basic testing was done by identifying isolates capable of leaching pyrite. The pyretic leaching observed in our case was based on the direct mechanism where the bacteria attaches to the surface of the pyrite and the ferric present in the exopolymer initiated pyretic leaching.
4.4 FUNCTIONAL PROTEIN SIGNATURES FOR THE BLUE COPPER PROTEINS

Transition metals are found at active sites of large number of proteins. One such set of proteins are the blue copper proteins involved in the electron transport chain of some of the eukaryotic and prokaryotic organisms. In blue copper proteins, the copper center is directly coordinated to amine acid residues. All the copper ions in the living cells are protein bound as it is toxic in its free form. In most copper proteins, the copper ion having the ability to change valence state is mainly involved in catalysis of biological process, or the transport of electrons different proteins in a cell. Blue copper proteins also known as cupredoxins, have a type 1 copper site. The coordination of copper in most of the blue copper proteins is determined by the conformation of its three closest ligands, two histidine nitrogens and a cysteine sulfur and of a fourth more distant ligand a methionine sulfur (Redinbo et al., 1994). In case of auracyanin, stellacyanin and umecyanin the methionine is substituted by a glutamine residue which binds as the fourth ligand to the copper atom. The green and purple copper protein have the same four ligands as the blue copper protein.

Specific signatures for each of the protein was designed from the conserved region around the active site based on multiple sequence alignment. The blue copper proteins chosen for the study include plantacyanin, plastocyanin, cucumber basic protein, stellacyanin, umecyanin, uclacyanin, and cusacyanin from eukaryotic source. The prokaryotic blue copper proteins chosen for the study were rusticyanin, sulfocyanin, halocyanin, azurin, pseudo azurin, auracyanin, amicyanin and blue nirite reductase. A common functional signature was also designed for a combination of blue and green and blue and purple copper protein. These signatures have high functional significance.

Protein signatures and peptides designed in this work picks out highly specific sequences of that particular protein from different source from the non
redundant database which has more than a lakh of sequences. The false positive picks is minimal and nil in most of the cases. These signatures can be very useful for the annotation of uncharacterized proteins. These signatures are different from the broad based generalized signatures already present in the databases. The signatures already available for the blue copper proteins like the Cyt b/b6, Photosystem 1 PSAGK, Rieske Iron Sulfur protein, and type 1 copper blue signatures are broad spectrum signatures.

Protein signatures are sequence length motifs diagnostic to a protein family indicating function. Signatures are matched to protein sequences in the non redundant databases and is scored using a dynamic programming algorithm which permits permeability in gap distance and residue type (Ison et al., 2000). Generating a signature involves identifying residues in a protein sequence that imparts functional properties to the protein. Protein signatures are efficient miners of related protein sequences having the same functional residues which belong to the same class of protein from the abundant sequences present in the non redundant databases.

By doing a keyword search, we get varied results from the different databases as indicated in table 2 owing to different levels of redundancy. On using a functionally related protein signature only relevant related sequences are picked out from the non redundant database as seen from table 5. Thus protein signatures can play a great role in extracting out highly related sequences from different databases than key word searches (Hofmann et al., 1999). Protein signatures designed taking into account the motif region will be very efficient for annotation of future uncharacterized proteins. The protein signatures in a way can be compared to primers used for amplification. The more specific and concise a primer the more specific is the amplification, similarly the more specific the protein signature the more significant are the picks from the non redundant databases. Specified signatures in a way reduce the time taken to pool related sequences from the abundantly available sequences from the non redundant databases. In this work, the concept of designing functional property
based signatures which have the amino acid residues binding to the copper atom has been successfully achieved and protein signatures for the Blue Copper Proteins and for a combination of rusticyanin with green copper protein and with purple copper protein respectively has been designed.

4.5 IDENTIFICATION OF *Serratia marsescens* USING SPECIFIC PRIMERS BASED ON 16s rRNA SEQUENCE CLUSTAL ANALYSIS

Rapid detection and quantitative assessment of specific microbial species in environmental samples is desirable for monitoring changes in ecosystems and for tracking natural or introduced microbial species during bioremediation of contaminated sites. Phospholipid fatty acid profiles have been found to be specific for microbial species, but varies with growth conditions (Pennanan et al., 1998). Sequencing of genes, such as rRNA genes, have been proven to be valuable in species identification (Wilson and Blichington, 1996). The 16s rRNA gene, is present throughout the microbial kingdoms, containing both highly conserved sequence regions and highly variable sequence regions (Woese, 1987). Vincent et al (1999), had chosen a set seven microorganisms which were environmentally important ubiquitous species known to degrade hydrocarbons. The microorganisms were *Escherichia coli* (E05133), *Klebsiella pneumoniae* (U33121), *Micrococcus lysodeikticus* (AF057289), *Pseudomonas aeruginosa* (X06684), *Serratia marsescens* (M59160), *Xanthomonas maltophilia* (M59158) and *Vibrio vulnificus* (X76333). Based on the clustal alignment of these sequences from the homologous regions a universal primer set UB16SC3 and UB16SDR9 was designed which amplified a 162 base pair product in all the bacterial DNA tested. The same set of primers gave a 162 base pair product when the DNA of *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Salmon sperm* and *Homo sapien* was tested.

Primer set Smar 16SV and Smar 16SWR was found to amplify the appropriate 417 base pair fragment in *Serratia marsescens*, but not in DNAs isolated
from the other bacteria's. This primer was thus chosen for the characterization of the isolate from soil used for this study. *Micrococcus* sp isolated from soil was used as the negative control. The 417 base pair fragment was seen in the PCR product where the DNA from the pigment producing isolate was used. These species specific identification procedures are simpler than previously reported molecular approaches that requires sequencing, pattern recognition, RFLPs, or probing of Southern blots. These methods are generally not directly applicable to the analysis of environmental samples, but the procedures are more complex and technically difficult (Shen et al., 1998). Species specific procedures based on the 16s rRNA sequences may be valuable in the analysis of human and veterinary clinical specimens. These species specific primers will also be valuable in analyzing environmental isolates. The PCR product gave a 417 base pair product for the genomic DNA of *Serratia marsescens*.

4.6 NOVEL MEDIA FOR ENHANCED PRODIGIOSIN PRODUCTION

Biopigments are synthesized by bacteria and possess enormous efficacy as medicinally important products. In order to increase the efficacy of the bacteria to synthesize large quantities of the pigment a comparative study of media of different composition, role of temperature and pH on the media, growth and pigment production had to be studied. The components of the media used were analysed and compared effectively to give a probable reason for the enhancement or the decline in pigment production. Keeping these objectives in mind it was thought of design a media which would be sumptuous for the bacteria but at the same time prove efficient to trigger high levels of pigment formation. The crushed peanut medium gave the highest yield of ~39 mg/ml over the nutrient broth and peptone glycerol broth compared in this work. In the experiment conducted by Jungdon et al (2001) the final yield in the bioreactor having an internal adsorbent was 13 mg / ml and the media used had dextrose in the culture broth and casein in production medium. Chang et al (2000) have quoted a medium containing ethanol and carbon source but the yield was 3 mg / ml. Nakamura in his patent describes the use of sodium oleate media and the
substitution of sodium oleate with oleic acid. Pure saturated and unsaturated fatty acids were substituted in the medium and triolein an unsaturated fatty acid gave the maximum of .69 mg/litre yield of the pigment.

In this work comparison was done between nutrient broth and peptone glycerol broth with sesame seed medium, peanut seed medium and copra seed medium. An enhanced pigment production was seen at 28°C in all the different media studied except in peptone glycerol broth where the maximum prodigiosin production was at 37°C. The reason could be that the viscosity of glycerol decreases at higher temperature thereby becoming more accessible to the culture for utilization of carbon source. In the case of the various seed medium tested at 37°C the yield is similar to what is seen in nutrient broth and peptone glycerol media at 30°C. In the case of peanut medium at 42°C a complete block in the pigment production was seen. One can assume that the culture did not grow at 42°C, because an OD reading of the cell culture broth could not be taken due to the interference of the peanut powder. This was overcome by the using the 42°C grown culture broth for reversion study. The inoculum from 42°C grown culture in peanut broth showed the reappearance of pink color in the culture broth at 28°C. The pigment regulation is not only temperature regulated but the temperature associated pigment regulation also varies with nutritional source in the medium. In case of the new peanut medium the pigment production was also the maximum and the loss of pigment was also seen only at 42°C.

The role of proteins did not play a major role in the pigment production nor on the cell growth. This has been inferred from this work based on the data obtained. The inherent concentration of protein was maximum in nutrient broth followed by sesame seed broth and peanut broth but the pigment production seen was the in the reverse order. Both in nutrient broth and peptone glycerol broth the major components are peptone, meat and yeast extract. Peptone is a commercially available digest of a particular plant or animal protein, made available to organisms as peptides and amino
acids to help satisfy requirements for nitrogen, sulfur, carbon and energy. Peptones also contain small amounts of various organic and inorganic compounds. But they may be deficient in certain minerals and vitamins. Yeast and meat extracts contain eukaryotic tissues (yeast, beef muscle, liver, brain, heart, etc.) that are extracted by boiling and then concentrated to a paste or dried to a powder. These extracts are frequently used as a source of amino acids, vitamins and coenzymes, growth factors by fastidious organisms. Trace elements, minerals and usually some sugar are also present. The seeds and oils contain metals, vitamins, saturated and unsaturated fatty acids and the concentration of these components are variable in each kind of seed or oil. Though the nutrient broth had the maximum amount of protein, it was only in the peanut broth that maximum pigment production seen. Even according to Kim et al (1998) casein proved to be a better nitrogen source than yeast extract, beef extract or peptone.

Based on the results got from the effect of sugars and glycerol on pigment production two points can be discussed to show the role of carbon source in pigment production. In nutrient broth the addition of maltose or glucose enhanced the pigment production, but there was no enhancement of pigment production seen in the case of sesame broth. Sesame by itself has a good composition of carbon source but nutrient broth is comparatively devoid of carbon source. Maltose and glucose when added in nutrient broth gave a better yield over nutrient broth or peptone glycerol broth alone. The second point is that enhanced pigment production was seen in the case of peptone glycerol broth when compared to nutrient broth which is similar in composition to nutrient broth in all aspects, except in having glycerol which is nothing but a carbon source. This clearly justifies the fact that carbon in any assessable form is necessary for the growth of the bacteria which in turn results in higher yield of pigment.

To an extent the role of fatty acids in enhancing cell density thereby enhancing pigment production can be justified from the data obtained in this work. The role of unsaturated fatty acids as a carbon source can be disproved to an extent in
this discussion. The oils are known for their high levels of unsaturated fatty acids and a very low percentage of saturated fatty acids. From the results observed the pigment yield is 15 times more in media containing seeds than in oils. According to Kim et al (1998) oil gave a better yield over the various carbon and nitrogen sources tested. In our case also oil has given a better yield when compared to nutrient broth and peptone glycerol broth but not when compared to the respective seed medium. Even this low level of pigment formation seen in oil medium could be due to the presence of low concentration of saturated fatty acid present in oils. If it is the unsaturated fatty acid which is contributing as major substrate to *Serratia marsescens* isolated, then the level of unsaturated fatty acid is higher (~47%) in sesame oil than peanut oil whereas the yield is higher in the case of the peanut oil medium. From this it can be proposed that the bonded fatty acids as carbon source is less accessible by *Serratia marsescens*

The role of saturated fatty acids as a better carbon source in terms of pigment yield and cell growth can be discussed with the following points. The overall saturated fatty acid composition is highest in copra, followed by peanut and then in sesame. In terms of yield peanut medium has given the maximum of ~39 mg / ml. The reason for this could be that ~50% lauric and 7% capric acid known for their antibacterial activity present in copra could have inhibited the growth of *Serratia marsescens* in the medium thereby giving a very low yield. The second point validating the role of saturated fatty acid is that as per literature peanut has a higher concentration of saturated fatty acid than than sesame and thereby the yield is also higher.

The final conclusion from this work could be that carbon in any form as substrates do show a marked enhancement of pigment production by *Serratia marsescens*. The saturated form of fatty acid as a carbon source could be a better source of carbon as the maximum yield of pigment which is approximately 39 mg/ml is seen in the case of peanut medium. The role of saturated fatty acid in enhancing cell growth and pigment formation needs little more investigation in future. A new,
cheaper and more pigment yielding medium has been successfully designed in this work and substrate is available universally.

4.7 EFFECT OF METALS ON GROWTH OF *Serratia marsescens* AND ON THE PRODIGIOSIN PRODUCTION

Bacteria encode resistance systems for a wide range of toxic metalloid ions including Ag⁺, AsO₂⁻, AsO₄³⁻, Cd²⁺, Co²⁺, Cr⁶⁺, Cu²⁺, Hg²⁺, Ni²⁺, Pb²⁺, Sb³⁺, TeO₂²⁻, Ti⁺ and Zn²⁺. Much information is now available on the genetics and mechanisms of action employed by bacteria to survive in the presence of these toxins. Resistance genes and operons may be plasmid and/or chromosomally encoded and are used to develop PCR systems to detect, and monitor strains in laboratory and field experiments. There is increasing interest in the potential applications of bacterial metal resistance determinants. Industrially and environmentally important bacterial strains such as the biomining strain *Thiobacillus ferroxidans*, and *Pseudomonas* strains used in bioremediation, are being adapted to continue their activities in the presence of increased levels of toxic metals. Additionally, the use of microbially based metal sequesterisation bioreactors is gaining increasing influence in metal bioremediation projects. Metal Bioremediation Proposal involve:

- Resistant strain identification and characterisation
- Genetic analysis of metal resistance determinants
- Analysis of metal bioremoval potential/methodology
- Development of metal toxicity bioassays
- Phytoremediation / Rhizoremediation of metals

Toxic heavy metals is a major threat to the environment, animals and humans. Human activities such as mining operations, metal processing, dyeing, fertilizer, petroleum industries are the major contributors of heavy metal pollution (Rapport and Muler, 1995; Benka and Ekundayo, 1995). Chemical oxidation,
reduction, precipitation, adsorption, solidification, electrolyte recovery and ion exchange are some of the physicochemical processes of metal removal. Bacteria, Yeast and fungi are efficient scavengers in cleaning heavy metal polluted environment acting as efficient adsorbants. The metal uptake process by microorganism is a complex area of research. The same metal ions are accumulated by different mechanisms in different microorganisms. Bioluminescent bacteria, *Photobacterium phosphorium* is used for toxicity testing (Shijin, 2003).

*Serratia marsescens* is known for the production of various important enzymes, but the presence of the red colored pigment was a hindrance for the assay activities for the other enzymes. It therefore became necessary to study the effect of different metals that could block pigment production totally in *Serratia marsescens* but not the growth of the organism. The temperature dependent pigment block was seen in nutrient broth culture at 37°C in peanut medium complete block of pigment production was at 42°C second generation. The second purpose of this work was on a species of *Serratia* found in acidophilic habitats. This ensures that the organism would definitely have tolerance to some of the metals. Further study on effect of metals on *Serratia marsescens* would pave way for us to use this organism in metal bioremediation. If the organism is considered a pathogen atleast the metal resistance genes could be identified and cloned in future.

As a first step to identification of the metal resistance characteristics of *Serratia marsescens* in our study nickel, copper, cobalt and chromium of 0.2 mg/ml showed a complete block of pigment production. Minimum concentration of metal capable of blocking pigment production was also studied. From this it was observed that above 0.115 mg/ml in all the four cases of metals pigment block was observed. The expression of the intracellular protein, in the metal grown culture was higher than the non metal grown culture. The extra cellular protein did show a decrease over the control in case of nickel and cobalt (6.8 mM), copper (6.3 mM) of 0.2 mg/ml. Chromium even at a concentration of 0.2 mg/ml (3.8 mM) showed a similar pattern of
extracellular protein like the protein expression in non metal grown culture. The metals effect variable effects on the protein biosynthesis. Copper and chromium at concentration of 0.5 mM did not affect protein synthesis in *Bacillus thuringiensis*, but on the other hand cobalt at 0.1 mM showed a significant inhibitor effect on protein biosynthesis (Hassen et al., 1998).

The growth profile could be monitored only in terms of protein expression since the presence of peanut interferes with cell optical density. The viability of the culture in metals was shown by reverting the culture back into non metal medium. The reversion experiment showed us that the metal did not do any permanent damage to the pathway of pigment production because with two steps of reversion the normal pigment production was achieved. Hence when wanting to work with other extracellular proteins like the lipase, chitinase etc. the pigment production can be blocked using metals in groundnut medium.

Metals play an important role in cellular physiology primarily through interactions with proteins including enzymes. Metals also interact with nucleic acids and lipids. In case of *Arthrobacter aurescens* RS-2 nickel and aluminium enhanced the expression of 68 kDa protein (Ram Scharf et al., 1993). A study on the pigment block of *Pseudomonas* has shown that cadmium supported only pyocyanin pigment expression and gene promoted only pyoverdin expression. Copper and Chromium inhibited pyoverdin biosynthesis and had no effect on pyocyanin expression (Hassen et al., 1998).

Nickel and Cobalt with a concentration of 1 mg/ml did not significantly block the growth of the culture, but blocked the pigment production. On reversion of this metal grown culture back to non metal medium the growth resumed back to normalcy and showed the reappearance of pigment both on culture plates and in peanut broth. Copper and cobalt at 0.2 mm, inhibited the growth of *Bacillus thuringiensis* by approximately 30% with respect to the control. The growth of
Bacillus thuringiensis appeared more sensitive to metals than that of Pseudomonas aeruginosa (Hassen et al., 1998).

In case of copper the tolerance limit was 0.2 mg/ml only and in higher concentration, on reversion of the metal grown culture the colony count was very few indicating the permanent damage the metal has done to the cell as seen on plates. Some bacteria like the E.coli, Proteus vulgaris, and Pseudomonas syringae can tolerate high levels of copper between 2.5mM to 12.5mM (Trevors, 1987). 0.2 mg/ml concentration of tolarance by Serratia marsescens is higher when compared to 0.1 mg/l concentration of copper provoking 50% reduction in growth in microalga Phaeodactylum tricornutum and 1 mg/l inhibited complete growth. Pigment analysis in Phaeodactylum tricornutum showed that copper affected the pigment pattern (Angeles et al., 1996). Wong and Chang (1991) have observed that 0.1 mg/l of copper produced a slight inhibition of photosynthesis in Chlorella pyrenoidosa, whereas 0.25 mg/l produced total inhibition.

The tolerance limit of Serratia marsescens to chromium was 0.6 mg/ml. The trivalent and the hexavalent forms of chromium are environmentally important and the hexavalent is of particular concern because of its greater toxicity. Broad screening of microbial cell mass for metal adsorption could serve as a basis for the development of efficient biosorbent materials. Aspergillus niger was found to be more efficient than Streptococcus equisimlis and Saccharomyces cervisiae in removing the higher concentration of chromium ions. In Saccharomyces cervisiae the cell mass from the logarithmic phase was more compact as compared to cell mass from stationary phase. The metal sorption after 24 hours of incubation was an enzyme linked energy dependent process whereas the adsorption obtained after 30 minutes of incubation resulted from surface phenomena. The adsorption of chromium (VI) increase with the decrease in pH of the medium (Goyal et al., 2003). The maximum metal ion concentration tolerable by Streptococcus equisimlis, Saccharomyces cervisiae and Aspergillus niger was 0.2 mg/ml, 0.1 mg/ml and 0.25 mg/ml.
respectively. Chromium at a concentration of 0.5 and 1mM, caused an inhibition of about 30% with regard to control in *Bacillus thuringiensis* (Hassen et al., 1998).

From the above comparative results of tolerance limit of some of the organisms, *Serratia marcescens* seems to have higher tolerance limit than the rest of the organism and can be considered for future work as a potential bioremediant.

### 4.8 Regulatory Protein of Prodigiosin in *Serratia marcescens*

A high molecular weight protein was observed from the SDS PAGE profile of the specifically in cell pellets of *Serratia marcescens* which showed pigment production. The prodigiosin pigment ran like a dye front in the gel. The cell pellets were from the samples of temperature, nutrition and metal regulated effect on prodigiosin biosynthesis. Block in prodigiosin biosynthesis was seen at higher growth temperatures which varied from medium to medium and due to effect of different metals. In the cell pellets where there was prodigiosin biosynthesis the high molecular weight protein was seen in the cell pellet as electrophoresed on SDS PAGE. In culture conditions which enabled a block in the biosynthesis of the pigment but not the growth of the organism, the high molecular weight protein was not seen in the cell pellet protein profile. Haddix and Werner (2000) have made an unpublished observation that a membrane permeable positive regulator is involved in the prodigiosin biosynthesis. At low cell density there is a low level expression of membrane permeable positive regulator of prodigiosin biosynthesis. The intracellular concentration of the regulator remains low at low cell density. However, as the cell density increases in a closed system, the intracellular concentration of the regulator increases to a threshold needed for the activation prodigiosin expression. Thus, high levels of prodigiosin are expressed in liquid culture only at high cell density. The authors have not talked about any protein with respect to the positive regulator. Based on the molecular weight marker the protein from *Serratia marcescens* was found to be of approximately 94kDa.
in size. The literature survey, helped in the identification of 94.2 kDa protein in *Streptomyces lividans*.

A 0.972-kilobase pair DNA fragment from *Streptomyces lividans* that induces the production of the blue pigmented antibiotic actinorhodine in *S. lividans* when cloned on a multicopy plasmid led to the isolation of a 4-kilobase pair DNA fragment from *Streptomyces coelicolor* containing homologous sequence. Computer-assisted analysis of the DNA sequence revealed three putative open reading frames (ORFs), ORF1, ORF2, and ORF3. ORF2 extends beyond the sequenced DNA fragment, and its deduced product shares no similarities with any other known proteins in the data bases. ORF3 is also truncated, and its 41-amino acid C-terminal product is identical to the *S. coelicolor* adenine phosphoribosyltransferase. The 847-amino acid ORF1 protein, with a predicted molecular mass of 94.2 kDa, strongly resembled the *relA* and *spoT* gene products from *Escherichia coli* and the homologs from *Vibrio* sp. strain S14, *Haemophilus influenzae*, *Streptococcus equisimilis* H46A, and *Mycoplasma genitalium*. Unlike these proteins, the ORF1 amino acid sequence analysis revealed the presence of a putative ATP/GTP-binding domain (Oscar et al., 1996).

A mutant was generated by deleting most of the ORF1 gene that showed an actinorhodine-nonproducing phenotype, while undecylprodigiosin and the calcium-dependent antibiotic were unaffected. The mutant strain grew at a much lower rate than the wild-type strain, and spore formation was delayed. When the gene was propagated on a low copy number vector, not only was actinorhodine production restored, but actinorhodine and undecylprodigiosin production was enhanced in both the mutant and wild-type strains and morphological differentiation returned to wild-type characteristics. The amino acid sequence of the ORF1 protein reveals a particularly well conserved ATP/GTP-binding domain (amino acids 458–465). This sequence motif, \( (A/G)\text{XXXXGK(S/T)} \), generally referred to as the “A” consensus sequence (83) or the “P-loop” (84), is not present in RelA or SpoT proteins (27, 43, 78–81) and represents a gap in these proteins when aligned with the ORF1 product.
The N-terminal region of the ORF1 protein is 90 amino acid residues longer than the homologous ones. Six nucleotides were different between *S. lividans* and *S. coelicolor* within the original fragment, while the corresponding products were almost identical with only a conserved change (leucine instead of valine at position 197). Further biochemical and genetic characterization of the ORF1 protein and the deleted mutant would provide some insight into the role played by (p)ppGpp levels in the onset of antibiotic biosynthesis as well as in other regulatory events in the cellular physiology of *Streptomyces* strains.

The Car- and non producers of the red pigment, prodigiosin led to the identification of a second regulatory gene affecting expression of the carbapenam antibiotic (Simon et al., 1999). The rap gene stands for the Regulation of Antibiotic and Pigment gene and is homologous to the Hor gene [Homologous of Rap] in *Erwinia caratovora*. The mechanism by which either Rap or Hor exert their influence and their nature of any potential interactions between the quorum sensing and Rp/Hor regulatory mechanisms is unknown. The Rap gene identified in *Serratia marcescens* was found to contain 146 amino acids and is found in almost all species of *Erwinia*, *E. coli*, *Shigella*, *Salmonella*, *Yersinia* and *Enterobacter* (Thomson et al., 1997).

Based on the report that the 847 amino acid protein identified in *Streptomyces coelicolor* has a unique 90 amino acid sequence in the N terminal region, primers were designed for the first 900 base pair of the gene sequence of high molecular weight protein of *Streptomyces coelicolor*. On PCR amplification, using primers designed for the *Streptomyces coelicolor* gene sequence for the high molecular weight protein and the template of plasmid and genomic DNA of *Serratia marcescens*, a 900 base pair product was seen. This finding suggests the presence of a protein similar to the 94 kDa regulatory protein in *Streptomyces coelicolor* (Oscar et al., 1996) that regulates prodigiosin biosynthesis in *Serratia marcescens*. This finding can be further substantiated by designing primers for the second half of the protein and sequence the PCR product.
4.9 IDENTIFICATION OF METAL RESISTANCE GENES IN PLASMID SEQUENCE OF *Serratia marsescens* USING BIOINFORMATICS TOOLS

The metal resistant systems have been found on plasmids, but frequently related systems are subsequently found determined by chromosomal genes in other organisms. There are also no resistant genes for Group VIIa halides, although halides are abundant in the environment and are toxic in higher concentrations. The isolate from soil was identified as *Serratia marsescens* and the strain was found to be resistant to up to 1 mg/ml concentration of nickel and cobalt, 0.2 mg/ml of copper and 0.6 mg/ml of chromium. The next study carried out was to identify metal resistance gene sequences in *Serratia marsescens* by doing a search for metal resistance genes from different organisms and look for the similarity using clustal W. Since the genomic sequencing is half way through for *Serratia marsescens*, the metal resistance genes were first searched for in the plasmid sequence of *Serratia marsescens*.

From the SWISS Prot database the copper, chromium, nickel and cobalt resistance genes from different sequences were blasted with the plasmid sequence of *Serratia marsescens*. The four copper resistance genes of *E. coli* was found to have 100% similarity with a portion of the plasmid sequence of *Serratia marsescens*. The cusRS genes in *E. coli* form a sensor / regulator pair that activates the adjacent but divergently transcribed gene, cusC and possibly two adjacent downstream genes, cusBA, in response to increasing copper (Wayne et al., 2000). From the Blast result, it was observed that in the *Serratia marsescens* plasmid sequence the cop A, B, C and D were found to be a continuous string of base pairs with probably 20 to 40 nucleotide gap. The cusCBA genes of *E. coli* are homologous to a family of proton cation antiporter complexes involved in the export of metal ions, xenobiotics and drugs. The four structural proteins determining copper resistance are the inner membrane protein copD, the outer membrane protein copB, and two periplasmic proteins copA and copC.
Resistance to chromate governed by bacterial plasmids appears to have nothing to do with chromate reduction. Plasmid determined chromate resistance results from reduced uptake of $\text{CrO}_4^{2-}$, by the resistant cells (Ohtake et al., 1987). The chromate A and B protein of *Pseudomonas* present in the plasmid pB4 was found to have partial homology with the plasmid sequence of *Serratia marsescens*. The Chr A is a membrane bound protein with various transmembrane spanning alpha helices which is encoded at the 3' end of the Chr. Deletion of Chr A leads to chromate sensitivity and loss of the mechanism leading to reduced accumulation of the metal ion. Genes with products homologous to Chr A have been found in *Alcaligenes eutrophus*, *Synechococcus*, *Synechocystis*, *Methanococcus jannaschii* and *Vibrio cholerae*. The chromate B protein is also essential for the chromate resistance. Expression of chr derivative with a deletion in the 5' end of chr B led to the hyperaccumulation of chromium (Nies et al., 1990). The ORF Finder was used to find a putative protein for the chromate resistance protein A in *Serratia marsescens* and the putative protein showed high percentage of homology with the Chr A of *Pseudomonas*. Next work at the molecular level would confirm the function of the putative protein deduced in this work.

The mercuric reductase gene of *Thiobacillus ferrooxidans* and *Serratia marsescens* have a high percentage of homology which has already been reported. A novel cysteine domain was identified in nearly 60 proteins and termed as TRASH because of its anticipated involvement in trafficking, sensing and resistance to heavy metals. TRASH is a metallochaperone like domain encoded by multiple archeal and bacterial genomes. In *Serratia marsescens* copper containing ATPases was found to contain these TRASH domains. Archae specific transcriptional regulators appeared to contain multiple copies of the cysteine signature constituting the TRASH domain. The cop A and cop C protein of *Serratia marsescens* identified in this work was also found to have a high percentage of similarity with rusticyanin protein of *Thiobacillus ferrooxidans*. This is also being reported for the first time in this work.
4.10 PRODIGIOSIN AS A POTENTIAL DYEING AGENT

The development of synthetic dyes at the beginning of the twentieth century led to a more complete level of quality and more reproducible techniques of application. The predominance of synthetic dyes hindered a continuous development and adaptation of natural dyeing to the changing requirements of modern dye houses. As a result, now a considerable gap exists, separating the knowledge about natural dyes from the demands of commercial dyeing processes. The dyestuff industry is suffering from the increases in costs of feedstock and energy for dye synthesis, and they are under increasing pressure to minimize the damage to the environment. The industries are continuously looking for cheaper, more environmentally friendly routes to existing dyes. Characteristic pigments are produced by a wide variety of fungi and bacteria and the chemical composition of natural dyes are elucidated. These pigments exhibit several biological activities besides cytotoxicity. Pigments are the building blocks of colour in the plastics world and it is through their many combinations that we get the shades we see around us.

The various medicinally important aspects of prodigiosin have already been reported in literatures. The use of this pigment as a suitable dye for textile, leather and plastic has been tried for the first time and it has been partially successful with some more refined treatments to be followed. The dyeing of plastic was found to be uniform and repeated washes did not result in fading of the color and only the pigment was used for the treatment at high temperature of 121°C. Liakopoulou et al (1998), has used a temperature of 90°C over 30 minutes and maintained at this level for 1 hour for dyeing cotton and wool fabrics. 10% sodium chloride or few drops of 40% acetic acid solution were added in the dyeing of cotton and wool fabrics.

The photofading and photostability of dyed and pigmented polymers is a commercial problem involving a complex interplay of phenomena and mechanisms many of which remain unresolved. Dye fading, for example, involves reactions not
only associated with the dye itself but also those involving the polymer as well as dye-polymer interactions and these, in turn, are further complicated by the nature of the environmental conditions. The interaction of the photoexcited dye chromophore with the polymer matrix is an additional process which will not only control the stability of the dye but that of the polymer as well. The interactions of the dye and polymer matrix may also involve energy transfer and quenching with photoactive units or impurities giving rise to phototendering and/or stabilisation effects. The state of aggregation and concentration of the dye are also important parameters which are interrelated in controlling dye stability with the former being controlled by the nature of the polymer matrix. In addition to these processes the stability of dyed and pigmented polymers is controlled by the nature of the atmosphere, e.g. presence of oxygen, the humidity which can often accentuate fading and degradation reactions, the light source and the temperature of the irradiation conditions. Many industrial developments in the dye and pigment worlds are hindered by such problems although some solutions are feasible in terms of effective stabilisation.

Prodigiosin solution did show decolorisation in the presence of direct sunlight and not when directly exposed to ultra violet rays. As reported by Akhira et al., (1997), the bluish purple pigment produced by *Janthinobacterium lividum*, also showed a similar effect of fading when the material was exposed to sunlight and found that a post treatment with thiourea solution reduced the fading of color due to light considerably. A similar procedure was followed with dyeing of different textile material and chamois leather with saturated salt solution and thiourea solution individually and was found to be effective. Common madder (*Rubia tinctorum* L.) produces anthraquinone pigments in its roots, one of them being alizarin (1,2 dihydroxy anthraquinone) which has been used for dyeing textiles since 2000 B.C. Industrial assays demonstrated good performance when using a weight of dry powder which is 30% of the weight of material to be dyed for dyeing cotton, wool and silk yarns. Resistance to fading appears to be fairly good for dyed wool (Lusiana et al., 1997). Cotton and wool fabrics were dyed with the natural dyes chlorophyll and
carmine after treatment with the enzymes cellulase, α-amylase and trypsin. Wash and light fastnesses of the dyed samples were studied. Enzymatic pretreatment resulted in an increase in pigment uptake in all cases compared with the corresponding untreated samples, and did not affect fastness properties (Tsatsaroni et al., 1995). The final conclusion focuses on the need to improve fastness properties of the natural dyes, which must be understood as an indicator for a distinct need for research to overcome these problems.

4.11 CONCLUSION

- A bioanalytical assay using ferron was designed to detect the oxidation of ferrous to ferric by acidophilic iron oxidizing bacteria. Owing to the low biomass of these acidophilic iron eating bacterias a direct measurement of growth was difficult and hence this assay can be used as an indirect measurement of growth.
- Agar based solid plates for the growth of iron eating acidophiles was designed and ammonium ferrioxalate plates were designed to exclusively detect iron oxidising acidophiles.
- Functional protein signatures were designed for the different blue copper proteins.
- Novel media containing fatty acids as the carbon source supported enhanced growth of Serratia marsescens and prodigiosin pigment production. From the media formulation experiments it was inferred that fatty acid was the best carbon source.
- Ni²⁺, CO²⁺, Cu²⁺, Cr⁶⁺ metals inhibited prodigisn pigment pain is Serratia marsescens without the interference of color from prodigiosin pigment.
• Approximately a 94 kDa protein playing a regulatory role in prodigiosin pigment production was identified from the studies of different temperature based growth of *Serratia marsescens* on different media and from the effect of metals on blocking prodigiosin production. A part of the high molecular weight protein was amplified using primers designed based on a similar protein from *Streptomyces coelicolor*.

• Prodigiosin was effective in dyeing plastic, textile samples and leather and this study signifies the importance of prodigiosin as a pigment.